PERSONAL CARE AND COSMETIC TECHNOLOGY

SKIN AGING HANDBOOK

An Integrated Approach to Biochemistry and Product Development

EDITED BY NAVA DAYAN

William Andrew

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This book is a memorial to my grandparents Shifra and Yehoshua Hershman, who raised me. They lost their families who were killed by the Nazis during World War II. They will never age. God bless their souls.

Nava Dayan

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Preface

Stress, Emotions and Skin Aging

The way we perceive or value a person's physical appearance is very complicated and contains numerous factors such as sociological background, age, personal experience and preferences. It also changes throughout our lifetime as does the weight we attribute to it in our preferential judgment. I remember that as a child I had two very close friends that were sisters. While in the view of many others they were not physically attractive, in my mind they were the most beautiful people I knew. This is because of their sincere care and affection. On the other hand, I had a very close family member that was only centered within her very own issues. Every few years she would perform a different type of plastic surgery, but no matter what the result was, I still never thought of her as a nice or pretty person.

In one of the bible books, the book of Kohelet (Ecclesiastes), which is a type of personal biography, the author says: "hevel hachen vesheker hayofi" meaning (from the Hebrew): charm is deceptive and beauty is vain. In essence, physical appearance may be misleading and illusory. Personally, I know that in time perspective, the way I remember people is not by their appearance, nor by their words, or what they have given or taken from me. In essence, I think and remember them by the way they made me feel.

In one of the books I read, I came across an interesting differentiation between public and medical journalism. While the daily public news wishes to bring the shocking, devastating news, medical journals will only rarely publish "bad" unexpected data. If a study failed to prove a hypothesis it will most likely not be published. In this sense, this book is staged beyond pure science–it provides cautious optimism combined with reality.

The protestant theologist Reinhold Niebuhr is communicating to God asking him the following: "grant me the courage to change the things I can change, the serenity to accept the things I cannot change and the wisdom to know the difference."

I am hoping that this book will provide the reader with a few tools to understand what can or cannot be changed in the process of skin aging and therefore will ease the acceptance of the unchangeable and encourage the further exploring of the processes that may be controlled.

In our society there are great social advantages to being attractive. Common stereotypes of elderly people depict them as physically unattractive and sexually undesirable (Koblenzer, 1996). This reflects a negative belief and drives aging individuals to seek ways to improve their appearance. By doing that, they find almost instant gratification in terms of enhanced self esteem and profound intra-psychic benefits. In that sense, the cosmetic industry exhibits key physical, emotional and economical benefits.

The scope of this preface is to illustrate the cycle of stress-aging-stress and review its components and implications in relation to skin. As we age our skin becomes dry, scaly, itchy, uncomfortable and less pleasant to touch. With the development of wrinkles, solar elastosis, seborrehic and actinic keratoses, solar lentigines and such, it also becomes less pleasant in appearance. The equation "ugly skin (appearance) = ugly person" may lead to "aging anxiety." This is a phenomenon that strongly contributes to negative responses affecting society's attitude towards the elderly population. Therefore, it is clear that a key to successful aging is good mental health. In fact, we know that aging is evolutionary and modifiable. In the past few decades humans were not only significantly successful in postponing the average age at death, but also in improving the quality of aging. Successful aging, therefore, can somewhat be controlled by the individual.

The relationships between psychological stress and skin condition have been recognized by modern medicine. In fact, it is at birth when the skin becomes the barrier between the individual and its environment (Koblenzer, 1988). It is an organ of perception and therefore responds to tactile cutaneous stimulation. In the first months after birth, the skin is the main route of communication between the mother and the newborn. Positive tactile stimulation is essential for the development of healthy physical and emotional characteristics. The physical mother-infant contact allows the baby to define himself through the recognition of his boundaries and develop a healthy ego and self esteem. Moreover, babies of mothers that were unable to provide an appropriate satisfaction to their needs, were shown to have a tendency to develop infantile eczema. As grown toddlers, they showed poor social adjustment, poor subject relations and difficulties controlling aggression or impulses.

In translating of the above psychological attributes to physiological ones we find that when cutaneous tactile stimulation is decreased, growth hormone levels are also decreased. These babies, in addition to the development

of emotional disabilities, will suffer retardation in skeletal growth and in central nerve system development. They will demonstrate delayed locomotor activity and poor learning performance.

This type of extreme stress and its effects on early development is destined to affect humans throughout life. Every aspect of the immune system can be influenced by stress and can be expressed in skin condition. Examples of hypophsiotropic hormones that are released as a result of stress and which can influence the skin are: thyroid stimulating hormone, growth hormones, adrenocorticotropic hormones, beta-endorphin, fibroblasts growth hormone and thymocyte stimulating factor. In addition, inflammatory mediators can be released. Examples of mediators that can be released directly or indirectly upon stress are histamine, kallikrein, bradykinin, prostaglandins, substance-P, vasoactive intestinal peptide, beta-endorphin and leukotrienes.

The definitions of biological stress and emotional stress are different, but their outcomes may collide or be subsequent. They are bound together, and can affect and nourish each other in continuous long cycles. An individual that grows with a positive self esteem will not only be able to cope better with stress situations, but will be able to maintain his high self esteem. This person will remain active and will take action to protect and nourish his body, skin included, from damage. When stress is not well managed, one will neglect his physical state, his body condition will deteriorate, and his appearance will become repulsive, and this can trigger a cycle of low self esteem as a result of the physical situation and additional neglect.

Emotional stress can be either acute or chronic, and the mental and physical response to it can vary. Among common acute stressors are noise, crowding, isolation, hunger, danger, infection, imagining a threat or remembering a dangerous event. Note that this list, composed by Hantman and Solomon (Hantman S, 2007), includes infection, which is a physical illness, as a trigger to emotional stress. Chronic emotional stress can manifest itself in a form of on-going, highly pressured work, long term problems in relationships, loneliness and persistent financial problems. Factors that were shown to increase susceptibility to chronic stress include: abusive behavior, genetic factors (inherent ability for enhanced or diminished efficient relaxation response), immune related diseases and traumatic experience.

As we age, we not only experience significant stressors such as medical problems and loss of a spouse and close friends, but the ability of the body to achieve relaxation and return to homeostasis after stress becomes more difficult. With the exploration of scientific evidence about the tight connection between our physical and emotional being it seems as if the key to its

understanding lies in the complexity of our immune system. In the skin, immune mechanisms or inflammatory cascades are involved with irritation, allergy, infections, degenerative conditions and observation of tumors. In connection to the emotional psychological aspects, anxiety levels are higher in patients with atopic dermatitis when compared to those in non-atopic type. The itch threshold is lower, and once itching is triggered, scratching becomes a conditioned response to stress.

While the biological response to acute stress is immediate, the response to chronic stress involves adaptation phases. In sub-chronic stress, the "relaxation phase" is eliminated and the body homeostasis is constantly challenged. The hypothalamic-pituitary adrenal system (HPA) will respond to acute stress by producing and releasing steroid hormones (glucorticoides). Cortisol is a key hormone that controls a variety of organs such as the heart, lungs, blood circulation, metabolism and immune system, and will be manifested in skin condition and appearance. It will also trigger the release of neurotransmitters such as dopamine, norepinephrine and epinephrine (adrenaline). These will activate the area in the brain, amygdale, which is responsive to a stressful emotional event.

As a result of this response, the blood flow will be diverted from peripheral organs such as the skin to support essential muscles such as the heart. Hence, the skin will be temporarily under-nourished; its enhanced secretory activity will create an appearance of a slimy, sticky and sweaty tissue.

If stress becomes persistent, body organs respond in either over or under activation. The cycle that starts with emotional stress, continues with physiological effects and may end with additional emotional disorders such as depression and anxiety. These can lead to physical neglect such as poor hygiene and malnutrition. In this way the cycle will be evident in one's appearance. Chronic stress also affects the immune system: It can lower white blood cell counts, and therefore increase the risk for infections. Upon stress, people that harbor viruses, such as HIV and Herpes (which may appear on the skin), may find them activated, and papiloma may become more susceptible to viral activation. Skin disorders that were shown to be activated by stress include: psoriasis, hives, acne, rosacea, eczema and unexplained itching.

Biological stress is defined in terms similar to emotional stress. Toussaint et al. (Toussaint O., 2000), define stress as a "general adaptation syndrome" that is triggered by shock, followed by a phase of counter shock that is built into a gradual development of resistance. It is the "non-specific response of the body to any demand made upon it." It can be caused by "any environmental factor potentially unfavorable to living organisms" and the result will depend on its intensity and the tolerability of the individual.

Permanent damage, or even death, will occur when the limits of tolerance are surpassed and the adjustment system is over worked.

Biological stress at the tissue and cellular levels is characterized by three phases: The first is the alarm reaction, which will result in a decline in vitality, an excess in catabolism and a decline in anabolism. This will be followed by the activation of repair mechanisms that aim to induce adaptation and restoration of the tissue, and finally there is either an end phase or a long term response when the stress intensity is too high or chronic. In cell biology measures, depending on stress severity and duration, cells may either fully regenerate and restore biological functions, or enter into senescence and die.

In real life, stress is not an occasional event that will appear and disappear. We are constantly exposed to both emotional and biological stressors. Any condition that results in modification of the fine balance we build between the capacity to repair damage and its accumulation is a stress. Moreover, the saying "whatever doesn't kill you makes you stronger" appears to be false in the context of stress, be it biological or emotional. The accumulation of damage due to stress might be a factor responsible for the decreased capacity to cope with new or additional insults.

In reality therefore, stress can be either reduced or managed, but not avoided. In relation to aging, we may attempt to delay the process and make it less painful, but we cannot prevent it. In fact, the classification of biological stress in thermodynamic measures includes "normal conditions" as the first class of stress. It appears in the form of constant, unavoidable steady state concentrations of reactive oxygen species (ROS). Aging under these conditions will develop over long periods of time and the biological system imbalance will develop gradually. The second class of stress in this model is chronic repeated stress of sub-lethal intensity. Physical conditions included are inflammation, anoxia-re-oxygenation phases, exposure to UV or other types of radiation, and exposure to pollutants and toxic chemicals. When the body's own immune system fails to efficiently and rapidly eliminate or repair the damage, a destabilization of the homeostatic steady state is reached. At the cellular level, this will lead to stress-induced premature senescence (SIPS) and can also provoke apoptosis due to a high level of internal damage and relatively low metabolic activity. The third type of stress is classified as "severe" and is attained when the cell fails to create a new equilibrium. In this case, cells will die by necrosis because of repeatable intercellular damage and a decrease in metabolic activity. Skin cells such as keratinocytes, fibroblasts and melanocytes are constantly exposed to a variety of types and degrees of non-cytotoxic stress. This can lead to the accumulation of senescent cells that will be

removed by immune cells or undergo apoptosis. Under normal aging conditions, the accumulation of senescent cells upon exposure to chronic, non-lethal stress will eventually lead to alterations in tissue function and initiation of pathological conditions. Phasing into senescence, the cells undergo morphological changes, expose specific biomarkers and lose their replicative ability.

The Darwinian theory of evolution suggests that a long life span is driven by better adaptation to stress. Genes that are involved in response to stress are those that encode for DNA repair enzymes and anti-oxidant enzymes. These are the genes that regulate aging. Senescence, therefore, according to this theory, occurs when the force of natural selection declines with age and longevity is only acquired at the expense of metabolic energy.

There are a variety of typical psychocutaneous disorders common in the elderly population that extends beyond the appearance of wrinkles and age spots. For example, elderly women with obsessive compulsive personality will tend to develop neurotic excoriations as a result of repetitive picking or scratching. Elderly women with obsessional worries who realize, at some level, that their fears are not real will still seek constant reassurance and often will develop worries and delusions about their skin. Interestingly, especially among lonely isolated elderly people, delusions of parasitic infestation or infections can be popular. These delusions become more common after the age of 50 and their male to female ratio is 1 to 3. Being psychotic in nature, these illogical symptoms that make perfect sense to the patient are being treated by anti- psychotic drugs. “Chronic cutaneous dysesthesia syndrome” is common in elderly women and is characterized by tactile sensations that can be wholly subjective. Therefore, they are less certain to be hallucinatory in nature. These are variable and their fundamental psychopathology is heterogeneous.

Psychocutaneous diseases can be classified into three categories, psychiatric, with psychiatric factor, and those which involve genetic or environmental factors. When a person who has a psychiatric disorder is exposed to stress he or she may develop skin related symptoms. For example, those with borderline personality disorder may develop dermatitis artefacta and find it difficult to accept their body image. Patients with obsessive compulsive disorder can develop compulsive concerns about their skin.

Urticaria (or hives) which is a condition characterized by red raised skin wheals can develop as a response to different types of stress. Being allergic in nature it can be mediated by mast cell degranulation and through stress-induced secretion of vasoactive intestinal peptide, substance P, beta endorphin and other mediators released by the hypothalamus.

Pruritus (or itch) can be psychogenic in nature (meaning, involving psychiatric factors). Higher histamine -induced itch response was observed in individuals with greater psychopathology. Flushing and sweating are well known cutaneous stress responses; therefore, dermatological disorders that are aggravated or triggered by these conditions will occur under stress. In relation to aging, autoimmune-related alopecia can be triggered and aggravated by chronic stress.

In a study conducted by Rexbye H. et al. (Rexbye H., 2006), 1826 Danish twins aged above 70 were evaluated for correlation between their apparent age, real age and rate of mortality. It was demonstrated that "looking old for one's age" is associated with increased mortality. Approximately 40% of the variation in the perceived age was associated with non-genetic factors that included sun exposure, smoking and low BMI. Younger look was associated with high social status, marital status (married individuals looked younger) and low depression score.

A clue to a partial resolution to this stress-aging-stress cycle is found in an interesting paper published by Pennebaker J.W. et al. (Pennebaker J.W., 1989) on the disclosure of stressful traumatic events by Holocaust survivors. Life in Nazi concentration camps during the Holocaust surly represents one of the most overwhelming traumas suffered and is by all means a long chronic psychological and physiological stress. Survivors suffer permanent scarring that is the result of prolonged starvation, disease, abuse and sub-human existence. It is, therefore, not surprising to find out that Holocaust survivors appear older than their chronological age, are certain to develop immune related psychiatric and physical illnesses and will die relatively young. Moreover, the generations raised by these survivors will most likely experience difficulties that are a result of their disabilities.

This study demonstrates that survivors who discussed their traumas with others and/or wrote about them were healthier both physically and mentally. Their physician visit rate was significantly reduced and their serum immune system factors were elevated.

In relation to skin, researchers measured the influence of traumatic events disclosures by measuring skin conductance. This was based on previous studies which demonstrated that when individuals actively restrict emotional expression they will develop short term increase in certain autonomic channels such as skin conductance.

Interestingly, here again, when the traumatic event is being experienced again through disclosure, the skin is the first organ to respond. Individuals who disclosed deeply traumatic experiences demonstrated significant drops in skin conductance levels, but no changes were observed in heart rate or blood pressure.

Although in principle this is a scientific book, it is uniquely designed to expand beyond the core scientific aspects of skin aging. It starts with an introduction chapter on skin aging social aspects and implications, followed by market trends that led to the creation of an entire growing industry around it. A special chapter describes the prediction of physical appearance utilizing computer science. The center of the book delves into recent scientific findings in skin aging and is followed by chapters on product development written by authors from leading cosmetic and personal care firms. Impressive newly designed technologies have been developed in recent years to measure and assess skin aging. These are described in two chapters on testing methodologies for further evaluation and claim substantiation. The book is concluded with the global regulatory aspects of anti-aging product development.

These chapters were all written by friends and colleagues. With some I go many years back into my graduate student years, some I acquired in recent years and some will hopefully become future close contacts. They are all professionals in their fields and working with them was a true pleasure and a wonderful learning experience. I thank them all sincerely.

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PART 1
SOCIOLOGICAL IMPLICATIONS, MARKET VIEW AND IT FRONTIERS

1

Aging Skin in Sociocultural Perspective

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1.1 Introduction

The process of aging begins with birth. In understanding aging, we utilize a cultural master narrative which views both childhood and old age as problematic in some way. For example, we refer to old age as a "second childhood," specifically in highlighting the need by some elders for physical or cognitive care. We tend to see old age as a time of decline. We also see midlife as a standard against which youth and old age are measured. Nevertheless, it is increasingly youth that is the gold standard for measurement of other ages. It is the existence of this gold standard that should be kept in mind in this chapter, which discusses the social and cultural factors regarding aging skin. We address the central question of why the concern with aging skin and skin appearance is permeating our social life. Why has this become so important? There are many factors related to this which we will discuss in turn. These include increased longevity; medicalization; the manner in which postmodern capitalism works; changes in individualism; pro-feminism; the notion of "lifestyle"; the onset of the "third age"; a focus on consumption; the invisibility of the aged face; and media and advertising.

Before we begin a discussion of these elements, several points need to be made. First, a very great deal of social science research in the past fifteen years has demonstrated that the body, while of course being a real thing, is an entity that is produced within society and culture and therefore has important sociocultural meaning. There are hundreds of studies that have explored the social construction and cultural meaning of the body and body parts, including skin, both in the West and in indigenous societies. The anthropologist Marshall Sahlins once remarked something to the effect that "just because the body is real, doesn't mean it's not symbolic." In focusing on the changing meaning of bodily entities, such as skin, we essentially situate ourselves at the nexus of what is real and socioculturally imagined (Balsamo, 2000)—possibilities that occur as the product of an empirically produced set of cultural structures (Bourdieu, 1977).

Second, changing attitudes towards aging skin are fundamentally situated in a particular shared ethos or morality about what the body means, about how the body "should" be. For example, in the past, when aging skin was seen as a negative marker of personal value, there was little that one could do about the situation. Ultimately that skin was seen as ugly. With technological change the array of choices about what to do with wrinkles

or other age-produced blemishes, represents a key moral stance, what is required in the image of the body for proper personhood. And of course such moral issues are engaged by their portrayal in electronic mass media.

Third, while technology has increasingly provided a means to treat a condition (e.g., wrinkles or other issues that concern aging skin), this solution has been provided for a condition that itself is manufactured within culture and society. At best, there is no *intrinsic* reason why elements of the body should have particular values attached to them; this is done as part of culture. In a sense, then, the condition of aging skin has developed as a problem that needs a solution.

Finally, a concern with aging skin also represents what Featherstone (1991) has called "the astheticization of everyday life," a concern with an increased modern focus on the form and condition of the body, of the home, of accessories, and so much else. Aging skin must be seen in this context; because of the concern for physical aesthetics, aging skin is increasingly important. We will turn next to a discussion of those factors that we believe have caused this.

1.2 Increased Longevity

People now live longer than ever before, with women living, on average, seven years more than men in the US. The cohort of elders aged eighty-five and older is the fastest growing segment of the population and the aging of the baby boomers will swell the population of the aged in the next few decades. Increased longevity has occurred in a context of measurably better health among the aged. Better health is itself seen in several ways. The dramatic changes in the technology of health have been critically important. Interventions to reduce infection as well as immunization have perhaps been the most important component of improved health over the last century. Acute illness can now be dealt with in ways that were not before possible. Even dramatic interventions such as organ transplant have become relatively common among people in their seventies and eighties. No one especially wants to continue to live in a way in which they also physically decline. Whereas cultural and technical manipulations have brought about increased longevity, increased longevity has also shaped cultural and technical manipulations so that the side effects of longevity, such as wrinkled skin, are increasingly treated.

1.3 The Spread of Medicalization

Along with the increase in the availability of sophistication of medical technology has come the increased medicalization of bodily states and practices that were once handled informally. For example, as Lock (1995) and others have noted, menopause is increasingly becoming the territory of medicine and medical intervention, when it was never this way in the past. This is also the case for skin. For example, birth has also become highly medicalized, with increasing rates of C-sections and drug intervention in delivery procedures. Baldness is now a "syndrome" with a variety of pharmaceutical treatments. Erectile dysfunction and similar conditions that effect older people are now medically treated. The cultural attitude that is a companion to these technologies is one that shapes our understanding of every aspect of the physical body, which can require "medical intervention" in many cases. It should be no surprise then that skin should be subject to similar procedures. A treatment such as Botox, for wrinkles, for example, is in essence a medicalized procedure. Cosmetic surgery and procedures for enhancing skin quality and youthful appearance are now commonplace.

1.4 The Mechanism of Capitalism

Along with these forces, market forces, especially the postmodern manner of capitalism, have transformed us from a production-oriented to a consumption-oriented society. Part of the mechanism here is to leave no niche market or possible or potential need unmet. It is often hard to tell whether "the need" makes the market or whether the market makes "the need." It is undoubtedly the case that these arrangements are complicated. But it does seem to be the manner of postmodern capitalism to search out every nook and cranny of the potential market for areas in which a need can be created. As we will see below, this has a lot to do with manipulations of the marketplace through media and advertising. Again, however, the mechanism of "need-creation" in consumer society is complex and multiply determined. The market motivation for enhanced skin care simply builds assiduously on the cultural attitude that nobody wants to grow old or, if they do, that its negative effects be muted.

1.5 Changes in Understandings of Individualism

Individualism and autonomy have been at the core of Western personhood for many centuries, but the nature of independence and autonomy has

changed with changing historical and economic circumstances. For several reasons, the nature of individualism has been reshaped in the latter part of the twentieth century, continuing through today. Traditionally, individualism was a property of the wealthy in Western societies and was tied to the ideology of individual possession, ownership, political mastery, juridical status, control, and autonomy. The need to control the self and one's own body has also increased since the fifteenth century, when manuals of individual deportment first became widely available to the wealthy. Under the cultural era known as modernism, the political and social rights of individuals were extended to ethnic minorities and other dispossessed persons. Individualism increasingly became the property of all citizens, not just some.

Most recently, in what has been called the postmodern era, individualism has increasingly focused, once again, on control of the self. In this postmodern pro-consumer period, there have been dramatic alterations in the sense of personal responsibility that a person should have for her own life including her own health care. These have also been important to the story of the social and cultural significance of aging skin. A particular set of requirements has emerged as the object of personal self-interventions. Never has the goal of appearing youthful and healthy, at any age, been so important to so many in the West and elsewhere. Further, with the emergence of a global culture and globalization, Western ideals of beauty have been widely disseminated and may form or influence standards for many throughout the world.

The powers of biomedicine—the perspective that sees medicine determined by powerful political and social forces and by the need of medicine to control people (Foucault, 1973)—were formerly seen in the form of authoritative medicine. Within authoritative medicine, patients followed doctors' orders unquestioningly and doctors appeared to be larger than life. In postmodernism, this approach to medicine has seen the authority of the doctor diminish, while individuals themselves, as consumers, have increasing responsibility for their own health and bodily condition through a proper lifestyle. Ironically, at the same time this has occurred, the medicalization—the need for medical authority—of culturally defined "ailments," such as baldness or wrinkles, has increased.

Essentially, what we have now is a system in which individuals must "make themselves." The person is a lifelong project, under their own

constant monitoring and manipulation. With changing standards for health and beauty, there is increased commercialization, and with increasing focus on the idea of a lifestyle, the individual must involve herself in a number of projects to constantly enhance the self. In old age, however, because physical change is ultimately the fate of everyone, there comes some point at which the management of individual self-projects must adapt to the changing realities of physical decline.

1.6 Pro-feminism

The effects of feminism and the women's movement have been profound in respect to law and legal rights. Some have argued that the feminist movement of the last forty years has largely influenced upper middle-class white women, and that its extension into the lower class has been more muted. Others have suggested that the feminist movement can be construed in different circumstances among minority populations, who have less access to wealth. Still some have argued that distinctive life histories of minorities have rendered their situations as different. For example, there has been recent publicity to the notion that "black don't crack," referring to the occurrence of skin suppleness and youthfulness among black older women.

Elements of the feminist movement have argued that women's interest in products such as cosmetics or fashion represent another way in which the male-centered society acts to control women (as well as their money). Be this as it may, it is apparent that many women buy into the ideology of cosmetics and fashion; these are multi-billion dollar industries. Further, newer cohorts of women have had little exposure to the feminist movement of the 1970s from which many ideas about female deportment emerged. While younger generations have been advantaged by the legal gains derived from political feminism, they may fully buy into the newer cultural ideas of feminine deportment. In this regard, it is interesting that many skin treatments present themselves as being "natural," that is, made from natural substances to effect a natural tone or look, rather than chemical in nature. Thus these natural treatments appear to remain part of the nonmedicalized portion of cultural intervention into beauty, although it is likely that some products will increasingly have a medicalized sensibility that requires experts to disseminate them, as new products are developed.

1.7 The Notion of "Lifestyle"

One important term that has come into vogue in the last two decades is "lifestyle," a term used to describe the intentional, chosen organization of attitudes, symbols of attitudes, and the social construction of the life course. Correlated with this has been a switch from production to consumption as a general political economic mode. Thus closely associated with "lifestyle" is the notion of "consumerism," an ideology also associated with postmodernism. Elements of lifestyle commonly include not only consumption but also how one behaves, and personal preferences for clothing, food, housing, social relationships, sexuality, bodily manipulation, and, of course, the symbolism and expression of hair and skin. Some scholars have discussed "lifestyle" in terms of its relationship to a general framework of health promotion. Having a healthy lifestyle is increasingly important at any age, and more specifically with regard to the obesity and diabetes epidemics. There is a more-or-less unstated folk belief among Americans that a healthy lifestyle should be manifest in the person's surface image. One should be able to *see* a healthy lifestyle as well as a poor lifestyle. Lifestyles, including exercise and proper diet, should generate a glow that can be seen on the skin. When this is not the case, there are now cosmetic products that provide such a glow. Thus appended in some way to consumerism is the goal of "natural beauty."

1.8 The "Third Age"

One intentional goal in gerontology and geriatrics has been to "square the curve," that is, to extend the active and productive part of the life course until the final terminal decline. Another way of expressing this goal has been the development of the notion of the "third age," specifically the period of time from retirement to the onset of terminal decline. Healthy elders now typically have many years to pursue interests other than the main ones that they had when working or parenting young children. New opportunities for the aged have developed. Part of these new conditions under postmodernism has been, as we noted above, that the onus of self-care has been placed on each older individual. As part of this, the need to stay healthy, to not be a "couch potato," to exercise regularly (perhaps join an exercise center), to eat healthfully, and most of all to stay young has been central. Starting in one's twenties, especially if one is a woman, the battle against wrinkles and other markers of age on the skin commences,

and continues for as long as possible. From a cursory glance at recent advertising, it appears that cosmetic companies are targeting younger and younger audiences for these forms of self-improvement. The noted sociologist, Anthony Giddens (1991), has written how, in postmodernism, the onus for self-care and self-management of life has been placed on the individual. It is not just self-care that is self-managed, but all forms of life are, including one's career, one's family life, and one's lifestyle choices. This is similar in a way to what Michel Foucault has called "technologies of the self," that armamentarium of self-interventions that people require to be who they hope to be.

There has been very little written on the relationship of the desire for youthful appearance and the onset of the third age. The third age is pictured more as a time for personal pursuits, education, volunteerism, and the like. It is probably the case, however, that as both the study and the public interest in this period continue to emerge, some link will be made between the third age and cosmetic interventions. In essence, there are two discourses at work here. There is the discourse of the third age, which is really about life span development, and there is the discourse of old age, which is related to cosmetic intervention. The two have not quite met. Nevertheless, a concern with the health and appearance of skin is relevant to both perspectives.

1.9 Consumption

As noted above, there has been a general switch from production to consumption as the major mode of the political economy. This means that as manufacturing and heavy industry have moved out of the Western nations to the developing nations, the goal of consumption has emerged as the single most important element, indeed, the driving mechanism of the global economy. There are many purposes to postmodern consumption, including being modern, being up to date, having the latest thing, such as consumer electronics, and possessing symbols of personhood. These consumed items are linked to issues of aging skin, in that the same concerns with youthfulness and being up to date emerge as significant.

The number and diversity of products that relate to appearance are increasing. Indeed, if one walks through a shopping mall, one would see that the majority of the products offered there are for appearance or "lifestyle." Consumption, in the form of shopping or interest in new items, has become

a major national sport. The increased focus on aging skin and its amelioration is part of this overall trend towards consumption.

1.10 The Invisibility of the Aged Face

Some gerontologists have described the phenomenon of old age as a mask, thus situated primarily in the face (Howson, 2004). The image of the mask suggests that despite what we may see, there is something else behind it. However, the aged are shielded not only by clothes and figurative masks but also by *social invisibility*. We suggest that there is a tendency to see to whatever degree the aged face and hands but that the aged body, and often the face, is socially invisible. We tend to believe that the faces of the elderly loose their individuality over time, as part of the general process of aging. In a sense, this represents the distinction between the face of the older person as it exists at the present time (the mask) from the person as they existed in the past. Up to a certain point, facial changes that appear with aging can in fact be disguised or covered up by commercial cosmetic preparations. These can cover wrinkles, discoloration, and some of the sagging that occurs on the face, as well as varicose veins, angiomas (small, visible blood vessels on the surface of the skin), and moles. Men tend to use fewer of these products, although hair dyes for men, as well as baldness regimens, are increasingly normative. It appears that the loss of hair is easier for women to confront than the loss of hair by men. Cosmetic surgery is increasingly normative for all classes, but especially the upper middle classes. Nevertheless, some have noted that even these treatments cannot reach beyond a certain point when full old age kicks in and that, beyond this, interventions do not really work well.

Medical geriatrics stresses the above changes and also others such as the thinning and drying out of skin, the skin's loss of elasticity and increase in subcutaneous fat (Wyatt, 1985). Old age is the great age of spots, warts, pimples, and wayward tufts of hair. Excessive exposure to the sun also promotes wrinkles. We also know that with age the skin has diminished physiological function and reserve capacity. With age, the skin cannot function any longer as effectively as the barrier it once was and it does not repair itself as well as it once did. Thus ultimately, the manufactured "fixes" for skin gradually run up against a wall, after which they will not really work. Some strategically placed wrinkles on men are said (by some) to help a man look distinctive or steely, although it is perhaps the case that a wrinkle free appearance is more the desired-for state. Additionally, some

wrinkles on healthy, fit women are also allowed. Nevertheless, for the vast majority of women, wrinkles are to be fought across a vast battlefield. These stigmata of aging are manageable through cultural and technical procedures. Socially, excess wrinkling—for the most part genetic—is said to be tragic and the opposite to be a blessing. ("Eighty-five years old and hardly a wrinkle on her!") No doubt the advertising agencies that produce aged skin cosmetics spend a very great deal of money on research on consumer behavior and on effective, proactive imagery. Whatever their discoveries, these will not have much meaning unless there is a widespread fear or disgust about aging to begin with. In reality, no one wants to get old.

1.11 Media and Advertising

While we have discussed this topic briefly above, a separate mention of this should be made. Dissemination of cultural images about youthfulness and perfect skin are largely the subject matter of media and advertising. The recent hyperconnectivity of media forms with and by means of the Internet, and the constant barrage of advertising images concerning youthful skin and flawed skin contribute to a burgeoning knowledge about and self-monitoring of one's skin situation.

1.12 The Social Significance of Aging Skin

Living up to the ideals of youth that our society defines as what is beautiful is understandably and logically difficult as we age. The idea that many people are concerned about the condition of their skin may stem from the fact that it is this part of our bodies which is first seen by others. From an individual's skin a person can generally glean a large amount of information—their race/ethnicity, their financial situation related to the time spent in the sun or working outside (indications of leisure activities and occupation), and also their age. This last bit of information is becoming increasingly more difficult to determine, however, as people use a variety of cosmetics, skin treatments, and surgeries to reduce signs of aging skin.

How has the use of these age-defining methods become so popular and, more importantly, why? It could possibly be because there is an innate tendency in some individuals to fight old age and to maintain the Western

ideal of youth and health as long as possible, and by whatever means necessary. When an individual looks in the mirror and sees wrinkles, these represent signs that one is aging and an affirmation that they are getting old. These cues of aging may run contrary to internal feelings of youthfulness and health. A fear of getting old, of getting sick, or of dying can all been seen in the attempts to hide wrinkles. This is even done, as we "hide the wrinkles" (i.e., the people with wrinkles) in nursing homes or other age-segregated housing such as assisted living. We are not accepting of the reality that we will all reach old age. And so, instead of revering wrinkles or cherishing our first gray hair, it has become necessary—and often expected—for individuals to conceal these age affirming cues and to modify ourselves to reflect the expectations of youth and beauty.

1.13 Conclusion: Skin as a Semiotic Language

Culturally, aging skin is the subject of a process of semiosis, based on the use of the body as a system of signs and symbols that persons can "read" and interpret. It must be recalled that the body has no intrinsic cultural meaning, but that the meaning or meanings are supplied within each cultural system. Within American culture, there is ample discourse about the nature and meaning of skin, and of aging skin in particular. Indeed, there is nothing about skin that is without cultural meaning. Everything about skin, from color to impurities, to scars, to wrinkles—everything—-has some kind of interpretable meaning.

There is also dispute, contestation, about what skin means and how it should be interpreted. There are different discourses, versions, and understandings about the story that skin tells us. Powerful, well-funded forces are attempting to portray one version of the meaning of skin, that signs of age are bad and need to be treated. There are several alternatives to this idea, but few that get as much media imagery and publicity. This is all based on the idea that aging is a bad thing and that no one wants to get old. There is a risk, however, in holding these points of view. The risk is this: that people who are old get tarnished in some ways, as people, because they are old. Are we looking at a public morality that thinks less of people because they *fail to* deal with or cover up outward signs of aging? We think, perhaps, we are. We see one possible scenario as this: that older people get divided into two kinds. The first kind is the wealthier elder who does everything possible to foster an active and youthful lifestyle, including manipulation of aging skin for purposes of beauty,

sexuality, and lifestyle. The second kind consists of those poorer elders, or those who can be bothered, who do not measure up to the increasingly exacting standards required to escape the manifestations of old age. It is likely that the first group will tend to believe that old age is not so great. Both groups, as well as the non-old, suffer from the prospect that they will label older people negatively and stigmatize them in some way. This is an acute possibility for those of the first category, who will put so much effort into staying young. All of this remains to be seen, however. What we do know is that there is a lot riding on the meaning of aging skin.

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2

Market Evolution of Topical Anti-aging Treatments

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Nava Dayan (ed.), Skin Aging Handbook: *An Integrated Approach to Biochemistry and Product Development*, 15–34, © 2008 William Andrew Inc.

2.1 Introduction

The ancient Egyptians applied plant essences, mud, milk, and kohl to protect, heal, and prolong youthfulness of their skin. Ponce De Leon was willing to travel the earth on a mission to find the fountain of youth. From the earliest records of civilization, humans have been on a constant pursuit to turn back the hands of time; in essence "anti-aging," the pursuit of prolonging youth, is ageless. Over the past century, life spans have grown longer and the population of elderly people is on the rise. As the quality of life improves people seek to look as young as they feel and unlike in the past, modern technology allows the luxury of not settling for "looking their age." The beauty industry answers the call and continually finds new and inventive ways to create innovative and efficacious anti-aging products.

How have we evolved on our quest? What have we learned through the years that can be applied to future endeavors and ensure victory over wrinkles and other signs of aging? This chapter will focus on the exploration of skin care preparations as a function of the changing outlook on aging. It will also evaluate marketing claims and consumer perception both in the United States and globally. Prior to delving into the subject matter, a brief review explaining changes in the skin when aging will be outlined to provide the basic understanding of the approaches needed to be taken.

2.2 Types of Aging

Aging can be divided into two categories: intrinsic or chronological aging and extrinsic also called premature or photo-aging.[1] Intrinsic aging is a natural occurrence in which numerous simultaneous mechanisms occur. Collagen and elastin production slows down; these are fibers that make up the dermal matrix and give our skin the ability to bounce back into its original position. As we age the capability of the skin to spring back to place is diminished.[2] Skin cell turnover slows down leaving excess dead skin cells remaining on the surface. The skin becomes thinner,[3] the dermal-epidermal junction compresses and the dermal structure begins to collapse.[4] The visual results of intrinsic aging are:

- dry/flaky skin
- fine lines
- wrinkles
- sagging/lax skin

Extrinsic aging is caused by outside factors such as cigarette smoke, exhaust and pollution but the most common culprit is over exposure to UV radiation. Continual sun exposure not only hinders the skin's ability to repair itself but continues to break down and debilitate the synthesis of new collagen. UV radiation can also lead to degradation of elastin fibers causing the premature decrease in skin flexibility.[2] The perceivable results of photo-aging are:

- hyperpigmentation
- leathery appearance
- dry skin
- deep wrinkles

While both intrinsic and extrinsic aging have existed since the beginning of time; our approach to fighting the visible signs of aging is new. Over the past two decades there has been a shift in the focus of skin care treatment regimens. Formulations started to focus on anti-aging claims and directly addressing the signs of chronological and photo aging. As the view on aging began to change from gracefully accepting the inevitable visible signs of aging to preventing and reversing the perceivable effects, there became a great need for a new generation of skin care products.

Skin care treatments were not a new concept to consumers; the use of home remedies was typically passed down from generation to generation. However, it wasn't until the early 1900s when Helena Rubinstein introduced the idea of a skin care regimen, did true skin care routines begin to be marketed and sold in pharmacies and hair salons.[5] But it was in the 1980s when the beauty industry actually started focusing on formulation technologies and claims to satisfy and/or create consumer needs.

2.3 The 1980s

The beginning of the decade was relatively simple in terms of skin care; the basic routine was to cleanse, tone, then moisturize with a cream that probably contained collagen proteins but the goal was enhancing beauty, not reversing time or creating an anti-aging effect. Collagen products were first introduced into the market place by major cosmetic firms in the 1970s and became a staple in creams for firming that is still used today. The focus of the beauty industry during this time period was an emphasis on moisturization and firming as beautiful women graced the pages of advertisements

evoking a sense of elegance, glamour and beauty. It was a decade where age was accepted and embraced. "40 and fabulous" was a popular ad slogan that exemplified the mood of the moment.[6]

2.4 The 1990s

As the 1990s began, the needs and wants of Baby Boomers were starting to become a significant factor in the marketplace and spurred an increasing demand for anti-aging products. Although developed in 1961 by R.J. Havighurst,[7] the concept of "successful aging" which described actively seeking and enjoying physical and mental health during the later years of life was never more relevant than the 1990s. Aging Baby Boomers were in better health and had more expendable income than their parents. The new "middle age" was no longer forty but late fifties and was beginning to be viewed as a new start in life rather than approaching the end of life.[8] This mindset translated into a new market category that produced a niche of skin care products that would revolutionize the way products were formulated and marketed. The new view of preventative aging opened doors for the beauty industry to create new demands for consumers who were previously concerned primarily with treating conditions such as balancing "oily" and/or "dry" skin and keeping skin moisturized. Now the Baby Boomer consumers wished to look younger and the industry responded with products that reduced the appearance of wrinkles and made the skin look healthier and more youthful. The late 1980s saw the emergence of true anti-aging products with Dior leading the way being the first to incorporate liposomes as a delivery system for ingredients.[9] Ingredients such as hyaluronic acid and petrolatum were used for moisturization while soluble collagen and elastin were used to help firm up the skin. There were products on the market for decreasing the appearance of wrinkles but individual ingredients and their effects were generally not discussed and the focus was the finished product.[9] Globally, the anti-aging phenomenon was beginning to take form.

As we moved into the 1990s the anti-aging category exploded. In 1993, anti-aging became a medical discipline in the US when the American Academy of Anti-Aging Medicine was formed by eleven physicians who saw a need to concentrate on older adults who sought "early detection, prevention and reversal of aging related diseases."[10] The new emphasis on anti-aging prompted formulators to take a new approach on formulating products. Scientists, chemists and formulators began to revisit the theories

on aging to take a closer look at the biochemical mechanisms leading to aging in an effort to counteract or slow down the process.

2.5 Common Anti-aging Theories

There are numerous anti-aging theories; the five most well known (Table 2.1) in the personal care and cosmetic industries are:

- Wear and tear theory
- Cross-linking theory
- Neuroendocrine theory
- Free radical theory
- Telomere theory

The wear and tear theory of aging was first formulated in 1882 by biologist Dr. August Weissman and suggests that accumulation of damage to cells, tissue and organs overtime eventually wear them out and kill them. This damage begins at the molecular level within our cells. The DNA in our genes sustains repeated damage from toxins, radiation and ultraviolet light. While our body has the capacity to repair DNA damage, not all of the repairs are accurate or complete therefore the damage progressively accumulates. With age the body loses its ability to repair damage leading to visible wrinkles and age spots.

The cross-linking theory of aging, also referred to as the Glycosylation Theory, was first proposed by Dr. Johan Bjorksten in 1941.[11] The cross-linking theory is based on the observation that as we age, protein and other molecular structures begin to cross link as they form covalent bonds between them to create larger polymeric molecules. This polymerization leads to a decrease in the elasticity and mobility of the molecules. When glucose and proteins are bound in the presence of oxygen and polymerization eventually occurs, the protein becomes damaged and inefficient in its tasks.[12] Increase of reactive oxygen species interacting with glucose and protein leads to the appearance of tough, leathery and yellow skin. When collagen is cross linked and is shown to be linked with the appearance of wrinkles and decreased skin elasticity.[11]

The neuroendocrine theory is a continuation of the wear and tear theory first addressed by Dr. Vladimir Dilman in 1954. It generally states that as we age, the hypothalamus loses its ability to precisely regulate the release

Table 2.1 Summary of Aging Theories

Theory Name	Theory Explanation
Free radical theory of aging[a]	• Free radicals attack the structure of our cell membranes, creating metabolic waste products, including substances known as lipofuscins (connected to "age spot"" formation) • Lipofuscins interfere with the cells' ability to repair and reproduce themselves, disturb DNA and RNA synthesis, inhibit protein synthesis, destroy cellular enzymes • Free radicals attack collagen and elastin causing the structures to break down but the body can repair itself. • With age, the effects of the accumulated free radical begin to slow down cell renewal therefore reducing the body's self-repair capabilities, leading to an increased breakdown of collagen and elastin eventually causing wrinkles.
Wear and tear theory of aging[a,b]	• Suggests that years of damage to cells, tissues, and organs eventually wears them out, leading to their death • Our bodies have the capacity to repair DNA damage, but not all of those repairs are accurate or complete; therefore, the damage progressively accumulates and leads to signs of aging such as wrinkles and sagging skin
Neuroendocrine theory of aging[c]	• This system governs the release of hormones that are altered by the hypothalamus located in the brain • The hypothalamus controls various chain reactions to instruct other organs and glands to release their hormones • As we age, the secretion of many hormones declines and their effectiveness is also reduced • One theory for the hypothalamus's loss of regulation is that it is damaged by the hormone cortisol • Cortisol, the hormone responsible for stress regulation, is produced from the adrenal glands (located on the kidneys) • It is known to be one of the few hormones that increase with age; increased cortisol levels lead to breakdown of muscle tissue and collagen

(*Continued*)

Table 2.1 Summary of Aging Theories (Continued)

Theory Name	Theory Explanation
Cross-linking theory of aging[a,d]	• Also referred to as the Glycosylation Theory of Aging • It is the binding of glucose (simple sugars) to protein (a process that occurs in the presence of oxygen) that causes various problems • Once this binding has occurred, the protein becomes impaired and is unable to perform as efficiently • Increase of oxygen meeting glucose and protein leads to the appearance of tough, leathery, and yellow skin • It is also theorized that sugars binding to DNA may cause damage that leads to malformed cells and thus cancer • Cross-linking of proteins may also be responsible for cardiac enlargement and the hardening of collagen • Cross-linking of the skin protein collagen has been shown to be at least partly responsible for wrinkling and other age-related changes in skin
Telomere theory of aging[c]	• Telomeres are sequences of nucleic acids extending from the ends of chromosomes • Telomeres shorten each time a cell divides • Shortening of telomeres is believed to lead to cellular damage due to the inability of the cell to duplicate itself correctly • Each time a cell divides, it duplicates itself with imperfections • This eventually leads to cellular dysfunction and aging

[a]Theories on Aging, health-cares.net, 2005.
[b]Dr. Ronald Klatz and Dr. Robert Goldman, "Stopping the Clock," Keats Publishing, New Canaan, CT, © 1997.
[c]International Anti-Aging Systems, "Theories of Aging," 2004.
[d]Marios Kyriazis, MD, "Cross-link Breakers and Inhibitors," www.anti-agingsystems.com, March 2003.

of hormones while the hormone receptors become less sensitive. The hypothalamus is a region in the brain that regulates certain metabolic cascades and autonomic activities of the body.[13] The secretion of key hormones decline and their efficacy is reduced. This popular theory states that the hypothalamus' deficiency is caused by an increase in the hormone

cortisol which is produced by the adrenal glands.[14] Cortisol is one of the few hormones that increase with age. Elevated levels of this hormone are responsible for enhanced catabolism, breaking down of muscle tissue as well as degradation of collagen.[15]

The free radical theory of aging is one of the deep rooted and most well known theories on aging. It was brought into view in the early 1950s by Dr. Denham Harmon.[16] Simply stated, "free radical" is a term used to describe any molecule that possesses a free electron and is highly reactive. Free radicals steal electrons from paired electrons in neighboring molecules thereby creating other free radicals. Oxygen is a powerful producer of free radicals known as reactive oxygen species (ROS). Examples of common ROS are superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), and nitric oxide (•NO). ROS attack the structure of our cell membranes, creating waste products, including pigments known as lipofuscins. An excess of lipofuscins in the body is shown as a spotting of the skin in certain areas perceivable to people in the form of "age spots." Lipofuscins interfere with the cells ability to repair and reproduce themselves, disturb DNA and RNA synthesis, inhibit protein synthesis and destroy cellular enzymes.[17]

Free radicals attack collagen and elastin causing the structure to break down. This kind of damage begins at birth but its effects are not detrimental in our youth due to the body's natural repair mechanisms. With age, the accumulated free radical effects begin to slow down the cell function therefore reducing the body's self-repair capabilities. Free radical attacks eventually lead to wrinkles, sagging skin and "age spots."

The telomere theory of aging is a more recent concept developed by the findings of Alexei Olovnikov and John Watson in 1972.[18] Telomeres are nucleic acid sequences located at the end of chromosomes. Their role is to provide extra buffer protection to the DNA from enzymatic degradation. Each time the cell divides the telomeres shorten; with continued division eventually the telomeres will become merely stubs and the cells will cease to divide leading to cell death.[14] Cellular senescence, a dormant state in which cells remain alive but can no longer divide is based on "Hayflick's Limit," a discovery by Leonard Hayflick in 1962 that proved human embryonic cells do not divide infinitely but have limits to the amount of times they divide.[18]

Knowing what biological and physical changes the body undergoes during the aging process is paramount when trying to develop ingredients to counteract the evidence of these changes.

2.6 Key Anti-aging Ingredients of the 1990s

With an increasing need to understand the causes of aging and a growing knowledge of the many mechanisms that lead to it, the focus on ingredient selections for anti-aging products was directed to manipulate the various theories mentioned above. The elegance of formulations became secondary in the development process, formulators and marketing departments shifted the focus to provide well formulated products that incorporated functional ingredients that affect the skin's biochemistry. Large companies began spending millions of dollars to research ingredients that will help to counteract the biological occurrences taking place on the cellular level to lessen the perceivable effects of aging and formulate them into their products. This is the birth of the "cosmeceutical" era. Cosmeceuticals refer to a class of products that merge cosmetics and pharmaceuticals meaning they contain topical attributes of cosmetics but include ingredients that influence biological functions of the skin.

2.6.1 Alpha Hydroxy Acids

An in-depth study was performed by Eugene J. Van Scott and R.J. Yu in the 1970s. This study showed the potential of alpha hydroxy acids, in particular glycolic acid, to accelerate skin cell turnover and allow new, young, less damaged cells to surface at the epidermis. Although the results of the studies were successful, alpha hydroxy acids did not become popular in skin care products for resurfacing the skin resulting in a smoother, brighter and less wrinkled appearance until the 1990s when Van Scott and Yu licensed the use of glycolic and lactic acid in skin care preparations for anti-aging purposes.[19]

2.6.2 Vitamins

Retinoids (vitamin A) are present in every living organism and are necessary for various biological processes. The term "retinoid" covers retinol, retinyl esters, retinaldehyde and retinoic acid.[20] In the 1980s Dr. Albert Kligman conducted research on the affects of retinoic acid on acne and discovered that it also showed positive results on repairing photodamaged skin.[21,22] Because retinoic acid is a regulated drug (Tretinoin) that cannot be used in cosmetics, this lead the personal care industry to study the effects of its derivatives, such as retinol (vitamin A). The two primary functions of retinoids in the body are the antioxidant activity on scavenging peroxyl radicals, quenching singlet oxygen, and their ability to affect specific genes to activate growth factors and keratin production.[23]

The primary claims for retinol are aiding in increased cell renewal leading to a decrease in the appearance of fine lines and wrinkles, toning the skin and brightening its complexion.

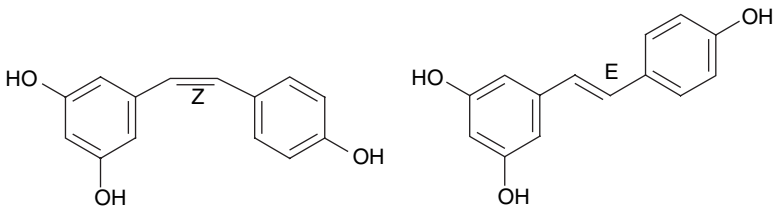
Ascorbic acid (vitamin C) was discovered in 1930s. Many studies were conducted to explain its biological functions.[23] Ascorbic acid has strong antioxidant activity and helps maintain vitamin E in its active form in the body. It has also been shown to stimulate the synthesis of collagen.[23] L-ascorbic acid has the greatest efficacy but poses certain difficulties due to its unstable nature in aqueous based formulation. Derivatives such as ascorbyl palmitate or magnesium ascorbyl phosphate were introduced to overcome stability issues. It was hypothesized that, once absorbed into the skin, the esters will hydrolyze releasing an active by-product ascorbic acid. A Canadian skin care company, Cellex-C, was one of the first to successfully launch an anti-aging product using vitamin C as the main functional ingredient in 1990.[24] The success of the product line encouraged other companies to follow suit. Claims attributed to the use of vitamin C include: stimulation of collagen synthesis therefore reducing the appearance of fine lines and wrinkles, improvement of skin tone for a healthy complexion and the neutralization free radicals to help protect against photoaging.

2.6.3 Botanical Antioxidants

In the mid-1990s, studies were conducted on polyphenols found in grape seed extract and green tea. The major polyphenolic compound in green tea is epigallocatechin-3-gallate (EGCG). It is purported to be 15–20 times more powerful as an anti-oxidant than both vitamin E and vitamin C.[25] Grape seed extract is rich in polyphenols, specifically proanthocyanidins (OPCs), which belongs to the bioflavonoid family. OPCs are strong antioxidants that when penetrate into the skin play a role in the stabilization of collagen and maintenance of elastin.[26] Resveratrol is an antioxidant with a phytoalexin structure (see Figure 2.1). It is found mainly in the skins of red grapes and has demonstrated hydroxyl-radical scavenging activity, in particular, quenching the superoxide anion and inhibiting hydrogen peroxide production. It has been found to have glutathione-like activity protecting the cells from oxidative stress.[27]

2.7 Cosmetic Claims of the 1990s

Cosmetic claims of the 1990s were fairly mild when compared to current claims made by skin care manufacturers. Most anti-aging products



cis-Resveratrol
($C_{14}H_{12}O_3$; mol. wt. = 228.25 g/mol)

trans-Resveratrol
($C_{14}H_{12}O_3$; mol. wt. = 228.25 g/mol)

Figure 2.1 Structure of resveratrol.

addressed firming, diminishing the appearance of fine lines and wrinkles, treating "sensitive skin" (i.e., inflammation/irritation prevention claims) and brightening the complexion. The ingredients mentioned above were the primary ones to support the claims. By the mid-1990s, propelled by the aging Baby Boomers, the US anti-aging trend was beginning to pick up momentum but global growth was much slower. The advertisements that displayed cosmetic claims during this decade changed from *prolonging* the youthful appearance and *preventing* premature aging to *reversing* signs of aging and *regaining* a youthful appearance by the mid- to late 1990s. The department stores were the main source for consumers to find promising "efficacious" skin treatment products. As the twentieth century was coming to a close, skin care manufacturers marketing strategy started to change. Manufacturers realized that not all consumers shopped at department stores due to location and price point; a large segment of the market was not being serviced. Products began showing up in mass market drug stores, supermarkets and specialty outlets that were relatively higher priced and provided perceived skin care benefits that started to rival the department stores and prestige outlets. Because the pricing for products sold in drug stores and mass market outlets was on the rise, manufacturers were able to afford more efficacious ingredients at higher percentages increasing the quality & efficacy of mass market skin care products.

2.8 2000s and beyond

The turn of the century brought about another shift in the anti-aging movement and globally, the movement began to flourish. In August 2001, the World Academy of Anti-Aging Medicine (WAAAM) was incorporated in England, Wales.[28] WAAAM was founded to be both a vehicle and a

forum for the advancement of anti-aging medicine in the UK and abroad. While the rest of the world was beginning to take notice of the anti-aging movement, US consumers were becoming more aggressive in their practices to regain their youth. Cosmetic surgery was on the rise with close to four million botox injections given in 2005.[29] There was a 38 percent increase in cosmetic surgery from 2000 to 2005; 10.2 million cosmetic procedures were performed totaling an astonishing amount of $9.4 billion dollars spent on cosmetic procedures in 2005.[30] To address the many needs of the population, the personal care industry segmented the skin care category and increased product offerings.[31] Procedures typically administered in the offices of dermatologists or estheticians have successfully migrated into the general market. Spa visits also increased and was no longer just a luxury for the upper echelon of society but a treat and sometimes refuge for working class women and men. This opened up another niche market for manufacturers, the "spa at home" concept, in which consumers can have spa-like treatments in the comfort of their own home. Such popular professional treatments such as peels and microdermabrasion have led manufacturers to duplicate the effects of these treatments and distribute the products through the many different retail channels.[32] Consumers of this decade have increased expectations of their skin care products, in particular of anti-aging products that make distinct claims. They are more sophisticated and their thought processes impact purchasing decisions. Consumers are looking for and expecting third-party validation of product efficacy claims.[33] Marketing departments and research and development groups of the personal care industry collaborate to develop products that will meet the new demands of the savvy consumer. Anti-aging products are expected by consumers to deliver more perceivable efficacious benefits as opposed to only providing the illusion of elegance and glamour as they have in the past.

Marketing campaigns have to walk a fine line to advertise all of the wonders of their ingredients and products while staying within the borders of cosmetic claims. With the increased use of bioactive functional ingredients, claims for products that contain these ingredients need to be "creatively" worded to avoid them being defined as "drugs" by the FDA. Common claims of ingredients that discuss adjusting cellular mechanisms in the dermal layer can be considered to be drug claims that fall out of the realm of cosmetics.

A new dimension in the anti-aging arena is the large number of dermatologists getting involved in offering their own product lines. While the term

"cosmeceutical" was introduced in the 1980s, the 2000s is when the term undoubtedly took flight. The term has no legal meaning and many companies have and continue to avoid its use so as not to have their products declared drugs and coming under increased FDA observations on the self-regulated personal care industry. The Food Drug and Cosmetic Act of 1938 clearly defines cosmetics and drugs as:

> **Cosmetics:** articles intended to be applied to the human body for cleansing, beautifying, promoting attractiveness, or altering the appearance without affecting the body's structure or functions.
>
> **Drugs:** Products that intend to treat prevent or mitigate disease, or otherwise affect structure or function of the human body.[34]

There is a fine line that companies who market cosmeceuticals walk when making claims on their products and care must be taken to keep claims on the cosmetic side of the line. For example, examine the two claims below, one of the claims is a cosmetic claim and the other is a drug claim.

> "Helps reduce the appearance of fine lines and wrinkles"
> "Reduces fine lines and wrinkles"

The former claim is a cosmetic claim because of two words, "helps" and "appearance"; this implies that product is altering the appearance without affecting the body's structure. The latter claim is making a drug statement because "reducing wrinkles" is done by altering the structure of the body, in particular the dermis, being affected or altered in some way.

Advertising for the new generation of anti-aging products center around science-based formulations and ingredients; an approach that manufacturers use to influence consumers as the demand for authenticity in these products increases with innovation.[35] In the age of botox, consumers seek products that give immediate benefits as well as long term. These products must demonstrate efficacy in a short period of time and the long term benefits should be substantiated by in properly run clinical studies.

2.9 Ingredients of the 2000s

Innovation is the name of the game for ingredients in the 2000s. New ingredients are largely based on molecular biology and biochemistry and

although claims made on the ingredients are cosmetic; the mode of action on many of the ingredients do appear upon examination to affect biochemical activities in the skin.

2.9.1 Peptides

Peptide is a buzzword that consumers recognize as an "anti-aging" ingredient almost like proteins or amino acids are to body builders. What are peptides and what is their mode of action? A peptide is any compound composed of amino acids with the chemical structure of an amide between a carboxyl group of one amino acid and an amino group of another amino acid. Peptides with fewer than 10–20 bonds are referred to as oligopeptides; if they include more amino acids then they are polypeptides.[36] As of today, there are many debates in the scientific community regarding peptides and proteins and how large a peptide has to be before it is considered a protein.

Peptides incorporated in anti-aging cosmetic products fall into three categories: signal peptides, carrier peptides and neurotransmitter blocking peptides.[37] Signal peptides mimic protein sequences found in collagen and elastin and stimulate the production of new collagen and elastin. Carrier peptides act as vehicles for other ingredients to aid in the enzymatic processes that increase collagen synthesis. Some peptides are fragmented and can block neurotransmission that can lead to reduction in facial contractions that would eventually breakdown the collagen and create the appearance of wrinkles.[37] Use of peptides in skin care formulations is boundless. Slight modifications in the sequence or differing feedstock can alter the functionality of the peptides (see Table 2.2). What are the implications to the consumer? Anti-wrinkle, skin firming and increased microcirculation are just a few claims made by the use of peptides due to the various modes of action they claim to have. (See Table 2.3 for more examples of peptides and their uses.)

Marketing department of skin care companies have trained the consumer to look for peptides in ingredient labels. However, when listed as a part of the ingredient label the term "peptide" does not reveal the exact structure or function of the compound used in the formulation. The nomenclature for peptides is not specific to function and may in fact have more than one function.

Table 2.2 Peptides and Their Modes of Action from Various Feedstocks

Feedstock	Claimed Mode of Action
Shark cartilage	MMP inhibition
Thymus (thymosins)	Immune stimulation
Spleen (glycoproteins)	Respiratory increase
Red algae (*Ahnfeltia concinna*)	Increased collagen synthesis
(peptides rich in hydroxyproline)	Cell turnover
	Skin firmness
	Retinoid receptor
Vegetable protein (hydrolysis with restrictive enzymes)	Stimulation of collagen synthesis
Soybean peptides	Proteinase inhibition, collagen synthesis
Rice bran peptides	MMP-1,2 inhibition

MMP, matrix metalloproteinase.
Source: Courtesy Raymond Sourial.

Table 2.3 Common Peptides and Their Claimed Functions

Common Peptides	Claimed Functions
Acetyl hexapeptide-8	Decrease expression lines
Dipeptide diaminobutyrol benzylamide diacetate	Decrease expression lines
Palmitoyl oligopeptide	Wrinkle reduction via cell communication and repair
Dipeptide-2	Decrease under eye bags
Palmitoyl tetrapeptide-7	
Acetyl tetrapepide-5	
Palmitoyl tripeptide-5	Increase collagen synthesis
Palmitoyl tetrapeptide-7	Decrease dark circles under eyes
Copper peptides	Skin renewal
Hexapeptide-11	Increase firmness
	Decrease fine lines
Tripeptide-1	Reactive carbonyl species "quencher," anti-glycation

Source: Courtesy Raymond Sourial

2.9.2 Phyto and Algae Extracts

Scientists began to study the biological affects of algae, phyto extracts and the compounds extracted from these species. Medicinal uses of plants date back to ancient civilizations. They relied on the foliage growing around them for healing purposes and beauty enhancements. In an effort to find new functional and innovative ingredients, companies searched from mountaintops to the depths of the seas—and every place in between—to find compounds and to identify the useful fractions that can be extracted for use in skin care products. This process has unearthed hundreds of new ingredients that brighten the skin, decrease the depth of wrinkles, stop muscle contractions, and stimulate collagen synthesis—or prevent its destruction—as well as inhibit tyrosinase to reduce the appearance of age spots and brighten the skin's complexion. (See Table 2.4 for a short list of phyto and algae extracts and their uses.)

Table 2.4 Sample of Phyto and Algae Extracts and Their Claimed Functions

Algae/Extract	Claimed Functions
Red algae	Increase cell proliferation Increase hydration Increase firmness Increase cell turnover
Green algae	Increase hydration Increase cellular energy
Micro-algae (micrometer size)	Free radical scavenger Increase collagen synthesis Increase ATP levels Increase cell turnover
Watermelon extract (specific)	DNA protection
Daisy extract	Skin brightening
Hibiscus (specific)	Expression line reduction
Corn extract (specific)	Increase healing mechanism Decrease wrinkles
Phytosterols	Increase cell proliferation Increase hydration Increase firmness Increase cell turnover Increase collagen synthesis Anti-irritation Free radical scavenger

The natural drug market for plant extracts is projected to increase annually by 10 percent in industrial countries or regions such as North America and the EU between 2005 and 2011.[38] In the time of increasing cosmetic surgery, the drift toward natural skin care is on the rise, making botanicals and marine derived ingredients even more in demand. The trend for "organic" skin care is carried over by the high publicity and demand for organic foods. Health conscious consumers and those who were opting for more natural skin care products prompted the personal care industry to create natural and organic skin care products. There is no clear definition on what makes a skin care product natural but many consumers have a preconceived notion on what to look for on the ingredient label in order to make an informed decision on which products to purchase. A common question is did the industry create the demand for natural products or are they responding to consumer needs? The answer goes back to the health conscious consumers who were not willing to compromise their internal well being with "tainted" food and at some point was no longer willing to compromise her external well being by applying "chemicals" on the skin. The personal care industry picked up on this trend early and did not miss a beat! The natural and organic skin care sector is the largest growing area in skin care and the forecast for this category is continued growth globally.

There are numerous ingredients used in anti-aging products that do not fall under the category of peptides, plants, or marine extracts. With all of the new technology introduced into the skin care market annually, it would take a book in itself to even graze the surface of new ingredients and their origins and functions. Table 2.5 provides a brief look into the broader picture of the ingredient technologies used in anti-aging products.

2.10 Summary

The cliché "What's old is new again" is a suitable anecdote for the aging population. Consumers desire skin care products that will bring new life to their skin and give them the appearance that matches their mental age and not chronological age. The shift in consumer attitude toward aging prompted skin care companies to place more emphasis on reversing the signs of extrinsic and intrinsic aging. This is focus of "anti-aging" skin care and cosmetics. Due to the types of compounds used in skin care products, many companies walk a fine line between making drug claims and cosmetic claims in order to attract the attention of the consumers. Phyto extracts that decrease dark spots on the skin, peptides that communicate

Table 2.5 Miscellaneous Ingredient Sources and Their Claimed Functions

Ingredient	Claimed Functions
Saccharomyces lysate	Boosts skin's oxygen content, consumption, and respiration
Pseudoalteromonas ferment extract	Helps stabilize proteins and lipids in extreme conditions
Pueraria lobata symbiosome	Free radical scavengers Enhances procollagen synthesis Improves skin tone and elasticity
Lotus japonica symbiosome	Free radical scavenger Promotes skin brightening
Yeast extract	Film-forming; binds moisture to skin
Glycine Max (Soybean) Symbiosome	Free radical scavenger
Lysate	Slows photo-aging of skin
Pyrus Malus (Apple)	Anti-inflammatory
Fruit Extract	Helps prevent the appearance of wrinkles
Soy isoflavones	Free radical scavenger Inhibits MMPs

with your brain to the affect the skins' biological processes or algae that protects the DNA. Ingredients have become more technologically advanced than that of simple emollients and collagen used in skin care products of the 1970s and 1980s.

People want to look younger for as long as possible and the personal care industry is up to the tasks by inventing and discovering new ingredients to turn back the clock and give consumers the opportunity to look as young as they feel. While the personal care industry offers hope in a jar it is also holds the responsibility to educate the consumers to realize the limitations of anti-aging products.

Acknowledgments

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3

Computer-Based Age Progression Methodologies

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Nava Dayan (ed.), Skin Aging Handbook: *An Integrated Approach to Biochemistry and Product Development*, 35–52, © 2008 William Andrew Inc.

3.1 Introduction

The question "How will I look like in the future?" is a question that most humans think about at some stage in their lives. Age progression is a process where the future facial appearance of a subject is predicted. Usually, age progression is done by artists who use a photograph of a subject as the input, and modify certain features in order to generate a picture of an age-progressed face [1,2,3]. The process of generating age progressed images relies on observations pertaining to standard age-related deformations of facial features (i.e., the introduction of wrinkles and bone growth) coupled with hereditary traits as observed from pictures showing close relatives of the subject in the target age. Where available, additional information such as lifestyle, health condition and occupation is also utilized in an effort to produce more accurate age-progressed faces. However, manual age progression systems cannot be used in everyday applications for the following reasons:

- The process of generating age-progressed pictures is time consuming.
- The cost of involving an artist can be high.
- The results of this procedure depend on the artistic impression of the artist hence the overall outcome is subjective.

Due to the factors outlined above the generation of age progressed images is restricted only for use in special cases, such as the generation of age portraits of missing children [3] and the generation of images showing the current facial appearance of wanted criminals [1,2].

3.1.1 Automatic Age Progression Systems

A number of researchers working in the field of digital image processing describe automatic age progression systems in an attempt to deal with the

limitations of manual age progression systems. The development of automatic age progression systems would enable the use of such technology in a wide range of different applications such as:

- Auto-readjustment of e-records: Face images appearing in personal identification documents (i.e., passports, driving licenses, etc.) need to be updated frequently. Automatic age progression methods can be used for automatic re-adjustment of such photographs.
- Age invariant face identification: Age-related facial deformations inhibit the process of face recognition. Automatic age progression/rejuvenation can be used for eliminating aging effects in order to develop age-invariant automatic face identification systems.
- Assist the development of anti-aging products: The operation of automatic age progression systems could be linked with the use of anti-aging products and cosmetics so that it will be possible to produce age-progressed images for different scenarios (i.e., predict the future facial appearance of a subject in the cases that an 'anti-aging' treatment is used and compare with the predicted appearance in the case that such treatment is not used).

3.1.2 Image Databases

The development of an automated age progression system requires the existence of training image sets containing images of subjects at different ages. Ideally images of the same subject at different ages should be available, in order to make it possible to isolate age-related changes in facial appearance. The generation of a suitable database is a difficult process because optimum pictures to be included in an aging database should display faces at high resolution, captured with neutral expression and approximately frontal view. Ideally no facial occluding structure such as hats, beards and spectacles should be present. When an image database is compiled using existing pictures it is practically impossible to fulfill all the requirements stated above.

Although few researchers used their own aging databases, currently there are two publicly available face databases that can be used for training age progression algorithms — the FG-NET Aging Database [4] and the MORPH Database (HYPERLINK "http://faceaginggroup.com/" \o "blocked::http://faceaginggroup.com/" \t "_blank" http://faceaginggroup.com).

The FG-NET Aging Database was developed as part of the European Union funded project FG-NET [5]. The database contains 1002 images from 82 different subjects with ages ranging between newborns to 69 years old. Typical images from the database are shown in Figure 3.1. Images from the FG-NET Aging database are not ideal for experimentation in developing age progression systems since the quality of images in the database is variable and in several cases images from the database show occluded faces with varying expressions and orientation. Because of the low resolution of the images in the database it is not possible to model efficiently subtle age related skin deformations such as wrinkle related deformations. The FG-NET database is more appropriate for modeling more distinct age-related facial transformations like shape variations and major texture variation. Although the FG-NET database is not ideal, currently more than 300 researchers around the world are using the database.

As part of an on going project, researchers from the Face Aging Group (HYPERLINK "http://faceaginggroup.com/" \o "blocked::http://faceaginggroup.com/" \t "_blank" http://faceaginggroup.com) at the University of North Carolina at Wilmington generated the MORPH database. The MORPH database contains more than 15,000 images captured within age intervals ranging from 46 days to 29 years. On average the MORPH database contains three samples per subject. The MORPH database has also been used extensively for age progression experiments.

The growing interest in performing research in the area of facial aging, dictates the need for generating improved aging face databases containing multiple high quality images per subject displaying extensive aging variation.

Figure 3.1 Sample images from the FG-NET aging database.

3.2 Computer-Based Age Progression Methodologies

Computer-based age progression methodologies reported in the literature can be divided into various categories as shown in Figure 3.2.

3.2.1 2D vs. 3D Age Progression Systems

In the case of the image format category, age progression methodologies are separated into the ones that use 2-dimensional (2D) images and the ones that use 3-dimensional (3D) faces. A 2D face image is simply a digitized picture that shows a face from a particular viewpoint. 3D face images are groups of 3D coordinate points that describe the geometry of the facial surface. Usually texture information is superimposed on 3D shape information so that full texture and shape 3D face models are obtained. Figure 3.3 shows an example of a 2D image and a 3D textured model of the same person obtained using a Konica-Minolta VI-9I 3D laser scanner. Three-dimensional face models provide a more detailed

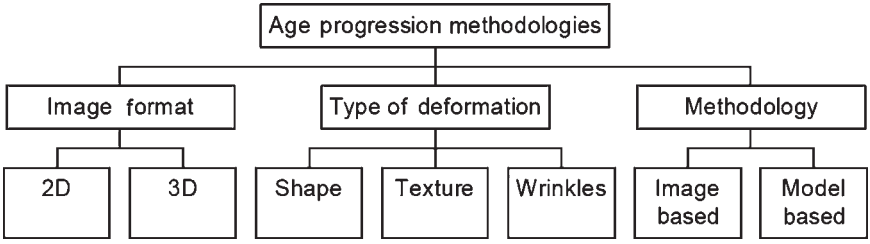

Figure 3.2 The major categories of age progression systems.

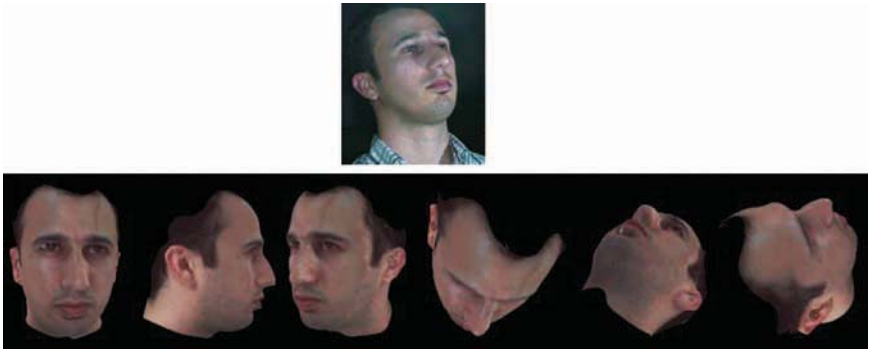

Figure 3.3 2D face image of a subject (top row) and a 3D model of the same person as seen from different viewpoints (bottom row).

description of the face structure and assorted texture; hence the use of 3D faces in age progression systems result in the generation of more accurate and realistic age-progressed images. However, 3D age progression systems require the existence of a 3D scanner in order to obtain a 3D scan of the person's face to be age-progressed. Due to the high cost of 3D scanners, the use of 3D age progression systems in non-specialized applications is limited. On the other hand simple and inexpensive digital cameras can be used for obtaining 2D face images to be used in conjunction with 2D age progression systems.

3.2.2 Shape vs. Texture Facial Deformation

Age progression systems reported in the literature can also be classified according to the type of deformation inflicted on face images. For example some systems cause only shape or only texture deformations. A special case of texture deformation usually encountered in relation with age progression is the addition of wrinkles; due to the importance of adding wrinkles in age progressed images, we classify the introduction of wrinkles as a different type of deformation. An integrated age progression system should be able to deal with all three types of deformation mentioned above. Depending on the application for which an age progression system is intended to be used and the availability of suitable training data, age progression systems reported in the literature may not deal with all three types of deformation mentioned above. Figure 3.4 shows examples of age progression for each type of deformation.

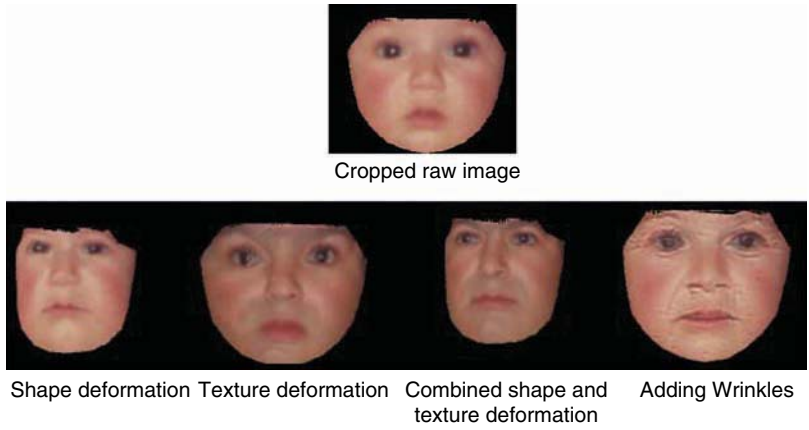

Figure 3.4 Age progression based on different types of facial deformation.

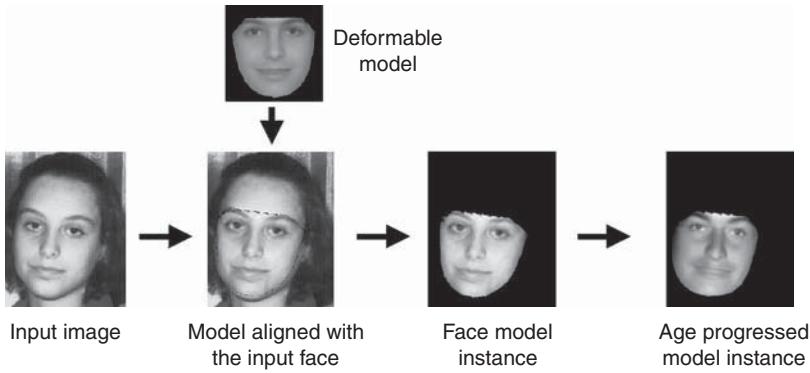

Figure 3.5 The basic steps in model-based age progression.

3.2.3 Image-Based vs. Model-Based Age Progression

Image-based age progression methodologies operate directly on the given image, in order to introduce age-related modifications. In the case of model-based age progression methods, a generic deformable face model that can undergo pre-computed shape and/or texture deformations is used. Given a new face image, the generic model is deformed in order to display the face of the person in the given image and subsequently the generated model instance is age-progressed. Figure 3.5 shows the basic steps in model-based age progression.

3.3 Typical Age Progression Systems

In this section we present typical age progression systems reported in the literature. This overview is by no means an exhaustive/in depth literature survey into the topic but aims to present the basic principles of few key approaches rather than presenting all the approaches reported in the literature. Additional, in depth technical details related to the algorithms described can be obtained in the corresponding references.

3.3.1 Age Prototypes

Rowland and Perret [6] calculate the average face among a number of face images belonging to the same age group in order to generate prototype faces for different age groups. The averaging process is carried out by

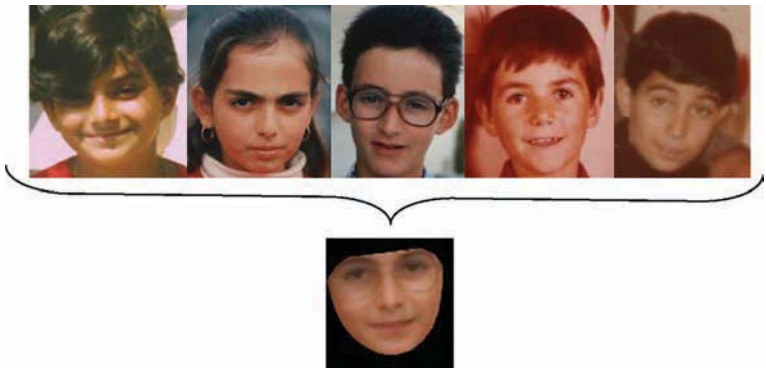

Figure 3.6 Face image showing 10-year-old subjects (top row) and the corresponding age prototype (bottom row) for these 10-year-old subjects. Because one of the subjects wears spectacles, traces of spectacles appears in the prototype.

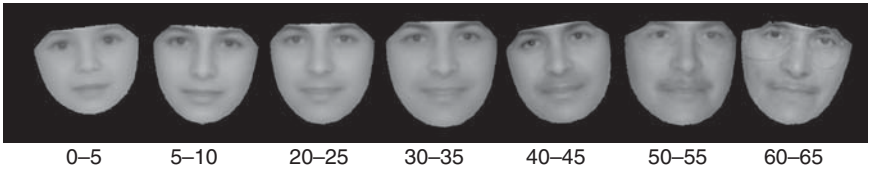

Figure 3.7 Age prototypes for the age groups 0–5, 5–10, 20–25, 30–35, 40–45, 50–55, 60–65 years. The age prototypes are derived using images from the FG-NET Aging Database.

calculating the average face shape and average image intensities among faces belonging to the same age group. Figure 3.6 shows a number of faces belonging to the same age group and the corresponding age prototype for that group. Figure 3.7 also demonstrates typical age prototypes for different groups. In age prototypes derived using a large number of samples, individual facial characteristics are suppressed in favor of typical facial characteristics of subjects belonging to the corresponding age group. Age progression is achieved by adding or subtracting to a face image differences between age prototypes from different age groups. Figure 3.8 illustrates the age progression methodology proposed by Rowland and Perret [6]. A major limitation with this approach is the elimination of high frequency artifacts (i.e., wrinkles) during the calculation of age prototypes resulting in smoothed age prototypes. As a result, age progressed faces

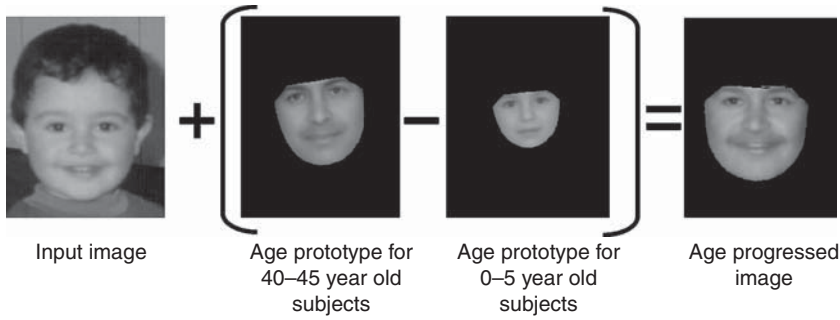

Figure 3.8 Age progression using prototypes. Age progression of a 4-year-old to 40 years is done by adding on the given image the difference between the age prototypes corresponding to 40-year-old subjects and 0–5-year-old subjects.

derived based on smoothed prototypes do not display wrinkles and other high frequency age-related artifacts. In a most recent approach [7] a method for adding high frequency details on age progressed images is described in an attempt to improve the appearance of the resulting images.

The method proposed by Perret *et al*. [6,7] is an image-based method that deals with all three age-related types of deformation (shape, texture and wrinkles). A variation of the basic method has also been used for performing age progression on 3D face images [8]. The quality of the age progressed images produced makes this approach attractive for a variety of applications such as the generation of age progressed portraits. The major disadvantages of this approach are the requirements for high quality face images showing faces with a standard pose and expression, without occluding structures such as spectacles and moustaches. Moreover, the method is based on the assumption that all subjects undergo similar types of aging transformations. Although this assumption is reasonable, individual factors such a person's lifestyle, gender and ethnic origin, could cause discrepancies from the standard aging pattern. One way of dealing with the diversity in aging variation is to generate different age prototypes for subjects belonging to different genders and/or ethnic origins.

3.3.2 Image Merging

Image merging techniques can be used for blending two images so that a novel image that combines the characteristics of the two input images is

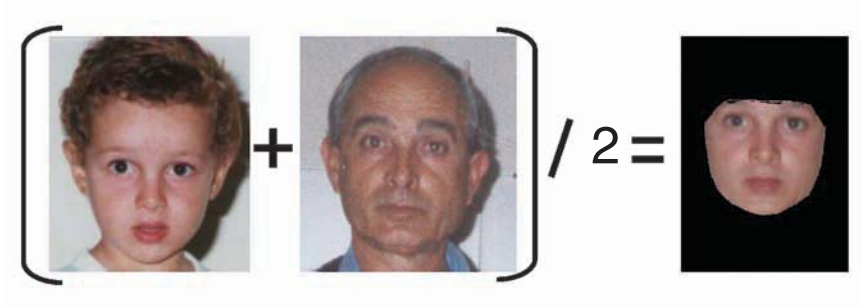

Figure 3.9 Age progression using image merging. An image of a 4-year-old subject is merged with an image of a 54-year-old subject. The resulting image shows an age-progressed image of the 4-year-old subject.

produced. Often different weights are given to each image composite so that in the resulting blended image facial characteristics of one of the two input faces are emphasized. In the case of age progression, image merging between an image of a young subject and an image of an elderly subject results in an age-progressed image of the young subject. Figure 3.9 demonstrates this approach.

Along those lines Lee *et al*. [9] obtained 3D face models of all members of a family (parents, daughter, and son). 3D image merging techniques are used for blending the models of the daughter and son with the model of their father, producing in that way age-progressed 3D images of the daughter and son. Wrinkles are also added on the blended images using a method that mimics the physics of wrinkle generation [9].

The main limitation of age progression systems based on image merging is the dependency of the results on the composite images showing the older subject, since in effect aging characteristics of the older subject are transferred to the younger face. Methods based on image merging are image-based methods requiring high quality input images in order to produce acceptable results.

3.3.3 Image Warping

Leta *et al*. [10,11] use images of 40 women aged between 25 and 65 years. On each image 26 distance measurements were recorded and following an

analysis procedure the correlation of those measurements with age is established. Based on functions that relate the 26 measurements with age, it is possible to predict how the 26 distance measurements are modified, as a person grows older. Image warping techniques are used for modifying the shape of a face in order to inflict age-related modification specified by the modification of the 26 distance measurements.

The method reported by Leta *et al*. [10,11] deals only with shape variation. Visual results presented do not demonstrate significant variations in facial appearance in the age progressed images. However, this approach is interesting since it provides a framework of relating modifications in facial measurements against changes in age.

3.3.4 Model-Based Approaches

Lanitis *et al*. [12] developed a model-based age progression methodology. In these experiments they use a face database containing images showing the same subjects at different ages. During the training phase they apply Principal Component Analysis (PCA) [13] on images from a training set, in order to generate a statistical face model [14]. Models of this type can be tuned in order to display the face of any subject similar to the ones appearing in the training set. One of the most important features of this type of models is the ability to represent faces using a small number of parameters; less than 100 numerical parameters are required for representing face images based on this approach. Model parameters control different types of variations in facial appearance such as variations due to change in facial expression, variation in skin texture, age related variations and variations in the shape of particular facial features and the spatial relationships between them. The coding achieved based on this methodology is reversible enabling the reconstruction of new faces once the values of model parameters are fixed.

In order to develop an age progression system Lanitis *et al*. [12] converted all training samples into the low-dimensional model-based representation. Since the age of each subject in each image is known, they define a polynomial function (the so called aging function) that relates the model-based representation of each subject to the actual age. Once an aging function is computed, it can be used for estimating the age of faces in images and also for generating typical faces showing a face at a desired age. Figure 3.10 shows synthetic faces at different ages produced by aging functions computed using images from the FG-NET Aging Database.

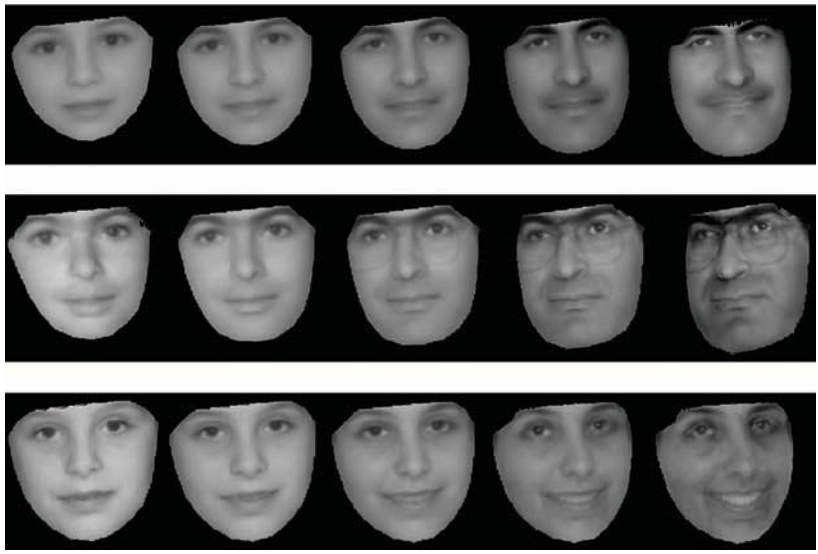

Figure 3.10 Images showing synthetic faces at ages between 0 and 50 years produced using aging functions. Images in the first row were generated based on an aging function trained using all images from the FG-NET Aging Database. Images in the last two rows were generated based on aging functions trained using images of two subjects.

Figure 3.11 Age progression using aging functions.

Given a previously unseen face to be age-progressed, Lanitis *et al*. [12] code the face into model parameters and based on the aging function they modify the model parameters in order to inflict the required change in age. The modified set of model parameters is used for generating the age-progressed face. The age progression process based on aging functions is

illustrated in Figure 3.11. Although the concept of coding faces into PCA model parameters has been exploited in a variety of face image processing applications, this is the first time that this approach has been used for modeling aging variation.

As an extension to this work, Lanitis *et al*. [12] train aging functions for different subjects in the database (see row 2 and 3 in Figure 3.10) in order to support the diversity of aging variation between different subjects. Given a new subject they select the most appropriate aging function to be used for performing age progression. The selection of the best aging function is based on the assumption that people who look similar tend to adopt similar aging patterns. In this context the aging function of the database subject who looks most similar to the given face is selected and used during the age progression process.

In an attempt to add lifestyle criteria in the aging function selection Lanitis *et al*. [12] establish a "lifestyle" profile for each subject in the database and in addition to face similarity criteria, lifestyle similarity criteria are utilized for the selection of the best aging function to be used in the age progression process. The lifestyle profile includes information about various factors that could influence the aging process such as gender, health, standard of living, economic situation, stress level, work conditions, marital status, place of living and exposure to extreme weather conditions. Profile information was gathered by asking all volunteers to fill appropriate questionnaires.

Along similar lines to the ones proposed by Lanitis *et al*., a number of researchers described 2D [15,16] and 3D [17] model-based age progression techniques.

In model-based approaches all the computations for establishing the age-related deformations are done in the model-parameter space enabling in that way the development of more robust methods when dealing with low quality face images. Also, model-based approaches can be used for other related applications such as automatic age estimation [18] and the development of age invariant face recognition systems [12]. However, because in model-based age progression methods face images are represented in a low dimensional space, fine details (such as wrinkles, scars) are eliminated. As a result model-based age progression systems tend to produce synthetically looking age progressed faces.

3.3.5 Adding Wrinkles

Systems that aim to simulate age effects by the introduction of wrinkles have been reported in the literature. Boissieux *et al*. [19] describe two approaches for adding wrinkles on 3D faces. In the first approach they use images of 80-year-old subjects in order to generate typical wrinkle-masks. A wrinkle-mask is an image showing the position and intensity of wrinkles in the facial area. In total, eight wrinkle masks are generated in order to cover for different sub categories of faces—faces of males, females, categories based on the shape of the face (round or long), and categories based on the expression of the face in the given image. In an attempt to establish a numerical model that relates wrinkle strength and age, Boissieux *et al*. [19] use quantitative data provided by L'Oreal. The data used relates the age with wrinkle depth and changes in skin biomechanical parameters such as skin viscosity and Young modulus. Based on the data given, they derive a mathematical formula that relates age and wrinkle strength so that it is possible to define the intensity of wrinkles to be added on a given face according to the desired age-progression target age. Given a new 3D face image, the category of the face is first established in order to select the optimum wrinkle-mask to be used for adding wrinkles. Then the wrinkle strength is defined based on the mathematical model that relates wrinkle strength and age. Wrinkles of the selected mask and strength are then superimposed on the given face in order to produce an age progressed face.

Boissieux *et al*. [19] also propose a model-based approach for wrinkle-generation. In this context skin is modeled as a two-layered elastic structure where the top (surface) layer is thin and stiff whereas the bottom layer is thick and soft. Each layer is represented as a triangular mesh, so that skin deformation can be enforced by deforming the triangular mesh. Different types of deformations and modifications of the layer thickness can be used for simulating different types of wrinkles on the facial surface. Also based on this method it is possible to simulate skin deformations influenced by external factors such as treatment using cosmetics and exposure to sun.

Gandhi [20] describes an approach for producing age progressed faces by adding wrinkles. The method proposed by Gandhi is based on age prototypes [6] for different age groups. During the generation of the age prototypes, a wrinkle transfer method is used [21] so that wrinkles from the training images are transferred to the prototypes rather than being

suppressed by the averaging process. Age progression is achieved by transferring wrinkles from the age prototype corresponding to the target age, to the input image. The key element in this approach is that an age estimation system is used in order to ensure that the wrinkle strength transferred to the input image is consistent with the target age. In this context wrinkles of different intensity are superimposed on the input face image and for each case the age of the resulting face is estimated—the optimum wrinkle intensity is chosen as the one for which the estimated age of the resulting image is consistent with the target age.

3.4 Discussion

In previous sections we have presented the basic categories of computer-based age progression systems and typical age progression systems reported in the literature. The key question to be addressed is whether the state of the art today is at adequate standard for using this type of technology in everyday applications and most importantly whether the use of computer-based age progression systems produces accurate results.

3.4.1 State of the Art

Currently there are commercial systems capable of receiving either a 2D or a 3D face and automatically produce an age progressed version of the input face. For example, in [22] a commercial system that can be used for applying aging effects on the appearance of 3D faces is available. Most age progression systems are capable of producing faces that look older than faces in the raw images. However, the most important aspect of this technology is not the generation of aesthetically correct results, but the generation of reasonably accurate estimates of the future facial appearance of subjects. On this aspect, the current technology of computer-based age progression systems is not at adequate standard for use in real life applications.

3.4.2 Future Directions

In order to develop fully functional and accurate age progression systems there are several directions that need to be further explored. Such directions are outlined in the remainder of this section.

3.4.2.1 Link with External Factors

It has been established that external factors such a lifestyle and health conditions affect significantly the process of aging. For example, it is well known that smokers tend to age faster whereas people who exercise regularly tend to look younger. In order to accurately predict the future facial appearance of a subject there is a need to take into account all possible external factors and use them to tune accordingly age-progressed images. An initial attempt to involve lifestyle parameters in automated age progression has been proposed by Lanitis *et al*. [12]—further work is required to establish the actual effect of different lifestyle parameters towards the aging process.

3.4.2.2 Age Progression Diversity

The aging process resembles similarities among different individuals. However, a number of researchers confirmed the existence of diverse aging effects between different genders [17] and different subjects [12]. Similarly subjects from different ethnic origins tend to adopt different aging patterns. A successful age progression system should be capable of dealing with the diversity of the aging process, in order to apply the most appropriate aging effects to a face of a particular subject. The main issue that prevents researchers to deal effectively with this problem is the requirement of a large number of high quality training samples showing subjects at different ages in their lives in order to be able to determine the aging pattern for individual subjects. Due to practical problems it is tedious to compile such an ideal database.

3.4.2.3 Hairstyles/Facial Occlusion

Most age progression systems do not deal effectively with hairstyles. Especially in the case of female subjects, hairstyles undergo enormous shape and texture variations making it difficult to model age-related hairstyle variations.

Most of the systems developed do not cope with occluding structures (i.e., spectacles) or facial hair (i.e., moustaches and beards). The development of age progression systems that can deal effectively with facial hair and occlusions is a requirement for further development in the area.

3.4.2.4 Performance Evaluation

The most important feature of a successful age progression system is the ability to produce accurate age progressed faces. In most cases researchers working in the area did not test their systems against this highly important aspect of age progression technology. Usually visual results showing age progressed images and true images at the target age are shown, leaving the reader of the paper to judge the accuracy of the system. Lanitis *et al*. [12], attempted to assess quantitatively the performance of an age progression system by estimating the differences between a set of age-progressed images against the real facial appearance of the same subject at the target age. Proper performance evaluation methodologies, preferably on a standard aging database should be formulated in order to support comparative evaluation of the performance of different age progression systems.

3.5 Conclusion

Automatic age progression systems could figure in numerous real-life applications. Although in many cases researchers produced promising results, more work is required for producing improved and most importantly accurate age progression systems. Based on the progress recorded in this area and the recent increase of interest for research in this topic, it is expected that in the near future automated, fully functional and reasonably accurate systems will be available.

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PART 2
BIOCHEMICAL, PHARMACOLOGICAL, AND ETHNICITY ASPECTS

4

Structural and Biochemical Changes in Aging Skin and Their Impact on Skin Permeability Barrier

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4.1 Introduction

Skin transformations are the most perceptible signs of aging. The multifaceted functions of the skin include acting as an entry barrier to compounds, regulating body temperature and fluid and electrolyte balance, and providing receptors for sensations such as touch, pain and pressure. Cutaneous aging is a mix of intrinsic aging (due to inherent genetics) and extrinsic aging (due to environmental conditions such as solar exposure). During this process of aging, the skin becomes thinner, wrinkled and saggy with graying of hair. This also has ramifications on the permeability characteristics and various functions of the skin. A brief synopsis of systematic and detailed studies on this topic is presented in the current chapter. An attempt is made to delineate the process of aging at the morphological and biochemical level and correlate it to changes in permeability.

4.2 Physiology of Human Skin

The development of skin as an organ commences during the fetal stage; however, its final development occurs only postnatally. In a human adult, the skin structure can be categorized into three main layers—epidermis, dermis, and hypodermis (Figure 4.1).

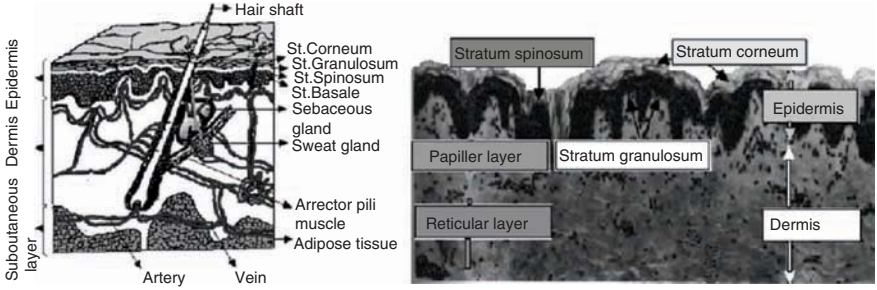

Figure 4.1 Structure and layers of skin. Reprinted with permission from ref.[108]

4.2.1 Epidermis

Epidermis is comprised of stratum corneum (horny layer) which is the outermost layer exposed to the environment, stratum granulosum (granular layer), stratum spinosum (prickle cell layer) and stratum basale (basal layer). The stratum corneum is a nonviable epidermal layer 10–15 cell layers thick, with anucleated keratinocytes (corneocytes) oriented like bricks in the surrounding lipid (that serve as a mortar) and forms the prime barrier to the transdermal delivery of actives. In spite of being a viable epidermal layer, the next layer, the stratum granulosum (1–3 cell layers thick) contains enzymes that have the potential to degrade vital cell organelles such as nuclei. By synthesizing keratin and degrading cell organelles, the keratinocytes in this layer gradually differentiate into the corneocytes of stratum corneum. The keratinocytes also synthesize membrane coating granules that carry the precursors for intercellular lipid lamellae of the stratum corneum. The stratum spinosum, the next viable epidermal layer consists of 2–6 layers of columnar keratinocytes that modify themselves into polygonal shapes. The keratin in this layer aggregates to form filaments called tonofilaments that on further condensation produce cell membrane connecting structures called desmosomes. The stratum spinosum together with the lower stratum basale layer is known as the Malphigian layer. The stratum basale is the layer with all the typical cell organelles and is the only layer that is capable of cell division. The keratinocytes in this layer are connected with the basement membrane (or dermo-epidermal membrane) by proteinaceous structures called hemidesmosomes and with cells of stratum spinosum layer by desmosomes. In addition to the keratinocytes, other specialized cells present in the basal layer are melanocytes, Langerhans cells and Merkel cells. Melanocytes secrete

melanosomes containing melanin (eumelanin or phaeomelanin) that protects the skin from ultraviolet radiations and free radicals. Langerhans cells are derived from bone-marrow and as part of the immune system function as antigen presenting cells (APC) of the skin. Merkel cells together with nerve endings are present in the dermis and are responsible for cutaneous sensation.

4.2.2 Dermis

The dermis (or corium) is 3–5 mm thick and is composed of numerous connective tissues especially collagen fibrils and elastic tissues that respectively provide support and flexibility to the dermis. It is supplied with a reticulate network of blood vessels, lymphatic vessels, nerve endings and numerous appendages. There are three main appendages namely, hair follicles, sebaceous glands and eccrine glands present in the skin that originate from the dermis. Hair follicles cover the entire body surface except soles of feet, palm of hands and lips. Sebum secreted by sebaceous glands associated with the follicles is composed of free fatty acids, triglycerides and waxes and plays a vital role in lubricating the skin surface and maintaining a surface pH of approximately 5. The eccrine glands (or sweat glands) originating in the dermis secrete sweat (a dilute salt solution of pH 5) in response to physical and emotional stress. Specialized glands known as apocrine glands are also present and are located in the dermo-epidermal layer in selective regions. All appendages act as "shunt routes" for permeants that can enter the lower layers of the skin without traversing the intact barrier of stratum corneum.

4.2.3 Hypodermis

The hypodermis (or subcutaneous fat layer) connects the dermis with the underlying organs. It provides insulation to the body and protects it from mechanical shock.

4.3 Structural Changes with Age

4.3.1 Epidermis

Hair graying, skin wrinkling, sagging and apparent thinning are some of the changes in the clinical appearances of the aging skin. However, experimental investigations have shown no correlation between the epidermal

thickness and age.[1] No differences in thickness are noted due to gender either. The decreased elasticity of the skin with age, however, promotes the apparition of a thinner skin due to reduced contractile ability of the epidermis and thus less crowding of epidermal cells. Ghadially *et al*.[2] assessed the integrity of the stratum corneum by tape stripping in aged vs. young subjects. A significantly lower number of strippings (18 ± 2 strippings) were required to disrupt the barrier for aged individuals than for young subjects (31 ± 2 strippings). The altered integrity also slows the barrier recovery in the elderly. The fine and regular epidermal surface patterns change to coarser and less regular ridges with aging leading to skin surface irregularity.[3] The paper-thin nature of aging skin sometimes approaching transparency has also been attributed to the loss of dermal thickness.[4]

The marked difference between young and old skin originates at the dermo-epidermal junction between the epidermis and the dermis. An undulating series of finger-like projections are observed between the dermis and the epidermis which serve to increase the area of contact between the two layers, and help to prevent the epidermis from being sloughed off.[5] The downward projections are termed rete pegs while the upward projections are called dermal papillae. These projections flatten with age leading to decrease in the contoured demeanor of the epidermis and making it prone to easy shearing. Thus, the epidermal area appreciably diminishes with fewer basal and keratinized cells per unit area. This effect eventually manifests itself as the characteristic dry skin in the aged.

At cellular levels, intrinsically aging skin also shows marked disparities in the differentiation and morphology of the basal layer cells. The development of cuboidal basal cells to spheroidal Malphigian cells and finally to flattened granular cells is disrupted and polarity is lost. A series of studies was conducted to investigate the effect of aging on epidermal Langerhans cells and on their response to a single ultraviolet exposure in skin biopsy specimens of healthy adults, aged 22–26/62–86 years. Study results indicated a reduced Langerhans cell density in the old.[6,7] Langerhans cells are responsible for recognition of foreign antigens and subsequent immune response. Further more, change was also observed in mast cells which play an important role in mediating allergic response. Using sunburn reaction as a marker for evaluating immune response, the authors observed fewer sunburn cells and less striking alterations of perivenular mast cells and endothelial cells in the old initially, but more pronounced effects at

72 hours relative to young skin. Older skin was observed to have a reduced immunological response. Number of enzymatically active melanocytes have also been documented to decrease probably due to reduction in actual number of cells or loss in functionality of the melanocytes.[8] Melanocytes are classified into two morphologically and functionally different types: DOPA-positive melanocytes characterized by active pigment formation, and inactive or DOPA-negative melanocytes. In the above studies, melanocyte density declined approximately 6–8 percent of the surviving population per decade. This decrease in melanocyte density was coupled with a simultaneous generalized increase in pigmentation with advancing age. This pigmentation is however uneven. This contradiction has been explained by the increased dopa-positivity of individual melanocytes in spite of reduced density in the chronically sun-exposed skin causing irreversible effects on pigmentation.

Another factor which has been studied to explain certain aging phenotypes includes telomere length. Telomeres are small stretches of DNA at each end of every chromosome. These protect the chromosome against degradation, and being termed as "damaged" DNA; thus, they guarantee chromosomal integrity. The telomeres however shorten with each replication until the chromosome is unable to replicate further. Telomere length reduces with age in both the epidermis and the dermis and the average shortening rates in the epidermis and in the dermis were 9 and 11 bp/year, respectively.[9] Such telomeric loss serves as the biological clock of aging. It is unknown if shortened telomeres cause aging or are a side effect, but shortened telomeres and age go hand-in-hand. A comprehensive review discussing the controversies relating to epidermal and dermal thickness and changes in blood flow, pH with age along with a complete listing of literature to date in this area is presented in a review by Waller and Maibach.[10]

Another factor which plays an important role in wound healing rates is the epidermal cell turnover which has been shown to decrease with age. The disappearance of the fluorescent dye, tetrachlorosalicylanilide from the stratum corneum has been used as an indicator of replacement rate of the cells.[11,12] The replacement time was seen to be 20–26 days in the 70 plus age group and 13–13.5 days in the below 40 age group. Another indirect measure of epidermal renewal—the number of corneocytes that can be scrubbed off the skin in a four day period—was observed to be two fold higher in younger vs. aged skin.[13]

4.3.2 Glands

An unappreciated advantage of old age is reduced sweating and body odor. Fewer number of sweat and apocrine glands coupled with lesser amount of sweat secretion leads to an overall diminished discharge.[14] Histologically fewer glands in sections of unexposed skin and disarray and shrinkage of the secretory coil sometimes with complete involution have been observed.[15] A more recent study conducted in Japan[16] comparing sweat production patterns in different seasons of the year among young and old males has shown that older skin has a decreased ability to maintain body temperature with passive heating of the extremities. However, regional differences exist in this age-related decrease of sweating. For instance, regional sweating rates were significantly lower on the thigh for older men but not much difference existed between the age groups when it came to sweating rates on the back.

Testosterone production controls the oil-producing sebaceous glands throughout life. A steady decline in sebum secretions of 23 percent per decade in men and 32 percent in women has been observed.[17] However, normally sebum production is also proportional to gland size. Studies have proven that the sebaceous gland almost doubles in size in older skin and hence ideally more sebum should be secreted in old than young skin. This paradox of decline in sebum production despite diffuse sebaceous gland hyperplasia has been explained by Plewig and Kligman[18] as one caused by sluggish movement of the cells, increased transit time and decreased turnover of sebum producing cells in the elderly, leading to decreased proliferative activity and reduced secretions. A noteworthy fact is that these glands are responsive to extrinsic hormonal stimuli. Sebum output was seen to have almost doubled with administration of fluoxymesterone, an anabolic steroid chemically and pharmacologically related to testosterone, to post menopausal women.[19] In another study on menopausal women,[20] skin surface lipids were seen to increase during combined HRT (estrogen and progesterone as opposed to estrogen administration alone), which may reflect stimulatory effects of the progesterone component on sebaceous gland activity, while estrogen alone has a sebum-suppressive action. After menopause, sebaceous gland activity gradually decreases in the female, while it remains unaltered in men until the seventh or eighth decade before a decrease is seen.[21] Thus, low production of sebum causing skin dryness and increased local over-production of melanin collectively makes the skin more prone to insult with age.

4.3.3 Appendages

Graying and thinning of the hair is the most proverbial sign of aging. Hair density and number of functional melanocytes undergo a progressive reduction and so does hair diameter. The graying has been noted to be an outcome of two factors: (a) changes in the production cycle of melanin pigments in melanocytes thus resulting in pigment dilution in each individual hair follicle and (b) percepted graying due to mixing of pigmented and white hair. In contrast to continuous epidermal melanocyte generation, melanogenesis in the hair is coupled with hair growth cycle. There are periods of melanocyte proliferation (during early anagen), maturation (mid to late anagen) and melanocyte death via apoptosis (during early catagen). This intrinsic rhythm of both melanocyte and hair growth can be influenced by gonadal, adrenal, and thyroid hormones.[22,23]

Tufts of hair have been observed on the faces of middle aged women injected with repeated doses of testosterone. Likewise, enhanced beard growth has been observed for elderly men treated in a similar fashion.[15] Hair cycles in older years fail to reconstruct new pigment units due to the decreased pigmentary potential of individual hair follicles as well as the fact that the remaining melanocytes in the hair follicle are either vaculated and have lost their melanin carriers, the melanosomes or melanocytes have reduced in number.[23] Tyrosinase is the rate limiting enzyme in the production process of melanin. Altered pigmentary potential of hair follicles (Figure 4.2) is due to a mix of reduction in tyrosinase activity of hair bulbar melanocytes, sub-optimal melanocyte-cortical keratinocyte interactions, and defective migration of melanocytes.[24]

4.3.4 Nails

In contrast to the aging process of the skin, the gerontobiological course of appendages has not been investigated to the same degree. Nails experience a marked reduction in growth rate as observed by Orentreich and Sharp,[25] in their study where thumb nail growth was seen to decrease on an average of 38 percent between third and ninth decade. Structurally, nail thickness may increase or decrease, they may appear pale, dull, and opaque, with the color ranging from white or yellow. At the cellular level, the nail plate keratinocytes increase in size and the nail bed experiences thickening of the blood vessels and degeneration of the elastic tissue. Calcium and strontium content of the nail is increased[15] and brittleness and undulations of the nail increase due to reduced hydration.[26] Nails in senile individuals

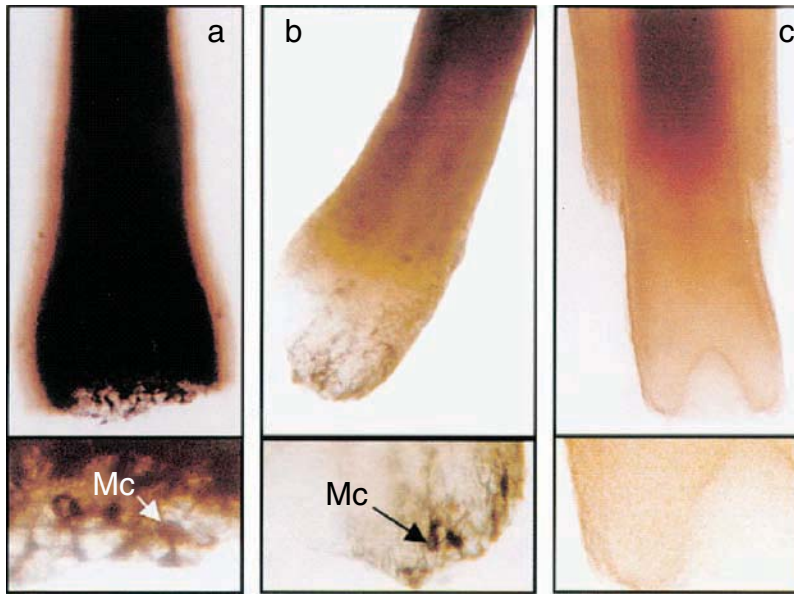

Figure 4.2 Pigmented, gray, and white anagen follicles plucked from the same scalp of a canities-affected individual after incubation with L-dopa. There is a reduction in the number and tyrosinase activity of hair bulb melanocytes (Mc) from (a) to (c). Reprinted from ref.[24] with permission from Elsevier.

can be affected with infections *such as* onychomycosis (fungal infection), onychauxis (localized hypertrophy of the nail plate manifested as hyperkeratosis, discoloration, and loss of translucency), onychogryphosis (enlargement and thickening of the great toe nail plate), onychocryptosis (ingrown nails) being the prevalent ones. However, no distinct correlation between infections and aging have been observed.[15]

4.3.5 Nerves

Cutaneous nerve network develops in two phases: first, a growth period through infancy which probably is completed in early adult life and a second phase with initial gradual decrease and then a more rapid decrease in the density of nerve structures and increase in their complexity during old age.[27] The number of Meissner corpuscles is seen to decrease with age from 80/mm^2 of tissue in infants to 5/mm^2 in the aged.[23] Thus a reduced sensory experience in the aged stemming from the above reasons is liable for the characteristic dulling of the senses.

4.4 Biochemical Changes with Age

4.4.1 Dermis

While most morphological changes occur in the epidermis, biochemical changes occur in the dermis. Atrophy of the dermis is manifested by changes in the dermal connective tissue. In order to understand the changes in the dermis with aging, it is first essential to understand its biochemical composition.

The dermis is a thick layer of elastic and fibrous tissue that gives the skin its strength, flexibility, and firmness and provides support to the epidermis as well as the nerve and vascular networks and appendages. It consists primarily of an extracellular matrix of connective tissue, which is composed of predominantly collagen and elastin. The collagen, which comprises 70–80% of the dry weight of the dermis, has high tensile strength and thus, prevents overstretching and tearing, while the elastin is an elastic protein that maintains skin tension, flexibility and resilience. The third and minor component of the dermal extracellular matrix are the glycosaminoglycan/proteoglycan macromolecules that account for 0.1–0.3% of the dry weight. The glycosaminoglycans in skin consist of hyaluronic acid (HA), dermatan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate. These compounds named natural moisturizing factors (NMFs) play a role in providing hydration to the skin, mainly through the water binding capacity of hyaluronic acid. The dermis is divided into two main layers, the papillary dermis which directly underlies the epidermis and the reticular layer that underlies the papillary dermis and is superficial to the hypodermis.[28] The papillary dermis is composed of thin bundles of collagen fibers that are interwoven with elastic fibers, such as elaunin and oxytalan fibers, forming an intermingled network. In the lower levels of the papillary dermis, the fibers run in a parallel orientation to the epidermal surface, while others ascend into the mid-papillary dermis and form an arcade below the dermo-epidermal junction (called elaunin fibers). Fine terminal elastic fibers (called oxytalan fibers) arise from this band and run perpendicular to the basal lamina of the dermo-epidermal junction. Some other fibers that arise from the papillary dermis split to form a brush like pattern of fine twigs that pass from the lower levels of the papillary dermis to the basal lamina of the dermo-epidermal junction, without forming a band or an arcade.[29] Elastic fibers are made of two components, a scaffold composed of fibrillar structures of 10–12 nm diameter called microfibrils that are embedded in a cement or a matrix composed of elastin, where the

elastin forms about 90 percent of the fibers.[30] While the oxytalan fibers are abundant in microfibrils, with some amorphous elastin, the elaunin fibers have a greater proportion of elastin associated with microfibrils. The reticular dermis is composed of thick undulating bundles of collagen fibrils that are almost parallel to the plane of the epidermis (Figure 4.3). The collagen bundles are connected to each other at various points due to interlacing of their fibers and are supported by a network of large, fully mature elastic fibers. The elastic fibers and the collagen bundles progressively increase in size toward the hypodermis.

Six different types of collagen have been detected in skin (type I through type VI) and additional collagens have been shown to be synthesized

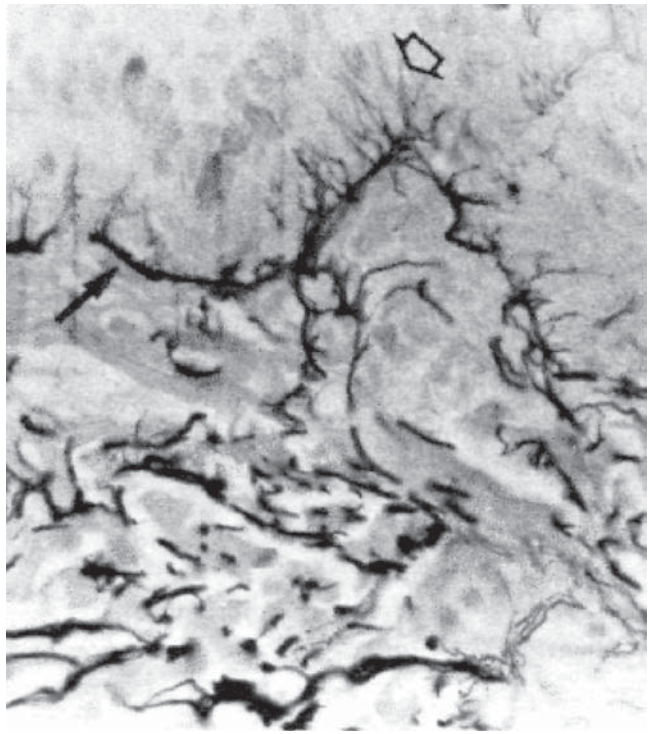

Figure 4.3 The elastic system of the dermis, seen after staining by Weigert's reaorcin fuchsin method, after oxidation with peracetic acid. Near the dermo-epidermal junction the oxytalan fibers (open arrow) radiate to the epidermis, starting from the elaunin plexus (solid arrow). In the reticular dermis thick elastic fibers are seen (×500). Adapted from ref.[29]

by fibroblasts *in vitro*.[31,32] In young adults, type I collagen represents 80 percent of the total dermal collagen, whereas type III collagen represents about 15 percent. Collagen is present as thick, regular, well-oriented fibers that are slightly wavy and assembled in parallel, where the fibers are composed of microfibrils that are built of individual collagen molecules. However, in the papillary dermis, the bundles are thinner and more flattened.[33] Collagen is synthesized as procollagen, which after secretion into the extracellular space, is converted to collagen molecules.

The major cell type in the dermis is the fibroblasts. These cells synthesize all components of the matrix- the collagen, elastin and ground substance, and are responsible for the remodeling of the collagen fibrils. The dermis also contains macrophages and mast cells. Fibroblasts or macrophages produce interstitial collagenase, an enzyme of a family called matrix metalloproteinases (MMPs), which can degrade collagen molecules. A second group of MMPs are called gelatinases and they degrade gelatin or denatured collagen.[34]

4.4.2 Connective Tissue

With increasing age, the dermis becomes thinner due to the reduction in the amount and organization of dermal connective tissue. Reduction in levels of collagen and elastin deprive the skin of its strength and resiliency and lead to loss of a youthful appearance and an increased tendency to bruise. Though a large part of the changes are caused by actinic damage, innate aging by itself contributes to the appearance of fine wrinkles, atrophy of the dermis and reduction of subcutaneous adipose tissue.[35] Collagen degradation is purported to be one of the major contributing factors to aging of the dermis, and leads to some of the major changes that cause the appearance of aged skin. In contrast to the dense and compact bundles in young skin, collagen bundles in aged skin are granulated, dispersed with separated bundles and fibers, or may appear densely packed in some areas. Though the stability of collagen increases with aging, due to the larger portion of non-reducible cross-links between molecules, the total collagen has been found to decrease with aging, due to increase in collagenase activity and due to the decrease in new collagen synthesis, as demonstrated to be measured as the rate of synthesis of radioactive hydroxyproline.[36,37] In this method, human skin slices are incubated in a medium containing radioactive proline, where the protocollagen proline hydroxylase converts peptide bound proline to hydroxyproline, and the

formation of radioactive hydroxyproline is taken as a measure of collagen synthesis.

4.4.2.1 Collagen Network

In the past years, photoaging and intrinsic aging were classified as different phenomena. However, in recent years, some common molecular mechanisms between the two have been discovered. One of these is the increase in the amount of MMPs in intrinsically aged and photoaged skin, though the increase is much higher in photodamaged skin. Varani *et al.*[38] studied the intrinsic dermal aging in four groups of individuals: (18–29 years, 30–59 years, 60–79 years, and 80+ years). They found a decrease in the number of fibroblasts with increasing decades accompanied by abnormalities in the connective tissue. Increased disorganization of the collagen fibers with subsequent thinning of the bundles, increased interfiber space and an increased depth to which this disorganization extended was observed. Individuals in the last two groups (60–79 years and 80+ years) demonstrated a significantly higher loss of fibroblasts and connective tissue abnormalities than those in the 18–29 and 30–59 years groups. The fibroblast growth potential was found to decrease with increasing age, while levels of MMPs, including MMP-1 (interstitial collagenase), MMP-9 (gelatinase- molecular weight 92 kDa) and MMP-2 (gelatinase- molecular weight 72 kDa) were found to be elevated by 40, 52 and 82 percent respectively in the 80+-year-old group as compared to the 18–29-year-old group. It was also found that the expression of type I and type III procollagen was decreased in aged skin, suggesting that changes in the connective tissue may be accompanied by reduced synthesis of new collagen.

Inhibition of procollagen synthesis has also been attributed to the degraded components of the extracellular matrix. Since synthesis of procollagen by fibroblasts is regulated by the interactions between the fibroblasts and the collagen peptides, presence of degraded collagen disrupts these interactions and reduces the procollagen synthesis.[39,40] Although this phenomenon is predominant in photodamaged skin due to the ultraviolet induced upregulation in MMPs and resulting collagen degradation, partially degraded collagen is also a feature of intrinsically aged skin. *In vitro* studies show that procollagen synthesis is reduced greatly in presence of partially degraded collagen as compared to intact collagen.[41] Fligiel *et al.*[41] found increased collagen fragments in sun-protected hip skin samples obtained from individuals aged 80 years or older as compared to

those in the age group of 18–29 years. Also, the total collagen content as a function of total protein was approximately 30 percent lower in skin samples from old individuals than in corresponding samples from young individuals. Similar collagen degradation was found when three-dimensional lattices of type I collagen were exposed *in vitro* to MMP-1.

Changes in the collagen network are accompanied by the aging of the collagen fibers itself. Collagen intermolecular cross-links are essential for providing stability and tensile strength to the skin, and this cross linking increases with age leading to an increase in stiffness of the skin. Two mechanisms involved are an enzyme-controlled process of development and maturation, and a non-enzymatic glycosylation process following maturation of the tissue.[42] The enzyme-controlled process converts the immature and reducible divalent cross-links into mature and stable trivalent cross-links of histidinohydroxylysinonorleucine (HHL), pyridinoline (Pyr) and deoxypyridinoline (DPyr), where HHL is the major mature cross-link in the skin.[43] The non-enzymatic glycosylation process of proteins, called the Maillard reaction leads to production of advanced glycosylation end products (AGE) that induce molecular damage by forming cross-links in collagen. So far only one AGE product, pentosidine (Pen) in collagen has been isolated and characterized, and is found in low concentration in the skin.[42,44]

4.4.3 Elastic Tissue Network

Age related structural changes in elastic fibers are also very pronounced, but are complex and variable. The elastic network is modified, becomes disorganized and the modifications vary from the dermo-epithelial junction to the reticular and the papillary dermis. While the oxytalan fibers become depleted, the elaunin fibers fray together in the reticular dermis. Increasing amounts of the microfibrillar component become incorporated into the amorphous dense matrix that appears as electron dense areas. One major manifestation of innate aging is the appearance of porous elastic fibers in the connective tissue.[45] The disappearance of oxytalan fibers and the increasing dystrophy and breakdown of the elaunic and elastic fibers is accompanied by the formation of lacunae or cysts, giving them a porous appearance.

Different kinds of changes in the elastin network have been observed in the subepidermal and the underlying reticular dermis. The changes during intrinsic aging in elastic fibers in the papillary dermis are marked.

The disintegration of the fibers becomes markedly apparent, and by the age of seventy, majority of the fibers are affected. The vertical fine elastic fibers in the subepidermal regions are practically lost in old skin, and this loss of fine fibers is thought to contribute to the superficial laxity of old skin and the finely wrinkled surface. Montagna and Carlisle[46] studied the sun protected areas of the axillae, breasts, and genitalia of women by light microscopy and found pronounced changes with aging (50 years or older) in the elastic fiber network. The terminal elastic fiber arcade becomes progressively and irregularly thicker and eventually the entire elastic fiber structure in the papillary dermis shrinks and sags. The distal branches from the dermis do not reach the epidermis and some are broken off and seem to remain attached to the dermis. In the underlying dermis, on the other hand, the fibers were found to become disorganized, thicker, more branched and increased in number.[47] Braverman and Fonferko[45] found a varying range of abnormalities in the sun protected skin of individuals belonging to a range of age groups. Abnormalities in the elastic network of individuals in the 30–70-year-old group were encountered more frequently in the papillary than in the reticular dermis, and presented in the form of microfibrillar dense zones with unilocular or multilocular cystic spaces. In persons between 50 and 70 years old, the age-related changes were more severe, as the cystic spaces had become larger forming lacunae that resulted in the separation of elastic skeleton fibers from one another, giving rise to a porous structure (Figure 4.4).

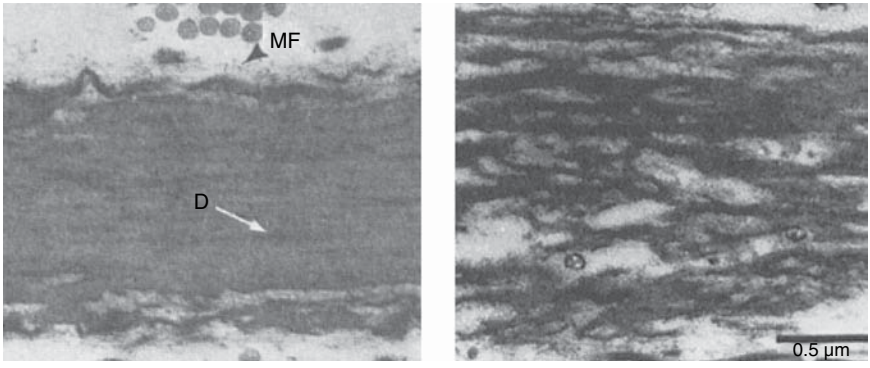

Figure 4.4 Age-related elastic fiber abnormalities: (a) mature elastic fibers with microfibrillar dense zones (D) in persons 30–70 years old; (b) elastic skeleton fiber separation with formation of lacunae in persons 50–70 years old. MF: Microfibrils. Reprinted from ref.[45]

To study the effects of intrinsic aging on the collagen and the elastin network, El-Domyati *et al*.[48] used biopsies of protected skin from healthy volunteers and examined their ultrastructure by transmission electron microscopy. They also used immunoperoxidase techniques with antibodies against type I and type III collagens and elastin to quantitatively evaluate changes in collagen and elastic fibers. The authors found that the transverse layer of elastic fibers in the dermis gradually thinned out with age and the oxytalan fibers shortened. The fibrillar nature of individual elastic fibers, however, was initially preserved. From the sixth decade on, the amount of oxytalan fibers progressively decreased until the ninth decade when only scanty oxytalan fibers could be seen. Also, the relative amount of elastin in protected skin significantly decreased from 49.2 ± 0.6% in the first decade to 30.4 ± 0.8% in the ninth decade. The type I and type III collagen staining was found to be altered after the eighth decade.

It is thus evident that the common setting in innate aging of the skin is the deterioration of the elastic tissue network which leads to the skin becoming looser and excessive, accompanied by a loss of ability to snap back to its original state after being deformed. This destruction of the elastic fiber architecture starts at age 30 years and becomes pronounced after the age of 70 years.[45,49]

Some studies have also addressed changes in the elastin fiber network that arise due to the changes in elastin gene expression with age. Development of recombinant DNA techniques have allowed determination of elastin messenger RNA levels and thereby elastin gene expression in cells. Fazio *et al.*[50] studied the elastin messenger RNA levels in skin fibroblasts obtained from persons of varying ages. Results obtained from Northern transfer analysis showed fairly constant levels of elastin messenger RNA in fibroblast cultures obtained from fetal skin (twelfth gestational week) and from skin of a 45-year-old person. Similarly, slot blot hybridizations revealed constant levels of elastin mRNA levels obtained from cells of persons varying from 3 days to 33 years. However in fibroblasts obtained from a person 61 years of age, the levels of elastin mRNA were only 12 percent of the mean of three other postnatal fibroblast strains. This implies that significantly different levels of elastin mRNA obtained from skin fibroblast cultures of fetal/adolescent/adult donors and 61-year-old person indicated that the consistent elastin mRNA levels may be lower in persons above 60 years of age.

4.4.4 Ground Substance (Glycosaminoglycans/Proteoglycans)

The ground substance of human dermis is composed of glycosaminoglycans (GAGs), glycoproteins and water. GAGs are polysaccharides, and when linked to a protein core, are called proteoglycans. The most commonly found GAGs in the human skin are hyaluronic acid (HA) and dermatan sulfate. Chondroitin 4- and 6- sulfate are also found in smaller amounts. The glycosaminogylcans and proteoglycans can bind a volume of water in the dermis up to 10,000 times of the size of the molecule itself.[51] Because of these water attracting properties, they are hypothesized to create a swelling pressure in the extracellular matrix which allows the rapid diffusion of water and water soluble molecules.[52] Thus, it may be responsible for maintenance of skin hydration and turgor as well as the transport of nutritional material in the matrix. Decorin, another major dermatan sulfate proteoglycan, binds to type I collagen fibrils and plays a role in the regulation of the size of collagen bundles.[52,53]

Significant reductions in the content of HA and other GAGs have been observed with aging. Fleischmajer *et al*.[54] found a significant reduction in the content of GAGs from newborns to infants, with the levels being stable during middle age and then reducing again during old age. Selective HA staining in the dermis of 9-year-old subjects has shown the presence of HA uniformly scattered in the cellular matrix and at the periphery of the collagen fibrils as well as between collagen and elastic fibers. Similar staining in the dermis of 30-year-old subjects shows presence of the matrix but loss of the continuity between the collagen and the elastic system. In the dermis of subjects aged 60 years, the HA precipitates were not found to be present in the intercellular or pericellular regions, nor on the surface of collagen fibers or between the collagen and elastic fibers. This reduction of the space between the collagen bundles and loss of water retaining capacity, could result in a more compact appearance, and account for the dried and wrinkled appearance of aged skin.[55]

Longas *et al*.[56] found that in human skin, the concentration of HA and dermatan sulfate decreased respectively from 0.03% to 0.007% of weight of wet skin and 0.05% to 0.026% of weight of wet skin between 19 and 75 years of age. In addition to HA, studies have been conducted with other GAGs to determine their impact on aging of skin. A chondroitin sulfate proteoglycan present in the basal lamina has been shown to be important in maintaining epidermal-dermal contact. Loss of the chondroitin sulfate

proteoglycan was found to begin at age 60, which may be a factor in the age-related changes that occur at the dermo-epidermal junction.[57] The same study found a notable increase in keratan sulfate in the epidermis beginning at age 50, whereas in the papillary dermis, the amount of dermatan sulfate increased after age 50. Increase in the proteoglycan decorin, a form of dermatan sulfate, can lead to decorin-collagen interaction that leads to collagen fibril disruption and decreased tensile strength leading to weakening of the tissue.[57,58] Thus, an overall decrease of the GAGs with age may lead to a reduction in water content and lead to changes in skin thickness with progressing age. It is to be noted though that these changes in GAGs may be a result of decreased fibroblast population with age.

4.5 Comparison of Neonatal Skin and Adult Skin

Neonatal skin has also been widely investigated in order to understand age-related modifications in the skin. Differences between adult and neonate skin and between preterm (gestation age/32 weeks) and full-term infant skin are found to exist on several parameters. The premature infant's skin is more transparent, gelatinous, free of wrinkles, and covered with fine lanugo hair than that of a full-term infant. Sexual hormonal effects are also less prominent in the premature infants.[59] Full-term infants, on the other hand, have high levels of maternal and placental hormones leading to localized pigmentation in genital areas and breast tissue. Preterm infants have epidermis composed of compressed thinner cells with fewer desmosomes and a thinner stratum corneum with more uniformity in cell size than adults.[60] The partial development of the skin and especially stratum corneum deprives the preterm infant of the essential barrier functions of the skin during the prenatal period and premature birth. The exact thickness of stratum corneum of neonates is however, elusive due to differences in criteria involved in stratum corneum measurement.[61] Neonates lack a strong immune system and therefore show reduced ability to fight against infections which is also evident by their low reactivity to allergens as compared to adults. Also, low production of melanin in neonates than in adults makes the former more susceptible to sun burning. Neonates have less mature and fewer elastic fibers than adults. Their skin is edematous (swollen with fluids) due to presence of excess water and sodium which are lost within first few days of life. This edematous nature of neonatal skin compensates for the absence of elastic fibers. There are

several types of collagen (I and III being the most predominant) present in the dermis and their amount varies with age. For instance, in fetal life there is large concentration of Type III collagen but soon after birth there is high ratio of Type I to Type III collagen that later reaches adult level by childhood. Similarly, chondroitin sulfate level is high in childhood, reduces at puberty and again rises in adulthood.[62] Preterm neonates have poorly developed fat supply at birth and a fat pad develops in subcutis several weeks after birth. However, full-term neonates have fat similar to that of adults.[63]

Lesser number of dermal papillae and fewer and smaller hemidesmosomes cause less cohesion between the layers in neonates than in adults.[63] Additionally, an immature dermis with weak organization of fibers is responsible for more risk in neonates to injury and stronger skin reactions in infants and children. Large and active sebaceous glands in newborns fade rapidly during infancy and resume their full formation at the time of puberty due to influence of intrinsically produced maternal androgens, fetal steroids and other steroids.[64] There is a lower ability to sweat in infants due to the low concentration of eccrine sweat glands.

In preterm neonates, hair termed as lanugo is fine, soft, poorly pigmented and unmedullated in nature and is normally shed in 7–8 months of fetal life. Neonates show synchronous hair growth whereas in adult dyssynchronous hair growth occurs. The vascular system is partially organized in preterm and full-term neonates and requires three months to develop. Subpapillary arterial network in dermis of neonates is disordered and attains order during the seventeenth week of life.

Stratum corneum hydration or capacitance measures the moisture content of stratum corneum and has major impact on mechanical and functional properties of stratum corneum including percutaneous permeation.[65] Full-term infants have lower stratum corneum hydration than adults, and this attribute increases postnatally. Okah *et al.*[66] showed that stratum corneum hydration depends on gestational age. Preterm infants (gestational age of less than 30 weeks) show greater stratum corneum hydration as compared to infants with gestational age greater than 30 weeks. Other authors have also reported that newborn skin is more susceptible to irritation as compared to adults and children because of their high skin pH and low stratum corneum hydration.[66]

Transepidermal water loss (TEWL) which represents a measure of water passing out of the epidermis not related to sweating can be a measure of the effectiveness of the barrier properties of stratum corneum. Absence of eccrine glands, vascular dilatation and photobiologic effects are responsible for low TEWL in newborn infants[67] as compared to adults. It is also observed that within the first four hours of life, TEWL value of infant skin was higher than few hours later, suggesting drying out of the skin which is a means of adaptation to *ex utero* life.[68,69]

Acid mantle or the skin pH also differs profoundly from neonatal to adult skin. A comprehensive review on this has been presented by Chiou and Blume-Peytavi.[61] The authors state that while skin pH in both children and adults is in the range of 4.0–5.9, it is above this range in neonatal skin. Green *et al*. describe that the physiologic acid mantle of the healthy human skin, which acts as a defensive mechanism against bacteria, is achieved soon after birth in full-term infants.[70] The skin pH of an infant is neutral or alkaline for 24–48 hours of birth then it drops to pH of 6.0 in the first week and finally reaches 5.5 at the end of the month. Term infants attain adult skin pH in the shoulder, axilla and abdomen by the first week of life[71] and maintained for the first month.[70] Studies have also observed that adult skin pH in volar forearm and buttock in infants is not even achieved by twenty-four months.[65]

4.6 Permeability Changes in Aging Skin

Structural and biochemical changes may help explain causation of the age-related changes in permeability of skin to actives**.** Prior to drawing any conclusions on the basis of the sparse literature available on these permeability changes, it is important to differentiate between alterations in permeability contributed by resistance offered by the epidermal stratum corneum termed as "percutaneous penetration" and the rate of clearance by the microvasculature in the dermis termed as "percutaneous absorption." *In vitro* studies suffice for investigating penetration parameters while intact local vasculature is used for study of the absorption via *in vivo* human studies. A comprehensive list of compounds that have been studied in aging adults includes water,[23,72,73,74] tetrahydrofurfuryl nicotinate,[75] testosterone, estradiol, benzoic acid , acetylsalicylic acid and caffeine,[76] hydrocortisone,[77] tri-*n*-propyl phosphate,[78] tetrachlorosalicylanilide,[23] ethanol, benzyl alcohol, decanol, cetyl

alcohol, fatty acids,[79] capsaicin,[80] sodium lauryl sulfate,[81] fluorescein,[23] and xenon-133.[82]

4.6.1 Factors Affecting Permeation

4.6.1.1 Stratum Corneum (SC) Barrier

All the *in vitro* studies conducted to understand age related permeation would aid in explaining the contribution of the SC to percutaneous permeation. Since the SC is the rate limiting layer for skin permeation, there is no role for vascular effects in such studies. Despite evident flattening of the rete ridges and visual thinning of the skin in the elderly, intact sheets of isolated SC have shown 15 cell layers in the mid-back of young and old individuals. There seems to be no difference in the thickness of the stratum corneum in the aged and the young. Tritiated water has been often used to examine the effect of aging on polar compound permeation.[72] Necropsy specimens from 73 individuals including 45 males and 28 females and with age groups ranging from 16 to 96 years were obtained from abdominal skin. Tritiated water was used in the donor in perfusion chambers mounted with these specimens. It was observed that permeability for water decreased in the elderly specifically after 60 years of age, and the effect was more pronounced in males than females (Figure 4.5). Previous studies correlated such permeability alterations with changes in epidermal lipids as hydrophilic solutes seem to be excluded more effectively from the skin. However, Squier *et al.*[72] and Kawashima *et al.*[83] showed that stratum corneum in the aged demonstrated an insufficiency of ceramides similar to atopic dermatitis. Ceramides are the major lipid constituent of lamellar sheets present in the intercellular spaces of the stratum corneum. These lamellar sheets are thought to play a key role in the barrier property of the epidermis. Ceramides are an important determinant for both water retention function and permeability barrier in the stratum corneum and a decrease in these would imply improved water permeation. It was observed that the total ceramide content significantly declined with increasing age. Each of six ceramide fractions simultaneously decreased with aging where ceramide I was reduced the most. This would mean increased water permeation should be observed in the elderly which is contradictory to the above findings of reduced water permeation in this group. This contradiction was later explained by the finding that there is a reduction in sebaceous gland secretion with age. It was observed that sebaceous lipids applied to the skin increased water permeation. As sebaceous fatty acids

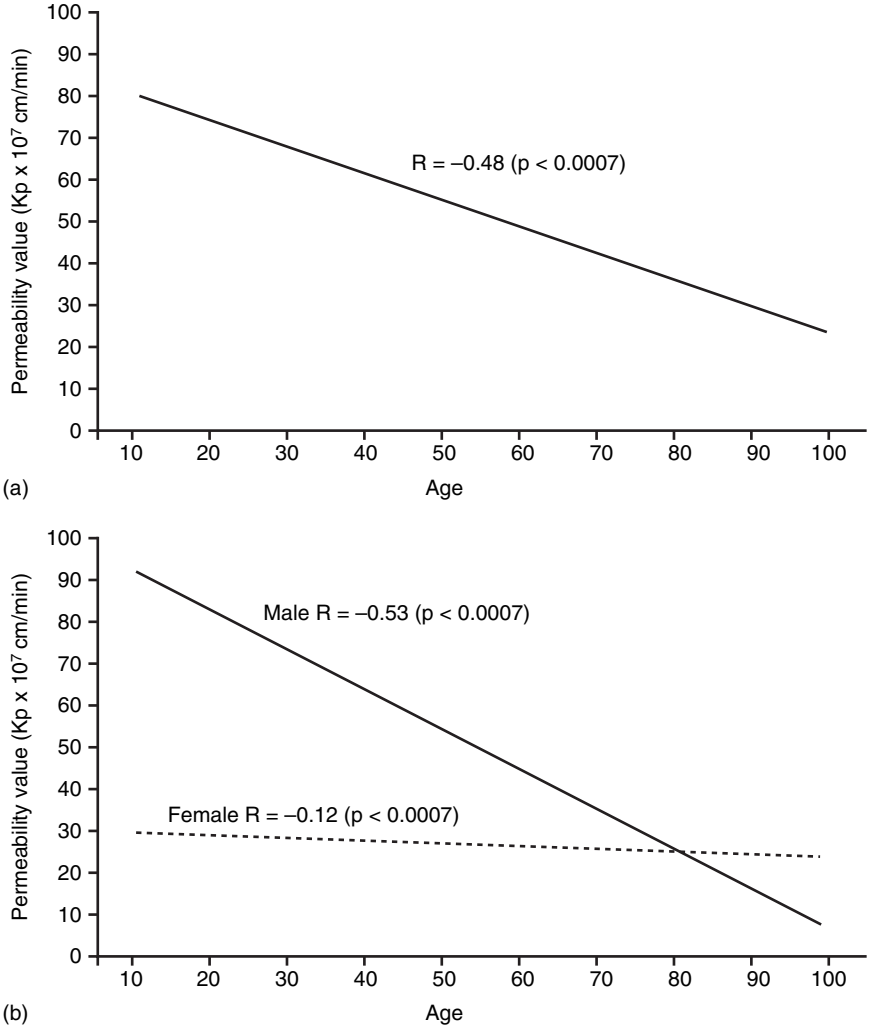

Figure 4.5 (a) Regression line for the permeability of skin with age; (b) regression lines for the permeability of skin in males and females with age. Reprinted from ref.[72]

reduce with age, it is possible that the above permeabilizing effect for water also diminishes.

4.6.1.2 Cutaneous Blood Flow

A difference in blood flow between ages is one of the factors responsible for altered absorption of compounds through the skin. *In vivo* studies using

different methods have shown no difference in cutaneous blood flow in children and adults. However, when it comes to the elderly compared to young men, regional variations exist in blood flow. For the lip, cushion of the third finger, nasal tip and forehead, blood flow decreased with age whereas in areas with low cutaneous blood perfusion, no clear variation occurred with age.[84] Peak vasodialator response to heat and vasoconstriction response to cold is blunted in the elderly.[85] In aged skin, it was found that the venular cross sections per 3 mm of surface were reduced by at least 30 percent. Effacement of the dermal papillae accompanied by disappearance of the capillary loops that supply them was observed.[80] Blood vessels around the appendages also became less prominent and may have been responsible for the decrease in glandular function, such as reduction in sweating and hair growth.

Histology and electron microscopy of the microvasculature have revealed deletion and derangement of small blood vessels and changes in vessel walls with regional variations. Biopsies from buttocks of 80- and 90-year-olds revealed abnormally thin vessels while actinically damaged forearm skin showed unusual wall thickening.[86]

Reduced clearance by the elderly and the difference between percutaneous permeation and absorption has been studied in detail by Christophers and Kligman.[87] *In vitro* water permeation studies conducted showed no difference in the capacity of SC to retard water loss in both young and old skin. However, *in vitro* permeability of sodium fluorescein and tetrachlorosalicylanilide was seen to be significantly higher in aged skin while *in vivo* permeation of testosterone was seen to be lower in older skin. To further understand the contribution of the horny layer and blood flow to the absorption process, clearance of radio-sodium and sodium flourescin was studied by the same group. Briefly Na^{22} or sodium fluorescein was injected intradermally into the midback of volunteers to circumvent the stratum corneum. The test site was covered with an atomic gamma ray probe and continuous plot of radioactivity provided when radiolabeled sodium was employed. For the fluorescein experiments, the disappearance of fluorescence from the skin under light was considered the end point. The clearance of both chemicals was markedly delayed in the elderly implying reduced clearance in the site of application. The results of all experiments by the group are tabulated in Table 4.1.

4.6.1.3 Enzyme Activity

An important influencing factor which has only recently gained attention in age related permeation studies is the role of skin enzymes in permeation

Table 4.1 Comparison of Permeation Parameters Obtained from *In Vitro and In Vivo* Studies on Young and Aged Skin[87]

Permeant	Study Type	Parameter Studied	Values for Young	Values for Aged	Conclusion
Water	*In vitro* (Flux)	Horny layer permeation	0.74 mg/hr cm^2	0.82 mg/hr cm^2	No difference in the age groups
Sodium fluorescein	*In vitro* (flux)	Horny layer permeation	0.93 μg/hr cm^2	6.78 μg/hr cm^2	Permeation increases with age
Sodium fluorescein	*In vivo* (disappearance from skin)	Cutaneous blood flow	96 min	133 min	Clearance reduces with age
Radio-sodium	*In vivo* (clearance)	Cutaneous blood flow	8.7 min	13.3 min	Clearance reduces with age
Testosterone	*In vivo* (flux and clearance)	Horny layer permeation and cutaneous blood flow	37.9 min	12.5 min	Clearance and/or permeation reduces with age

All studies were conducted in human subjects.

of actives. It is now imperative to differentiate between percutaneous permeation through stratum corneum, blood levels due to absorption through cutaneous blood vessels and altered permeation due to increase/decrease in activity and number of skin metabolic enzymes. An interesting study was conducted by Ngawhirunpat *et al.*[88] to understand the age-dependent characteristics of transdermal permeation of ethyl nicotinate (EN) and its metabolism to nicotinic acid (NA) in rats (ages from 21 day fetus to 360 days adult). Ethyl nicotinate (an ester prodrug of nicotinic acid) was used in different concentrations in side-by-side diffusion cells containing phosphate buffered saline using rat skin as the membrane and permeation parameters were recorded. Enzyme activity was studied by conducting hydrolysis studies of skin homogenates. Intact EN permeating across the stratum corneum was hydrolyzed to NA by viable skin esterases, and then both EN and NA diffused simultaneously into the receiver solution. Thus,

$$J_{\text{EN through SC}} = J_{\text{EN}} + J_{\text{NA through total skin}}$$

where J = flux (μmol/cm^2/hr) for respective actives.

The total flux from EN-saturated solution was the highest in rat skin at full-term gestation, a fetus at 21 days, and decreased gradually with increase in age. In contrast, NA flux was almost the same low level (0.7 μmol/cm^2/hr) in a fetus at 21 days and a rat at 3 days, drastically increased from 3 days (0.706 μmol/cm^2/hr) to 50 days (3.01 μmol/cm^2/hr), then became constant again (6.0 μmol/cm^2/hr) after 50 days. The total flux decreased gradually with aging; however, the degree of this decrease was lower than that of EN. Interestingly, the alteration in NA to total flux ratio, NA flux and enzyme kinetics showed the same tendency of triphasic (slow increase of parameters up to 3 days in the first phase, a marked increase was in the second phase from 3 to 50 days, and a constant increase was obtained in the third phase). The studies conclude that skin esterases of rats gradually develop after birth, with an intermittent spurt in development and then reach a constant at the adult period.

4.6.1.4 Electrophysiological Properties

A variety of physiological changes in the skin with age such as moisture content in the skin, lipid composition, lipid content, dermis thickness, and density of skin appendages may also affect the mechanisms of the age dependency in dermal and transdermal absorption of drugs. When skin

impedance data across ages using rat skin (ages from 21 day fetus to 360 days adult) as the model membrane was compared, it was observed that the resistance of the stratum corneum (R_{S1}) was markedly higher than the resistance generated by the viable epidermis and dermis (R_{S2}).[89] R_{S1} reflects the properties of pathways for hydrophilic permeants or pore pathways in skin permeation. To further differentiate between components affecting R_{S1}, thickness of stratum corneum and water content were measured. An increase in thickness of stratum corneum (L_{SC}) from fetal to adult stage was observed. However, the ratio of R_{S1}/L_{SC} increased with age implying L_{SC} is not the only component contributing to increase R_{S1} with age.

It is possible that the volume of pore pathways (which include pores through hair follicles and sweat glands) or porosity of the skin might be decreasing with increasing age. A decrease in water content which reduces the fluidity of SC lipids has been observed and might be the other factor affecting R_{S1}. R_{S2} also increased with aging. Increased thickness of the viable epidermis and dermis (L_{VS}) with aging but constant values of R_{S2}/L_{VS} at all ages indicate that L_{VS} is a major factor in the age dependency in R_{S2}. Capacitance (C_S) which represents insulating properties of the lipid-cellular matrix decreased with age. Thus decreased water content and increased SC thickness which defines the aqueous pathways is responsible for reduced *in vitro* permeation of hydrophilic permeants. The decreased C_S may be responsible for reduced *in vitro* permeation of lipophilic test compounds.

4.6.2 Permeability Studies in Animals

A significant amount of literature with updated research is available on age related permeability issues in animals. Intersubject variability and site variation coupled with difficulty in obtaining human skin in the desired age groups either *in vitro* or *in vivo* has led researchers to use animal models in this area. While pig skin is the most comparable to human skin in terms of permeability, mouse skin is frequently used in this aspect for the following reasons:[90] (a) permeability characteristics of the model to human skin are comparable, (b) easy and economical to obtain, (c) offers similar pathways of test compound absorption as human skin, and (d) short life span of the animal model.

Behl *et al.*[90] studied permeability of low molecular weight solutes covering a wide range of lipophilicity (water, methanol, ethanol, butanol,

hexanol, octanol, hydrocortisone, and phenol) through hairless mouse skin. Increase in skin permeation is found over the first 4 weeks followed by a steady decline for the next 5 weeks and not much change thereafter. Hair follicle cycle was seen to increase the permeation coefficient three to five fold. Polar solutes showed a lower permeation in thicker dorsal skin which compares well with permeation trends in human skin. Compaction of the stratum corneum in the epidermis at older age makes it more difficult for such compounds to permeate.

Age influenced changes in permeation of hydrophilic (deuterium oxide and diclofenac sodium) and lipophilic (ketoprofen and isosorbide dinitrate) compounds have also been studied in rats.[91] The results showed that the permeability coefficients through intact skin decreased with increasing age, and the extent of these decreases was higher for lipophilic permeants than for hydrophilic permeants. Rhesus monkeys have also been employed as study models for skin permeation variations with age.[92] The systemic absorption of testosterone was seen to be three fold higher in newborn as compared to adult monkeys.

Transdermal iontophoresis is a promising method of transdermal drug delivery of drugs. It involves application of low currents to propel drugs through the skin. An interesting study was conducted by Kanikkannan *et al*.[93] to investigate the effect of age of rats on the delivery of timolol maleate into the skin, with the help of electric current. Briefly, freshly excised skin from Wistar rats (1–8 months old) was mounted in diffusion cells filled with 1mg/ml phosphate buffer and formaldehyde solution (preservative). Anodal iontophoresis with current application of 0.375 mA/cm^2 was carried out for two hrs and sample collected at different intervals up to 12 hours. No significant differences either in the amount of test compound delivered during iontophoresis or the cumulative amount delivered at the end of 12 hours were seen across the ages. It was concluded that as the electric current is the dominant driving force for drug delivery in iontophoresis, changes in thickness and composition may not be affecting the iontophoretic transport of drugs.

4.6.3 Permeation Studies in Neonates and Infants

The measurement of percutaneous permeability of several permeants through infant skin has been performed using *in vitro*, *in vivo*, and animal model methods. Indirect methods such as transepidermal water loss have also been used to measure the extent of skin permeability with TEWL

values below 10 g/m^2/h indicative of low percutaneous permeability and levels above 20 g/m^2/h showing high permeability.[94] The skin permeability in case of full-term infants is similar to that of adults. Harpin and Rutter[95] reported that skin of mature neonate is an effective barrier against loss of water and absorption of drugs. The study involved measurement of skin water loss and percutaneous absorption of phenylephrine (as a function of blanching response) in 70 newborn infants of gestation age between 25 and 41 weeks and post natal age ranging from 1 hour to 26 days. Infants of 37 weeks gestation showed minimal drug absorption and very low water loss (<10 g/m^2/hr) whereas in case of immature infants of gestational age <30 weeks, maximum permeability of phenylephrine and highest transepidermal water loss (65 g/m^2/hr) was measured. The contrast in barrier properties of skin becomes extremely remarkable when skin of preterm neonates is compared to that of adults. It is corroborated by a study performed on percutaneous permeation of tritiated water through human cadaver skin (site-abdomen) obtained from three age groups: adult (50–76 years), full-term infants (37–40 weeks gestation; 1–3 days postnatal) and premature infants (26–30 weeks gestation:1–3 days postnatal).[96] It was observed that permeability coefficients in case of adults, full-term infants and premature infants were 4.9×10^{-5} cm/min, 6.6×10^{-5} cm/min and 4.6×10^{-4} cm/min, respectively. The data suggested existence of similarity in percutaneous permeability of adult and full-term infant skin but depicted a remarkable difference in permeability of the preterm infants. In another separate study *in vitro* permeation of a series of alcohols (ethanol, benzyl alcohol, and cetyl alcohol) and fatty acids (caprylic acid, oleic acid, stearic acid, and lauric acid) through preterm infant, full-term infant and adult human skin was compared.[79] The permeability of alcohols was observed to be highest through the preterm infant skin, intermediate in case of adult skin and least permeation was reported through full-term infant skin, thus supporting excellent barrier properties of the full-term infant skin. Similar release order was obtained in case of fatty acids except in case of caprylic acid where full-term infants showed more percutaneous permeability of caprylic acid than adults due to the higher presence of lipid in full-term infant skin than the adult skin. Despite the similarity in cutaneous permeability through full-term infant and adult skin, caution should be exercised in application of certain chemicals, such as hexachlorophene,[97] aniline dyes used to mark diapers,[98] certain steroids,[99] iodine,[100] isopropyl alcohol,[101] industrial methylated spirits,[102] to the infant skin. This is due to the fact that several studies have shown relatively

high permeability of actives through infant skin due to multiple reasons. *In vitro* and *in vivo* studies have been performed to evaluate the efficacy of permeants in infant skin for a particular therapeutic indication. A trend of high permeability through preterm infant skin has been observed and is explained by the underdeveloped barrier properties of stratum corneum. As the development of keratinized horny layer occurs in the middle of third trimester, preterm infants lack the presence of fully developed stratum corneum.[95] Higher surface area to body weight ratio in neonates than adults is another reason for higher permeability.[103] Also the reduced thickness of preterm neonate stratum corneum reduces diffusional distance required to cross the region and increase the percutaneous absorption of the permeant. The permeation of testosterone has been investigated in newborn rhesus monkey model, a model that shows percutaneous permeability similar to humans.[92] The study involved application of C-14 labeled testosterone in concentrations 4 or 40 μg/cm^2 on ventral forearm of newborns that resulted in testosterone systemic levels of 0.9 and 2.7 μg/cm^2 respectively, indicating a three fold increase in systemic absorption with 10 fold increase in drug. The study suggested that if equal application of testosterone is applied on equal area of newborn and adult skin, the systemic availability of the drug in newborn will increase 3–4 times more than adults as the surface area of neonate is four times that of adults.[104] Amato *et al.*[105] performed *in vivo* studies to evaluate the therapeutic efficacy of topical caffeine in low birth infants (weighing less than 1500 g) for the treatment of neonatal apnea. It was observed that after 10 topical applications of 10 mg of caffeine citrate, 97 percent low birth neonates attained serum levels within the therapeutic range. This implied that application of 10 mg doses of the active was sufficient to treat the indication in low birth infants which was not the case when same amount of caffeine citrate was administered topically to adults. In another *in vivo* study, Evans *et al.*[106] measured theophylline in blood samples of 20 preterm infants of gestation age ≤30 weeks after 17 mg topical application of anhydrous theophylline in gel formulation to 3.14 cm^2 area of the upper abdomen. A negative correlation was observed between amount of theophylline absorbed and the postnatal age of the infant. It was also observed that barrier function matures rapidly after birth even in the most immature neonate after 2–3 weeks. A similar correlation was observed when permeation of lidocaine through excised skin from 24 infants of gestational age 25–40 weeks with post natal age ranging from 0 to 7 days was studied and results indicated an inverse correlation between gestational age and skin permeability.[107]

4.7 Summary

As skin ages, it undergoes certain morphological and functional changes which lead to altered drug permeability, increased susceptibility to irritant contact dermatitis, and in addition possibly severe xerosis. From the published literature it seems that the thickness of the stratum corneum does not change significantly with age but there is an increase in the surface area of the corneocytes. Other major changes include reduced elasticity of the epidermis, a decrease in the number of sweat glands and an increased loss of collagen and elastin. There is also an increased disorganization in the collagen and elastic tissue network within the dermis. Stratum corneum water content has been reported to either not change or decrease slightly, yet permeability of drugs has been found to alter significantly. For example, the permeability of fluorescein, a water soluble dye, increases while the permeability of testosterone, a lipid soluble drug, decreases. The cause for these changes is the fact that there is an overall deficiency of all stratum corneum lipids in aged skin, resulting in decreased stratum corneum lamellar bilayers. This in turn results in major changes in the barrier properties of the skin. While these are the more significant changes, there are a number of smaller variations occurring structurally and biochemically with age such as the reduction in skin lipids and a reduction in cell turnover. The complex nature and high number of changes occurring simultaneously as well as the experimentally introduced variations due to use of subjects of different races, genders, ethnicity, ages makes it difficult to draw conclusive correlations between such changes and alterations in permeation of drugs through aged skin. Overall, these changes in the aged human skin make it prone to higher drug permeation and susceptible to other exogenous and environmental insults.

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5

Changes in Epidermal Lipids and Sebum Secretion with Aging

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5.1 Epidermal Lipids

5.1.1 Historical Perspective

The principal function of the epidermis is to provide a barrier to water loss. Without this water barrier life on dry land would not be possible [Attenborough, 1980]. The epidermis also provides a microbial barrier by maintaining surface conditions that are not amenable to microbial growth and colonization. The favorable conditions include relatively limited water, limited phosphate, low pH, and the presence of the sebaceous lipid film that contains some antimicrobial components and can inhibit adherence of some organisms. In addition, epidermal keratinocytes are able to produce both antimicrobial lipids and peptides. They can also release cytokines that result in recruitment of neutrophiles. This chapter will focus on skin lipids and aging.

5.1.2 Metabolic Pathways

In general, the rates of lipid synthesis and catabolism are unaltered in aged epidermis compared to young epidermis; however, the ability to increase lipid synthesis in response to epidermal damage becomes impaired with advanced age [Elias & Ghadially, 2002].

5.1.2.1 Fatty Acids

Free fatty acids in human stratum corneum consist mostly of straight-chain saturated species ranging from fourteen to twenty-eight carbons in length. The major species are longer than 20 carbons with the 22- and 24-carbon entities being most abundant. The fatty acids are the only ionizable major lipids in human stratum corneum, and may be necessary for formation of lamellae.

Acetate from the circulation appears to be the main source of carbon for lipid synthesis. Within the viable epidermal keratinocytes acetyl-CoA is produced through the action of acetate thiokinase at the expense of one ATP. Biotin-dependent acetyl-CoA carboxylase converts acetyl-CoA into malonyl-CoA at the cost of one ATP. This is the rate limiting step in fatty acid synthesis. Malonate and acetate are transferred from the CoA thioesters to acyl carrier protein (ACP) in the cytosolic fatty acid synthetase complex. This complex converts seven malonates and one acetate into palmitate. One carbon dioxide is liberated and two NADPHs are used in each condensation step.

The palmitoyl group is either transferred to CoA to make palmitoyl CoA or hydrolyzed from ACP yielding palmitic acid. The fatty acid chain of palmitoyl-CoA can be extended in length through the action of a fatty acid elongase system located in the endoplasmic reticulum to produce the longer fatty acids [Peroera et al., 2003]. A p-450 mediated hydroxylation of the terminal carbon is thought to be responsible for production of the ω-hydroxyacids found in the acylceramides and acylglucosylceramides. It has been suggested that this hydroxylation takes place when chain extension has resulted in a fatty acid just long enough to span the endoplasmic reticular membrane. Double bonds are introduced as appropriate through by fatty acid desaturases in the endoplasmic reticulum [Munday, 2002].

Acetyl-CoA carboxylase is regulated by phosphorylation with the phosphorylated enzyme being inactive and the dephosphorylated form active. Citrate is also an allosteric activator.

5.1.2.2 Cholesterol

The only major sterol in human and porcine stratum corneum is cholesterol. This is not true of rodent models, where cholest-7-ene-3-β-ol is also present in significant amounts. In the final stages of the keratinization process, oleic acid from phosphoglycerides is transferred to cholesterol to produce cholesteryl esters. These unsaturated cholesteryl oleate molecules are thought to separate into liquid phase pockets within the intercellular space of the stratum corneum, and this may provide a mechanism for keeping unsaturated fatty acids from permeabilizing the lamellar phase domains.

Keratinocytes on the basal layer have low density lipoprotein (LDL) receptors and are able to take up cholesterol from the circulation; however, on moving off of the basal layer the LDL receptors are internalized and degraded. All cholesterol accumulated after this event must be synthesized *de novo*. The biosynthesis of cholesterol is far more complicated than depicted in biochemistry textbooks. There are many enzymatic steps beyond the formation of squalene. The first part of the cholesterol biosynthetic pathway involves the conversion of three molecules of acetyl-CoA into one β-hydroxy-β-methylglutaryl CoA (HMG-CoA). This is followed by the rate limiting step of reducing HMG-CoA by HMG-CoA reductase to mevalonate-CoA at the expense of two molecules of NADPH. This CoA thioester is hydrolyzed to release mevalonic acid, which is then phosphorylated at the cost of two ATPs to produce 5-pyrophospomevalonic acid. One additional phosphorylation step produces an unstable intermediate

which spontaneously releases one phosphate group yielding 3-isopentenylpyrophosphate. This metabolite equilibrates with its isomer, 3,3′dimethylallyl pyrophosphate, and the two isomers undergo a condensation with the release of pyrophosphate to form trans, trans-geranyl pyrophosphate. A second molecule of isoprenyl pyrophosphate is condensed with this metabolite, again with loss of pyrophosphate, to produce trans, trans-farnesyl pyrophosphate, which equilibrates with its isomer, nerolidol pyrophosphate. These intermediates are reductively combined in a reaction that requires NADPH to produce squalene. After oxidation of squalene to squalene 2,3-epoxide, lanosterol, a tetracyclic triterpene is rapidly produced. The transformation of lanosterol to cholesterol involves nineteen discrete enzymatic steps, most of which are not documented in textbooks. The enzymes involved in this conversion are all membrane associated. One of the intermediates in this conversion is 7-dehydrocholesterol, the precursor of vitamin D in the skin. The conversion of 7-dehydrocholesterol to vitamin D requires exposure to ultraviolet light and is nonenzymatic.

As with the rate limiting enzyme in fatty acid synthesis, the activity of HMG-CoA reductase is regulated by phosphorylation [Beg et al., 1978]. The dephosphorylated form of the enzyme is active, while phosphorylation inactivates the enzyme.

5.1.2.3 Sphingolipids

The rate limiting enzyme in sphingolipid synthesis is serine palmitoyl transferase [Radin, 1984]. It combines serine and palmitoyl CoA in an NADPH-dependent reaction to produce 3-ketodihydrosphingosine. An NADPH-requiring reductase converts this intermediate to dihydrosphingosine, and a ceramide is then produced by N-acylation of the long-chain base. Dihydrosphingosine can then be hydroxylated to produce a phytosphingosine-containing ceramide, or a trans double bond can be introduced between carbons 4 and 5 to yield a sphingosine-containing ceramide. The mechanism by which 6-hydroxysphingosine is produced has not yet been elucidated. Fatty acid hydroxylations result in the production of α- and ω-hydroxyacids. Vitamin C is required for the hydroxylation reactions that produce the more polar ceramides [Ponec et al., 1997].

Conversion of ceramides to glucosylceramides occurs in the Golgi apparatus within keratinocytes. This is mediated by ceramide glucosyltransferase, which utilizes UDP-glucose and is located on the cytosolic surface of the organelle. The likely site of lamellar granule origin is the Golgi apparatus.

Hydrolytic enzymes extruded from the lamellar granules deglycosylate glucosylceramides and cleave phosphorylcholine from sphingomyelin to produce ceramides at the interface between the uppermost granular cells and the stratum corneum to produce ceramides. Phospholipids are also enzymatically processed at this time to produce free fatty acids and cholesterol esters.

5.1.3 Chemical Structures

Ceramides, cholesterol, and free fatty acids are the major lipids of human stratum corneum, comprising approximately 50 percent, 25 percent, and 10 percent of the total lipid mass, respectively [Wertz & Norlen, 2003]. This was established by the pioneering work of Gray and Yardley in the mid- to late-1970s [Gray & Yardley, 1975; Gray & White, 1978; Yardley & Summerly, 1081]. These workers established that the ceramides were structurally heterogenous and contained long-chain normal and alpha-hydroxyacids and sphingosine and phytosphingosine bases as component building blocks. They established that porcine skin was a good model for human skin.

Detailed structures of the porcine ceramides were subsequently determined [Wertz & Downing, 1983], and all of the same structural types were found in human stratum corneum [Ponec et al., 2003]. Chemical structures of human stratum corneum ceramides are shown in Figure 5.1. In addition to the long-chain base and fatty acid components identified by Gray and White, human ceramides include ω-hydroxyacids [Wertz & Downing, 1983] and 6-hydroxysphingosine [Robson et al., 1994] as constituent building blocks. All combinations of sphingosine, phytosphingosine and 6-hydroxysphingosine bearing amide-linked normal fatty acid, α-hydroxyacid and ω-O-linoleoyl fatty acid have been identified.

Figure 5.1 includes ceramide nomenclature as proposed by Motta et al [1993]. In this system, amide-linked normal fatty acid, α-hydroxyacid or ω-hydroxyacids are designated by N, A or O, respectively. Sphingosine, phytosphingosine and 6-hydroxysphingosine are indicated by S, P and H, respectively, and the presence of ester-linked fatty acid is indicated by the prefix E. In the normal acylceramides (EOS, EOP & EOH) the major ester-linked fatty acid is always linoleate in normal, healthy epidermis.

The linoleate-containing acylceramides are the most unusual lipids of the stratum corneum. This type of lipid is found only in epidermis and is structurally unique. The ω-hydroxyacids in these ceramides are 30- to 34-carbons in length, and the most abundant ester-linked fatty acid is linoleic acid.

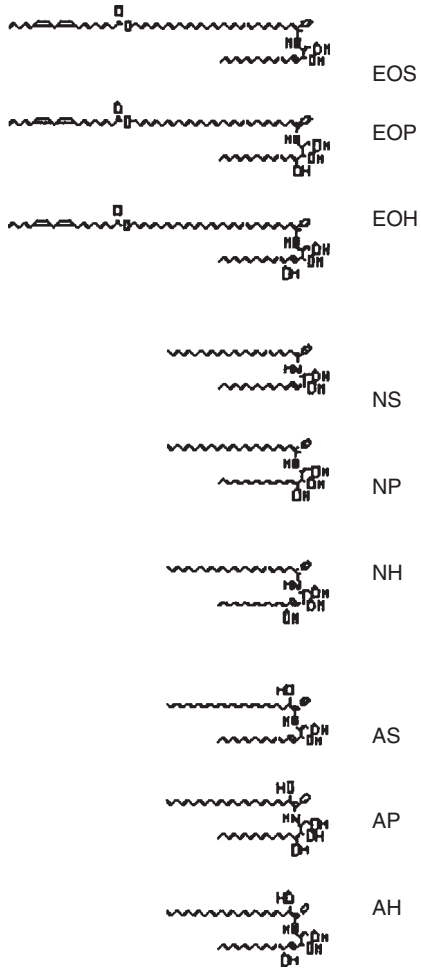

Figure 5.1 Representative structures of the ceramides found in human stratum corneum.

It has been shown that the same complex pattern of lipid lamellae seen in native stratum corneum using transmission electron microscopy can be reproduced from reconstituted stratum corneum lipids [Kuempel et al., 1998]; however, if CER EOS is removed from the lipid mixture, only simple bilayers are generated. There is no information on possible functional roles or relative significance of CER EOH and CER EOP.

5.1.4 Covalently Bound Lipids

It was originally reported that porcine epidermis contains significant amounts (approximately 2 percent of the dry weight of stratum corneum)

of covalently bound lipid consisting mainly of ω-hydroxyceramide (OS) with small proportions of ω-hydroxyacids and normal fatty acids [Wertz & Downing, 1986]. It was soon realized that this covalently bound lipid was attached to the outer surfaces of the cornified envelopes in the stratum corneum [Wertz & Downing, 1987; Swartzendruber et al., 1987]. Lamellar granules are associated with a linoleate-containing acylglucosylceramide [Wertz et al., 1984], and it is thought that about two-thirds of this glycolipid are in the bounding membrane of the lamellar granule with the glucosyl moieties directed inward [Slater et al., 2003]. As the bounding membrane fuses into the cell plasma membrane the linoleate is cleaved and recycled. The glucosylated ω-hydroxyceramide then becomes ester-linked to acidic amino acid side chains on the surface of the nascent cornified envelope, possibly through the action of transglutaminase 1 [Kalinin et al., 2001]. The glucose is then released through the action of a glucocerebrosidase. The fatty acid that is covalently bound is thought to be either ester-linked to hydroxyls of serine or threonine side chains or possibly attached through thioester linkages [Lopez et al., 2007]. The covalently bound ω-hydroxyacid is thought to reflect ceramidase action on covalently bound ceramide to release sphingosine. In human stratum corneum, the situation is similar to that found in porcine epidermis; however, in addition to covalently bound ceramide OS there is also OH and a small amount of OP [Hill et al., 2006].

5.1.5 Minor Components

A minor stratum corneum lipid that has been implicated in the regulation of the desquamation process is cholesterol sulfate [Elias, 1983]. With both mouse ear skin organ culture [Ranasinghe et al., 1986] and human skin *in vivo* [Long et al., 1985], it has been demonstrated that hydrolysis of cholesterol sulfate accompanies cell shedding. Also, in the genetic disease recessive X-linked ichthyosis where sterol sulfatase is defective desquamation is defective [Elias et al., 1988], and the skin can become very rough and scaly. It has been demonstrated that cholesterol sulfate can inhibit the serine proteases [Sato et al., 1998] that normally degrade desmosomal proteins as part of the desquamation process.

Free long-chain bases are thought to be released through the action of ceramidases on ceramides [Wertz & Downing, 1990a, b; Steen Law et al., 1994]. Acid, alkaline and neutral ceramidases and an alkaline ceramidase have been detected, and there is a sphingosine gradient across the epidermis [Steen Law et al., 1994]. This gradient from the stratum corneum to the inner layers of the epidermis could be significant both for providing

an antimicrobial agent to the skin surface and, through the ability of sphingosine to inhibit protein kinase C, for regulation of the keratinization process.

5.1.6 Physical Organization

The lipids in the intercellular spaces of the stratum corneum form unique lamellar structures as shown in Figure 5.2. Because of the relative paucity of reactive functional groups in the stratum corneum lipids, the lamellar structure cannot be visualized by transmission electron microscopy (TEM) using the usual osmium tetroxide fixation. The use of ruthenium tetroxide [Madison et al., 1987], which is more reactive, or cryopreservation [Norlen, 2001], does permit visualization of these lamellar structures. Although there is some disagreement over the interpretation of these images, it is generally agreed that the intercellular lamellae are organized into trilamellar units with an overall dimension of 13 nm [Madison et al., 1987; Bouwstra et al., 2000]. The lucent bands within these units appear to be broad-narrow-broad. In one interpretation of the images revealed using ruthenium tetroxide and supported by the cryoTEM is that the individual membranes are uniformly 4.3 nm thick [Hill & Wertz, 2003]. The alternating broad-narrow-broad pattern is thought to reflect a nonuniform distribution of the linoleates from the acylceramides (EOS, EOP & EOH).

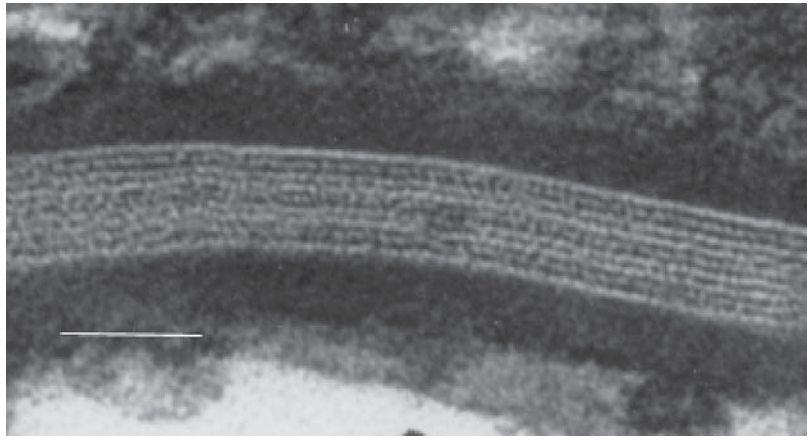

Figure 5.2 Intercellular lipid lamellae in human stratum corneum. The bar represents 50 nm.

5.1.7 Effects of Aging

Extensive work on aging skin using a murine model has been reviewed elsewhere [Elias & Ghadially, 2002].

Regarding human skin, there is general agreement that the water barrier of the skin actually improves with age as judged by transepidermal water loss measurements [Thune et al., 1988; Wilhelm et al., 1991] or by measurement of permeability to tritiated water [Squier et al., 1994]. However, after damage, recovery of barrier function is impaired. The improved barrier function with age does not reflect an increase in lipid content. It appears to be related to larger corneocytes [Marks, 1981]. There is also a general increase in dry skin with advancing age [Kligman, 1979]. Attempts to relate xerosis to changes in the proportions of stratum corneum lipid have failed.

Although some investigators have found no age-related changes in stratum corneum lipids [Saint Leger et al., 1988], the most extensive and technically sophisticated studies both found, on average, a statistically significant 30 percent decrease in all major lipid classes in going from 20s to 60+ [Imokawa et al., 1991; Rogers et al., 1996]. The first of these two studies used a Japanese population (N = 65), while the second used a Caucasian population (N = 49). This was interpreted as a reflection of slower general metabolism and lipid synthesis in older keratinocytes.

5.2 Sebaceous Lipids

5.2.1 Distribution and Physiology of Sebaceous Glands

Pilosebaceous units are found in all regions of the skin except for the palmar and plantar surfaces [Montagna, 1963]. Pilosebaceous units are most dense on the scalp, followed by the face, neck, and shoulders. They are relatively sparse on the torso and limbs. The sebaceous glands are multilobular holocrine glands. As sebocytes move from the periphery of the gland toward the lumen they undergo differentiation. Gradually, all of the carbon of the cell becomes converted into lipid. Initially, this appears as lipid droplets within cells, but in the lumen there is only a lipid mixture with no cell boundaries. In the pilosebaceous units the gland is connected to the hair follicle by a short sebaceous duct. Sebum flows through the duct, out through the follicle and over the skin surface. A second kind of

sebaceous gland is the sebaceous follicle. These are less abundant and are not associated with major hairs. Their distribution is similar to the pilosebaceous units. They are found surrounding every orifice of the human body, suggesting a protective function. They produce sebum with the same composition as the pilosebaceous units.

5.2.2 Composition of Sebaceous Lipids

Sebum composition is species specific [Lindholm et al., 1981]. Human sebum as it is found in the lumen of the gland consists of 57 percent triglycerides, 25 percent wax monoesters, 13 percent squalene, 3 percent cholesterol esters, and 2 percent cholesterol [Downing et al., 1969]. The small amounts of cholesterol and cholesterol esters found in human sebum are thought to be residual from the basal sebocyte plasma membrane. Squalene is normally present in trace amounts as an intermediate in the biosynthesis of cholesterol. In human sebaceous glands, the enzymes beyond this point in the cholesterol synthetic pathway are not expressed, so squalene becomes the end product. As sebum flows through the follicle and over the skin surface, variable portions of the triglyceride fraction undergoes hydrolysis to release fatty acids, glycerol and small amounts of 1,2- and 1,3-diacylglycerols [Downing et al., 1969]. This hydrolysis is mediated, at least in part, by microbial lipases [Kellum & Strangfield, 1967]. There is reason to believe that the epidermis may also contribute to this process.

5.2.3 Effects of Aging on Sebum Secretion Rates

Sebaceous glands are highly active *in utero* under the influence of maternal hormones [Agache et al., 1980], and sebaceous lipids contribute significantly to the vernix caseosa [Rissman et al., 2006]. Within days after birth, sebaceous lipid secretion rates become very low. The glands atrophy and remain so until the onset of puberty [Stewart & Downing, 1985]. At that time, under androgenic stimulation, the glands enlarge and become active [Jacobsen et al., 1985]. Maximum rates of sebum secretion are generally found in the mid to late teen years, but some individuals do not peak until early twenties. During this time of peak sebum secretion, the most active sebum secretors can develop severe acne [Strauss, 1998]. Those with the lowest sebum secretion rates do not develop acne, and those individuals with sebum secretion rates between the extremes may develop mild to moderate acne. After the early twenties, sebum secretion rates decline in an exponential manner such that individuals older than about

sixty-five have low sebum secretion rates resembling those of prepubertal teens [Jacobsen et al., 1985]. Coincidentally, the incidence of skin infections becomes much elevated in this older population [Kligman, 1979].

5.3 Summary

A mixture of ceramides, cholesterol and fatty acids organized into multilamellar structures in the intercellular spaces of the stratum corneum provide a barrier function. In aged skin, the lipid content of the stratum corneum is decreased compared to young skin. Nevertheless, the permeability of aged skin to water is superior in aged skin due to the increased size of the corneocytes. It is yet to be determined if aged skin also has a superior barrier to compounds other than water. Aged skin recovers from damage more slowly than young skin. Human sebaceous lipids consist of squalene, wax esters, cholesterol esters, and triglycerides. At the skin surface, some of the triglycerides are hydrolyzed to release fatty acids, some of which are antimicrobial. Sebum secretion rates peak in the teen years and gradually decline thereafter. People over the age of about sixty-five years have very low sebum secretion rates. This decrease in sebum production with the concomitant reduction in antimicrobial fatty acid at the skin surface may contribute to the increased occurrence of skin infections in the elderly.

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6

Skin Disorders of Inflammation and Immune Suppression in Young and Elder: A Special Role for Mast Cells

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6.1 Introduction

Diseases of the skin are regarded as critical health problems of our current society [1]. There is a growing frequency of skin diseases in the modern world, their variety being of 3000 types, and their incidence being estimated at 0.5–5 percent in the general population. In particular, many skin diseases are age-dependent, occurring mostly in elderly. Accordingly, it is of the utmost importance to identify and characterize the vital components of skin aging and disease, in order to enable detection of therapeutic targets and progress drug development.

Most dermatological disorders can be categorized as skin diseases, yet within this sub-group they differ in many aspects, including cause, phenotype, and severity. Infectious, inflammatory, and neoplastic pathologies are viewed as the most frequent and severe skin diseases. Though occurrence of some of these cutaneous disorders is linked with genetic predisposition, it is widely accepted that environmental factors and chronological aging are essential facilitators of pathological skin alterations. In fact, the majority of skin diseases are associated with changes in immune surveillance, structure, continuous exposure to carcinogenic and external factors such as ultraviolet (UV) wavelengths in sunlight, and co-existence of other diseases [2–4].

The common ground of the age-associated disorders is their stringent association with the cutaneous immune response. Skin immunity undergoes a milieu of modifications with age, rendering the skin immune system of elderly less susceptible to certain allergic and inflammatory diseases, but more susceptible to cancer, infections, and immunosuppressive diseases. In particular, there are many indications that the tissue-residing cells of the immune system, known as mast cells, are implicated in pathogenesis of skin diseases. Mast cells contain pre-formed and newly synthesized mediators, express many cell surface receptors and adhesion molecules, and can respond to both specific and non-specific stimuli. It is currently believed that the mast cell function, whether beneficial or detrimental to the host, is highly influenced by its surrounding microenvironment. Skin mast cells can have a beneficial role in rejection of infectious parasites and in tissue repair and remodeling yet, conversely, they may very well function in both the evolution of skin aging and pathogenesis of skin diseases [5,6].

In this chapter, up-to-date epidemiology and pathogenesis of skin diseases that are linked with immune alterations and age will be presented. We will review the current knowledge regarding skin immunity in general and mast

cell function in particular. In addition, the manner by which deterioration of these immune mechanisms promotes both skin aging and skin disease progression will be examined.

6.2 Epidemiology and Prevalence of Immunological Skin Diseases

Although pathologies of the skin tissue have an extensive history of research, and despite the known existence of wide arsenal of skin diseases, epidemiology is still a subject of much obscurity in this field [3]. The controversy surrounding this issue is suspected to be a result of diversity in population characteristics (i.e., age, origin, genetic background) and in methods of analysis. Notwithstanding, some general statements can be made with regards to a number of recent studies [2–4,7–11]. The epidemiology of the most frequent immune-related disorders are summarized in Figure 6.1, and described hereafter.

Aging populations suffer from higher occurrence of infectious skin diseases, such as bacterial and viral infections [11,12]. Infections of gram-positive bacteria (such as cellulitis, erysipelas, necrotizing fasciitis, folliculitis, impetigo, and furunculosis) are more common in the elderly

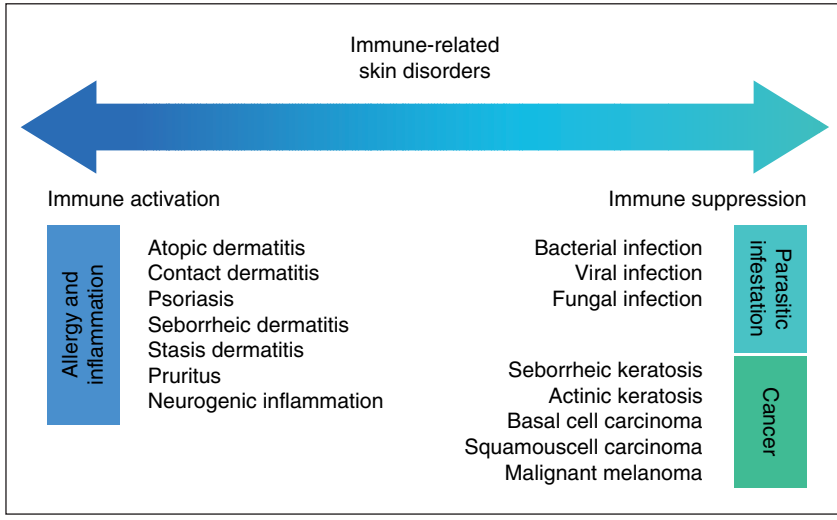

Figure 6.1 Immune related disorders, classified according to immune activity.

than in younger persons [12]. Herpes breakouts are four times more frequent in the age of eighty years, than in that between twenty and fifty years [11]. In one study, 6.2 percent, 7.9 percent, and 10.2 percent of the elderly patients in the corresponding groups aged roughly seventy years, eighty years, and over eighty-five years were virally infected, demonstrating an age-dependent increase [4]. The increased frequency of the fungal infections is estimated between 2-fold and 6-fold in patients in their sixties as compared to those in their thirties [2]. For example, dermatophytosis is estimated to occur in 80 percent of individuals over sixty-five years [11].

Some inflammatory and allergic disorders, such as pruritus and stasis dermatitis, are found to be elevated in elderly populations [4]. Pruritus (itching) that is often associated with skin inflammation, is also highly elevated with age, increasing 2-fold in patients over eighty-five years as compared to those between sixty-five and seventy-four years [4]. The association between this inflammatory syndrome and elderly is further intensified by the fact that it is also induced by psychological factors of aging, such as anxiety, stress, and depression, especially in patients with an atopic background [13]. Stasis dermatitis, inflammation of the lower legs that is instigated by chronic impairment in venous flow, was shown to be twice as prevalent in patients over the age of seventy years, as compared to those below this age [14]. This high occurrence of stasis dermatitis can be rationalized by it being a consequence of certain bacterial infections, such as cellulites and impetigo, which have been shown to highly infect the aged population [12].

However, most inflammatory syndromes are found to be of higher prevalence in the very young, and gradually reduce with age. For example, atopic (eczematous) dermatitis, an inflammatory response of the skin due to exposure to external and/or internal agents, presents primarily in infants and children. This disorder is observable in patients who are atopics. In some cases, it accompanies the development of asthma which is also higher in the younger ages. Comparing between infants (aged less than 1 year) and geriatric patients (aged 65 years), prevalence of this disorder is reduced by 15-fold (i.e., from 45 percent to 3 percent, respectively [2]. Indeed, findings of a different study coincided with the age-dependent decrease; in three groups (aged roughly 70 years, 80 years, and over 85 years), 21.6 percent, 17.3 percent, and 14.1 percent of the elderly patients in each group, suffered from atopic dermatitis, respectively [4]. Other studies show similar age-dependence of atopic dermatitis occurrence [15]. Likewise, psoriasis, a chronic inflammatory syndrome which can be triggered by multiple factors

(such as genetic predisposition, parasitic infection, stress), is an adult disease peaking at the third decade of life, which subsequently decreases. It is worth mentioning that some forms of skin inflammation, such as seborrheic dermatitis, are relatively indifferent to age-associated changes; in this chronic inflammation disorder, prevalence stands at a stable value of 10 percent for all ages [2].

In contact dermatitis, another form of inflammation following physical skin contact with an allergen, there is also an age-dependent decrease in incidence. Allergic sensitization in elderly patients exposed to contact-mediated allergens was found to be delayed in a number of studies [15–17]. Still, with respect to some allergens, higher sensitization rates are observed in old age, as shown in reports of increased contact dermatitis in elderly [16]. This suggests that the occurrence of this type of dermatitis is more dependent on the allergen than on age.

Skin cancer accounts for roughly 50 percent of all malignancies in the United States, and exceeds the collective occurrence of other malignancies in Australia [5]. Cutaneous cancers are typically divided into two groups; first, the common non-melanoma cancers, such as squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), constitute 77 percent and 20 percent of cancers, respectively. Secondly, the malignant melanomas and rarer cancers, which are considered less frequent but more aggressive due to their metastatic abilities [18]. Indeed, malignant melanoma has been stated to comprise only 1 percent of skin cancers, but accounts for greater than 60 percent of skin cancer deaths [9]. Benign forms of cancer, such as seborrheic keratosis and actinic keratosis, which in some cases precede the development of carcinoma and melanoma, also exist. The incidence of these skin cancers is very predominant within the elderly community. Collectively it is thought that prevalence of cancer doubles with every nine years of age [19]. The occurrence of seborrheic keratosis is remarkably intensified with age, as a monotonic increase in prevalence was observed in the 20- and 60-year age group, with values of 10 percent and 80 percent, respectively [2]. Beyond the age of seventy, prevalence has been shown to further increase significantly [4]. In another study focusing on BCC and SCC, the incidence of cancer was increased in the 60–69 age-group as compared to the 40–49 age group, by 2-fold (for BCC) and by 4-fold (for SCC) [10]. In the case of melanoma, age was characterized as an independent prognostic factor; increased mortality rates and lower survival were well documented in elderly patients especially over sixty years of age [7,8].

Whether initiated by parasites, viruses, allergens, inflammatory substances, or cancer inducers, it is clear that development of skin diseases is tightly linked with the intactness of skin immunity. The myriad of skin pathologies that occur in situations of increased immune activation, or alternatively, in immunosuppressed conditions, reinforce the importance of immune surveillance for normal homeostasis of the skin. Consequently, complete characterization of skin immunity is essential for therapeutic intervention in skin pathology.

6.3 Skin Immunity

The skin, being the largest and most exposed organ of the human body, is known as a first line of host protection against external pathogens. The cutaneous immune system is composed of a wide array of mechanisms and responses, spread across the epidermal and dermal layers of the skin. In this section, we will describe the different components and mechanisms of innate and adaptive skin immunity, with a special focus on mast cells, as documented in current literature [20–24]. These are summarized in Figure 6.2. Modifications in this system that are mediated by aging will be presented as well.

6.3.1 Innate and Adaptive Immunity in the Skin: Cellular and Neuro-Endocrine Mechanisms

Innate immunity of the skin is manifested first by the stratum corneum, the external skin barrier composed of keratinized cells (keratinocytes) and lipids, which confer selectivity and physical defense against exterior pathogens. Yet the majority of host protection of the skin consists of active defense mechanisms. Non-cellular immune surveillance includes lipid components of the epidermis, resident microflora, and skin-secreted antibodies, which have all been shown to actively protect the skin in bacterial challenge. Cell-mediated mechanisms, generally activated upon failure of the first defense line to eradicate the pathogen, are characterized by a higher level of complexity, and involve a broad array of cell types and molecular interactions. These include a variety of antigen-presenting cells (APCs), such as Langerhans' cells (LCs), dendritic cells (DCs), and macrophages, as well as keratinocytes, fibroblasts, mast cells, and the cytokines and chemokines produced by these different cell-types. Lymphocytes, present in the epidermis and dermis at different subclasses, constitute the adaptive

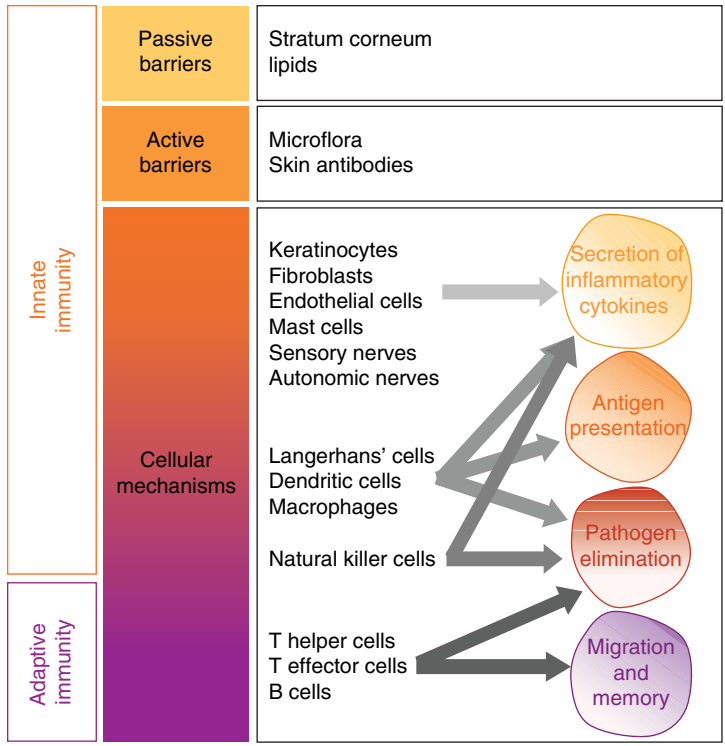

Figure 6.2 Innate and adaptive immune mechanisms of the skin.

arm of skin immunity which is antigen-specific and responsive to memory. These will be described later on.

Keratinocytes that constitute the majority of cells in the epidermis do not classically belong to skin defense mechanisms. Nevertheless, these cells possess immune features which cannot be overridden. Keratinocytes react to temperature changes, UV radiation, microbial insult, and various mediators. In turn, they release primary inflammation-associated cytokines, enhancing the immune response. These include interleukin (IL) 1α and tumor necrosis factor (TNF) α. Skin fibroblasts and melanocytes have similar abilities, secreting IL-6, TNFα and keratinocyte growth factor (KGF) that further facilitate an immune response initiated by keratinocytes. Inflammatory expression patterns of these local skin cells are also sensitive to immune cell-products such as interferon (IFN) γ, aiding the perpetuation of the immune response.

While these resident skin cells contribute to host defense, specialized immune APCs are chiefly responsible for guarding the skin from exterior challenges. In the skin, these are primarily LCs, bone marrow-derived APCs that constitute 2–8 percent of epidermal cells and co-exist alongside the keratinocytes. In addition to being the first line of antigen-presenting cells to encounter, process and present a pathogen to immune cells in lymphatic regions, LCs express a considerable level of major histocompatibility complex (MHC) class II antigens, cell-surface antigens, and membrane-bound co-stimulatory molecules. The expression pattern of the surface molecules varies according to the location, maturation state, and function of the LCs. Dermal DCs and macrophages are phenotypically and functionally similar to LCs, but reside in a more internal region of the skin. Keratinocytes themselves can present antigens, yet this is a local and limited effect since these cells do not migrate to lymph nodes, and lack many standard APC functions.

Skin APCs can be activated following recognition of antigenic determinants by bacterial-associated toll-like receptors (TLRs) and other signaling receptors, or by exposure to inflammatory cytokines, initiating a number of activation events. Inflammatory cytokines (such as IL-1α, IFNγ and TNFα) are released into the microenvironment; these act in an autocrine fashion to enhance self-antigen presentation and secretion of additional cytokines, and in a paracrine fashion to increase the expression of cytokines, chemokines, and cell adhesion molecules. The latter are expressed by endothelial cells, keratinocytes, and lymphocytes, and include intercellular adhesion molecule (ICAM) 1, endothelial cell-leukocyte adhesion molecule (ELAM) 1, vascular cell adhesion molecule (VCAM) 1, and P- and E-selectins. The consequence of this upregulation is the recruitment of additional immune cells, such as natural killer (NK) cells and lymphocytes, to the area of inflammation. Importantly, in the case of bacterial challenge via TLRs, nitric oxide and other reactive oxygen species (ROS) are often released by endothelial cells, thereby leading to direct pathogen elimination.

APCs are also involved in acquisition of adaptive immune responses. Epidermal and dermal APCs (LCs and DCs) migrate into the cutaneous draining lymph nodes and unveil the pathogenic component to resting T lymphocytes, thereby initiating their clonal expansion and enabling their specific recognition of the pathogen. The resulting cutaneous lymphocytes are mostly helper (CD4+) T cells and cytotoxic (CD8+) T cells, capable of eliciting an immediate attack against the intrusive pathogen. This is co-stimulated by the milieu of pro-inflammatory factors secreted by skin APCs

and resident cells. Additionally, a certain population of lymphocytes in the skin is identified as memory T cells, providing means to initiate a more efficient response upon secondary antigenic stimulation. This lymphocytic subtype, also produced in the lymph nodes, migrates to other tissues as well.

Whether intended for immediate effector functions or long-term memory functions, mature antigen-specialized T cells must undergo extravasation from the peripheral blood into the skin in order to carry out their tasks. The surface molecules cutaneous lymphocyte-associated antigen (CLA), CC chemokine receptor (CCR) 4, and leukocyte function-associated antigen (LFA) 1, expressed by the majority of skin T cells, mediate their attachment to adhesive molecules, ultimately leading to cell migration. Notably, it has been suggested that constant lymphocytic migration exists in the skin at low amounts, ensuring basal immune surveillance.

Immune and inflammatory processes in the skin are mediated also by neuronal mechanisms. The dermal layer of the skin is highly innervated by sensory and autonomic fibers, and responds to a milieu of neuropeptides in both normal and pathophysiological conditions. Sensory nerves, present in the skin layers, secrete numerous neuropeptides such as substance P, calcitonin gene-related peptide (CGRP) and somatostatin, which cause increased secretion of inflammatory mediators and leukocyte adhesion to blood vessels. Autonomic nerves in the skin secrete acetylcholine and vasoactive intestinal peptide, which both act as anti-inflammatory agents through down-regulation of IL-1α and TNFα. It is thought that a complex loop of autocrine and paracrine feedback is present, where these neuropeptides further affect receptor expression and cytokine/chemokine secretion capabilities of resident skin cells, immune cells, and blood vessels. In particular, several of these factors are stringently associated with the regulation of mast cell function, as will be discussed in section 6.3.2. The effects of many other neuropeptides, reviewed in [23], are beyond the scope of this chapter.

6.3.2 Mast Cells in Skin Immunity

Mast cells, tissue-dwelling granular immune cells, are best known for their role in allergy or type-I (immediate) hypersensitivity reactions. However, they have also been implied in a number of other inflammatory diseases (i.e., autoimmune diseases and cancer). They derive from CD34-positive precursors under the specific influence of stem cell factor (SCF), and localize and mature in the serosal and mucosal surfaces, usually in close proximity to blood vessels and nerves. Mast cells express high affinity plasma membrane

receptors binding IgE antibodies (Fc RI), and contain prominent cytoplasmic granules storing biogenic amines, proteoglycans, cytokines and neutral proteases [6,25,26].

Upon IgE-dependent and independent activation, mast cells release a variety of mediators. These include pre-formed granule constituents such as tryptase, histamine, and heparin, as well as newly synthesized lipid mediators, like prostaglandin (PG) D_2, and leukotriens (LT) C_4 and B_4. Many cytokines, chemokines and growth factors are also products of mast cells. These include interleukins 1, 3, 4, 5, 6, 8, 10, 13, as well as IFNγ, TNFα, transforming growth factor (TGF) β, RANTES, monocyte chemoattractant protein (MCP) 1, eotaxin, nerve growth factor (NGF), macrophage colony stimulating factor (M-CSF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) 2, 5,7, 10, and vascular endothelial growth factor (VEGF). In the absence of IgE, mast cells may be activated directly by a series of basic agonists, such as certain neuropeptides (i.e. bradykinin), eosinophil-derived major basic protein (MBP), or by factors involved in differentiation, survival, proliferation, maturation, adhesion and chemotaxis processes (i.e., SCF and NGF). Release of the described mediators is accomplished either by classical MC exocytosis of granules (i.e., compound degranulation), or by differential release or intragranular activation. The secreted mediators initiate a wide array of events, which usually result in both immediate and late-phase inflammatory reactions [6,25,26].

In the skin, mast cell numbers reach approximately 10,000 per mm^2 (corresponding to roughly 0.5 percent of the dermal area), and are located near hair follicles, sweat glands, and blood vessels. Skin mast cells are known to contain both high amounts of tryptase, chymase, histamine, carboxypeptidase, β-hexosaminidase, and cathepsin G, and they synthesize prostaglandins and (to a lesser extent) leukotriens [20,21]. These phenotypes are common for most connective tissue mast cells. Mast cells of the skin are centrally involved in carrying out IgE-dependent immediate hypersensitivity reactions, i.e., atopic dermatitis (which will be further discussed in section 6.5). As mentioned above, it has been shown that these cells are also responsive to non-IgE-dependent stimuli [27].

Although skin mast cells are implicated in pathogenesis of inflammatory skin diseases, they are valuable in cases of parasitic infections. The immediate, IgE-dependent (type 1) hypersensitivity response is then critical for eradication of parasites [6]. Chronic inflammation mediated by mast cell activation and degranulation has been shown to lead to tissue damage and

subsequent repair [28,29]. With respect to tissue degradation, mast cell proteases such as tryptase activate (and may produce) matrix metalloproteinases, enzymes which degrade the extracellular matrix, facilitating fibrolysis and tissue breakdown [6]. Yet some of the mast cell mediators are characterized as growth and differentiation factors for keratinocytes and fibroblasts, thus mediating tissue repair. In this respect, tryptase, histamine, FGF-2, EGF, TGFβ, LTB_4, LTC_4, LTD_4, IL-3, -6, and -8, act on keratinocytes, and PDGF, tryptase, chymase, carboxypeptidase A, TGFβ, histamine, IL-4, and FGF-2 act on fibroblasts [6,26,28–30]. The functional importance of these factors is evident following damage to the skin by intrinsic and extrinsic factors (see section 6.4.1). Examples are hypertrophic scars, keloids, sclerodermoid chronic graft-versus-host disease (cGVHD), and scleroderma. In particular, in cGVHD, a syndrome which is characterized by dermal fibrosis and loss of cutaneous appendages, the effects of mast cell activation on tissue remodeling have been extensively defined in murine models and humans. In cGVHD patients who were treated with ketotifen, an anti-histamine, mast cells had stabilizing properties and skin manifestations were ameliorated [28–31]. Likewise, profibrotic mechanisms are observed in cancerous diseases, since they are necessary for tissue remodeling associated with malignancy (see section 6.5.2).

Mast cell functions in skin are associated in an interactive communication network to cutaneous neuropeptides [6,23,26,32]. CGRP and substance P, both potent neuropeptides, stimulate the release of most pre-formed granules and inflammation-associated cytokines from mast cells. Similarly, corticotropin-releasing hormone, pro-opimelanocortins, endothelin-1, somatostatin, vasoactive intestinal peptide, α-melanocyte stimulating hormone, and β-endorphins also promote mast cell activation and secretion of these factors, predominantly histamine. NGF elevates mast cell numbers and activation, and can also be expressed by these cells themselves. However, mast cells also express receptors for endocannabinoids and endovanilloids, which collectively attenuate the inflammatory response. Moreover, mast cells are capable of actively affecting sensory nerves and the cutaneous functions of neuropeptides. Tryptase activate sensory nerves, while histamine can either up-regulate or down-regulate nervous stimulation. Mast cell-secreted proteases can reduce the vasodilatory effects of CGRP and increase cytokine and neuropeptide clearance. Conversely, mast cell-derived LTB_4 is involved in some inflammatory activities of substance P [6,23,32].

Mast cells numbers and activation status are vastly higher in cancerous diseases, and inflammatory diseases such as rhinitis, dermatitis, urticaria,

and asthma. Yet the end effect of the mast cell-immunologic and/or -neurologic interplay in pathological conditions is clearly not straightforward, but rather depends on the specific profile of peptides and cytokines within the microenvironment [6]. Such dependences may be the reason for the different end effects of mast cell activation in various pathologies. This is further discussed in the next sections.

6.4 Skin of the Elderly

Aging, a process biologically defined as the accumulation of molecular damages on both the genetic and phenotypic levels, is a topic of wide discussion. Though not necessarily affecting the life span of a human being, aging is known to affect virtually all tissues and organs of the human body, including the skin tissue. Skin aging is a result of intrinsic decline of regulatory cell-maintenance processes, combined with extrinsic and environmentally-induced stressors, such as UV type B (UVB) radiation, nutrition, smoke, stress, and physical trauma [32]. In this section, we will explain how immune mechanisms of increased inflammation are exploited to promote the aging and remodeling of the cutaneous tissue, and how the established aged skin bears altered and compromised immunity. Specifically, we will present the changes that are manifested in mast cell functions, and the implications these modifications bear on the health of the skin. These are also summarized in Figure 6.3.

6.4.1 Aging of the Skin Due to Immune Modulation: Mast Cell-Induced Inflammation

Whether inflicted by endogenous or exogenous factors, it is accepted that skin aging is a downstream effect of inflammation [32]. The decreasing cellular integrity that accompanies intrinsic aging (i.e., cellular damage) causes the generation of ROS (such as superoxide) in the process of oxidative phosphorylation. This leads to mitochondrial damage, causing an increased production of prostaglandins and leukotriens. Inflammatory processes are thereby instigated; this is primarily manifested by mast cell activation and degranulation. The subsequent release of histamine and TNFα promotes vasodilation, enhanced blood flow and ICAM-1 expression by endothelial cells on nearby blood vessels, which facilitate the binding of immune cells to the vascular walls and their extravasation to the tissue, resulting in inflammation. Infiltrating immune cells, such as APCs and lymphocytes, secrete enzymes such as myeloperoxidase, collagenase, and different

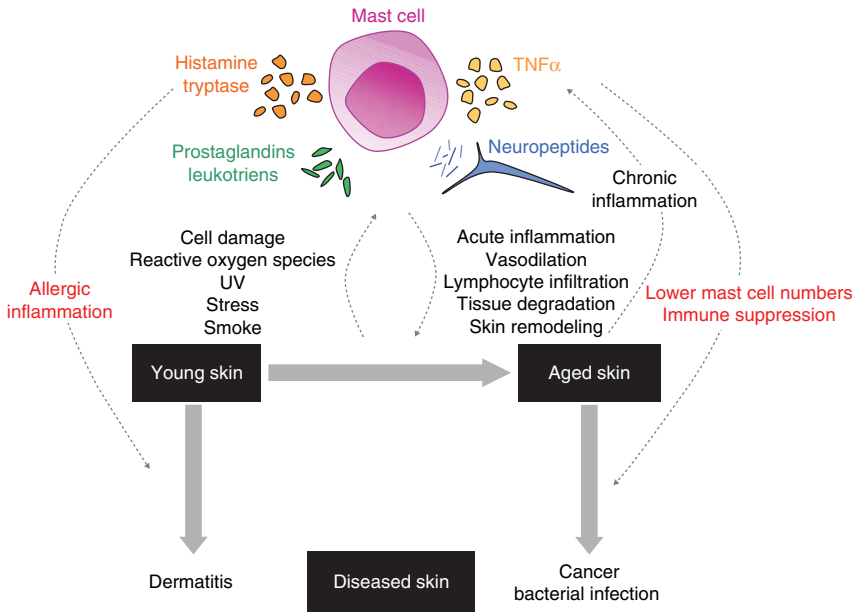

Figure 6.3 Mast cell involvement in skin aging and pathogenesis.

metalloproteinases, which directly degrade the extracellular matrix and cause structural damage. Mast cells contribute to this stage as well, by further activating metalloproteinases and other enzymes to degrade the tissue. The process does not cease at this point; rather, these immune cells promote chronicity of the inflammatory response by further secreting prostaglandins and leukotriens and activating surrounding immune and resident cells to produce the above-described enzymes. Thus, inflammation mediates manifestations of aging, such as induction of skin wrinkling and remodeling [32].

Extrinsic causes of skin aging appear to employ mast cells for generating inflammation as well. Environmental inducers of skin deterioration have been shown to lead to mast cell activation and histamine release by a variety of skin cytokines and neuropeptides. UV radiation, for example, was shown to cause the secretion of SCF from epithelial cells, and causes human keratinocytes to release β-endorphins and NGF, collectively leading to mast cell proliferation and degranulation [32,33]. Such radiation is not only implicated in aging and remodeling processes of the skin, but is also one of the primary causes of skin cancers, connecting mast cells to pathogenesis of

these diseases as well (see section 6.5). Exposure to electromagnetic fields, another source of skin deterioration and wrinkling, was previously correlated with increased mast cell numbers, levels of neuropeptides (CGRP, somatostatin, neuropeptide Y, etc.) and occurrence of clinical inflammatory symptoms, such as itch and edema, in screen dermatitis patients. Interestingly, psychological stress (which is associated with skin remodeling as well) has also been correlated with increased mast cell degranulation, in a mechanism mediated by CGRP and substance P [32].

Collectively, it appears that both intrinsic and extrinsic factors lead to aging and deterioration of the skin tissue, via instigation of inflammatory signals from a number of immune cells, especially mast cells. Accordingly, therapeutic targeting of mast cells and their mediators is continuingly being evaluated. Examples include the use of mast cell stabilizers and inhibitors [28,34,35], as further discussed in section 6.6.

6.4.2 Alteration of Immunity in Aged Skin

The aging of the skin brings about an array of mechanical, structural, and immunological failures. The response to damaging effects of extrinsic factors, such as UVB radiation and wounds, as well as of intrinsic molecular modifications, is significantly diminished in elderly [19,32]. Physical properties of the skin are modified, impairing the barrier functions of this tissue. As in the rest of the body, in the skin both innate and adaptive immune mechanisms malfunction; LCs, T cells, and B cells have lower reactivity [19]. It is possible that the numbers of these cells are lowered as well, yet this is an issue of controversy [36]. Secondary mechanisms of skin immunity are also compromised, since resident epidermal and dermal cells are decreased and have altered function [19,32]. For example, the amount of melanocytes in the skin decreases as a function of age, with an estimated 10 percent reduction over a period of ten years [37]. Since these cells possess the ability to reduce levels of free radicals, it is clear that their reduction with age creates conditions that allow for the accumulation of ROS and their downstream detrimental effects [38].

In addition to immune-related effects, the vasculature and lymphatic drainage deteriorate in old age, alongside the thinning of the epidermis. Besides hindered skin nutrition, these alterations further contribute to the reduced defense and healing abilities of the skin. In fact, the increasing difficulty of antigen presenting cells to migrate to lymphatic regions is viewed as one of the leading causes for immune failure in the aging skin [3,38].

Age-associated occurrence of immune-related diseases in other physiological systems also inflicts a certain degree of damage to skin immunity [12]. Diabetes, a known autoimmune disorder of the elderly, brings about immune deterioration, and is also accompanied by functional deterioration of the extracellular matrix, which includes collagen cross-linking and ROS-induced damage. Hyperlipidemia and hypertension, also diseases of increased incidence in aged populations, devastate blood flow and thus abrogate wound healing and impair mechanisms of systemic immunity. Consequently, initiation of such diseases together with the naturally harmful aging processes may have a synergistic damaging effect in the geriatric community [12].

Like their immune counterparts in the skin, mast cells are also subjected to alterations in old age. In healthy adults, aging is accompanied by a decrease of up to 50 percent in skin mast cell numbers [20]. Yet the modified mast cell response in elderly is better reflected in characteristics other than mast cell numbers. It has been suggested that the modifications in the functional phenotype of these cells are of more significance. A recent murine study [39] hypothesized that with age, mast cells undergo behavioral changes, in that they become less responsive to FcR (IgE-induced) activation and more susceptible to non-FcR activation pathways. In other words, dermal mast cells of elderly may be more easily activated, as evident in their higher ability to be stimulated by innate mechanisms rather than by adaptive mechanisms [39]. Similarly, in another study, peritoneal mast cells from aged mice exhibited lower expression of the receptor FcγRIIB (a negative regulator of mast cell activation) and higher IgG-mediated degranulation, than that observed in young mice [40]. Notwithstanding, there are indications that under certain conditions, mast cells may be increased in elderly. C-kit, the receptor for the primary mast cell growth factor SCF, has been shown to be elevated in aging mice. Some cutaneous diseases, i.e., neoplasms of the skin (see section 6.5.2), which are characterized by high occurrence in old age, are associated with elevated numbers of dermal mast cells [33]. Studies focused on mast cell prevalence and responsiveness in elderly are needed in order to clarify these issues, yet current evidence supports a central role for these cells in the alteration of aged skin.

In whole, the above-described modulations in aged skin disrupt the immune equilibrium in this organ. This imbalance is accompanied with manifestation of various disorders (as detailed in section 6.2). Specifically, mast cell modifications are considered to be of primary importance in skin disease development, as will be discussed in the following section.

6.5 Skin Pathogenesis: Mast Cells as Key Elements

Skin diseases are diverse in origin and pathogenesis, but share common characteristics of immune modulation and imbalance. Prevalence of such diseases, primarily associated to inflammation and malignancy, has been shown to be influenced by aging, as we have elaborated in section 6.2. Here, we will describe the general immune system alterations and specific mast cell modifications which are involved in development of these disorders.

6.5.1 Inflammatory Disorders

In old age, susceptibility to infectious agents such as viruses, bacteria, and fungi, are increased, as described in section 6.2. This is consistent with age-dependent impairment of cell-mediated immunity (described in section 6.4.2). Specifically, the modulations in mast cell function could allow for the parasitic pathogen to overcome innate immunity, since these cells are shown to be critical for host defense responses to dermal and non-dermal bacteria [6]. Bacterial components and released factors are known to stimulate mast cell activation, as facilitated by direct binding of a mast cell surface molecule (CD48), and a bacterial component. The corresponding protective effects of mast cells in an intact skin are manifested by initiation of innate responses, primarily via secretion of TNFα and related factors, and by inducing acquired lymphocyte responses, through mast cell presentation of antigens to these cells. In addition, mast cell-neuropeptide crosstalk is stimulated by bacterial toxins. Importantly, mast cell-deficient mice fail to eradicate bacterial infections [6]. Nevertheless, bacterial infections may prevail simply by virtue of reduced mast cell numbers in aged skin; indeed, there are indications that mast cells can eradicate bacteria by physical contact [6]. As in the case of bacteria, viral proteins can also induce mast cell activation.

As we have already illustrated in section 6.2, most inflammatory and allergic disorders are manifested in younger populations. The intact immune system, with mast cells at its center, allows for high rates of inflammatory activation. This is primarily facilitated by the mast cell secretion of multiple mediators, which act to create a type 1 hypersensitivity response mediated by IgE activation (as portrayed in section 6.3.2). For example, tryptase-positive mast cells have been found in abnormally high rates in lesions of atopic dermatitis patients, localized in proximity to the area of inflammation [41,42]. Interestingly, this is also true for non-lesional skin of these patients, suggesting one mechanism for the predisposition of these patients

to this inflammatory syndrome. Similarly, a decrease in chymase was found in mast cells of these patients, creating a lack in the capability to suppress the inflammatory state, as chymase is known to degrade pro-inflammatory proteins [42]. As inflammatory skin diseases are often accompanied by the remodeling of the tissue (as in aging processes, see section 6.4), it is clear why pathogenesis often involves abnormal expression of factors associated with tissue degradation and repair. For example, matrix metalloproteinases are induced in inflammatory circumstances, both in culture and in various forms of dermatitis [34,35,43,44]. As stimulation of these factors is recognized as a downstream event of mast cell activation (see section 6.3.2), this could indeed be another mechanism by which mast cell pathogenesis manifests in allergic and inflammatory disorders.

Mast cell-activating neuropeptides, described in sections 6.3.1 and 6.3.2, are also found in elevated concentrations in several inflammatory diseases. Nerve fibers secreting substance P, which is a potent mast cell stimulator in the skin, are increased in certain inflammatory human skin diseases, such as urticaria, psoriasis, atopic dermatitis, and contact dermatitis. Enhanced expression of NGF was found in mast cells and keratinocytes of patients with atopic dermatitis and psoriasis. Somatostatin was previously implicated in the pathophysiology of atopic dermatitis, and is indeed found to stimulate mast cells. It should be noted that immunosuppressive effects have also been observed in the presence of somatostatin, which is important in the pathogenesis of skin cancers, where abrogation of cell-mediated immunity plays a role (see section 6.5.2). Collectively, it seems that mast cell-neuropeptide cross-talk is an important facilitator of skin diseases [23].

As discussed, inflammatory diseases are not as abundant in old age as they are in youth, presumably due to lower activation ability of the aging immune system (described in the previous section). Still, elderly populations tend to develop certain inflammatory disorders of the skin, such as pruritus and stasis dermatitis, and according to some researchers, contact dermatitis as well (see section 6.2). These, however, are likely downstream effects of parasitic infestation [12], or of factors inducing aging [13]. The function of mast cells in inducing these inflammatory syndromes is supported by the fact that substance P and CGRP, known mast cell stimulators (see section 6.3.2), provoke occurrence of pruritus. Moreover, this disorder is abrogated by anti-histamines, indicating a central role for mast cells in its evolution [23]. Thus, even the few inflammatory syndromes that are observed in old age implicate mast cells as primary effectors of this pathogenesis.

6.5.2 Neoplastic Diseases

Cancers of the skin are perhaps the most discussed cutaneous diseases in the context of aging, as specified in section 6.2. Development of these cancers is thought to be stringently associated with skin immunosuppression initiated by UVB-exposure. UVB is known as a highly potent carcinogenic factor, which delivers its detrimental effects to the skin not only by molecular damage and mutagenesis of cutaneous cells, but also by suppressing skin immunity. UVB has been found to compromise the ability to respond to viral and bacterial infections, and substantially reduce occurrence of contact hypersensitivity responses. With regards to cancer, recent studies support the notion that UVB-induced immunosuppression is more influential than cellular transformation of skin cells in causing this disease, as UVB-associated cancers are very immunogenic and easily eradicated by non-UVB-exposed immune systems [5,45]. Moreover, patients treated with immunosuppressive therapy show increased rates of SCC linked to sun exposure [18,33].

The mechanisms through which UVB exerts immune suppression are multiple. Irradiation can weaken APC activity, impairing the ability to form antigen-specific cytolytic immune cells. UVB has also been found to create conditions favoring the Th2 responses over those of Th1, elevating the humoral immune response at the expense of cell-mediated immunity. Since the latter is functionally more important for rapid eradication of infectious agents and malignant cells, the link between UVB and immunosuppression is evident [45].

Although these mechanisms are all of value, a large body of evidence implicates mast cells as the primary effectors, on which the radiation-induced immune suppression is dependent [33]. Mast cell numbers are collectively increased around many cutaneous tumors, especially BCC and aggressive melanoma [5]. The amount of mast cells in the chronically-irradiated skin correlates directly with the severity of the UVB-inflicted immune reduction. In highly exposed skin, such as that of the hand, mast cells numbers and their expression of the SCF receptor (c-kit) are also augmented as a function of age, a marker of accumulated UVB exposure [33]. Interestingly, mast cell-deficient rodents fail to develop immunosuppression in response to UVB, but reconstitution of these cells by engraftment leads to phenotypes of suppressed skin immunity [33].

Attempts to establish a more defined correlation between mast cell numbers, and development of UVB-inflicted cancers, have been made. In BCC

and melanoma, mast cell counts in the non-irradiated buttock skin, but not in the UVB exposed skin, are found to be increased. In contrast, in SCC there are indications that mast cells are elevated in the irradiated parts of the skin tissue. This difference is rationalized by the diverse nature of the development of cancers; BCC is connected with early-life solar radiation, while SCC and melanoma are more associated with photo-aging, as it is increased with age, sun exposure, and immunosuppressive therapy [18,33].

Mast cells in the UVB-exposed skin are not only subjected to modifications in number, but also in function. UVB-induced factors such as NGF and cis-urocanic acid serve as stimuli for mast cell degranulation, and it is thought that additional neuropeptides do the same. TNFα, one product of this degranulation, locally induces immunosuppression, apparently by altering morphology of LCs and DCs, and suppressing their function and migration. Histamine, another factor released by mast cells, acts in a more global manner to modulate the immune environment. This is done by increasing Th2 cytokines such as IL-10, and suppressing Th1 cytokines like IL-12, thereby systemically advancing the response towards reduced cell-mediated immunity. Histamine also stimulates PGE_2 formation in keratinocytes, and both histamine and PGE_2 abrogate expansion of Th1 lymphocytes, thereby suppressing cytolytic effector cells. IL-10, another cytokine secreted by UVB-activated mast cells and histamine-activated monocytes and lymphocytes, is considered to have a role promoting humoral immunity and inducing immune tolerance [5,33,45]. Immune evasion in cancerous states may also be achieved by reduced lymphocyte expansion exerted by somatostatin, a neuropeptide that is co-localized with mast cell activation [23].

Importantly, mast cell secretion of angiogenic factors, predominantly VEGF, FGF-2 and IL-8, may constitute another contribution of these cells to tumor development, by aiding degradation of the extracellular matrix, and facilitating the required remodeling and vascularization in the tissue [5,28–30,33,45]. This is supported by the higher mast cell amounts observed in metastatic and vascularized melanoma, as opposed to benign melanoma. Moreover, it is claimed that the mast cell accumulation in the vicinity of the tumor is highest when the tumor acquires its angiogenic ability [5]. Mast cells may also directly enhance tumor proliferation, since FGF-2 and IL-8 are growth factors for melanocytes, the cells from which melanoma arises [5].

The above findings support a central involvement of mast cells in formation of an immunosuppressive environment aiding cancer evolution. Yet this appears to be somewhat contradictory to the fact that mast cells

are usually involved in immune activation rather than suppression, as demonstrated by their instigation of inflammatory processes that cause skin aging. Furthermore, mast cells are considered to be reduced with age, rather than increased with age (as we have mentioned in section 6.4.2). The documented discrepancies can be rationalized by the following considerations: First, dermal mast cell numbers and distribution varies within different areas of the skin tissue, due to diversity in intrinsic characteristics (such as tissue humidity and thickness) and environmental exposure between the areas [33]. Thus, it is difficult to standardize with regards to their density in the tissue. Secondly, it seems that the downstream effects of mast cell activation, whether immunosuppressive or immunostimulating, may vary as a function of age and/or modification of the immune cells and cytokine profile of the microenvironment [5]. For instance, it is probable that mast cell mediators have a pro-inflammatory effect only when skin immunity is intact. Accordingly, the impaired mast cell functions in aged skin may actually drive the opposite effect—immunosuppression.

Alternatively, the opposite scenario is also feasible: with age, mast cells themselves may undergo modifications in their ability to be activated and/or regulated by different stimuli (as described in section 6.4.2), assigning them different functionality. This could be manifested in the easier mast cell stimulation in aged skin (i.e., inflammation). Moreover, genetic diversity and differences in exposure to the sun create the general difficulty to establish a reliable "aging" model. Collectively, in aging-induced modulation of skin immunity, as well as in diseased populations, mast cells can have both stimulatory and inhibitory effects on the surrounding immune system, and this depends on the many factors described above.

6.6 Conclusions

The steady increase in the percentage of aging populations, mainly in developed countries, is gradually presenting challenges for the medical community. One of the most acute problems in the elderly is the high incidence of skin diseases. Most cutaneous pathologies classified with high prevalence, morbidity, and mortality, are age-dependent. Likewise, the majority of these skin diseases are immune-related, presenting in individuals in which skin immunity is no longer intact. Accordingly, the increased immune disregulation and breakdown in old age is viewed as the leading cause of skin disorders.

Management of these skin disorders, namely parasitic infections, allergic inflammation, and cancer, therefore necessitates the targeting of factors at the core of the malfunctioning immune system. In the case of cancer and infectious diseases, current treatments target the hazardous element (by chemotherapy or antibacterial therapy, respectively) and/or intensify the immune response against it (by immunotherapy). Conversely, in skin inflammation the detrimental effects are a result of immune upregulation, thus its suppression is the primary therapeutic goal. This can be attained by targeting the immune system as a whole (e.g., by glucocorticoids). Yet, more specific approaches are usually applied; since mast cells are key players in the development of these disorders and abrogation of normal, cell-mediated immunity, these cells and their mediators are deemed as proper therapeutic targets. Histamine, one of the primary mast cell mediators which is implicated in pathogenesis of inflammation, has in fact been a therapeutic target for the past thirty years; many antagonists of histamine receptors, termed antihistamines, are continuously being developed for the purpose of abrogating allergic inflammation [6,25,26,33,45].

Yet the current treatments in the field are still unsatisfactory. Conventional treatment approaches, both in cancer and in inflammation, yield significant toxicity and high variation in patient response, thereby limiting drug dosing and disease control. Newer avenues are therefore being developed. One example is the targeting of neuropeptides, as they are thought to hold a significant place in perpetuating inflammatory responses in the skin. Agents affecting secretion and clearance of these peptides will likely be developed in the near future [32]. Yet, even more promise exists in therapeutic targeting at the cellular, rather than molecular, level. In concurrence with the central role of mast cells in skin induced pathologies, it is hypothesized that inhibition of total mast cell degranulation would yield most efficacious prevention and/or reversal of disease. By means of hindering activatory signaling or stimulating inhibitory signaling, mast cells can theoretically be driven to stability and senescence, affording long lasting abrogation of disease pathogenesis. Recent studies have characterized the Ig superfamily receptor IRp60 (CD300a) as an inhibitor of mast cell degranulation and survival [46]. Indeed, binding of this receptor using a bi-specific antibody has been shown to abrogate asthma and airway inflammation [47,48]. Similar methods can be used to modulate mast cell functions in the skin, thereby giving rise to development of novel therapeutic pathways for treating immune-related skin disorders.

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7

Cellular Senescence and Skin Aging

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7.1 Introduction

Most if not all of our body cells possess a limited capacity to proliferate. Human cells enter a stage of altered phenotype known as senescent phenotype at the end of their replicative life span. Senescent phenotype is characterized not only by the inability of cells to divide despite appropriate proliferative signals but also by secretion of matrix degrading enzymes by senescent cells. Accumulation of senescent cells is thought to compromise tissue regenerative capacity, and contribute to chronological (time-dependent) and physiological aging (time-dependent or time-independent) of tissues in an organism. Human skin, a protective layer made of various cell types and matrix undergoes conspicuous changes over the chronological age, such as increased loss of elasticity, wrinkling, sagging and coarse, rough and deep lining. These cosmetic changes are exacerbated by extrinsic factors such as long accumulative exposure to sun light. Because of altered phenotype, accumulation of senescent cells, at least in part could contribute to these undesirable perceived cosmetic changes. This chapter will review the role of cellular senescence in skin aging, and potential impact of skin stem cells and their senescence on skin aging.

7.2 Skin Histology and Skin Cell Types

Mammalian skin is a complex organ, which covers most of body. It is the first line of defense to protect the body from dehydration, external injuries and bacterial or viral infections. To exert these vital functions, skin has evolved an elaborate structure comprised of tissues of various origins. The skin consists of three layers—the epidermis (and its associated appendages, pilosebaceous follicles, and sweat glands), the dermis, and the hypodermis [1,2].

7.2.1 The Epidermis

The tissue that forms the interface between the organism and its environment is a stratified squamous, keratinized epithelium called epidermis (Figure 7.1). It contains various cell types such as keratinocytes, Langerhans cells, melanocytes and Merkel cells [3].

7.2.1.1 Keratinocytes

Keratinocytes are arranged in continuous layers consisting of basal (single layer), spinous (5–15 layers), granular (1–3 layers), and cornified or horny

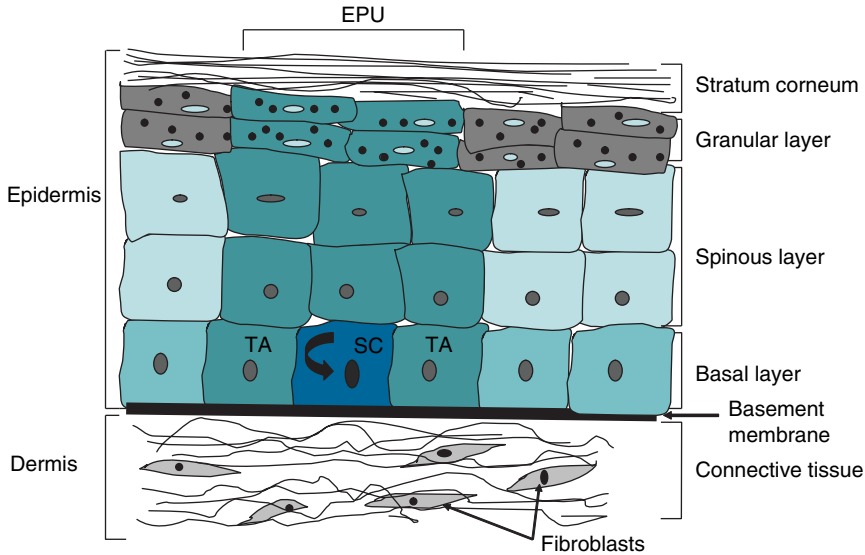

Figure 7.1 Diagramatic representation of skin histology. The epidermis consists of basal, spinous, granular, and cornified or horny cell layers. Each layer is defined by its position, shape, polarity, morphology, and state of differentiation of the keratinocytes. The basement membrane separates the epidermis from the dermis in which fibroblasts are located among collagen and elastic fibres. The middle column of cells represent an epidermal proliferation unit (EPU), which is considered the progeny of a single stem cell [3,5,42,44]. SC represents a putative keratinocyte stem cell, which is able to self-renew and give rise to TA cells. TA denotes a transit-amplifying keratinocyte cell. Not shown in the figure are epidermal appendages, and hypodermis, which primarily consists of adipocytes. For the simplification of figure, melanocytes, Langerhans cells and Merkel cells (of epidermis), and macrophages and mast cells (of dermis) are also not shown.

(5–10 layers) cell layers (Figure 7.1). Each layer is defined by its position, shape, polarity, morphology and state of differentiation of the keratinocytes. The basement membrane, which lies in between the dermis and epidermis, serves as a support for keratinocyte migration during wound healing. The morphology of keratinocytes varies with the epidermal layers in which they reside. The basal layer which directly contacts the basement membrane contains proliferating and stem keratinocytes. Keratinocyte cells of the basal layer are columnar or cubical and possess a network of 10 nm keratin intermediate filaments that reaches the hemidesmosomes and cell-cell junctions, called desmosomes [4].

The population of basal keratinocytes expands because these cells are mitotically active. Eventually, some keratinocytes detach from the basement membrane, lose their proliferative potential and begin to move outward towards the skin surface. When the basal cells enter the spinous layer, the first change to occur is the strengthening of their intermediate filaments network to increase the cellular tensile strength. Cells achieve this by synthesizing new sets of keratins, which assemble into intermediate filaments that aggregate into more resilient bundles or cables. Intermediate filament cables anchor to desmosomes, thus distributing force not over individual cells but over the entire tissue [4].

Suprabasal cells connected by desmosomes, move in tandem towards the granular layer where they produce the epidermal barrier. Granular keratinocytes are flattened and lay parallel to the skin surface. The granular layer is the last of the viable epidermal layers. Granular cells synthesize a number of structural proteins that form the cornified envelope of cornified layers. As the cells enter the final phase of terminal differentiation, cells cease transcriptional and metabolic activity and undergo programmed cell death, which is a different physiological response than cellular senescence. In the later process, cells cease to divide but remain metabolically active. The corneocytes of cornified layers have the greatest dimensions of all the keratinocytes and are devoid of nucleus and cytoplasmic organelles. They are made of a dense filamentous keratin matrix and a thick cornified envelope consisting of cross-linked proteins.

7.2.1.2 Melanocytes

Melanocytes originate from the neural crest and migrate into the epidermis. They are distributed regularly among basal keratinocytes. Their main function is represented by the synthesis of melanin, the natural pigment of the

skin. Mature melanosomes, which are intracellular specialized membrane-bound organelles containing melanin are transported along the dendritic processes and transferred to basal keratinocytes. The epidermal melanin unit consists of one melanocyte, which delivers melanosomes to thirty-six associated keratinocytes. Melanization is responsible for skin tone tanning and provides protection to skin against UV damage.

7.2.1.3 Langerhans Cells

Langerhans cells are epidermal antigen-presenting dendritic cells originating from CD34+ haematopoietic precursors of the bone marrow. These cells represent 3–6% of all epidermal cells and are usually found in the spinous layers. Their rounded body contains unique granules, known as Birbeck granules, which are substructures of the endosomal recycling compartment. These cells have dendritic processes extending between adjacent keratinocytes. Langerhans cells uptake exogenous antigens encountered by the skin and process them.

7.2.1.4 Merkel Cells

Merkel cells display both neuroendocrine and epithelial features. These cells are localized in the basal layer of the epidermis and function as mechanoreceptors.

7.2.2 The Dermis

The dermis makes up the bulk of the skin and provides its structural strength. It protects the body from mechanical injury and functions in thermal regulation and as a receptor of sensory stimuli. It interacts with the epidermis in maintaining normal skin structure during embryogenesis, repair, and remodeling. The dermis is an integrated system of fibrous, filamentous, and amorphous connective tissue that accommodates several cells such as fibroblasts, monocytes/macrophages, mast cells, neutrophils and lymphocytes, nerve and vascular networks, and the appendages formed by the epidermis.

7.2.2.1 Fibroblasts

Dermal fibroblasts produce and organize the extracellular matrix of the dermis. They also communicate with each other and other cell types.

Fibroblasts play a crucial role in regulating skin physiology and cutaneous wound repair. Normal adult human skin contains at least three distinct subpopulations of fibroblasts—papillary, reticular, and follicular. Phenotypic differences between these fibroblasts population are manifested in extracellular matrix production and organization, production of growth factors/cytokines, and participations in inflammatory responses.

7.2.2.2 Macrophages

Macrophages are derived from precursor cells of the bone marrow that differentiate into monocytes in the blood and become terminally differentiated in the dermis as macrophages. They function in processing and presenting antigens to lymphoid cells.

7.2.2.3 Mast Cells

Mast cells are specialized secretory cells of bone marrow origin, sparsely distributed around vessels of the dermis. They are responsible for immediate-type hypersensitivity reaction in the skin and are involved in the production of subacute and chronic inflammatory disease. Mast cells contain characteristic cytoplasmic granules and respond to exogenous stimuli by releasing the content of their granules.

7.2.3 The Hypodermis

The Hypodermis is a fatty tissue representing the deepest part of the skin. It plays an important role in thermoregulation, provision of energy, and protection from mechanical injury. The main cells of the hypodermis are the adipocytes, which present a lipid-laden cytoplasm compressing the nucleus against the cell membrane. They are of mesenchymal origin and are organized in lobules defined by septa of fibrous connective tissue, where the nerves vessels and lymphatics are located.

7.2.4 Epidermal Appendages

Epidermal appendages such as hair follicles, sebaceous glands, and sweat glands are specialized epithelial structures, connected to the surface of epidermis and are located mainly in the dermis and hypodermis [1,2,5,6].

7.3 Cellular Senescence

Most if not all human cells irreversibly arrest growth with a peculiar large and flat cell morphology after a limited number of cell divisions in culture [7,8]. This process known as cellular senescence was first described by Hayflick and colleagues in cultured human fibroblasts [9]. Since the time of Hayflick's discovery, cellular senescence has been described in other cell types such as keratinocytes, epithelial cells, endothelial cells, and melanocytes. It is likely that in higher organisms, all cell types capable of undergoing mitotic divisions undergo cellular senescence in culture and possibly *in vivo*. Since the predominant cause of cellular senescence in culture appears to be mitosis or repetitive cell divisions, cellular senescence is also described as replicative senescence (Figure 7.2).

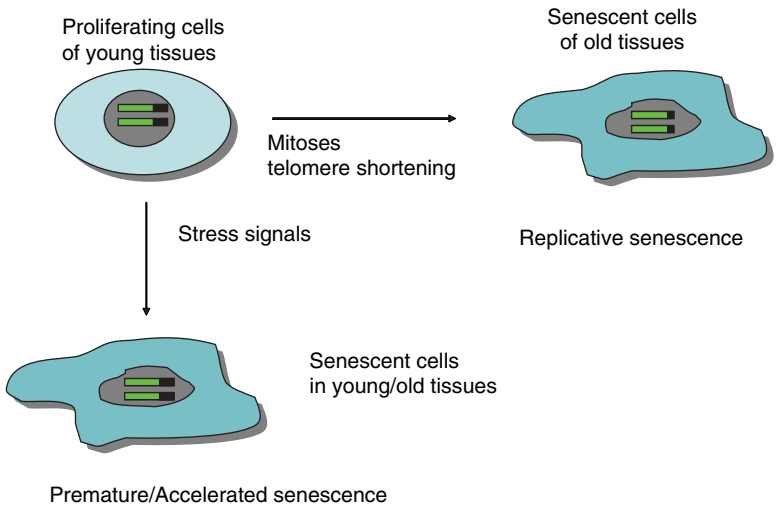

Figure 7.2 Senescent cells are end result of two types of senescence replicative and premature senescence. Replicative senescence is caused by cell divisions (mitoses), during each cell division, telomeres (in black color) at the end of each chromosome (in green color) shortens about 50–100 bp (base pairs). When telomeres length gets critically short to 1–2 kb (kilobase) from 8–12 kb, cells stop dividing and acquire a distinctive morphology. On the other hand premature or accelerated senescence is caused by stress signals. During premature senescence, telomere length remains unchanged; however, the distinctive morphology is still acquired by the cells.

It is thought that cellular senescence in culture reflect an aging process *in vivo*, and hence is also known as cellular aging [8]. Human cells have linear chromosomes, each chromosome shortens from its ends, or telomeres, during every round of cell division. Because human somatic cells lack enzyme telomerase, which rebuilds telomere ends, human chromosomes keep shortening, eventually sending a DNA damage signal to cells and withdrawing permanently from the cell cycle, leading to replicative senescence [7]. Recent evidence suggests that cells also undergo senescence in response to various stress signals [7], such as inappropriate activation of oncogenes, strong mitogenic signals, direct DNA damage caused by genotoxic agents and radiation, and chromatin remodeling agents [8]. Cellular senescence induced by stress signals is known as premature or accelerated senescence (Figure 7.2). Thus cellular senescence refers to replicative and premature senescence, the former type of senescence is caused by repeated cell divisions, while the later type of senescence is caused primarily by stress causing agents (Figure 7.2). Both type of senescence display overlapping phenotypes and may be equally important for tissue or organ aging in an organism.

Different proteins are involved in the maintenance and generation of senescent phenotype. One such protein is p53, a tumor suppressor that is mutated in large number of human cancers. It is a transcription factor and acts as a tumor suppressor, in part by inducing its targets such as p21 protein, which is an inhibitor of cell cycle progression. In human fibroblasts, dysfunctional telomeres signal via tumor suppressor p53 and its target p21 to stop cell proliferation and set up the early stage of cellular senescence. The late stage of cellular senescence is maintained by retinoblastoma tumor suppressor pRb, which is also mutated in human cancers. pRb acts via another cell cycle regulatory protein p16 which blocks activity of cyclin-dependent kinases (CDKs), and allows cells to permanently withdraw from the cell cycle. In other cell types such as human mammary epithelial cells, prostate epithelial cells and keratinocytes, pRb-p16 pathway plays the predominant role in inducing cellular senescence [7,10]. Stress signals also induce senescence via p53-p21 and/or pRb-p16 pathways [7]. Using senescence-associated β-galactosidase (SA-β-gal) as a biomarker to detect senescent cells, we have shown that in many tissues, senescent cells accumulate with advanced age [8,11]. Senescent cells are also detected using other markers such as markers of DNA damage, which occur frequently in senescent cells [12].

7.4 Cellular Senescence of Various Skin Cell Types

As described above, various cell types such as fibroblasts, keratinocytes, melanocytes, and Langerhans cells are integral part of the human skin, and they all undergo cellular senescence when cultured *in vitro*. It is very likely that cellular senescence of these different cell types contributes to overall deterioration seen in aging skin. Here, we give a brief updated overview about the cellular senescence of these various cell types in culture and aging skin *in vivo*.

7.4.1 Fibroblasts

Senescence in fibroblasts derived from dermal layer of the skin has been reported in several studies. Although, the data are not very conclusive, in general, dermal fibroblasts from older donors possess less replicative potential and senesce much earlier compared to dermal fibroblasts from young donors [13,14]. However, in culture, dermal fibroblasts regardless of donor's age always undergo cellular senescence. Previously, we have studied senescence in skin biopsies from young (<40 yr of age) and old (>69 yr of age) donors [11]. While the dermis layer in young donors was mostly negative for SA-β-gal marker, which suggest absence of significant number of senescent cells. On the other hand, the dermis layer from old individuals scored detectable number of SA-β-gal positive cells, indicating that the cells in dermis (fibroblasts), undergo senescence *in vivo* [11].

Accumulation of senescent fibroblasts also has been reported in skin biopsies from *Macaca mulatta* species of primates [15]. In this study, it was shown that the number of SA-β-gal positive cells increased with age in skin biopsies from ad libitum fed monkeys. Using a different set of biomarkers of senescence, as high as 15 percent of cells, presumably fibroblasts, has been reported to be senescent in aging skin from baboons [12]. Increase in the number of SA-β-gal positive fibroblasts has also been reported in elderly patients with pathogenic conditions such as chronic skin wounds such as venous leg ulcers, diabetic ulcers and pressure sores [16–21]. These studies suggest a potential role of cellular senescence in the development of chronic skin ulcers.

7.4.2 Keratinocytes

Epidermal keratinocytes have been used as a model to study growth, proliferation and senescence in culture [22,23]. It is known that serial cultivation of

epidermal keratinocytes eventually results in replicative senescence [24–27]. Using SA-β-gal marker of senescence, we have reported that skin biopsies from old donors contain increased number of senescent keratinocytes in the epidermis [11]. Similarly, it has been reported that compared to young donors, keratinocytes and skin equivalents from old donors exhibit less growth potential and undergo senescence rapidly in culture [28]. The proportion of epidermal stem cells, which reside in the basal compartment also decreases with age in vivo and passage in culture [29]. We have recently suggested that decrease in proliferation and induction of senescence in keratinocytes result due to accumulation of p16 and that its downregulation maintains stem cell features, bypasses senescence and immortalizes keratinocytes [30].

7.4.3 Melanocytes and Langerhans Cells

In contrast to fibroblasts and keratinocytes, very little is known about the senescence of two other cell types- melanocytes and Langerhans cells found in skin epidermis. We have reported that neonatal and adult melanocytes undergo cellular senescence in culture and can be identified using SA-β-gal staining. However, correlation between senescence in melanocytes and donor's age has not been studied in detail. Senescence in melanocytes requires p16 but not ARF tumor suppressor, and senescence in general is not regulated by p53 in p16 expressing melanocytes [31]. Cellular senescence in Langerhans cells is not reported but decrease in number and density of melanocytes and Langerhans cells in skin with advanced age has been reported [32].

7.5 Altered Gene Expression Pattern of Senescent Skin Cells

To gain insight into aging process in general and skin aging in particular, gene expression analyses of dermal fibroblasts undergoing senescence in culture has been performed. It was found that during senescence, fibroblasts switch from extracellular matrix (ECM) synthesizing to ECM degrading phenotype by upregulating matrix metalloproteinases (MMPs) and downregulating the expression of tissue inhibitor of metalloproteinases (TIMPs), collagen I and other matrix proteins [33–35]. More recently, microarray-based analyses have been performed to uncover the gene expression signature of aging skin. These studies have compared dermal fibroblasts that are proliferating or undergoing cellular senescence in culture [36,37].

Dermal fibroblasts from individuals with premature aging syndromes such as Werner Syndrome and Hutchinson-Gilford Syndrome also has been used in such comparative gene expression profiling studies [38,39]. The appearance of skin tissue of the individuals with premature aging syndrome resembles naturally aged skin of old individuals, hence these aging syndromes also offer a suitable model to study skin aging. A more recent study has used dermal fibroblasts from young and old individuals, and dermal fibroblasts that have been made replicatively senescent in culture [40]. In general, the genes that were found to be misregulated in these studies belong to two functional groups.

1. Genes which encode proteins related to cell cycle progression such as c-Fos, c-Myc, Cyclin A, Cyclin B, polo kinase PLK, centromere-associated protein CENP-A and CENP-F, and c-Myb. In general, these genes are downregulated in senescent fibroblasts and dermal fibroblasts derived from old donors and progeria patients.
2. Genes which encode proteins associated with maintenance and remodeling of ECM, such as Stromelysin 1, Stromelysin 2, Collagenase, Gelatinase, Human Macrophage Metalloproteinase (HME), tPA, uPA, PAI 1, PAI 2, Cystatin M, Thrombospondin, Dermatopontin, Fibromodulin, Collagens VI and XV, Elafin, Cathepsin D, Cathepsin L, Serpin b2, and other ECM associated proteins. These proteins are upregulated in senescent fibroblasts and dermal fibroblasts isolated from aging skin. On the other hand, Elastin is downregulated in senescent dermal fibroblasts.

Other genes, which are upregulated in senescent fibroblasts are related to inflammation such as the one that codes for IL-1β, IL6, IL-15, MCP1, and Cox2. Study by Ly et al. suggest that compared to dermal fibroblasts from young donors, Cox2 transcript levels are decreased in fibroblasts from middle aged donors but then are increased in fibroblasts from old donors and progeria patients [39].

While the aforementioned studies have focused on dermal fibroblasts, a recent study compared the transcriptome of proliferating, and senescent keratinocytes, which are either undergoing accelerated senescence due to confluency or becoming replicatively senescent [41]. Although the accelerated senescence due to confluency is likely to occur only *in vitro*, it may occur also *in vivo* due to potential DNA damage causing agents. It was

reported that similar to dermal fibroblasts, cell cycle regulatory genes, such as CDC2, Ki67, Cyclin A2, Cyclin B1, and Cyclin B2 are downregulated in replicatively senescent keratinocytes and keratinocytes undergoing accelerated senescence due to confluency. Among keratinocytes differentiation related genes, SPR1A, SPR1B, Envoplakin, Involucrin, Keratin1 and 15, Profillagrin, and Transglutaminase were found to be upregulated in both accelerated and replicatively senescent keratinocytes [41]. However, few keratinocyte differentiation related genes such as LAMC2, WNT7A, DKK1, LAMC2 and KRTHA4 were downregulated in senescent keratinocytes when compared to proliferating keratinocytes in culture. The third category of genes that were differentially regulated in proliferating and senescent keartinocytes was genes related to inflammation. Among this category, most genes such as TRAIL, SAA, OAS-1, STAT-1, Il-6, IL-1β, IFI-27, 35 and 44, MX1, and UPA were upregulated in replicatively senescent keratinocytes but downregulated in keratinocytes that were undergoing accelerated senescence induced by confluency. Lastly, activated Notch (N^{IC}-1), a regulator of stem cell phenotype was downregulated in replicatively senescent keratinocytes but was upregulated in keratinocytes undergoing accelerated senescence caused by confluency [41].

7.6 Skin Stem Cells and Their Relevance to Skin Aging

Keratinocyte stem cells typically divide to maintain homeostasis of epidermal tissue. As a stem cell daughter commits to differentiate, it first enters a transient state of rapid proliferation (Figure 7.3). These rapidly dividing cells are called transiently amplifying (TA) cells (Figure 7.3). Following several cycles of division, the TA cells withdraw from the cell cycle and execute a terminal differentiation program [42]. A TA cell can also give rise to senescent cell or undergo apotosis (Figure 7.3). Adult stem cells reside in specific niches that provide a microenvironment that is important in protecting and perpetuating the undifferentiated state of resident cells [5]. When histological evaluation is performed, there is no obvious morphologically distinct region or niche found in the epidermal basal layer where stem cells might be located. Histological analysis and lineage marking experiments, based on genetically-modified xenografts of human foreskin transplanted onto nude mice, demonstrated that descendents of a single stem cell formed a column of cells stretching from the basal layer to the surface. This column of cells forms a distinct spatial unit termed as epidermal proliferation unit (EPU) (Figure 7.1) [42–44].

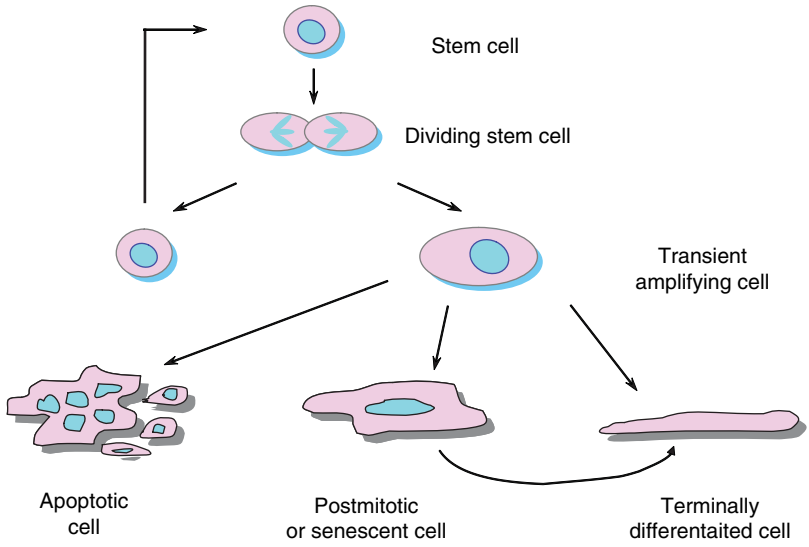

Figure 7.3 A simplified view of stem cell fate in the skin. A skin stem cell asymmetrically divides and gives rise to a self-renewing daughter stem cell and a transient amplifying (TA) cell. TA cells differentiate and give rise to spinous, granular and stratum corneum layers. After several rounds of cell divisions, these TA cells may become senescent TA cells, which may not differentiate into cells of spinous, granular and stratum corneum layers. Senescent cells could also arise due to stress signals, to which skin is inadvertently exposed such as excessive UV radiation. These stress signals can also cause TA cells to undergo programmed cell death or apoptosis. Both senescence and apoptosis can compromise the integrity and appearance of the skin tissue.

Keratinocyte stem cells are thought to be dispersed along the basal compartment, residing in both rete ridges and over dermal papillae, and do not appear to be clustered at any specific location in the basal compartment [43]. Early studies demonstrate that primary culture of skin keratinocytes derived from skin epithelium can be maintained for hundreds of passages without undergoing senescence suggesting the presence of keratinocyte stem cells in the primary culture [25,26]. Detailed analysis of primary culture of keratinocytes suggest that the stem and TA keratinocytes are present in it, and that stem and TA keratinocytes can be identified according to their different proliferative characteristics *in vitro*. Once a keratinocyte clone has been derived, its growth potential can be estimated from the resulting colony type. There are three types of keratonocyte clones- holoclone,

meroclone, and paraclone (Figure 7.4) [45]. Holoclone can give rise to meroclone and paraclone but reverse does not happen. The holoclone is the smallest colony-forming cell. It has the highest proliferative capacity, and is likely to be the keratinocyte stem cell (Figure 7.4). The meroclone and paraclone are considered young and old TA cells respectively. Meroclones become senescent after few rounds of passaging in culture and give rise to paraclones. The paraclone displays large flat morphology reminiscent of senescent fibroblasts or terminally differentiated cells [45–47]. Thus, in culture, holoclone, meroclone and paraclone possess highest, intermediate and lowest colony forming capabilities respectively (Figure 7.4).

Skin keratinocyte stem cells are involved in tissue homeostasis as well as skin regeneration and repair processes. As skin shows an age-related decline in the rate and/or efficacy of normal cellular turnover and regeneration in response to injury or stress, this functional decline could be ascribed to the intrinsic aging of stem cells or to the impairment of stem-cell function in the aged tissue environment with which they dynamically interact. Compared to skin biopsies from old donors, the skin biopsies from young donors contain more cells that stain positive for various skin stem cell associated markers such as p63, PCNA, CD71, and α6 integrin [29]. Keratin-19 (K-19) also has been proposed to be a skin stem cell marker. It has been shown that the number of K-19 expressing keratinocytes decreases with increasing donor age, suggesting a chronological depletion of skin stem cells [28,48].

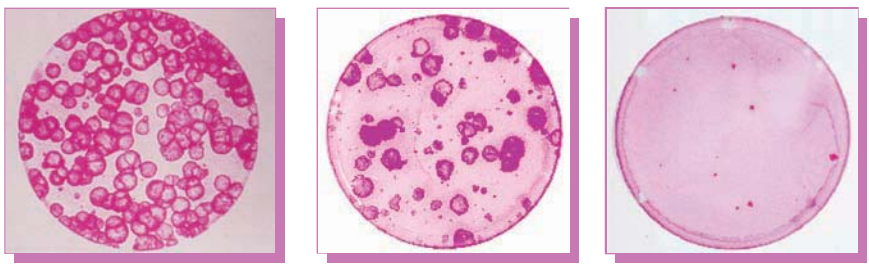

Figure 7.4 Holoclone, meroclone, and paraclone. Colonies obtained from secondary cultures, each originated from a single cell were seeded into indicator dishes and cultivated for fourteen days. After fourteen days colonies were fixed, stained with rhodamine, and photographed. Note that the dish conatining holoclone contained the most number of colonies indiacating the highest prolifeartive potential, and paraclone dish coantined the least number of colonies indicative of minimal or no proloferative potential.

Within their niche, stem cells respond to the extracellular matrix signals, to other cells within the niche and to paracrine factors. Alteration of these signals could also decrease the propensity of keratinocyte stem cells to generate sufficient functional progeny for effective regeneration of skin. Moreover, within the tissue, the stem cell/niche unit is influenced by soluble factors derived from parenchymal cells. Secretion of these factors is influenced by systemic immunological and neuroendocrine signals. In fact, human skin aging is thought to be affected by modifications in growth factors and various hormones that decline with age. Also, external environmental stimuli act on organs or tissues to influence stem-cell function. In human skin aging, chronic sun exposure induces cellular changes, which are superimposed on chronological skin aging. Thus, the age-related functional decline of stem cells could be ascribed to chronological depletion of stem cells, various paracrine factors, external factors such as chronic sun exposure, and aging of tissue environment in which stem cells reside [49–51].

7.7 Possible Contribution of Cellular Senescence to Skin Aging

As we discussed here, cellular senescence is accompanied by several undesirable changes in gene expression, which can directly or indirectly influence and exacerbate skin aging. Skin aging has two components—extrinsic aging and intrinsic aging [52,53]. The major cause of extrinsic aging is UV induced damage to skin during exposure to sun. Because of its relation to sun exposure, extrinsic aging is also known as photoaging. On the other hand, intrinsic (or chronological) aging is caused by progressive time-dependent changes in skin tissues. This type of aging is complex and has multiple genetic and epigenetic components, which are common to other bodily tissues. In mitotically active somatic cells, these genetic and epigenetic changes occurring over time may results in cellular senescence. Since skin contains mitotically active or mitosis competent cells, it is very likely that cellular senescence and gene expression changes associated with it directly contribute to aging of skin.

Cellular senescence-associated genes contribute to intrinsic aging of skin primarily by affecting ECM structure. As described above, activity of two key matrix metalloproteinases MMP1 (Collagenase) and MMP3 (Stromelysin) is substantially upregulated in senescent fibroblasts, while activity of TIMP1 and TIMP3 is reduced due to downregulation of genes encoding these proteins. This imbalance of TIMPs and MMPs, coupled

with low collagen and low elastin biosynthesis by senescent cells can cause dermal thinning and increased wrinkling as seen in aging skin.

This differential gene expression pattern of senescent cells can also augment the effects of photoaging by further increasing the activities of MMPs that are also induced by UV radiation. In fact, it has been shown that chronic UV irradiation induces premature senescence accompanied by upregulation of MMP1 in dermal fibroblasts. Thus, accelerated senescence in skin cells by UV radiation may also directly contribute to photoaging [54]. Furthermore, the reduced proliferative capacity of senescent cells due to downregulation of cell cycle regulatory genes ensures that dermal repair is impaired and cells that are lost due to excessive photoaging are not replenished.

Progressive depletion of skin stem cell pool, which has been postulated to occur with advanced age possibly due to induction of cellular senescence, may also affect skin healing and exacerbate intrinsic and extrinsic aging.

7.8 Concluding Remarks

In this chapter, we have reviewed current literature about cellular senescence and aging in skin and skin derived cells such as dermal fibroblasts and keratinocytes. Different cell types present in skin contribute to skin aging in various ways depending on the natural function of a particular cell type. Apart from cell type-specific functions, a common phenotype termed cellular senescence, which all these cell types undergo contribute to a differential gene expression profile, which is detrimental to well being of skin such as physical appearance of the skin, and its ability to withstand natural wear and tear. Cellular senescence may also contribute to the depletion of skin stem cell pool, which could affect the regeneration of various skin cell types, which differentiate to generate various skin layers.

Recent work from various laboratories including our's suggest that p16 inactivation and/or Bmi-1 overexpression can maintain stem cell characteristics in keratinocytes and other cell types. These studies offer intriguing possibilities of maintaining stem cell pools in skin and delaying senescence and ultimately skin aging by using reagents that either downregulate p16 expression or upregulate and maintain Bmi-1 expression [30,55,56]. Along these lines, constitutive expression of hTERT (catalytic subunit of telomerase), another widely expressed gene in stem cells, has been shown

to restore dermal integrity in a reconstituted skin model *in vitro* [57]. Similarly, recently it has been suggested that expression of various defined transcription factors such as Oct3/4, Nanog, Sox2 and Tcf4 can maintain stem cell-like characteristics and can even induce de-differentiation of adult fibroblasts and keratinocytes [58–60]. These studies provide new and exciting avenues of research in skin aging and offer possibilities of postponing aging and its unwanted effects on skin.

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8

Prevention and Treatment of Aging Skin with Topical Antioxidants

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8.1 Introduction

According to zoologist Desmond Morris, flawless skin is the most universally desired human feature. Skin is not only the body's most aesthetic organ, it is also the most pervasive—indeed, skin is the largest organ of the body. Our skin is our calling card, our presentation to others. There is no way to be beautiful without healthy, unblemished skin.

The state of our skin influences our psychologic outlook. Numerous studies have proven that we feel more confident and happier when we look good—for which the appearance of younger, undamaged skin is essential. We actually do *feel* younger, more energetic, and more optimistic when we *look* younger by treating the unattractive wrinkles and dark spots of aging skin. Baby boomers want to look as young as they feel. New products exist which do indeed revitalize aging skin. This chapter describes some of the most effective, scientifically proven formulations.

As the outermost organ of our body, our skin is a shield protecting us from harmful exposure to solar ultraviolet (UV) radiation and air pollutants. This exposure results in direct damage to the nucleus of the cell itself and in formation of reactive oxygen species (ROS) and other free radicals that subsequently react with important molecules in connective tissue and cell membranes. Clinically, the *acute* UV-induced skin damage is seen as the erythema, edema, and blistering in sunburn followed by peeling, then tanning (the mark of long-lasting damage). *Chronic* UV-induced damage results in the appearance of premature aging of the skin (photoaging) with wrinkles, mottled dark spots, a dry, leather-like texture, and loss of elasticity as well as in precancers and cancers that not only leave deforming scars, but can also be fatal if not treated.

These medically damaging and unattractive results of sun damage can be prevented. Obviously, applying sunscreens frequently is essential, though not enough. The skin naturally uses a variety of antioxidants which interact at different levels of oxidative processes to scavenge and remove free radicals and oxidatively damaged molecules. However, this antioxidant defense is overwhelmed by the oxidative stress of excess UV exposure, as well as cigarette smoke and other airborne pollutants. Fortunately, applying high concentrations of natural, topical antioxidants can overcome and correct this damage. Applied topically, far higher concentrations are attained in the skin than ever possible, even by taking high doses orally. Furthermore, once absorbed, the antioxidants cannot be washed, perspired,

or rubbed off—giving protection for up to several days, thereby enhancing sunscreens that must be applied frequently. And regular use of topical antioxidants can actually *reverse* pervious photodamage.

The challenge is to know which antioxidant products at which concentrations and in which combinations to apply. We are bombarded with advertising promoting many commercial preparations of antioxidants; however, few are truly effective. Antioxidants are by nature quite unstable molecules when exposed to oxygen in air: Think how quickly an apple turns brown after being cut—that is oxidization. Also many products contain ester derivatives of the antioxidants or non-natural isomer forms that cannot be absorbed and/or metabolized by the skin. Furthermore, most preparations contain such low concentrations of the antioxidant that, even if the correct molecule were used, there is not enough for protection from or treatment of free-radical damage.

This chapter focuses on the most prevalent natural water-soluble, intracellular antioxidant L-ascorbic acid (vitamin C), the most important lipid membrane antioxidant d-α-tocopherol (vitamin E), and the essential trace mineral selenium (the required cofactor for several important free-radical-quenching enzymes), formulated for topical application as L-selenomethionine.

The requirements for (1) stability, (2) bioavailability—delivery of the correct form to give optimal absorption and metabolism after topical application, and (3) effectively-high concentrations for each antioxidant will be described. Synergy in activity by specific combinations of antioxidants will be discussed. Research demonstrating effective protection from UV and other free-radical damage will be presented.

8.2 Vitamin C

Vitamin C (L-ascorbic acid) (see Figure 8.1) is the body's most important intracellular and extracellular aqueous-phase antioxidant. It is also required for the regulation of many cellular and enzymatic activities and is therefore essential for life. Most animals synthesize vitamin C; only humans and other primates, the Indian fruit-eating bat, and the guinea pig lack the enzyme L-glucono-gamma-lactone oxidase to synthesize vitamin C from glucose-derived glucaronic acid. A 59-kg goat synthesizes 13 g of vitamin C per day, almost 200 times the American Food and Drug Administration (FDA) requirement.[1] Not only do other animals make hundreds of times

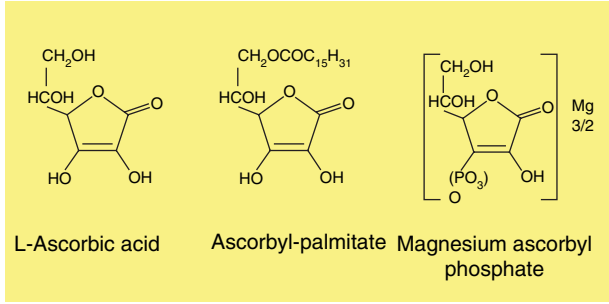


Figure 8.1 The molecular structure of L-asorbic acid, L-ascorbyl-6-palmitate, and magnesium ascorbyl phosphate.

the vitamin C we ingest, but they also synthesize more than ten times their normal amount when under stress.

We humans must obtain vitamin C solely from our diet—citrus fruits, black currants, red peppers, and leafy green vegetables. Because active transport from the gastrointestinal tract is limited, unfortunately even massive oral doses do not increase the concentration to the optimal levels for photoprotection in the skin, levels that can be attained through topical application.

However, sufficient dietary vitamin C does provide vitamin C to both the dermis and epidermis of animals and humans. In human skin, the epidermis contains about fivefold higher levels than the dermis.[2] This difference may reflect an increased utilization for the regulation of collagen and elastin biosynthesis,[3] or facilitated transport for vitamin C from the dermal blood vessels to the epidermis. The epidermis is not only exposed to the environment, but also requires vitamin C for efficient formation of the stratum corneum barrier.[4] Isolated human stratum corneum contains only very low ascorbate levels, as compared with levels in subjacent epidermal layers,[5] possibly because the stratum corneum is hydrophobic and highly exposed to the environment.

Indeed, exposure to sunlight and pollution actually depletes vitamin C from the outer layers of the skin. Even minimal UV exposure of 1.6 minimal erythema dose (MED) decreases the level of vitamin C to 70 percent of the normal level, and exposure to 10 MED decreases the vitamin C to only 54 percent.[6] Exposure to 10 parts per million of ozone in city pollution decreases the level of epidermal vitamin C by 55 percent.[7]

8.2.1 Formulation

Increasing the level of vitamin C in the skin by topical delivery can be extremely beneficial. However, active L-ascorbic acid is such an excellent antioxidant that it is inherently unstable, turning brown as it is oxidized to dihydroascorbic acid when exposed to air. Therefore the shelf life of most formulations containing pure vitamin C is short. Although many products with vitamin C in lotions, creams, serums, and patches are sold, few are effective.

To overcome the problem of instability, the more stable esterified derivatives ascorbyl-6-palmitate and magnesium ascorbyl phosphate are often used (see Figure 8.1), but these derivatives are not well absorbed[8] and are only minimally metabolized by the skin to the active, free ascorbic acid form.

To achieve the benefits to the skin with topical vitamin C, the formulation must (1) contain L-ascorbic acid (2) in a high enough concentration (at least 10 percent), (3) be stable, and (4) be at an acid pH—less than the pKa (=4.2) of vitamin C. (The optimal pH of a vitamin C formulation is 3.5.)

Effective skin levels of vitamin C can be attained if the above criteria are met. Radioactive labeling studies in pigs proved successful topical absorption. After treatment with 10 percent vitamin C cream, 8.2 percent was found in the dermis, and 0.7 percent was in the blood.[8] Formulations containing 5 percent, 10 percent, 15 percent , 20 percent , or 25 percent vitamin C were tested: 20 percent resulted in the highest skin levels, with maximized concentration in the skin after 3 days of once daily application.[9] In fact, levels of vitamin C after topical application of 15 percent serum are a factor of about 27 times that which could ever be attained by even very high oral intake. If topical application is discontinued after skin saturation is achieved, effective levels still exist in the skin for more than three days.[9]

8.2.2 Actions and Efficacy

Vitamin C provides many benefits to the skin—most significantly, increased synthesis of collagen and photoprotection.

Vitamin C is absolutely essential for synthesis of collagen. Vitamin C is the essential cofactor for the two enzymes required for collagen synthesis: prolyl hydroxylase (to stabilize the collagen molecule) and lysyl hydroxylase (to give structural strength cross-linking).[10] Recent research has further

demonstrated that vitamin C acts directly on DNA to increase the transcription rate and to stabilize the procollagen messenger RNA, thus regulating and maintaining the intercellular amount of collagen.[11]

By enhancing collagen synthesis, vitamin C also has anti-aging effects. Studies *in vitro* compared newborn with elderly fibroblasts (derived from newborn foreskins after circumcision and from 80–95 year-old patients, respectively).[12] Elderly cells proliferate *in vitro* at only one-fifth of the rate of newborn cells. However, when vitamin C is added to the culture medium, the elderly fibroblasts increase proliferation by a factor of six to propagate even faster than normal unsupplemented newborn fibroblasts. Even the newborn fibroblasts proliferate almost four times better when exposed to vitamin C.[12] (see Table 8.1)

Not only do fibroblasts increase proliferation in the presence of vitamin C, but they also synthesize more collagen. Newborn fibroblasts synthesize a larger percentage of collagen than elderly cells, but again, when elderly cells are exposed to vitamin C *in vitro*, they double their collagen production, outperforming normal, newborn fibroblasts.[12] Surprisingly, even the newborn cells double the amount of collagen synthesized.[12] (see Table 8.1)

Since vitamin C is essential for collagen synthesis, it is critical for wound healing. With vitamin C deficiency, fibroblasts produce unstable collagen, providing a weak framework for repair. It is well documented that vitamin C-deficient animals show prolonged wound healing, but whether supplemental oral vitamin C can improve wound healing is uncertain. Although severely ill patients with depleted baseline levels certainly benefit from extra vitamin C, one study showed no enhancement of wound healing.[13]

Table 8.1 Anti-Aging Effects of Vitamin C *in Vitro

	Increase in Fibroblast Proliferation			Increase in Collagen Synthesis		
	Cells $\times 10^6$ (day 17)			% Collagen/Total Protein		
Fibroblasts	– Vit C	+ Vit C*	Increase	– Vit C	+ Vit C*	Increase
Newborn	1.0	3.7	×3.7	10.5	19.9	×1.9
Elderly	0.2	1.2	×6.0	6.2	12.4	×2.0

*Adapted with permission from Phillips, C.L., S.B. Combs, and S.R. Pinnell. "Effects of ascorbic acid on proliferation and collagen synthesis in relation to donor age of human dermal fibroblasts." *J Invest Dermatol* 103:228–232, 1994.

In 20 patients with pressure ulcers, vitamin C (500 mg per day vs. placebo) did reduce the area of ulceration compared with controls.[14] Whether topical vitamin C could accelerate wound healing should certainly be further investigated.

In contrast to the increased synthesis of collagen, *in vitro* studies suggest that vitamin C may inhibit elastin biosynthesis by fibroblasts.[15] This may be advantageous in reducing the solar elastosis of photodamage.

Topical vitamin C has also been shown to enhance collagen production in human skin *in vivo*.[16] Postmenopausal women who applied 5 percent vitamin C to one arm and half of the neck with placebo to the other side showed an increase in mRNA of collagens I and III.[16] Tissue levels of the inhibitor of metalloproteinase–I (MMP-I) were also increased, thus decreasing UV-induced collagen breakdown. However, mRNA levels of elastin, fibrillin, and tissue inhibitor of MMP-2 remained unchanged.[16] Clinically, a significant decrease was observed in deep facial furrows and substantiated by silicone replicas. Histology showed elastic tissue repair.

This synthesis of collagen and inhibition of MMP-I contribute to clinical reversal of photoaging, as seen in Figure 8.2. After one year of once-daily

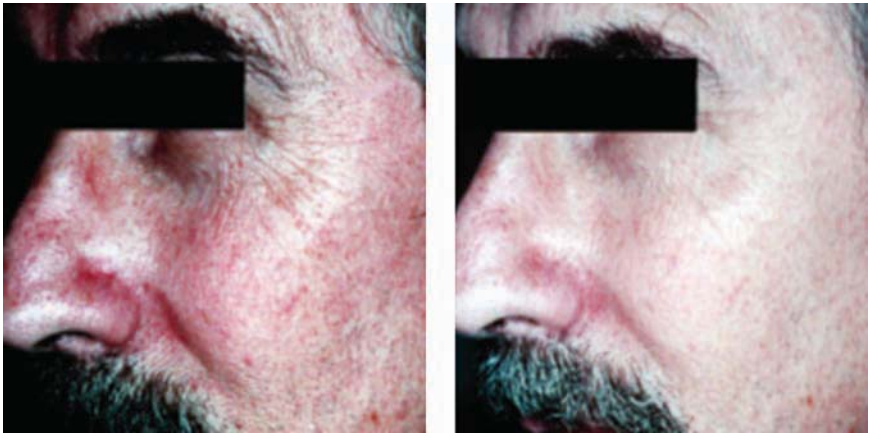


Figure 8.2 Correction of photoaging after one year of once-daily treatment with 15 percent Vitamin C Serum (SkinCeuticals).

Notice the improvement of fine periorbital wrinkles, lightening of solar lentigines, and reduction in redness (i.e., correction of rosacea). (These photographs were provided by SkinCeuticals, Garland, TX, USA and are printed with their permission for Burke KE, Chapter 74. Photodamage of the Skin: Protection and Reversal with Topical Antioxidants. In *Textbook of Cosmetic Dermatology.* R. Baran, H. Marbach, eds. London: Martin Dunitz, Taylor and Francis Group, 2004: 725–736.)

treatment with 15 percent topical vitamin C, wrinkles were clearly reduced and mottled pigmentation resolved. The skin acquired a healthy, more youthful glow.

A new formulation of topical vitamin C (10% percent) with the lipid soluble derivative tetrahexyldecyl ascorbate (7 percent) in an anhydrous polysilicone gel base was shown to improve photoaged skin[17] in a double-blind, half-face study in ten patients. Comparing the vitamin C combination with control gel, statistically significant clinical improvement was noted on the vitamin C-treated cheeks and the perioral area after twelve weeks of treatment. Histological analysis of biopsies showed increased repair in the upper dermis ("Grenze zone") with fine, fibrillar, new collagen and increased staining for mRNA for Type I collagen in the ascorbic acid-treated side. The periorbital areas improved on both sides, which the authors attribute to improved hydration.

The second major action of topical vitamin C is that it has been proven to be photoprotective. Vitamin C is itself not a sunscreen since it has no UV absorption spectra in the UVA or UVB range; however, as an antioxidant it deactivates UV-induced free radicals and decreases UVB erythema in porcine skin by 52 percent.[8] In human volunteers, a less intense erythematous response to UVB was noted on the volar forearms when treated with 10% vitamin C, as compared with controls.[18] Protection by topical vitamin C from chronic UV-induced photodamage and skin cancers was demonstrated by Bisset *et al*[19] in mice. In another mouse study, Dunham *et al*[20] found decreased UV-induced tumors with increased oral vitamin C. This protection was confirmed histologically: Treatment with topical 10 percent vitamin C decreased the number of abnormal 'sunburn cells' by 40–60 percent[8] and reduced the UV damage to DNA 8-hydroxydeoxyguanosine (8-OHdG) by 62 percent in porcine skin.[8]

Photoprotection is enhanced by the anti-inflammatory properties of vitamin C. *In vitro* studies with human cells in vitamin C-enriched media demonstrated decreased activation of the transcription factor NF-κβ (nuclear factor kappa beta), the factor responsible for many pre-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins Il-1, Il-6, and Il-8.[21] In fact, topical vitamin C has been used by dermatologists to treat acne and rosacea.[22] In a split-face study, Alster and West[23] showed a significant decrease in post-CO_2-laser resurfacing erythema after eight weeks of topical treatment with a solution of vitamin C (10 percent), zinc sulfate (2 percent), and tyrosine (0.5 percent) as compared with the untreated side.

By directly decreasing inflammation, post-inflammatory hyperpigmentation is reduced. Also, vitamin C is itself an excellent depigmentating agent because it inhibits the action of the enzyme tyrosinase by reducing *o*-quinones,[24] thereby decreasing melanin production. In the experience of this author, topical vitamin C (15 percent) demonstrated clinical lightening of melasma and solar lentigines even after only two months of daily application. Figure 8.2 shows lightening of solar lentigines and resolution of mottled, uneven pigmentation in an elderly woman after one year of daily application of 15 percent vitamin C serum.

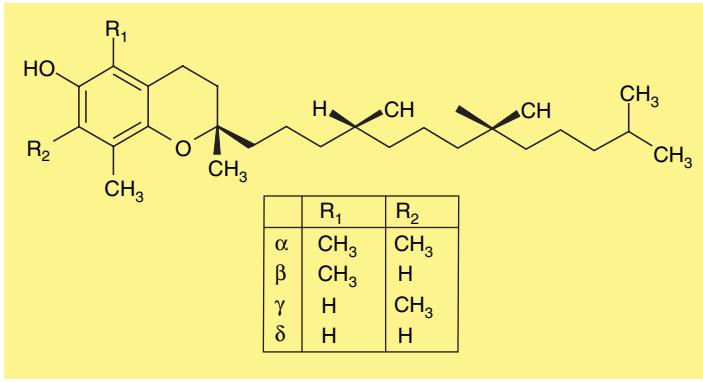
Furthermore, Kameyama *et al*[25] showed suppression of melanin formation by inhibition of tyrosinase in melanocytes and in melanoma cells by magnesium-L-ascorbyl-2 phosphate. When a 10 percent magnesium-L-ascorbyl-2-phosphate cream was applied to human skin, significant lightening of melasma and of lentigenes was observed in 19 of 34 patients.[25]

All of these proven functions of topical vitamin C contribute to reversal of the appearance of photoaging: Photoprotection over many months allows the skin to correct previous photodamage, the synthesis of collagen and inhibition of MMP-I was proven to decrease wrinkles,[16] and the inhibition of tyrosinase and anti-inflammatory activity result in depigmenting solar lentigines.

Another important action of vitamin C on the skin is that topical vitamin C increases the synthesis of several specific lipids of the skin surface.[26] Thus, not only does vitamin C help the natural moisturization of the skin, but it also was shown to enhance the protective barrier function.[27]

8.3 Vitamin E

Natural vitamin E (see Figure 8.3) is the most important lipid-soluble, membrane-bound antioxidant in plasma, membranes, and tissues. Like vitamin C, vitamin E is supplied to the body solely by the diet. Fresh vegetables, vegetable oils, seeds, cereals, nuts, some meats, and some dairy products provide vitamin E. In the skin, vitamin E is especially abundant in the stratum corneum, delivered there by sebum.[28,29] Its concentration is highest at the lower levels of the stratum corneum, with a decreasing gradient outward. As the outermost defense of the body, the stratum corneum is first to absorb the oxidative stress of sunlight and pollution. With this exposure, vitamin E is depleted, though not as dramatically as is



	R_1	R_2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Figure 8.3 Molecular structure of tocopherols.

vitamin C. Exposure to 10 MED depletes vitamin E by only 4 percent.[6] Also, the concentration of α-tocopherol was found to be significantly lower in the epidermis of photoaged and aged skin (56 percent and 61 percent, respectively, of young skin's concentration).[30] Thus, topical application could be particularly advantageous. The lipophilic structure makes vitamin E an especially attractive for topical application and absorption.

8.3.1 Formulation

Several forms of vitamin E exist in natural dietary sources. The most abundant form in mammalian tissues with by far the greatest biologic activity is pure, non-esterified RRR-α-tocopherol (or d-α-tocopherol),[31,32] which has three methyl groups on the 6-chromal ring. Humans use predominantly α-tocopherol because a specific α-tocopherol transfer protein selectively transfers α-tocopherol into lipoproteins.[33] The other natural forms are β, γ, and δ which contain only one or two methyl groups on the 6-chromal ring (See Figure 8.3). Relative to the α form, the β, γ, and δ RRR-tocopherols give only 42 percent, 72 percent, and 40 percent, respectively, of the protection against post-UV edema.[34] The synthetic form is 'dl' or 'all-*rac*', a mixture of eight stereoisomers. These synthetic isomers are esterified (to acetates and succinates) for use in commercial vitamins and some topical formulations because the esters are far more stable. This ester must be hydrolyzed before there is any biological activity, a reaction which readily occurs in the stomach after oral ingestion or in cell and organ culture, but is very slow after topical application. The skin has only a limited capacity to cleave the esterified forms of vitamin E to the active free tocopherol form, so the antioxidant potential of the esters is minimal.[35,36] In fact, in a mouse

model the acetate and succinate esters were found not only failed to protect, but also enhanced the UV-induced damage.[35] Furthermore, the all-*rac* form of vitamin E has been reported to cause allergic contact dermatitis[37] and erythema multiforme[38] when applied topically. No such adverse reactions have been reported with d-α-tocopherol.

8.3.2 Actions and Efficacy

It is primarily as an antioxidant responsible for quenching lipid peroxyl free radicals that vitamin E protects against acute and chronic UV-induced damage. In addition, unlike vitamin C, vitamin E does absorb UVB (λ_{max} = 292 nm), though less intensely than most sunscreen ingredients. Thus vitamin E may provide some UV protection directly as a sunscreen.

Furthermore, both α-tocoperhol and α-tocopheryl acetate were found to modulate cellular response to protect from UVB damage through action on the cell signaling pathways of NF-κβ activation.[39] Treatment of normal and neoplastic mouse epidermal keratinocytes with UVB markedly decreased viable cell number and caused DNA fragmentation. When both forms of vitamin E were applied to cells before and after UVB, an increase in viable cells and a concomitant decrease in apoptotic cells was noted, with vitamin pretreatment providing better protection than posttreatment. Simultaneous pre- and post-treatment of irradiated cells restored cell viability to control levels, and α-tocopherol reduced apoptotic cells by half. Pretreatment with both forms of vitamin E significantly inhibited UVB-induced NF-κβ activation.

Photoprotection and decreased postinflammatory hyperpigmentation after injury are enhanced by the anti-inflammatory action of vitamin E. Vitamin E decreases the biosynthesis of prostaglandin E_2,[40] possibly by preventing release of arachidonic acid by phospholipase A_2.[41] In a dose-dependent manner, α-tocopherol depresses lipo-oxygenase function in thrombocytes[42] but enhances lipo-oxygenase function and biosynthesis of prostacyclins in neutrophils.[42,43] These modulations to the eicosanoid system result in a visible anti-inflammatory effect by α-tocopherol.

There is extensive literature documenting photoprotection with topical vitamin E. The difficulty in comparing the many studies is that different isomers of vitamin E are used, alone or mixed with other antioxidants, as well as different concentrations, vehicles, treatment schedules, and experimental

models (*in vitro* or animals). In reviewing these many publications, it is evident that even forms of topical vitamin E which are less metabolically potent than nonesterified d-α-tocopherol demonstrate protection from the *acute*[44–45] UV-induced damage of inflammation (erythema, sunburn) and hyperpigmentation (tanning) as well as protection from the *chronic* UV-induced damage of skin cancer.[44,46–47] The following discussion will concentrate only on the most biologically active form of vitamin E—d-α-tocopherol and its esters, as they meet the criteria described in optimal formulation.

In an experimental mouse model, oral d-α-tocopheryl acetate and topical d-α-tocopherol were equally effective in preventing UV-induced pigmentation; topical d-α-tocopheryl succinate was less protective, as shown in Figure 8.4.[44] Interestingly, both dl-α-tocopherol in lecithin and dl-α-tocopheryl ferulate in lecithin were found to inhibit the enzyme tyrosinase to decrease melanogenesis in human melanoma cells.[48] Both also decreased UV-induced DNA damage as measured by reduced 8-OHdG.[48] Indeed, topical vitamin E has been used effectively to treat cholasma and pigmented contact dermatitis, as demonstrated anecdotally and in one multiclinical double-blind study (through the combination of vitamins E and C was better, as described below).

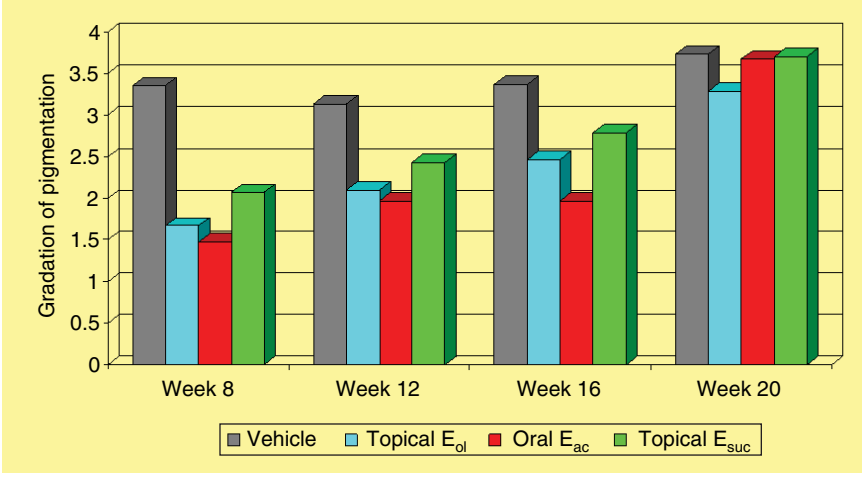


Figure 8.4 Inhibition of ultraviolet (UV)-induced pigmentation by vitamin E.

Values are averages of 15 Skh:2 mice in each treatment group. 0, No hyperpigmentation; 4, maximal hyperpigmentation. Vehicle alone (grey bar); E_{ol}=RRR-α-tocopherol (5%, topical) (blue bar); E_{ac}=d-α-tocopheryl acetate (oral) (red bar); E_{suc}=d-α-tocopheryl succinate (5%, topical) (green bar).[29]

As seen in Figure 8.5, topical d-α-tocopherol (5 percent) was far more effective in protecting against all acute and chronic UV-induced damage than topical d-α-tocopheryl succinate (5 percent) in mice; oral d-α-tocopheryl acetate was about as effective as topical d-α-tocopherol.[44] Other studies substantiate this photoprotection: In rabbits, topical α-tocopheryl acetate was less effective than α-tocopherol in decreasing UV-induced erythema[45] and UV-induced photoaging in mice.[49] However, one other mouse study contradicted these conclusions: Topical α-tocopheryl succinate and α-tocopheryl acetate failed to inhibit UVB-induced immunosuppression and carcinogenesis and actively appeared to enhance carcinogenesis.[50]

Measurement of MED in human volunteers with a solar simulator demonstrated a dose-dependent increase in protection from acute erythema with topical d-α-tocopherol, with concentrations below 1 percent not significantly effective; concentrations of 2 percent to 5 percent did protect, with 5 percent optimal (Burke KE, et al, unpublished research). Higher concentrations

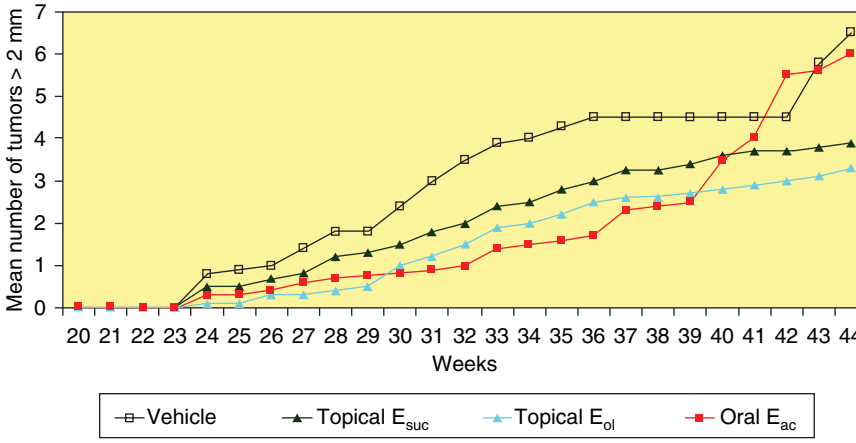


Figure 8.5 Inhibition of UV-induced skin cancer by vitamin E.

Mean number of tumors ≥2 mm in UV-irradiated Skh:2 mice. Beginning 2 weeks before UV exposure, 15 mice in each treatment group were treated thrice weekly throughout duration of experiment with vehicle lotion (open grey squares and filled red squares), topical d-α-tocopherol (5%) (filled blue triangles, blue line), or topical d-α-tocopheryl succinate (5%) (filled black triangles, black line) ≥30 min before UV exposure. In addition, 1 group (filled red squares, red line) was fed a diet supplemented with d-α-tocopheryl acetate (comparable to a human dose of about 600 IU/day). Animals were exposed to UV radiation thrice weekly for 24 weeks, and topical and oral treatments were continued through and thereafter. Number of tumors ≥2 mm on each mouse was counted, and mean number of tumors per mouse was calculated based on total number of 15 mice in each treatment group.[44]

did not substantially further enhance photoprotection. Concentrations over 15 percent were quite oily and difficult to formulate as an elegant, attractive, luxurious cosmetic. Furthermore, this higher concentration could be comedogenic because of the high vitamin E oil content.

Clinically, topical vitamin E does decrease wrinkles and solar lentigines of photoaging. Reversal of photoaging was confirmed histologically in mice. Microscopic examination of skin biopsies showed correction of the UV-induced epidermal hypertrophy, thickened stratum corneum, increased 'sunburn cells' in the basal layer, and disruption of dermal collagen and elastin after 8 weeks of topical treatment (KE Burke, L Ricotti, EG Gross, unpublished research). Further electron microscopic analysis confirmed correction of collagen and elastin fiber damage and demonstrated repair of UV-induced disruption of the basement membrane anchoring fibrils. Interestingly, another study found the derivative α-tocopheryl sorbate (5 percent) to be more effective than α-tocopherol in decreasing free radicals and in protecting against chronic UV-induced photoaging, as measured by decreased wrinkling in mice.[50]

Oral vitamin E has been used to treat many dermatologic diseases—including yellow nail syndrome, epidermolysis bullosa, claudication, ulcers, and wound healing. The efficacy has not been convincing because the reports are anecdotal rather than placebo-controlled prospective studies with specifically the natural d-α-tocopherol form. In a placebo-controlled study of ninety-six atopic dermatitis patients treated with placebo or 400 IU of oral vitamin E for eight months, there was improvement (and sometimes almost remission) in atopic dermatitis with a 62 percent decrease in IgE levels.[51] Topical vitamin E may also prove to be effective in this very common dermatologic disease.

There are conflicting reports about the efficacy of topical vitamin E in preventing and treating hypertrophic scars. Again, few controlled studies were done and the most effective natural d-α-tocopherol form in high concentration has rarely been used.

The effects of topical and oral vitamin E on wound healing remain controversial, despite many studies. Parsa[52] reviewed the literature and concluded that vitamin E may enhance wound healing, probably through its anti-inflammatory actions. In a randomized double-blind study in 57 patients with chronic lower extremity stasis ulcers, Lee[53] found the 28 individuals receiving oral vitamin E (400 IU/day) healed faster and more frequently

than the 29 receiving placebo. Weiser[54] reported accelerated healing of superficial wounds with topical α-tocopherol acetate (5 percent) in humans. In a placebo controlled study of wound healing in porcine skin after CO_2-laser abrasion, topical d-α-tocopherol (5 percent) did increase healing and decrease inflammation (KE Burke *et al*, unpublished research).

8.4 Vitamin C with Vitamin E

In cells, vitamins C and E interact synergistically to provide antioxidant protection. In membranes, vitamin E is oxidized as it quenches peroxyl free radicals. Since the molar ratio or tocopherols to polyunsaturated phospholipids, the first-line targets of oxidative attack, is less than 1:1000, the continued regeneration of tocopherol is essential to maintain its antioxidant activity. The neighboring, plentiful, intracellular vitamin C (with its lower redox potential) reduces the oxidized vitamin E to regenerate its activity so it need not be replaced in the membrane.[55] Furthermore, vitamin C alone and in the presence of transition metal ions, such as iron, may act as a pro-oxidant. However, in rat studies, Wefers and Sies[56] showed that the presence of significant amounts of vitamin E abrogated this pro-oxidant activity of ascorbic acid, shifting to a protective antioxidation.

Combination of vitamin C with vitamin E has proven effective for photoprotection. Oral vitamin C with E in high doses protects against UV-induced erythema in humans, whereas either vitamin alone is ineffective.[57] Topical L-ascorbic acid (15 percent) with α-tocopherol (1 percent) gives four-fold protection against UV-induced erythema, decreasing the number of damaged "sunburn cells" seen histologically and decreasing thiamine dimer formation in porcine skin,[58] compared to twofold protection for either vitamin alone. This protection from UV-induced erythema by vitamins C and E combined with melatonin[59] was further demonstrated in humans. Fortunately, mixing these hydrophilic and lipophilic antioxidants in a topical formulation stabilizes each[58] in a cosmetically appealing serum.

8.5 Vitamin C with Vitamin E and Ferulic Acid

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, Figure 8.6) is a potent antioxidant present in cell walls of grains, fruits, and vegetables where it is conjugated with mono-, di-, and polysaccharides and other compounds. Like vitamin E, ferulic acid interrupts UV-induced peroxidative chain reactions in phospholipid membranes.[60]

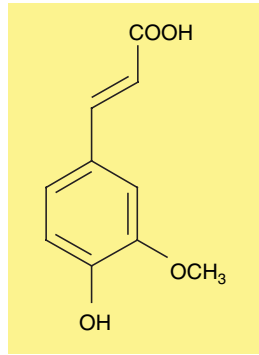


Figure 8.6 The molecular structure of ferulic acid (4-hydroxy-3-methoxycinnamic acid).

Ferulic acid alone absorbs some UV and therefore is itself a weak sunscreen. Topical application has been shown to protect the skin from UVB-induced erythema.[61] When mixed with vitamin C and vitamin E, ferulic acid stabilizes the formulation and acts synergistically to double the photoprotection from fourfold to eightfold.[62] The increase in MED is clearly seen in Figure 8.7. This triple antioxidant combination has been made into the

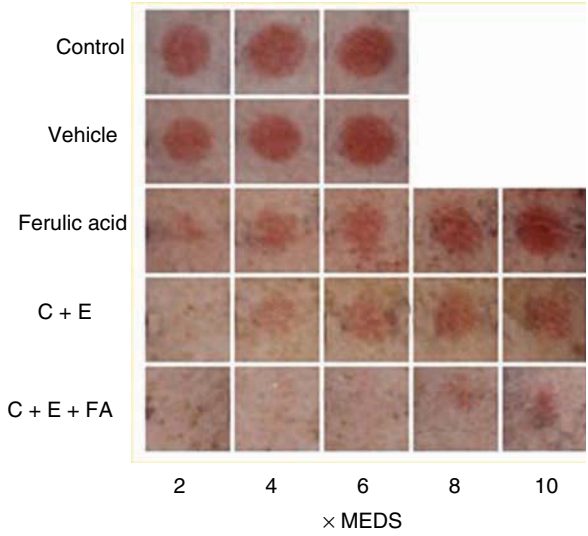


Figure 8.7 Minimal erythema dose after application of antioxidants.

Skin was pretreated with vehicle, ferulic acid (0.5%), vitamin C (15%) with vitamin E (1%), and vitamin C + E + ferulic acid and irradiated with solar simulated radiation of 2× to 10× MED. The increase in MED with topical antioxidant treatment is clearly seen.[62]

SkinCeuticals product "C E Ferulic" (with 15 percent vitamin C, 1 percent vitamin E, and 0.5 percent ferulic acid).

8.6 Selenium

Selenium is an essential trace element in humans and animals. Selenium is unevenly distributed throughout the world. Selenium-rich soils are thought to result from ancient volcanic eruptions and subsequent leeching to ancient inland seas long since evaporated. The southeast United States, the United Kingdom, the Netherlands, Canada, Switzerland, and the Scandinavian countries have quite low levels of selenium; South Dakota and parts of China have such high levels that ruminant cattle sometimes develop metabolic toxicity. Good food sources of selenium include whole grain cereals, seafood, garlic, liver, and eggs. Foods from animal sources are generally richer than those from vegetable sources, so vegetarians should supplement their diet with selenium.

The primary function of selenium is as the required cofactor for the intracellular antioxidant enzymes glutathione peroxidase and thioredoxin reductase.[63,64] Selenium has other protective activities, as described below.

8.6.1 Formulation

Topical preparations containing selenium sulfide are frequently used for the treatment of tinea versicolor,[65] seborrheic dermatitis,[66] and dandruff.[67] However, the selenium from these preparations is not absorbed by the skin.[67] Because the atomic structure of selenium is similar to that of sulfur (selenium is just under sulfur in the chemistry periodic table), selenium can be substituted for sulfur in the amino acid methionine. (see Figure 8.8) Topical selenomethionine is absorbed transdermally, as first demonstrated in guinea pigs:[68] topical application to mice was shown to increase skin and liver levels, confirming absorption.[69] Topical application of L-selenomethionine (0.02 percent) to rats gave 12.0 percent absorption in radioactive labeling experiments (Combs GF and Burke KE, unpublished data). After application of topical L-selenomethionine to volar forearms of human volunteers, tape stripping demonstrated that about 9.3 percent was absorbed (Burke KE, unpublished data).

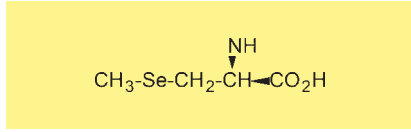


Figure 8.8 The molecular structure of selenomethionine.

8.6.2 Actions and Efficacy

Extensive literature documents that both cancer incidence and mortality are lower in areas with intermediate to high levels of selenium. Decreased cancers of the lung, colon and rectum, bladder, esophagus, pancreas, breast, ovary, and cervix have all been reported, as reviewed by Clark *et al.*[70]

In animal tumor models, moderate selenium supplementation at levels above the dietary requirements decreases the number of tumors induced by chemical carcinogens[71] and viruses,[72] and reduces the incidence of spontaneous mammary tumors.[73] In addition, selenium supplements inhibit the growth of human tumor cell lines in *vitro*[74] as well as the growth of transplanted tumors in mice,[75,76] and decrease the mutagenic activity of several known carcinogens.[77,78]

Some, but not all, epidemiological studies have found a reduced risk for several kinds of cancer associated with a higher blood concentration of selenium.[79–80] A decreased selenium concentration and glutathionine peroxidase activity in blood and, interestingly, an increase of these parameters in malignant tissue was found in lung cancer patients.[80]

Skin cancers have also been studied. Patients with malignant melanoma, mycosis fungoides, and Sezary syndrome had lower blood levels of both selenium and glutathione peroxidase.[81] A study of 240 non-melanoma skin cancer patients in good general health demonstrated a significantly lower mean plasma selenium concentration than control subjects without skin cancer.[82] In fact, those patients whose blood concentrations were in the lower decile had 4.4 times the incidence of skin cancer as those in the highest decile.[82]

In a 10-year prospective study of 1312 patients with a history of basal cell or squamous cell carcinomas of the skin, oral selenium supplementation of 200 µg/day did not protect against further development of such skin cancers; however, it did reduce total cancer incidence (by 37 percent) and the incidence of lung, colorectal, and prostate cancer (by 45 percent to 58 percent, and 63 percent respectively) as well as total cancer mortality (by 50 percent).[83]

Selenium is thought to be effective as an anti-neoplastic agent primarily because it is the required cofactor for the intracellular antioxidant enzymes glutathione peroxidase and thioredoxin reductase. These enzymes are as essential as vitamin C for quenching intracellular, aqueous phase free radicals. Furthermore, selenium has been shown to have other protective effects that may not involve selenium-dependent glutathione peroxidase activity,[84] such as protecting[85] and repairing DNA,[86,87] reducing the DNA binding of carcinogens,[88] inhibiting neoplastic transformation,[89] and suppressing gene mutations at the lysine and histidine loci.[90]

These mechanisms of cancer prevention by selenium may be through regulation of the p53 protein.[91] This p53 protein acts as "the guardian of the genome" since it regulates signal pathways of the cell cycle, DNA repair, and apoptosis to suppress tumors. P53 is the most frequently mutated gene in cancer: About 70 percent of all human cancers have defective p53. Mutations of p53 are high in UV-induced precancerous actinic keratoses and even higher in squameous cell carcinomas.

Different selenium compounds regulate p53 by distinct mechanisms:[91] *selenomethionine* reduces the –SH of two p53 cysteines in the region of DNA binding; *methyl-selenic acid* phosphorylates two p53 threonines; and *sodium selenite* phosphorylates three p53 serines in the region of mediation of apoptosis. With knowledge of these proven antineoplastic machanisms and of the epidemiologic and experimental efficacy, topical L-selenomethionine was studied in a mouse model.[69] Indeed, both oral and topical (0.02 percent) L-selenomethionine eliminated UV-induced edema and blistering and decreased tanning, as shown in Figure 8.9.[69] Initially, oral supplementation was more effective than topical, but by twelve weeks, efficacy was equal. Both forms of L-selenomethionine prolonged the onset and decreased the incidence of UV-induced skin tumors, as seen in Figure 8.10. Topical supplementation proved more effective than oral.[69]

Pretreatment of human volunteers with topical L-selenomethionine before exposure to UVB was also found to increase the MED.[92] Increasing concentrations of topical L-selenium were more protective, with a plateau attained at concentrations between 0.02 percent and 0.05 percent.

Topical L-selenomethionine is highly effective not only in preventing but also in reversing photoaging.[93] After mice were exposed to UVB for 6 weeks and photoaging was clinically and histologically confirmed, the skin was treated with placebo, topical retinoic acid (0.05 percent)

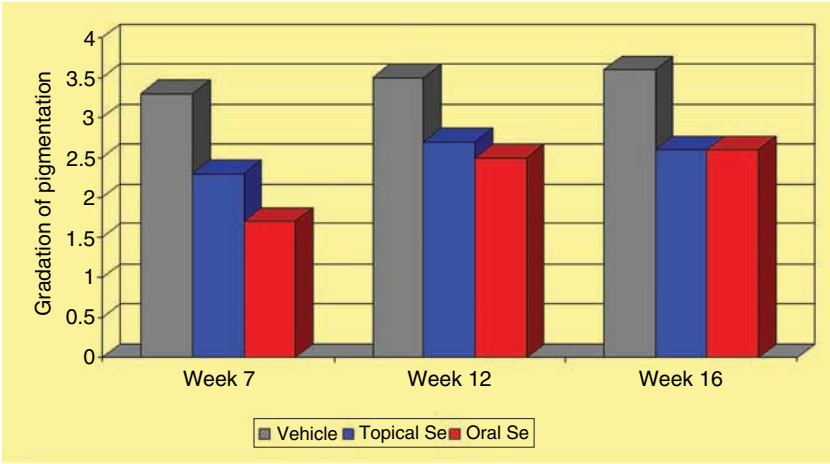


Figure 8.9 Inhibition of UV-induced pigmentation by L-selenomethioinine.

Values are averages of 15 Skh:2 mice in each treatment group. 0, No hyperpigmentation; 4, maximal hyperpigmentation. Vehicle, topical (grey bars); L-selenomethionine: topical: 0.02% (blue bars), oral: 1.5 ppm in water (red bars). (Adapted from Table 1 of reference 69.)

(the "gold standard" of reversing photoaging of the skin), and topical L-selenomethionine (0.02 percent). Parameters of photodamage were evaluated histologically: Epidermal thickness, hyperkeratosis, collagen disruption, and solar elastosis. The L-selenomethionine was equal or more effective in reversing each of these manifestations of photoaging than the retinoic acid.[93] Electron microscopy confirmed enhanced repair of dermal collagen and of derma-epidermal junction anchoring fibril disruption.

Reversal of photoaging was also demonstrated by clinical observation of improvement in periorbital wrinkles in ten women (aged 55 to 72), treated for 4 months with daily application of topical L-selenomethionine (0.05 percent). All patients improved. The significant decrease in periorbital wrinkles of a 77-year-old volunteer can be seen in Figure 8.11.

8.7 Selenium with Vitamin E

In many biologic systems, vitamin E and selenium act synergistically. Borek *et al.*[94] demonstrated that selenium and d-α-tocopheryl succinate act alone by different mechanisms to prevent radiogenic and chemically induced transformation *in vitro.* They further showed that there was additive protection when both were used together.[94]

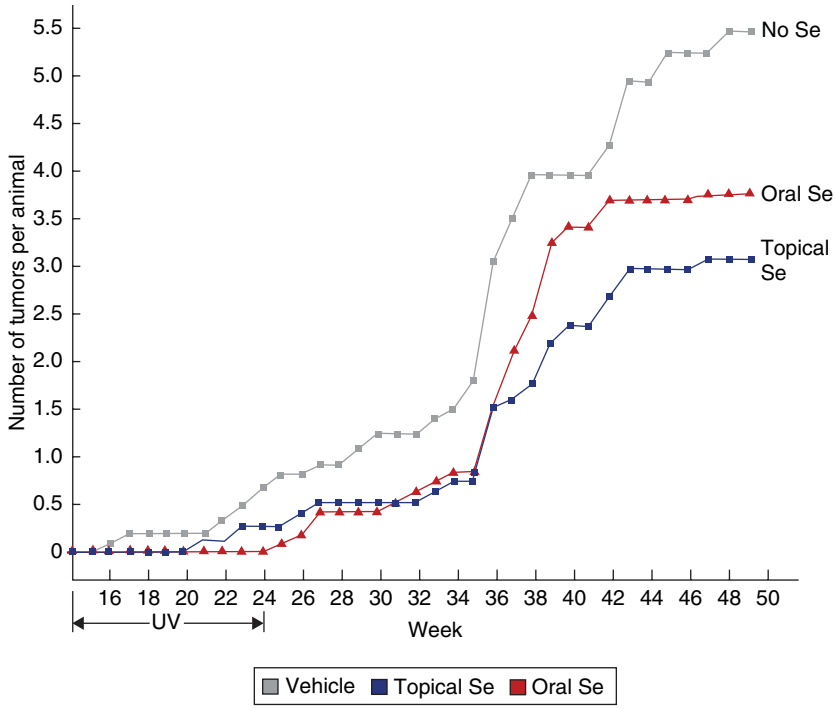


Figure 8.10 Inhibition of UV-induced skin cancer by L-selenomethioinal.
Mean number of tumors ≥2 mm in UV-irradiated Skh:2 mice. Beginning 2 weeks before UV exposure, 15 mice in each treatment group were treated thrice weekly throughout duration of experiment with vehicle lotion (top line, grey squares), oral L-selenomethionine (1.5 ppm in drinking water) (middle line, red triangles), and topical L-selenomethinoine (0.02%) (bottom line, blue circles). Animals were exposed to UV radiation thrice weekly for 24 weeks, and topical and oral treatments were continued through and thereafter. Number of tumors ≥2 mm on each mouse was counted, and mean number of tumors per mouse was calculated based on total number of 15 mice in each treatment group.[64]

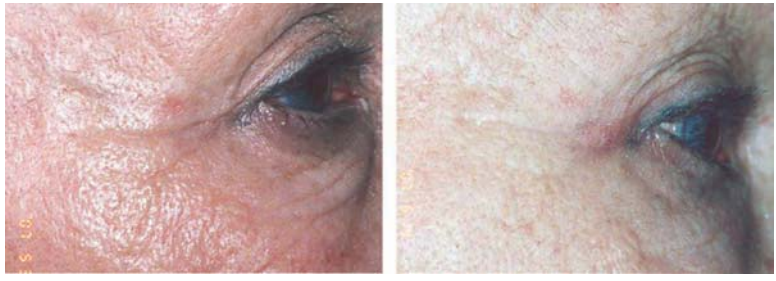


Figure 8.11 Correction of periorbital wrinkles after 4 months of once-daily treatment with 0.05% L-selenomethioinine lotion.

In experiments in mice comparing and combining topical L-selenomethionine with topical d-α-tocopherol,[95] the topical combination was no more effective than topical vitamin E alone. In prolonging the onset and in decreasing the incidence of UV-induced skin cancers,[95] topical L-selenomethionine with oral vitamin E was more effective than either alone. Topical L-selenomethionine (alone or with either form of vitamin E) was most effective in preventing acute UV-induced inflammation (100 percent effective!).[95] In reducing UV-induced pigmentation, topical L-selenomethionine with topical or with oral vitamin E was more effective than any one of these antioxidants alone, particularly during the first eight weeks of UV exposure.[95]

8.8 Conclusion

There are two great advantages to applying an active formulation of topical antioxidant(s) to the skin. First, the skin attains far higher levels of each antioxidant than can be achieved by only taking these supplements orally. For example, the level of vitamin C attained in the skin by topical application is 20–40 times the level achievable with oral vitamin C.[9] With topical application, the concentration of vitamin E increases by a factor of 10.6[44] and selenium, by a factor of 1.7.[69] Second, topical application arms the skin with a reservoir of antioxidant(s) that cannot be washed or rubbed off, a protection which stays in the skin up to several days after application.[9]

While sunscreens are still mainstay for protecting skin from photodamage, they are not enough. Because most of us actually apply only about one-fourth of the amount of sunscreen needed to give the designated SPF (as specified by the Federal Drug Administration (FDA) for laboratory measurement of UV protection),[96] we attain, for example, only an effective SPF of 2.3 for a sunscreen labeled SPF 30. Frequent application is absolutely necessary even for "highly water-resistant" formulations because sunscreen is washed off not only by swimming and sweating, but also by imperceptible perspiration.

Furthermore, while sunscreens reduce UV-induced erythema and the DNA damage of 8-OHdG and thymine dimer,[5] they only block about 55 percent of the free radical production.[97] As discussed above, antioxidants do better. Because even once-daily application of correct formulations of topical antioxidants provides a *long-lasting reservoir* in the skin for protection not only against post UV-induced erythema, hyperpigmentation, photoaging,

and skin cancer, but also against other free-radical damage, they are indeed a valuable adjunct to frequent sunscreen application. This protection over time not only *protects* the skin by diminishing the ongoing free radical insult and inflammation (giving respite for natural repair), but also *reverses* the unattractive appearance of previous photodamage by directly enhancing collagen synthesis and elastic tissue repair. We all can truly "look as young as we feel" by applying topical antioxidants.

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9

Skin Aging in the Asian Population

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9.1 Introduction

People with "skin of color" or "pigmented skin" comprise the vast majority of the world's population. Interestingly much of the published data about skin biology, structure, and function is gathered from studying predominantly Caucasian skin types. Caucasians have, in the past, represented a large consumer market interested in skin care, especially in antiaging, perhaps partly driven by the West's cultural obsession with the prevention of aging and the desire to maintain a youthful appearance. In addition, the wealthy baby boomer generation are aging, one person in the USA turns fifty years old every eight seconds (1). This group is a major and growing demographic segment interested in preserving youth by buying antiaging products. In comparison, the population of Asia is a relatively young population, but one sharing and increasingly aspiring to the Western ideal, for example using skin whitening products to lighten a dark complexion (Figure 9.1). As this population ages they will likely show similar concerns over aging. Coupled with this, the Asian population, accounting for more than half the total population of the world, is becoming increasingly affluent and is becoming a prominent global force in future world economies. The largest face care markets are in Japan and Korea and it is surprising how little we know about the biology of skin and attitude towards skin care in these societies. Additionally, in the West, demographic groups are changing in the twenty-first century and in the US projections to 2050 indicate 47 percent of the population will have skin of color (2). This chapter summarizes some of the current available scientific information on aging in Asian skin: based on the above observations a rapid growth in this knowledge base is expected in years to come.

9.2 Skin Aging

In general, all races are susceptible to changes in skin appearance with age as a result of two processes. First, there are intrinsic changes resulting from hereditary factors along with biological modifications occurring inherently in the structure, physiology, and mechanical properties of the skin, caused for example by hormonal changes. Second, photodamage occurs as a result of the cumulative exposure of the skin to UV irradiation (3). On the skin surface it is possible to differentiate between the effect of intrinsic and

Figure 9.1 The Asian ideal—flawless, "white" skin.

photodamage due to the way these changes are manifested: intrinsic aging gives rise to smooth, dry, pale and finely wrinkled skin while photoaging gives rise to severe coarse wrinkling accompanied by pigmentary changes such as solar lentigo and mottled pigmentation. Both types of aging can be seen within a single individual if one compares an area of skin commonly exposed to the sun, for example the face and forearms, with an area commonly masked from the sun, for example buttock skin. Studies have characterized aging and investigated the biochemical sequences underlying intrinsic aging and photodamage including histological and *in vitro* examinations although mostly in Caucasian skin (4). In addition, prophylactic and preventative treatment of skin aging with retinoids, antioxidants and sun protection (SPF) has been an area of great interest and study (5).

9.3 How is Asian Skin Different?

Skin type is typically classified by the Fitzpatrick score on the predicted reaction of skin to sunlight and ultraviolet radiation (6). The majority of

the Asian population has relatively darker skin, usually type IV and above (Table 9.1), compared to Westerners. Furthermore, there are distinct differences between different ethnic groups in Asia. Asians can be subdivided into North East Asians (Chinese, Japanese, Koreans), Southeast Asians (Indonesians, Malaysians, Singaporeans, Thais, Cambodians, Vietnamese) and South or Central Asians (Indians, Pakistanis, Sri Lankans, Bangladeshis). The skin of people from Northeast Asia tend to be lighter and exhibit more seasonal variation compared with Southeast Asians who inhabit countries receiving more sunlight all year round since they are geographically closer to the equator. For example, people with skin type II, commonly associated with Caucasians, have been reported in Korea (7) and even in Thailand (8).

In fact, this diversity only originates from two different skin types. Indians and inhabitants of Central Asia are Caucasian in origin, while Japanese, Koreans, Chinese, and South East Asians are primarily of Mongolian descent, with typical Mongol features. Thus, at the very basic level, differences in skeletal structure exist which could affect the progress of intrinsic aging. Along with this, Asian skin is reported to have a thicker dermis containing more collagen (9) and epidermally, there is an increased pigmentation in skin providing greater protection against UV exposure (4). Conversely, Asian faces are subjected to greater gravitational force due to the weaker skeletal support and heavier soft tissue which could make this group more prone to skin sagging during aging (9). Although Caucasian skin is firmer, the better photoprotective property of Asian skin, owing to the high pigment content, preserves Asian skin, with it showing fewer signs of wrinkling and less sagging.

Table 9.1 Table of Fitzpatrick Score and Natural SPF

Fitzpatrick Assigned Score	Definition	Natural SPF
1	Celtic—burns easily	1
2	Peaches and cream	1.5
3	Olive	2.5
4	Light Asian	4
5	Far Eastern	6
6	Light black	9
7	Dark black	10+

Ethnic differences have also been found in the stratum corneum and the barrier properties it provides to skin, between blacks, Asians and Caucasians, although the data are contradictory. Some scientists find Asian skin to have a thinner stratum corneum (10,11), indicating that as a consequence it can be compromised more easily and is more susceptible to barrier damage (12). Others, however, report finding no ethnic differences in Japanese compared with German women (13) or a better barrier function in Asians (14) related to the higher ceramide content.

9.4 Differences in Skin Color and the Role of Melanin

Natural color or "pigmentation" of skin is determined by the quantity, type and arrangement of melanin in the skin. These factors also determine how the skin will appear after UV exposure, for example during tanning. Thus, the more melanin in the skin, the darker the overall skin tone is and the darker the skin can become upon exposure to UV radiation. Importantly, melanin pigment provides photoprotection, with black epidermis having the highest inherent SPF, up to 13.4, compared to 3–4 or even less for Caucasian skin (15). For UVA, the mean sun protection score of black epidermis was significantly greater than for white epidermis. *In vivo*, UV protection and filtration in white skin mainly occurs at the stratum corneum level, whilst in darker skin more photoprotection is provided by the viable epidermal layers containing high quantities of melanin. No differences were detected in the number of viable epidermal layers between different skin types (16), although, as mentioned earlier, there is conflicting information on the number of layers of stratum corneum.

Transmission of UVB light through black epidermis was found to be lower—5.7 percent in black skin, versus 29.4 percent for white epidermis. Similarly the mean UVA transmission through black epidermis was 17.5 percent compared to 55.5 percent for white indicating that 5 times more UVB and 3–4 times more UVA reaches the upper dermis of whites compared to those with darker skin. Thus considerably more UV radiation is able to reach the dermal layers in lighter skin and consequently individuals with higher Fitzpatrick scores (Table 9.1) are less susceptible to photoaging. It is a commonly held belief that skin wrinkling does not occur in Asian populations until after fifty years of age, and signs of skin aging are more frequently manifested as pigmentary changes.

9.4.1 Types of Melanin Pigment

Two types of melanin exist in human skin, both are produced by the melanocyte in melanosomes from tyrosine. There are no racial differences in the number of melanocytes (17,18) and it is the amount and relative ratio of each type of melanin which contributes in part to the skin's overall color. The two types are:

- *Pheomelanin*—yellow/red color, alkali soluble
- *Eumelanin*—brown in color and further subdivided into lighter brown, alkali soluble eumelanin, enriched in 5,6-dihydroxyindole-2-carboxylic acid and dark brown/black color, alkali insoluble eumelanin enriched in 5,6-dihydroxyindole

In darker skin types V and VI, insoluble, darker eumelanin predominates (about 60–70 percent), followed by soluble eumelanin (25–35 percent), with pheomelanin content being marginal (some 2–3 percent) (19). Lightly pigmented skin types of different ethnicity (European, Chinese, Mexican) commonly have approximately half as much melanin compared with darker skin types (Indian and African) (20). Epidermal melanin content is significantly higher in chronically UV-exposed skin compared to UV protected skin (up to two times) irrespective of ethnicity. Additionally, at sun exposed sites, there is a further increase in the concentration of dark brown eumelanin (19).

Once produced, packaged melanin in the melanosome is conveyed to keratinocytes, through dendrites via exocytosis. Perceived skin pigment not only depends on the type of melanin and intensity of melanin in the melanocytes but also melanosomes vary in arrangement, size and number across different skin types. Melanosome variation most likely plays a large influence on determining the resulting skin color. For example, African skin contains the largest melanosomes, followed by Indian, Mexican, Chinese, and European (20). Distribution of melanosomes occurs as a spectrum with large, individual melanosomes predominating in dark skin (approximately 88.9 percent), a mixture of single and smaller, clustered melanosomes in Asian skin (62.6 percent and 37.4 percent, respectively) and in Caucasians, groups of melanosomes are the predominant arrangement (84.5 percent) (21). Skin color is also determined by the duration in which melanosomes persist in the epidermis, thus most lighter Asian skin types contain melanosomes grouped together in complexes of two or more, dispersed and degraded in the spinous skin layer (2). In the much darker

skin types the larger, singly dispersed melanosomes with melanin granules remain well into the layers of stratum corneum (20). The number of melanosomes was found to be higher in African-American and African blacks and Australian aborigines.

9.4.2 Melanin Production

Melanin is produced by melanocytes situated in the basal layer of the epidermis. The melanocortin 1 receptor (MC1R) is regulating the production of both eumelanin and pheomelanin, and the gene encoding MC1R has been sequenced from different ethnic groups (21). Both types of melanin are synthesized from tyrosine by hydroxylation to dihydroxyphenylalanine (DOPA) and subsequent oxidation to dopaquinone, both reactions are catalyzed by the enzyme tyrosinase, which is the rate limiting step. Differences in levels of melanin production have been reported between the different skin types and *in vitro* cultures of melanocytes obtained from black skin donors produce higher levels of melanin when compared with those obtained from Caucasian skin. However, the level of tyrosinase was found to be equal in the different skin types (22).

Additional enzymes are involved in the production of melanin from dopaquinone. These are poorly understood although differences in these enzymes may explain constitutive ethnic variation. For example, tyrosinase-related protein-1 (TRP-1) is reported to be 2.6 times higher in darker African and Indian skin types compared with lighter Chinese, Mexican, and Caucasian skin (22). Transient increases in pigmentation as a result of, for example, sun exposure, appear to be a consequence of activating both tyrosinase and TRP-1, with no change in the density of melanocytes at the dermal-epidermal junction and no differences detected between different ethnic groups. Activation of the enzymes results in only a modest overall increase in melanin pigment, with a 4 percent increase in melanin measured 7 days after sun exposure for Asian skin and only a 1 percent increase measured for Caucasian skin (23). The most dramatic difference between skin types subjected to UV exposure is in the distribution of melanin from the lower layers of skin upwards which is most striking in darker skin types (24). In chronically sun-exposed darker skin, there can be an overall increase in skin color which is largely the result of a constitutive increase in the number of tyrosinase positive melanocytes and an irreversible accumulation of insoluble melanin.

9.5 Asian Skin Aging

9.5.1 Protective Mechanisms in Darker Skin Types

The changes in skin following UV irradiation vary from erythema, scaling, and peeling in individuals with a Fitzpatrick skin type I and II, to an immediate and delayed darkening of skin, the so-called "tanning" response, due to upregulation of melanin production and increased transfer of melanosomes to keratinocytes in darker skin types. This higher melanin content contributes to the increased ability of darker skin to resist the damaging effects of the sun in many ways. UVA induced cytotoxicity was lower and thus resistance to UVA damage was greater in cultured melanocytes possessing a higher melanin content (25,26). A study of melanocytes from different pigmentary phenotypes (skin types I-IV) showed that those with the highest melanin content had the greatest capacity to resume proliferation following UVB exposure. All melanocytes had the same survival rate, but those with least melanin seemed to pause for longer in growth phases of the cell cycle, perhaps to repair the more extensive damage suffered. *In vivo* constitutive melanin content inversely correlates with amount of DNA damage incurred and rates of removal (23). This susceptibility to greater UV damage in lighter skinned subjects is exacerbated since in lighter skin there is only a modest increase in melanin compared to the significantly greater increase in melanin content in heavily pigmented cells (27). Furthermore, data show that darker skin may have other protective mechanisms capable of conferring a greater degree of photoprotection and provides increased resistance to photodamage. For example, p53 is a tumor suppressor gene known to regulate cell cycle progression, upregulate DNA repair enzymes and induce apoptosis in damaged cells. Although all skin types show signs of photodamage caused by UV, increased p53 levels have been demonstrated to be higher in darker skinned individuals after a single minimal erythemal dose (28) and in Chinese subjects 24h after 1 minimal erythemal dose. In individuals with lightly pigmented skin, induction of p53 appears to be less efficient, combined with a significantly higher propensity to burn and reduced tanning response. Similar data were obtained with cultured melanocytes from skin types I-IV irradiated with UVB. The cells with the higher melanin content exhibited a quick increase in p53 levels peaking at 24h after UVB exposure, compared with 48h for in lighter pigmented cells (27). UV-induced DNA damage in the lower epidermis (keratinocyte stem cells and melanocytes) is greater in light skinned individuals and UV-induced apoptosis is significantly higher in darker skin, suggesting that UV-damaged cells may be removed more efficiently in pigmented epidermis (29).

Rijken *et al* (30) compared the response of black with white skin and showed that in the black population there was an increased resistance to the deleterious effects of sun damage in darker skin. In all white skinned subjects in the study, exposure to UV radiation induced DNA damage in epidermal and dermal cells, an influx of neutrophils and activated proteolytic enzymes. Additionally, three of the subjects showed further extensive signs of inflammation including interleukin-10 positive neutrophil infiltration into the epidermis. Except for the DNA damage, none of these changes were detected in the dark skinned individuals. In conclusion, darker skin appears to have several mechanisms present: increased levels of melanin protect against UV radiation combined with a more efficient removal of damage to DNA and cells, which may help to protect against photoaging, sunburn, and skin carcinogenesis. Collectively, this may result in less accumulated photodamage compared to lighter skin types. A summary of differences between Caucasian and Asian skin is given in Table 9.2.

9.5.2 Pigmentary Changes

The different levels of melanin are obviously manifested clinically to give the skin its color. Skin types IV and above have naturally higher melanin

Table 9.2 Summary of Differences between Caucasian and Asian Skin Which may Underlie the Lower Incidence of Wrinkling in Asian Skin

Skin Characteristic	Reported Difference	Implication
Melanin	Differ in type and amount	Asian skin is darker and tans more intensely on UV exposure
Melanocyte number	No racial differences	
Melanosomes	Differ in number, size and arrangement in epidermis	Skin has more "built-in" resistance to sun
DNA repair mechanisms induced by UV	Appear to be more efficient in darker skin types	Damage to DNA can be quickly and more effectively removed
Melanocyte proliferation	More efficient response to UV radiation by proliferation	Melanocytes appear to resist UV radiation and respond more rapidly by producing melanin

content with a darker skin color and therefore respond to acute sun exposure with intense tanning response. The higher melanin content appears to make darker skin types more susceptible to pigmentary changes as features of photoaging and skin aging in darker skin types often manifests itself more in pigmentation changes than in the occurrence of wrinkling (31,32) (Figure 9.2). Culture, habits and weather play a major role when considering photoaging. In Asia there is a wide variation in sun exposure, for example Asians inhabiting the northern part will have less exposure to sun compared to South East Asia, which is closer in proximity to the equator (Singapore for example is 80 miles from the equator) and where the UV index is high all year accompanied with 12h of daylight year round. Thus signs of photodamage may be expected to be higher in South East Asians (Indonesia, Thailand, Vietnam etc) compared with North East Asians (Japan, China, Korea, Taiwan etc) exposed to a typical seasonal variations of autumn, winter and spring seasons.

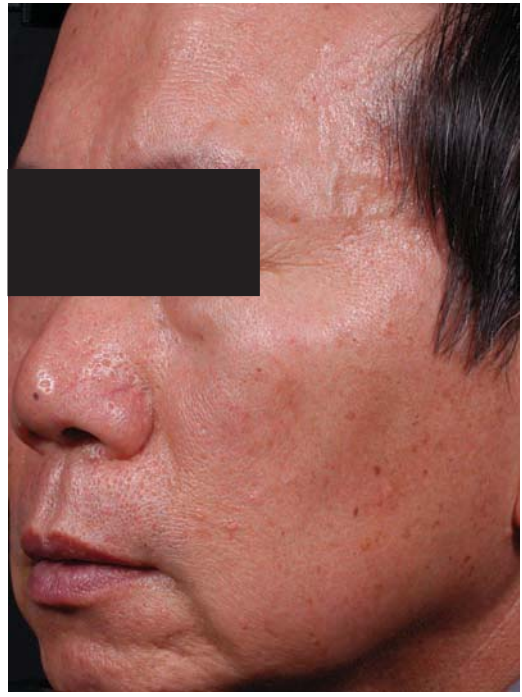

Figure 9.2 Aging on an Asian face—few wrinkles are evident, but there are several changes in skin pigmentation.

With chronic sun exposure, Asians develop primary, discrete pigmentary changes as common signs of photodamage: including actinic lentigines, flat pigmented seborrhoeic keratoses (SK) and mottled hyperpigmentation (Figure 9.2). Solar-induced melasma is also common. However, hyperpigmentation leading to freckles are more frequent in Caucasians compared to Asians. These different types of hyperpigmented lesions can be differentiated by their clinical and histological appearances. Freckles are characterized by hyperpigmentation of the basal cell layer with no increase in number of basal melanocytes and no elongation of the rete ridges. In contrast, a lentigo is a distinct, pigmented macule, with elongated rete ridges and increased number of melanocytes. Mottled hyperpigmentation is characterized by diffuse, mottled irregular areas of pigment and may be explained by an increased DOPA activity in chronically irradiated melanocytes. SK vary from pale brown to pink to black lesions. In brown skin, most SK are brown to black and may represent a benign skin lesion. Finally, besides these pigmented lesions, acquired bilateral nevus of Ota-like macules or Hori macules is another pigmentary condition triggered by UV radiation and is found in a small percentage of Asian females (33).

In some races, for example in Koreans, the pattern of pigmentary changes has been shown to be gender dependent, with lentigo being most common in women and seborrhoeic keratosis being found more often in elderly men (34). The incidence of SK was 79 percent at 40 years and 94 percent at 50 years, with increasing area covered by SK. This study included 407 Koreans between 30–92 years of age. On the other hand, SK were found to be equally common, if not more common in Caucasians (for example one study reported that 81–100 percent of Caucasian males over the age of 40 years have at least one SK) (35), and were distributed equally among Caucasian males and females. Additionally, SK were found to be less common in darker skinned Asians inhabiting Western countries (United Kingdom) (36). These results suggest ethnicity and gender have an influence on the prevalence of this lesion type, although melasma has been found to affect Asian males and females equally (37). It is not clear whether these differences reflect the different types of sun exposure during life or are truly gender related, but clearly more work needs to be completed on the changes which occur in skin pigmentation as a result of age in darker skin types.

At the histological level, a study of Thai skin showed extensive disorganization of melanin with abnormal differentiation appearing by the age of forty years. Additionally basal keratinocytes were highly melanized with

clusters of melanosomes; usually these only occur as a feature of extensive chemical and physical trauma to skin (38). Over time, with continued sun exposure, both Caucasian and Korean skin show the same trend in accumulating a greater number of melanocytes and increased melanin content with higher DOPA activity in sun-exposed photoaged facial skin compared to sun-protected skin, where the number of melanocytes and melanin pigment were found to be reduced with age (34).

9.5.3 Dermal Changes

Histological findings of aged skin show distinct differences in skin intrinsically aged versus photodamaged skin, most strikingly in the dermis. Intrinsic aging of Caucasian skin shows a general decrease in the extracellular matrix proteins with reduced elastin, disintegration of elastic fibers and degradation of collagen (3). In contrast, the histological findings of photoaged skin showed prominent features referred to as solar elastosis, and an accumulation of dystrophic elastotic material in the reticular dermis. In addition, increased fibrillin expression and deposition in the reticular dermis have been reported. Together these changes result in loss of elasticity, skin sagging and reduction in skin firmness.

Despite the higher level of protective melanin and reduced transmission of UV through the epidermis, dermal changes in darker skin types are extensive and very evident with age. In the Thai study mentioned above (38), severe, marked elastosis with significant collagen damage was detected equivalent to end-stage photodamage in white skin. Seo *et al* detected mild solar elastosis in Koreans as young as twenty years of age in sun-exposed facial skin, severe accumulation of elastotic material was found by forty years. However, in sun protected areas, solar elastosis was absent (39). Another study showed that both Caucasian and Asian skin had similar levels of collagen cross links which occurs spontaneously with aging. Measured elasticity decreased with age, although Caucasian skin showed greater loss of elasticity and firmness compared with Asian skin (14).

In Korean skin, both intrinsically aged and photoaged skin showed an age-dependent reduction of cutaneous vessel size in the dermis (40) which will result in less nutrition and oxygen provided to the skin, possible further causing age related changes. Photoaged skin also exhibited a significantly reduced number of dermal vessels in particular in the dermal areas showing extensive matrix damage. Linear regression analysis of the data revealed an inverse relationship between vessel number and age in sun-damaged,

but not sun-protected skin. This suggests that at least in this ethnic group there are different changes in the vasculature depending on whether aging is caused by intrinsic factors or versus photodamage.

9.5.4 Clinical Scales and the Occurrence of Wrinkling

The occurrence, degree and severity, of wrinkling as a measure of photodamage is most frequently assessed clinically using objective scales and a trained expert grader. In Caucasian populations several scales exist for clinically assessing wrinkles *in vivo* (31,32). Due to the increased pigmentation and lower incidence of wrinkling, it is not known whether these scales are relevant for use in Asian populations and in fact, the developers of these scales suggest that they may indeed not be suitable for assessing Asian skin aging. Thus, unique Asian scales need to be developed with perhaps even different ones for the diverse skin types in Asia. The first such scale for Asian skin wrinkling was published by Goh (37) who concluded that wrinkling in Asians is not noticeable until the age of about fifty and even then the degree of coarse wrinkling was lower compared to age matched Caucasian counterparts. In contrast, pigmentary changes such as hyperpigmentation, were an early and prominent feature. In this paper, the patient base was extensive (over 1500 subjects), including individuals from Singapore, Malaysia, or Indonesia and were Mongoloid in race (37). A more recent study conducted by the same clinician using identical standardized photographic equipment to compare the pattern of wrinkling in two different populations (160 Chinese versus 160 French age-matched women, of age range 20–60 years) in their respective countries, agreed with this finding. Results showed that for each facial skin area, wrinkle onset was delayed by about ten years in Chinese women compared to French women and the rate of occurrence for facial wrinkles was linear for French women, but Chinese appear to experience a faster aging increase between the ages of forty to fifty years. Pigmented spot intensity was a much more common sign of aging in the Chinese subjects (41). In both populations, wrinkles around the eye area appeared as the first signs of wrinkle development, followed by the forehead area and lastly the perioral area. Identical findings are reported in a multicenter study conducted in China on 2000 women (42) and Koreans (43).

Scales developed on Korean subjects were reported by Chung *et al.* (44), who developed separate eight-point photographic scales for wrinkles in Korean males and female subjects, and also a six-point scale for pigmentation changes. Using the scales, the authors confirmed that a different

wrinkling pattern existed in Asians compared to Caucasians. Asians had coarser, thicker and deeper wrinkles, particularly on the forehead, perioral and crows-feet areas. In contrast, Caucasians usually have relatively fine wrinkles on the cheeks and crows feet areas. The reasons for these differences are not known and need to be further investigated. Korean subjects in their sixties were found to have a twelve-fold higher risk of wrinkling and subjects in their seventies had a fifty-six-fold higher risk versus the younger age group of 30–59 years. Thus Chung et al. concluded that in Korean populations wrinkling was still a prominent feature associated with aging. The scales also indicated that wrinkling patterns in both sexes were very similar, although more severe wrinkling was found in women. This may be due to reductions in estrogen levels after menopause, since wrinkling increased significantly following menopause and more severe wrinkling was associated with an increased number of full-term pregnancies (45). In agreement, hormone replacement therapy was associated with a significantly lower risk for the development of facial wrinkling in postmenopausal Korean women. It is interesting to speculate the effect in Asia of diet, which is enriched with soy products noted for their significant phytoestrogen content, on wrinkling.

In Japanese subjects, a correlation of increased wrinkles with age has been detected, in the following order: eye area > lower eyelid > upper eyelid > cheek > forehead > mouth area > nasolabial grooves > forehead (46). Individuals living in Tokyo had fewer wrinkles and less facial sagging compared with age matched Caucasian individuals in Cincinnati (47). However, within Japan, surprisingly, individuals living further north showed greater signs of photodamage compared to southern Japanese, the latter had fewer facial wrinkles, less hyperpigmentation and less yellow skin, and showed an average a younger skin phenotype varying between 8–16 years (48). A study comparing different ethnic groups living in the same area (Los Angeles) reported increased incidence of skin wrinkling in the order Caucasian > Hispanic > African American > East Asian, with Asians also exhibiting the fewest hyperpigmented spots (49). In summary, overall these studies show a trend to less wrinkling in Asian skin types, although more robust studies need to be completed in detail.

In Caucasian populations, certain lifestyle habits are associated with an increased propensity for developing premature aging, including wrinkles. Risk factors showing a strong correlation include smoking (50,51) and sun exposure. Interestingly, however, smoking has been demonstrated not to play a significant role in wrinkling in black skin (52), but in Asians,

prominent facial wrinkling was significantly increased among smokers compared to non-smokers. Koreans were found to have an eleven-fold increase in wrinkling in smokers compared to non-smokers (44). A similar trend was observed for Japanese subjects, who had a twenty-two times higher risk of developing more severe skin wrinkling than those who had never smoked and had lower sun exposure (53). In Koreans, sun exposure of 5h per day was associated with a 4.8-fold increase risk or wrinkling compared to those with 1–2h of sun exposure.

Overall skin structural differences may also play a role in changes in aging relating to skin sagging and lack of firmness. Asians have a thicker dermis, with greater amounts of collagen, but have a weaker skeletal support, heavier soft tissue, a larger amount of fat, and a weaker skin structure (9). Differences in facial muscle positioning, content and movements may also contribute, as well as diet. Dietary carotenoids from a healthy unsupplemented diet accumulate in the skin and their level significantly correlates with sun protection (54). Eating large quantities of fish oil appears to provide a sun protective effect, in some cases up to an SPF of 5, and may reduce the UV-induced inflammatory response by a lowered prostaglandin E2 levels (a mediator in the arachidonic acid cascade for inflammation) (55). It is interesting to speculate whether this can also moderate the response of melanocytes to UV radiation.

9.5.5 Other Changes Measured with Aging

Characteristic features well documented for Caucasian skin also include reduction of cell turnover rate and accumulation of larger corneocytes at the skin surface, reduction in sebum levels, and loss of skin moisture. These changes have also been documented to occur similarly in Asian skin types (14,56).

9.6 Treatments of Photoaging in Asian Skin

Another area with limited published work is treatment of aging skin and its response in Asian skin population, in terms of efficacy and adverse effects. As demonstrated there are significant biological differences between Caucasian and Asian skin. It is expected, therefore, that treatments suited for Westerners may not be optimal for skin of color. There is a growing awareness in Asia of various treatment options for aging skin, for example microdermabrasion, Botox injections, and laser treatments offered in many

dermatology practices and in independent skin clinics throughout North and South Asia. Additionally, the demand for effective products is high considering the Asian ideal of white, flawless skin.

Treatment for photoaging in Asian subjects differs in several important respects. Asians with photodamage tend to have more pigmentary problems and less wrinkling than whites and these differences in clinical manifestations lead to different consumer expectations and, if possible, treatment goals need to be clarified with the subject prior to treatment. Asian skin responds differently to treatments and is often reported to be more sensitive than Caucasian skin, which affects the use and treatment regimens of many antiaging compounds, such as retinoids and α-hydroxy acids, which are known to be irritating.

9.6.1 Treatment with Hypopigmenting Agents

Since the key features of aging in Asians are increased focal areas of pigmentation it is perhaps surprising that this approach is not more commonly described for the treatment of Asian signs of photodamage. This could be due to the overall predominance of Western treatments for aging in the marketplace. Nevertheless, a few studies exist showing that this approach provides good clinical relief for photodamage in this ethnic group. The use of 5 percent niacinamide significantly decreased hyperpigmentation (57) and iontophoresis of vitamin C was used for melasma (58). Other skin whitening ingredients have also been shown to provide some benefit (59).

9.6.2 Retinoids

Topical retinoids include retinol and derivatives of retinol and retinaldehyde, used frequently in cosmetic products (for example retinol and retinyl palmitate), and retinoids only available on prescription—of these only all-*trans* retinoic acid (tretinoin) is currently approved for the treatment of photoaging and is marketed in the US as Renova®. Retinoic acid has been proven to provide excellent clinical results in the treatment of photodamaged skin for Caucasians and has shown similar results in South East Asians. A study conducted on 23 Chinese and 22 Japanese subjects showed that 0.1 percent topical tretinoin cream significantly lightens the appearance of hyperpigmented lesions and reduces the epidermal pigmentation assessed using histology (40), with minimal adverse effects. However, in this study, the subjects had too few wrinkles for any assessment to be made on the effect of retinoic acid on wrinkling. Success using 0.1 percent retinoic

acid for treating melasma has also been reported, although this is for Caucasians (60) and for African-Americans (61).

A regimen consisting of application of 0.05 percent tretinoin at night and 0.1 percent tretinoin in the morning was found to be effective in reducing the visible signs of aging in Asians, including wrinkling, skin texture, and overall skin appearance which changed from a sallow yellow to a rosy glow. A better result was observed for longer treatment regimens with higher concentrations (37) and there was a noticeable improvement in hyperpigmentation as assessed by both the dermatologist and subject receiving treatment. However the incidence of side-effects was high and the subjects reported increased sensitivity to cosmetics. In combination with 5 percent hydroquinone (a skin lightening treatment) 0.1–0.4 percent retinoic acid was found effective in reducing melasma, which has been noted to be difficult to treat (62). Korean patients with therapy resistent melasma applied a combination of 0.1 percent tretinoin, 5 percent hydroquinone and 1 percent hydrocortisone twice a week, instead of the usual daily application, in an attempt to reduce the side effects. The treatment provided statistically significant depigmentation on a time scale similar to that observed for Caucasians receiving daily treatment. Additionally, increased collagen synthesis was noted in the dermis which may also help to reduce wrinkling (63). A variety of other pigmented lesions, including senile lentigines, nevus spilius, and postinflammatory hyperpigmentation present in Japanese has also been reported to respond well to a combination of 0.1–0.4 percent retinoic acid and hydroquinone-lactic acid ointment (64). A different isomer, 13-*cis*-retinoic acid, (isotretinoin) is normally used orally to treat acne. An investigation of a topical treatment using 0.05 percent 13-*cis*-retinoic acid in Thai subjects showed no benefit on melasma when compared to use of a sunscreen alone (65).

Tazarotene is a newer receptor-selective retinoid designed in order to provide the benefits of retinoid therapy with reduced irritation. It is only available on prescription, but studies have shown good efficacy for the treatment of photodamage in Caucasians (66). Similar studies on photoaging have not been conducted for Asians, only one study reported that tazarotene is effective on Asian skin in reducing acne vulgaris (67).

9.6.3 α-Hydroxy Acids

α-Hydroxy acids (AHA), particularly glycolic and lactic acids, have been used extensively at high concentrations as peeling agents in the treatment

of photodamaged skin. Even at lower concentrations, AHAs induce exfoliation of the outer skin layers stimulating a wound healing reaction. Because of potential side effects, such as the production of irregular pigmentation, deeper chemical peels are often not recommended for dark-skinned subjects. Additionally, (and as with retinoids) topical use of AHAs increases photosensitivity and tanning response to UVB and UVA. This is of special concern for those populations living closer to the equator, for example in South East Asia (68).

Despite the above there are a number of published studies on the use of AHAs in pigmented skin (69,70). When used at 3 week intervals, in a study in Singapore, 20–70 percent glycolic acid peel in combination with 10 percent glycolic acid and 2 percent hydroquinone produced a subjective improvement in melasma, when compared to 10 percent glycolic acid and 2 percent hydroquinone used alone, which also provided improvement in melasma and facial wrinkling (71). Similar results in pigmented skin were obtained showing no additional benefit of a 20–30 percent glycolic acid peel in hyperpigmentation when used with 4 percent hydroquinone (72) or when compared with 1 percent tretinoin (73). Thus these studies indicate that the risk of using high concentrations of AHAs as peels in pigmented skin may not outweigh the clinical benefits. Other studies support the combination of glycolic acid peels as an adjunct to other therapies in Indians with only few side effects such as increased pigmentation (74,75).

Polyhydroxyacids are similar to AHAs but do not provoke the sensory irritation response and provide humectancy and moisturization properties. Limited data indicate that polyhydroxyacids are compatible with Asian skin types and provide skin lightening benefits as well as improvement in characteristic signs of aged skin such as reduced wrinkles and improved skin texture (76).

9.6.4 Treatment for Aging Skin Using Lasers

Recent developments have led to the availability of a variety of laser treatment practices, but these should be used with caution in Asian subjects due to the high risk of postinflammatory hyperpigmentation which may be permanent. Therefore, nonablative lasers have generated much interest in Asia versus the use of ablative lasers which cause extreme epidermal damage. The ideal nonablative laser for Asian skin should have a long wavelength with adequate cooling and provide long term improvement (77). Lasers operating in the infrared region such as 1320-nm Nd:YAG and

1540-nm erbium glass lasers appear to be effective in improving wrinkles and skin texture in Asian subjects. Intensed pulse light (IPL) has also been shown to be effective in the treatment of pigment reduction, thus a combination approach of IPL with for example 1320-nm Nd:YAG laser may be an attractive treatment choice. In contrast, the longer wavelength 1450-nm diode laser showed improvement for facial photoaging in Asians, but this treatment regimen was accompanied with a high degree of postinflammatory hyperpigmentation (78).

Treating lentigines with a 510-nm pulse dye laser, QS Nd:YAG 532-nm laser, QS ruby laser and QS alexandrite laser gave good results, although studies with QS lasers have indicated that the risk of post inflammatory hyperpigmentation is as high as 20 percent in Asian subjects (79). QS lasers are well suited for melanin reduction since they emit light which is well absorbed by melanin. Hyperpigmentation is believed to develop from the follicular melanocytes which may not be destroyed by the emitted light but are stimulated to produce more melanin.

Radiofrequency treatment offers a significant advantage for Asian skin, since the operator can exert more control over depth of penetration and energy applied; thus, skin type does not influence the results and there is less risk of unwanted side effects. The method involves the passage of an electrical current leading to tissue heating and to protect the skin, simultaneous contact cooling is provided to the epidermis. Further work needs to be done on this modality and Asian skin.

9.7 Skin Sensitivity

One issue which needs to be addressed is that of increased skin sensitivity reported amongst Asians compared to Caucasian counterparts, especially in Japanese populations (80). The reason for this increased irritation is not clear and unless this can be overcome, it presents a barrier to large scale use and popularity of many antiaging treatments in Asia. Studies testing the response of different skin types to irritants often give conflicting results, even when the same population is used. For example, Asians seem to be more reactive than Caucasians in some studies and less in others, even when the studies were done under the same investigator and protocol (81). It is known however that irritation responses can vary from subject to subject and therefore any given test will show a range of reactivity to the same stimulus. Certainly, the perception of Asians is that they have very

sensitive skin, although again conflicting data exist. A study investigating perception of Asians in comparison to Caucasians did not reveal a higher level of self-perceived irritation amongst Asians (82).

In a patch-test study, Japanese and Chinese subjects showed a more rapid and higher irritation reaction compared to Caucasians exposed to 20 percent sodium dodecyl sulfate (SDS), 10 percent acetic acid or 100 percent decanol and male subjects were directionally or significantly more reactive to each of the test chemicals than female subjects (83). In agreement, other studies have reported that Japanese subjects were found to have significantly stronger subjective sensations compared to German subjects (13) and greater irritation response compared to Caucasians (80), but this could not be explained by differences in barrier function and no ethnic differences in innervation have been detected (84). Methyl nicotinate assessment of the vasoactive response suggests that there may be ethnic differences in skin permeability (10). Increased percutaneous absorption of benzoic acid, caffeine and acetylsalicylic acid was demonstrated in Asians compared to Caucasians (13). It is possible that the increased penetration may be due to a greater number of sweat glands in Asians (85).

9.8 Conclusions

Skin biology and understanding has progressed rapidly over the last few decades mainly from studies in Western populations. It is clear that Asians and Caucasians manifest different phenotypes of aging. Asians are more prone to changes in pigment with age, with wrinkles developing later in life. In comparison, Caucasians develop wrinkling earlier and more extensively. External influences, such as differences in diet, exposure to sunlight and culture undoubtedly play a role in this.

Biologically, differences between Caucasian and Asians are due to the melanocytic pathways operating in either skin type, although the data seem to suggest that number of melanocytes and melanin production is only marginally different. More dramatic differences are observed suprabasally with melanosomes distribution and size. Along with the greater protection from the increased melanin levels and differences in melanosomes, Asian skin additionally demonstrates differences in the way it responds to UV exposure compared to Caucasian skin. Notably there are better and more efficient mechanisms for coping with damage caused by UV-exposure. Over time this may lead to the clinical differences in observed in skin aging.

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PART 3
PRODUCT DEVELOPMENT

10

The Use of Natural Compounds and Botanicals in the Development of Anti-Aging Skin Care Products

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10.1 Introduction

The desire to prevent the ravages of aging skin is an ancient phenomenon, and natural products have figured heavily in traditional treatment regimens. Even before the fifth century BC, the Egyptians utilized mineral powders like kohl from antimony for painting the eyes, and henna leaves for coloring hair and cheeks.[1] Belladonna was instilled into eyes to dilate the pupils rendering a woman more beautiful. Plant psoralens that increase the skin's sensitivity to UV light have been used to repigment skin for thousands of years.[2] Plant oils and extracts remain a popular element of modern cosmetics, but with the advent of cheaper, more convenient synthetic ingredients, does the continued use of extracts and compounds isolated from plants make a meaningful contribution to efficacy? The answer to that question will depend on many factors including quality, preparation, and amount of the plant material as well as the intended use of the product; however, plants can undoubtedly contribute to product benefits in a real and measurable way.

Higher plants have been evolving on earth for more than 350 million years. With conservative estimates of more than 250,000 distinct species on earth, plants represent a spectacular diversity of natural chemicals. The proteins, sugars, and fats in plants are readily assimilated when ingested as part of the diet; these primary metabolites provide the foundation of nutrition for healthy growth and metabolism. The "secondary metabolites" found in plants are molecules that are not associated directly with plant structure, growth, or metabolism.[3] Some are recognized to be chemicals of defense against microbes, viruses, or oxidation while the function of others is obscure or unknown. These "phytochemicals" often explain the basis for traditional hygienic, medicinal, or cosmetic uses of plants.

Our ancestors used the plants around them as their pharmacy. They learned which plants had the power to cure infections, to stop bleeding, to ease pain and inflammation, to soothe burns and dry skin. All around the world, plants have been incorporated into daily use regimens to keep the body healthy as well as to treat disease or injury when it occurs. It is estimated by the World Health Organization that 80 percent of the global population still uses plants as their primary source of medicine.[4] Further, in industrialized nations like the US, a significant percentage of drugs in current use is derived from plants.[5] The distinguishing difference between use of plants in the developing versus the developed nations is that industrialized countries use purified compounds from plants instead of crude or whole plant extracts. While many plants may have one predominantly active compound, many more have numerous active components with various biochemical actions, and this complexity is often overlooked or disregarded in the interest of potency.

It is intriguing to consider the multiplicity of biochemical activities found in plants within the context of skin aging, itself the sum of many distinct but often interrelated biochemical events. There is little disagreement that exposure to UV light leads to skin aging, yet the damage is mediated through multiple, concerted insults, in various cells, structural components and strata of the skin. Thus, until it is conceivable to turn back the clock or achieve 100 percent protection from sunlight, it may be possible to apply the diverse chemistry of plants and other natural products toward the attenuation of some of the biochemical consequences of aging that contribute to detrimental changes in the appearance and/or function of the skin. There are numerous biochemical and physiological points at which it may be possible to intervene with the aging process in the skin. This chapter will discuss the evidence for the application of botanicals and natural products to modulate several of these targets, namely, oxidation, inflammation, the skin barrier, the extracellular matrix (ECM), and DNA repair.

10.2 Oxidation and Skin Aging

There is a large body of evidence to suggest that oxidative stress plays a major role in the intrinsic as well as the extrinsic process of skin aging.[6–9] The biochemical process of oxidation is critical to aerobic life itself, but all living organisms must keep the generation of oxidative molecules in balance to avoid consequences that are detrimental to health and even survival. The free radical theory of aging has been discussed and debated for decades,[10,11] and while free radical generation is certainly not the only contributor to the aging process, there is ample evidence that oxidative damage to cellular and extracellular components is responsible for many of the degenerative changes observed with age.[12] UV irradiation continuously bombards the skin with profound oxidizing effects, and many other factors such as ozone, cigarette smoke, and environmental pollutants also contribute to the oxidative assault.[13–16] As the organ with the greatest exposure to such external sources of oxidation, the skin has evolved a complex network of endogenous and assimilated antioxidants for protection; however, these are not always sufficient to prevent or repair damage due to reactive oxygen species (ROS). Exposure of a human skin model to as little as one minimal erythemal dose (MED) of UV irradiation has been shown to deplete cellular antioxidant levels as well as antioxidant enzymes like catalase.[17–19] Antioxidant compounds such as vitamin E, glutathione and coenzyme Q10 (CoQ_{10}) are known to decrease significantly in skin with age,[8,20,21] lowering the antioxidant capacity of the epidermis. With respect to aging, it is also of interest to note preliminary evidence that graying hair follicles have a higher level of oxidative stress than more pigmented ones.[22]

Proteins such as those of the ECM, intercellular lipids of the epidermal barrier, and DNA in the nucleus and mitochondria are all highly susceptible to damage by ROS.[8,23] Furthermore, proteosomes in skin cells that are responsible for removing oxidized proteins from the cell become less efficient over time[24,25] leading to an accumulation of damaged and dysfunctional molecules. Oxidation of the phospholipids in cell membranes initiates a chain reaction that results in lipid peroxide formation; these compromise the integrity of the membrane, initiate inflammation and have also been shown to be involved in the up-regulation of the degradative enzyme, matrix metalloproteinase-1 (MMP-1, collagenase).[26]

Oxidative stress also affects the transcription of other genes through mitogen-activated protein (MAP) kinase pathways, ERK, JNK and p38. UV irradiation initiates a cascade of events including phosphorylation of protein

kinases of the MAPK family as well as activation of transcription factors such as nuclear factor κB (NFκB) that mediates an inflammatory response.[27] The extracellular signal-regulated kinase (ERK) pathway that mediates the anabolic response to growth factors is reduced in aged skin, while the c-Jun amino terminal kinase (JNK) that mediates the oxidative stress response is increased in aged compared to young skin,[28] one consequence of which is the increased degradation of the ECM. Many plant antioxidants have been found to inhibit the UV-induced activation (i.e., phosphorylation) of MAP kinases.

10.2.1 Botanical Antioxidants

Antioxidant activity is an excellent example of a functional benefit that plant extracts can deliver. Plants are known to contain a variety of natural antioxidants that protect and preserve their physical and metabolic integrity as well as their heredity by way of their seeds. Many of these extracts and compounds from plants are emerging as candidates for moderating the effects of the aging process on skin by limiting biochemical consequences of oxidation.

Compounds such as vitamin C, vitamin E and rosmarinic acid (RA) are commonly used in foods as well as cosmetics for their potent antioxidant activity that aids in product stability. Reduction in oxidation has a clear benefit for the product as well as for skin, and the consumer perception of antioxidants is a positive one, making them particularly attractive as cosmetic ingredients. The danger is that the use of a single antioxidant is often positioned as a panacea. The phenomenon of the product that contains the "most potent antioxidant ever discovered" belies the scientific understanding that antioxidants work in synergy. The physiological codependence of water soluble vitamin C and lipophilic vitamin E is well accepted. Plant antioxidants differ not only in redox potential and solubility, but also in their mechanism of action. Some quench one or more ROS such as superoxide, hydroxyl radicals, or singlet oxygen. Others inhibit activity or expression of oxidative enzymes, or enhance activity or expression of antioxidative compounds or enzymes like catalase, or chelate oxidizing metal ions, or act by other mechanisms, known and unknown. Given the variety of chemical structures and biological mechanisms of antioxidants described from plants, it is not surprising that not all antioxidants confer the same degree of functional protection to the skin.

The small molecular weight antioxidants that are naturally found in skin include compounds synthesized by skin cells such as glutathione and ubiquinol as well as those assimilated from plant sources in the diet such

as vitamin E, vitamin C, and retinoids. They function synergistically in some cases but also operate as part of independently-regulated systems to address challenges to the redox status of the cell or the tissue.[20]

Since many relatively simple bioassays are readily available for assessment of antioxidant activity, a large number of plant compounds and extracts have been shown to act as antioxidants *in vitro*, and many have also demonstrated the capacity to reduce oxidative stress in skin in vivo as well as skin cells *in vitro*. This activity may well be expected to protect aging cells; however, as will be seen in the examples below, many antioxidant compounds display additional biological activities such as inhibition of inflammation or modulation of gene transcription that may not be exclusively related to antioxidant activity, and this uncertainty can thwart simple attempts to associate antioxidant activity in and of itself with a predictable clinical benefit.

10.2.2 Endogenous Antioxidants

Vitamin E and vitamin C are among the small molecular weight antioxidants in the skin that provide it with natural protection, and their concentrations are known to decline with age. It was shown that restoration of these compounds to the levels found in younger skin might impart an anti-aging benefit.[29] Recent publications have reviewed the numerous studies showing that topical application of L-ascorbic acid (vitamin C) or d-α-tocopherol (vitamin E) or mixed tocopherols can inhibit many of the consequences of UV irradiation of skin, including erythema, sunburn cell formation (UV-induced apoptosis) and DNA damage.[30,31] Many skin care products contain the more stable, esterified forms of the vitamins, and efficacy of these forms is also supported by the literature as some level of bioconversion to the active forms occurs in the skin. Kashino *et al.* 2003[32] showed that Ascorbic acid phosphoric ester magnesium salt treatment reduced oxidative stress in human adult fibroblasts and delayed senescence by reducing the rate of telomere shortening. A water soluble derivative of γ-tocopherol has recently been demonstrated to be effective in reducing UV-induced inflammation in mouse skin. Gamma-tocopherol was more active than α-tocopherol in this regard, although the latter was a more potent inhibitor of UV-induced lipid peroxidation.[33] Combined with the fact that plant-derived vitamin E consists of a mixture of tocopherols and tocotrienols and that skin notably has a higher level of γ-tocopherol than α-tocopherol,[34] this illustrates that much research remains to be done to identify the most efficacious combination. Since humans are dependent upon plants as foods to provide vitamin E and it occurs naturally as a

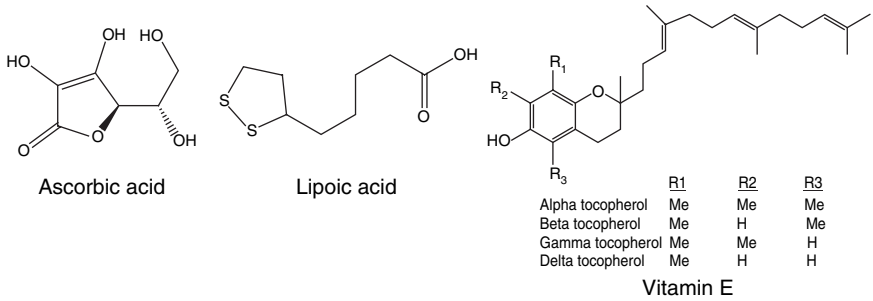


Figure 10.1 Examples of endogenous antioxidants.

mixture of forms, there may be biological advantage to the natural mix that has yet to be elucidated.

More recent studies in skin cells have confirmed additive or synergistic effects with combinations of antioxidant vitamins. The combination of vitamins E and C was more effective than each alone at reducing UV-induced matrix MMP-1 expression in human fibroblasts.[35] This research team also demonstrated that micromolar levels of the rosemary polyphenol carnosic acid significantly inhibited UV-induced MMP-1 expression. The combination of vitamin C and E was also demonstrated by Lin *et al.* to reduce UV-stimulated thymine dimer formation[36] and to attenuate both erythema and sunburn cell formation in UV-irradiated pig skin.[37] Expression of the catabolic protease enzymes, caspase 3 and 7, was significantly inhibited; a further synergistic effect was observed with the addition of 0.5 percent ferulic acid, a caffeic acid derivative.

Oral supplementation with relatively high doses of vitamins E and C has led to some photoprotection in the form of mild to moderate increases in the amount of UV irradiation required to produce the MED.[38,39] In another combination study, vitamins E and C, lycopene, β-carotene, selenium and proanthocyanidins were given as oral supplements to young human volunteers and found to decrease MMP-1 expression,[40] although other parameters such as MED did not reach significance. Other studies have shown no effect in spite of supplementation sufficient to raise the plasma levels of both vitamins.[41] This is in contrast to reports of efficient topical delivery of vitamins E and C to the skin.[42]

Another endogenous antioxidant with a quinone structure related to vitamin E is ubiquinol or $CoQ_{10.}$ Beyer 1990[43] noted an age-related decrease in

CoQ_{10}, so restoring the levels of this molecule may be worth investigating as a means of reducing oxidative damage. Hoppe *et al.* used human keratinocytes and fibroblasts to demonstrate that topically applied CoQ_{10} protected cells from UV-A-induced DNA damage, spared glutathione and suppressed collagenase expression.[44] They also observed a significant reduction in the depth of wrinkles around the eyes of 20 elderly volunteers with daily application of 0.3 percent CoQ_{10} for 6 months. Another study failed to demonstrate an effect of CoQ_{10} on MED or reduction of sunburn cells formation in pig skin.[45]

Alpha-lipoic acid is another endogenous antioxidant, required for the efficient biochemical function of vitamins E and C. This molecule has been shown to restore the age-dependent loss of reduced glutathione in brain tissue.[46] Lipoic acid was found to spare tocopherol and ubiquinol in keratinocytes following UV-A irradiation,[47] but no photoprotective benefit was noted upon topical application to skin.[48]

10.2.3 Polyphenols

Plant phenols and polyphenols constitute another large and important group of naturally-occurring antioxidants by virtue of the fact that the phenolic group can stabilize oxidative radicals.[49] They are widely dispersed throughout the plant kingdom. Flavonoids, flavonoid glycosides, catechins, proanthocyanidins, flavanolignans, and phenylpropanoids are all examples of plant phenolic compounds for which there is much supporting evidence for an antioxidative benefit to skin.[50,51] Plant phenolic compounds have shown direct antioxidation in quenching superoxide anion, oxidative radicals and lipid peroxidation. Many members of this group have also demonstrated anti-inflammatory and antimicrobial activities, and influence on gene expression (such as downregulation of collagenase) by mechanisms that may be only indirectly related to their inherent antioxidant activity.[52,53] Phenolic antioxidants can induce the expression of genes encoding antioxidative and detoxification enzymes through interaction with the antioxidant response element that is found in the promoter region of the enzyme's gene.[54] While as a whole, the evidence suggests that flavonoids and other phenolics are antioxidant and protective, it should also be pointed out that prooxidant activity has been reported for individual compounds used at high concentrations[55,56] or under particular cellular conditions.[57]

Genistein is an isoflavone that is found in legumes including red clover, but soy is the most prominent source since it is such a staple in the Asian diet.

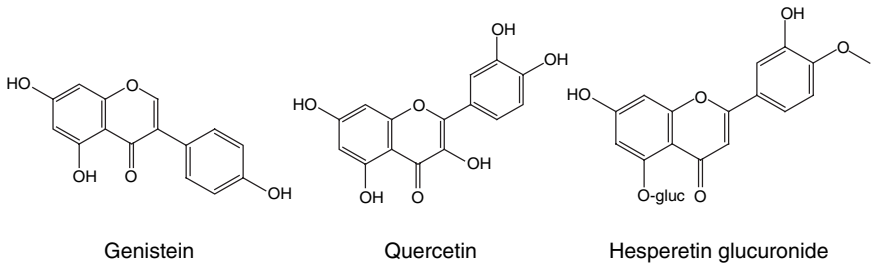


Figure 10.2 Flavonoid antioxidants.

It has been shown to inhibit skin carcinogenesis in a number of experimental models, and to reduce UV-mediated skin aging.[58] Topical application of genistein to human skin was found to inhibit induction of collagenase mRNA by inhibiting UV-induction of the MAP kinase pathways, ERK and JNK. No decrease in UV erythema was noted.[59] Moore *et al*. 2006[60] demonstrated that pretreatment of reconstituted human skin with genistein prior to UV-B irradiation substantially protected the cells from DNA damage, specifically from pyrimidine dimer formation. Topical treatment of hairless mice with quercetin was demonstrated to inhibit increases in myeloperoxidase and protease activity as well as reduced glutathione (GSH) depletion secondary to UV-B irradiation.[61]

Another flavonoid, α-glucosylrutin, has also been reported to prevent oxidative damage to skin in clinical settings.[62]

Hesperetin glucuronide, but not hesperetin, protects human skin fibroblasts against UV-A-induced necrotic cell death.[63]

10.2.4 Tea (*Camellia sinensis*)

Although green tea is largely regarded as a popular beverage rather than a medicinal herb, *in vitro* studies have demonstrated that a group of flavonoid derivatives called catechins have powerful antioxidant capacity. Extracts of both black (fermented) and green (non-fermented) tea were found to be potent scavengers of hydrogen peroxide in vitro and significantly inhibited UV-induced DNA damage (formation of 8-hydroxy 2′deoxyguanosine).[64] One of the major tea catechins, (–)-epi-gallocatechin 3-gallate (EGCG) has been most frequently studied.[50,65] EGCG moderates oxidation and the inflammatory process at several cellular levels including free radical

scavenging, inhibition of cyclooxygenase (COX-2), 5-lipoxygenase (5-LOX), nitric oxide synthase (NOS), and transcriptional modulation.[66–72] Exposure of cells or whole animals to green tea or individual catechins demonstrates that cells experience less damage from oxygen free radicals, and the fact that DNA suffers less damage is particularly relevant to anti-aging effects and a lower risk of carcinogenesis.[65,73–76]

Incubation of cultured keratinocytes with EGCG protected them from oxidative damage caused by UV-A irradiation.[77] In an investigation involving topical application of EGCG to guinea pig or mouse skin prior to UV exposure, EGCG was found to significantly inhibit lipid peroxide formation as well as erythema, and to reduce roughness and sagginess. Using human fibroblasts, the researchers confirmed inhibition of collagenase expression and inhibition of activation of the pro-inflammatory transcription factors NFκB and AP-1.[78] In cell culture models as well as hairless mice, EGCG as well as theaflavins from tea were shown to inhibit the activation of MAP kinases.[79,80] EGCG was also found to enhance DNA repair through an interleukin-12-dependent mechanism.[81]

Several human clinical studies relevant to the use of tea for skin benefits have been conducted, with some promising results. Green tea (oral or topical administration) was shown to protect the skin of mice from ultraviolet radiation-induced changes to DNA that are known to be associated with the development of skin cancer.[82,83] In an additional experiment, the forearm skin of two human volunteers was exposed to the same dose of UVA radiation (2.5 J/cm^2) with or without pre-treatment with topical green tea extract; the skin was protected from inflammation and redness in the presence of green tea. Application of EGCG was demonstrated to reduce UV-induced skin inflammation,[84] and a combination of dietary and topical green tea improved elastic tissue content in a small clinical trial.[85]

There is considerable evidence[77,86] that green tea catechins may act synergistically, and green tea contains many other flavonoids and polyphenols that likely contribute to its anti-aging benefits. Other compounds of note include caffeine, a xanthine that has demonstrated antiinflammatory effects through the inhibition of a phosphodiesterase enzyme, and caffeic acid. Caffeic acid and a multitude of natural derivatives thereof are phenylpropanoid polyphenols that possess potent antioxidant and antiinflammatory activities.[87] Caffeic acid was found to inhibit IL-10 expression and activation of the MAP kinases, JNK and ERK, after UV-B treatment of mice. The implications are that caffeic acid application can reduce immunosuppression and oxidative and inflammatory responses subsequent to UV exposure.[88]

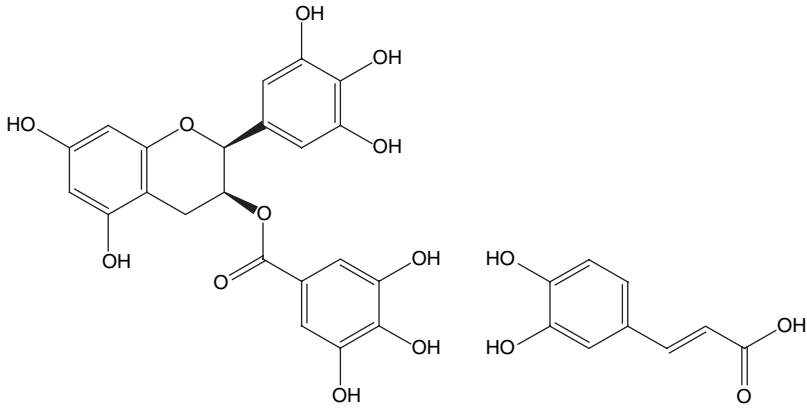


Figure 10.3 Antioxidants in tea.

10.2.5 Grape (*Vitis vinifera*)

Like green tea, grapes are not generally regarded as medicinal herbs, but they are associated with many important health benefits.[89–91] Unlike green tea, there is little published data to support topical use of grape extracts. Research into the "French paradox," the observation that moderate red wine consumption in conjunction with a diet that is relatively high in saturated fat leads to positive effects on cardiovascular health, has uncovered numerous biochemicals and specific biological benefits of red grapes.[92–94]

Grapes contain some of the same catechin polyphenols that occur in tea, including epicatechin and EGCG as well as the related molecule, cyanidin, that yields the red/purple color. Particularly in the skin and seeds, these simple flavonoids are polymerized into larger molecules called condensed tannins or oligomeric proanthocyanidins (OPCs). The catechins and OPCs demonstrate strong antioxidant and antiinflammatory activities[95] as well as chemopreventive effects.[96] Oral supplementation of hairless mice with grape OPCs was reported to prevent UV-B-induced skin carcinogenesis, and the authors correlated this to a proportional decrease in lipid peroxidation.[97] A subsequent study with human epidermal keratinocytes showed that the photoprotective mechanism of grape OPCs involved inhibition of UV-B mediated activation of MAP kinase proteins, ERK, JNK and p38, as well as the NFκB pathway of inflammation.[27]

Many other flavonoids and flavonoid glycosides such as rutin, quercetin and myricetin are present in grape[98] and are known to exhibit abundant

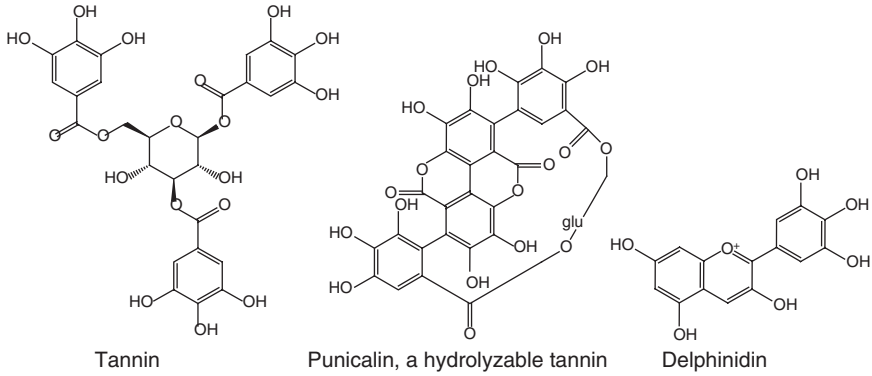


Figure 10.4 Antioxidant tannins.

biological effects including antimicrobial, free radical scavenger, and anti-inflammatory activities.

The story of the French paradox renders red grapes the best recognized source of the polyphenolic compound, resveratrol, though it occurs in many other plants as well. Resveratrol is an intensely studied natural chemical that has been shown to have a variety of biological activities such as antioxidant, inhibition of several aspects of inflammation, and chemoprevention.[99–103] Application of resveratrol to hairless mouse skin prior to UV-B irradiation resulted in significant decreases in lipid peroxide formation, edema, and leukocyte chemotaxis as well as inhibition of the inflammatory enzyme cyclooxygenase and an enzyme associated with tumor promotion, ornithine decarboxylase.[104] Protective effects of resveratrol against short-term markers of photocarcinogenesis were also reported in this mouse skin model.

Although a recent review presents much data to support a chemopreventive action of resveratrol[105] one study sounds a cautionary note, indicating that resveratrol was found to enhance DNA damage in keratinocytes after UV-A exposure.[106]

10.2.6 Other Tannins

French maritime pine bark (*Pinus pinaster* ssp *atlantica*) yields an extract commercially called pycnogenol that is rich in OPC tannins. It has been shown in topical as well as oral human studies to reduce UV-induced erythema. It also inhibits NFκB-dependent gene expression thereby reducing inflammation.[107,108]

Hydrolyzable tannins are another type of tannin in which several gallic acid units or dimers are linked to glucose forming highly hydroxylated compounds of mw 1000 or greater. Pomegranate (*Punica granatum*) fruit is a botanical source of hydrolyzable tannins such as punicalagin and punicalin as well as the polyphenolic anthocyanins that yield the deep red color. The fruit extract has been found to inhibit phorbol ester-induced skin tumorigenesis in a mouse model, inhibiting phosphorylation of MAP kinase proteins and also the activation of NFκB-mediated inflammation.[109] A similar result was obtained in normal human epidermal keratinocytes irradiated with UV-B, implying photoprotection.[110] Further studies in human keratinocytes and mouse skin on delphinidin, the major anthocyanin in pomegranate and common in many other deeply colored fruits and vegetables, confirmed a role for this compound in the protective effects that had been observed. Delphinidin inhibited UV-B induced lipid peroxidation and reduced DNA damage thus preventing apoptosis.[111]

Emblica officinalis is another example of a plant with potent antioxidant activity attributable to hydrolyzable tannins. The fruit of this tree has been employed in Ayurvedic medicine for millennia, and it is central to many important healing preparations both internal and external.[112] A commercial preparation has been characterized for use in skin care, and was found to protect fibroblasts from superoxide-induced oxidative stress and to be quite stable over time, especially since the products of oxidation of these tannins retain antioxidative activity .[113] The absence of prooxidant activity is attributed to the capacity of the active tannins to chelate Fe^{3+} and Cu^{2+}.

10.2.7 Lignans

Silymarin is actually not a single entity but a mixture of several flavonolignan molecules including silybin and silychristin that are isolated from the seeds of the milk thistle plant, *Silybum marianum*. In a number of studies, topical application of silybin to mouse skin significantly inhibited UV-induced sequelae including skin edema, sunburn cell formation, depletion of endogenous catalase activity, phosphorylation of MAPKs, formation of thymine dimers in DNA, induction of cyclooxygenase activity and activation of NFκB.[114–117] Silymarin also led to increases in the tumor suppressor p53 in these studies, and more recent experiments found it to increase the levels of the immunostimulant IL-12 in the skin.[118] In work done with SENCAR mice (sensitive to carcinogenesis), pretreatment with silymarin prior to the tumor promoter, TPA, resulted in strong antioxidant effects, with significant inhibition of lipid peroxidation and myeloperoxidase, as

well as sparing of epidermal superoxide dismutase (SOD), catalase, and glutathione peroxidase activities. Significant anti-inflammatory effects were also observed with inhibition of cyclooxygenase and epidermal lipoxygenase activities as well as induction of IL-1α.[119] Feeding studies with hairless mice found strong evidence for protection of silybin against UV-B-induced skin damage including DNA damage and sunburn cell formation.[120]

10.2.8 Phenylpropanoids

Caffeic acid and ferulic acid are very commonly occurring phytochemicals, and have already been introduced above as active examples of the phenylpropanoid class of phenolics. Several additional examples of phenylpropanoid derivatives with demonstrated benefits to skin are of interest.

Turmeric (*Curcuma longa*) has been cultivated for thousands of years in India. The roots have been prized for culinary purposes as well as for their medicinal virtues. In Ayurveda, turmeric is a veritable household remedy used internally for colds, fever, liver disorders, and GI distress, but is also applied externally for skin and joint inflammation, wounds, eczema, and other skin disorders.[121]

The antioxidant and antiinflammatory compounds in turmeric are known as phenylpropanoids. Curcumin, or diferuloylmethane is the bright yellow compound that gives turmeric its color as well as much of its biological activity.[122] Much scientific research has been conducted on curcumin and the related compounds called curcuminoids that are found naturally in turmeric. Potent antioxidant activity has been documented for curcumin,[123] and treatment of keratinocytes and fibroblasts with low levels of the compound have been shown to protect the cells from hydrogen peroxide.[124] Topical application of this compound enhances levels of the cellular antioxidant, glutathione, and reduces arachidonic acid metabolism that leads to inflammation.[50,125] Curcumin has also been demonstrated to inhibit the NFκB pathway of inflammation.[126] It has been found to enhance tissue repair and wound healing[127] and cancer preventive activities have also been described for curcuminoids.[128,129] Curcumin inhibits UV-induced activation of JNK,[130] and topical application was found to potently inhibit the UV or UV and TPA-induced expression of metallothionein or ornithine decarboxylase.[131] Curcumin up-regulates the tumor suppressor p53 in human basal cell carcinoma cells.[132] Studies with oral curcumin treatment

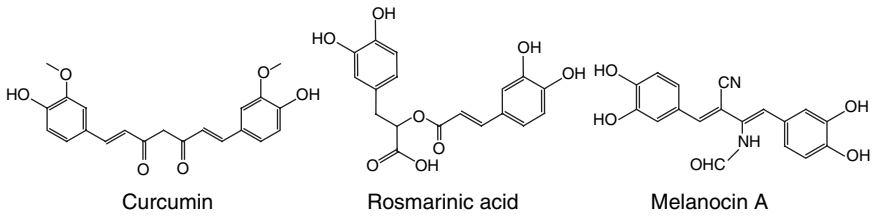


Figure 10.5 Phenylpropanoid antioxidants.

of diabetic mice show that the compound inhibits the generation of collagen cross-linking as well as cataract formation, and also lowers blood lipid and glucose levels, and promotes wound healing.[133,134] Since these areas are of concern in aging, it is interesting to note that a recent study found curcumin to inhibit lipid peroxidation and prevent protein glycosylation caused by elevated blood glucose levels.[135] The authors also found evidence to support direct scavenging of superoxide radicals by curcumin and significant enhancement of cellular metabolism of glucose. One group reported natural, mixed curcuminoids to be more protective against superoxide than the same concentration of pure curcumin.[136]

Rosmarinic acid is a phenylpropanoid compound found in rosemary leaves as might be suspected from its name, but it is also common in many other species of plants and culinary herbs. It has been long used as a food preservative and its well-established antioxidant activity is being increasingly exploited in the modern prepared food industry. RA also possesses antimicrobial, antiviral, and anti-inflammatory properties.[137] It has been shown to inhibit lipopolysaccharide-induced nitric oxide generation and inducible nitric oxide synthase (iNOS) synthesis, phorbol ester-induced superoxide formation and peroxynitrite-mediated cell damage.[138] RA has been investigated as a potential detoxifying partner for the anti-tumor agent adriamycin (ADR).[139] It was found to substantially inhibit ADR-induced apoptotic cell morphology and caspase activation by reducing cellular ROS. RA restored the downregulation of SOD and glutathione levels as well as mitochondrial membrane potential. RA also reduced the ADR-induced activation of the MAP kinases ERK and Jun. Another phenolic rosemary constituent, carnosic acid, was already mentioned above as an inhibitor of UV-induced MMP-1 mRNA.[35]

Melanocin A is a phenylpropanoid molecule elicited by the fungus *Eupenicillium shearii* F80695. It has recently been reported to inhibit UV-induced

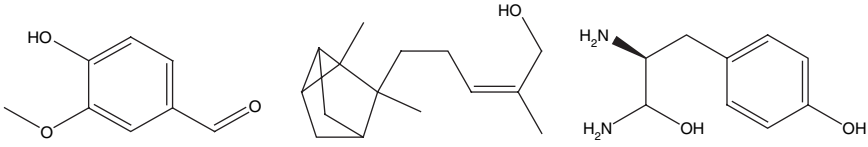
expression of matrix metalloproteases MMP-2 and MMP-9 in cultured human keratinocytes as well as in a hairless mouse model.[140] UV-induced increase in skin thickness and wrinkling was significantly reduced by melanocin A.

10.2.9 Miscellaneous Natural Antioxidants

Application of psoralen in combination with UVA light (PUVA therapy) is commonly used to treat skin conditions such as psoriasis and vitiligo. An extract of the fern, *Polypodium leucotomos*, administered orally, reduced phototoxicity due to PUVA therapy and also decreased UV-induced skin damage in normal healthy subjects.[141] The main antioxidant compounds in the extract were small phenolics like hydroxybenzoic acid, hydroxycinnamic acid, ferulic acid, and caffeic acid.[142] In *in vitro* experiments with cultured fibroblasts and keratinocytes, *P. leucotomos* decreased UV-induced cell damage due to the inhibition of lipid peroxidation and MMP-1 expression, along with enhanced elastin expression.[143]

The phenolic aldehyde compound, vanillin, has been found to inhibit the nitration of tyrosine by peroxynitrate in a cell-free system, suggesting that it may act as an effective peroxynitrite scavenger.[144] Alpha santalol is another terpenoid and is a major component of sandalwood oil. It has been found to be effective in preventing experimentally induced skin cancer by stimulating apoptosis of damaged cells in a mechanism that involves activation of caspase-3.[145]

Mannitol was found to be an effective inhibitor of UV-B-induced DNA damage as measured by the marker, 8-oxo-7,8-dihydro-2′- deoxyguanosine. The protection of DNA from oxidative damage was attributed to the ability of mannitol to scavenge hydroxyl radicals.[146]



Vanillin Alpha-santalol Hydroxytyrosol

Figure 10.6 Other natural antioxidants.

Hydroxytyrosol is a small phenolic compound that is a major antioxidant in olive oil. It has been reported to inhibit markers of oxidative stress as well as protein damage induced by UV-A irradiation of human melanoma cells.[147] Olive phenols are peroxynitrite scavengers, and their effect can be persistent since their oxidation products are also functional antioxidants.[148]

10.2.10 Carotenoids

Topical retinoids are approved as drugs to treat photodamaged skin. By enhancing dermal collagen synthesis, limiting matrix degradation by inhibiting MMPs, inhibiting activation of JNK, and stimulating activity of ERK, tretinoin (retinol) is effective in reducing fine wrinkles, skin roughness, and skin laxity. Use of this compound is associated with a high incidence of dermatitis and skin dryness, although newer generations of topical retinoids have lessened such side effects.[149–152]

Retinol is by far the most prominent carotenoid substance studied for skin aging, but other natural compounds have also been examined. In human fibroblasts, lycopene, β-carotene and lutein were all capable of significantly reducing lipid peroxidation caused by UV-B; however, dose was of critical importance as higher doses led to prooxidant effects.[153] Offord et al.[35] found lycopene and β-carotene to enhance the expression of MMP-1 in cultured, UV-irradiated human skin fibroblasts; this detrimental effect was reversed in the presence of vitamin E, and the authors suggest that the carotenoids may require their own antioxidant protection for stability.[35] This inherent instability even compared to other antioxidants, along with their deep coloration imparted to the skin, may account for the lesser use of carotenoids in topical products. Wertz et al. found that UV-A-irradiation of human keratinocytes nearly obliterated β-carotene levels, but if test levels of the compound were maintained by subsequent supplementation, significant suppression of MMP-1, MMP-3 and MMP-10 was detected, with no synergistic effect of vitamin E.[154] The mechanism was dependent upon quenching of singlet oxygen, and independent of retinoic acid regulatory pathways.

Astaxanthin, the oxygenated carotenoid found in red marine microalgae, was determined to reduce UV-induced DNA damage and spare the antioxidants SOD and GSH in human skin fibroblasts and melanocytes.[155] Dietary supplementation with lutein plus zeaxanthin (both also oxygenated carotenoids) was also effective in reducing UV-B-induced skin edema and DNA damage in hairless mice.[156] Mice fed lutein in their diet were

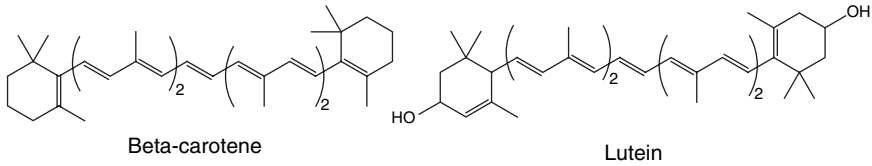


Figure 10.7 Carotenoids.

shown to exhibit less UV-induced inflammation, immunosuppression and generation of ROS in skin.[157]

One clinical study reported significant improvements in skin thickness, density, roughness and scaling after twelve week oral supplementation with lycopene, lutein, β-carotene, α-tocopherol and selenium.[158] Another human trial with oral administration of lycopene β-carotene, α-tocopherol and selenium reported decreased UV-induced erythema, lipid peroxidation and sunburn cell formation.[159]

10.2.11 Other Mechanisms for Reducing Oxidative Cell Damage

A few other examples of natural products which counter oxidative damage through mechanisms other than antioxidation are of interest. Proteosome assemblies within a cell's nucleus and cytoplasm are responsible for eliminating dysfunctional and obsolete proteins from the cell. Since proteosome competence decreases with age, enhancement of the activity of this organelle may decrease accumulation of oxidatively damaged proteins. Proteosomal peptidase stimulation by an extract of the algae, *Phaeodactylum tricornutum,* reduced the level of oxidatively damaged proteins in human keratinocytes subjected to UV-A and UV-B irradiation.[160] Curcumin has also been found to stimulate activity of the proteosome.[161] Shifting from elimination of oxidized proteins to repair, the enzyme Met-S-sulfoxide reductase has recently been identified as an enzyme in human skin that can repair oxidative protein damage.[162] The enzyme is reported to be up-regulated by UV-A; it would be of interest to screen natural products for the ability to induce or enhance the activity of this enzyme.

Increasing the level of creatine in normal human skin fibroblasts protected the cells from oxidative damage caused by UV-A irradiation.[163] Stimulation of cellular energy with exogenous creatine essentially eliminated the generation of the mitochondrial common deletion mediated by singlet oxygen as well as the increase in MMP-1 following UV-A irradiation.[164]

10.2.12 Summary

There is mounting evidence that antioxidants can help to prevent and even reverse some of the biochemical consequences of skin aging that lead to physical changes such as wrinkles, laxity of tone, and skin roughness. Much of the scientific work in this area relies on models of UV-induced damage, tending to skew the significance more toward photoaging than intrinsic aging; however, sun exposure is unquestionably a factor in the aging of the face, the target of most cosmetic intervention with respect to skin aging.

The current scientific evidence tends to support improved stability as well as efficacy for formulations containing lower levels of multiple antioxidants as opposed to higher concentrations of a single compound that may exert an undesired prooxidant effect.[55] A healthy diet that includes antioxidant-rich fruits and vegetables may help to maintain the general health of the skin. Supplements are being explored as potential therapies for skin diseases as well as skin aging, and some have been shown to increase skin concentrations of the supplemental antioxidant with a measurable benefit to skin. Topical application of an antioxidant often appears to be a more effective strategy in studies published to date, but systemic/topical combination protocols may hold future promise.

10.3 Inflammation and Skin Aging

10.3.1 Causes and Consequences of Inflammation

Inflammation is a local, protective response of the body's immune system to an insult, chemical or microbial invasion. This process consists of the release of a large variety of oxidants, cytokines and proteolytic enzymes by various cells of the immune system such as neutrophils, mast cells and macrophages. The role played by these oxidants and proteolytic enzymes is obviously to neutralize and eliminate all the bacteria and viruses which have invaded the skin, or to cope with antigens that are recognized as "non-self" and are at the source of the activation of this inflammatory reaction. This process must be fine tuned to reduce the risk of excessive or deficient responses. Clearly, an insufficient response will lead to elevated microbial colonization, and other pathological conditions such as sepsis, whereas excessive responses results in autoimmune disease such as rheumatoid arthritis, arteriosclerosis, and other debilitating conditions.

The perfect balance between an excessive and an insufficient response is rarely achieved in the real life situation, and more often one observes the

development of a virulent inflammatory response even in the absence of any true bacterial invasion. For example the well known sunburn reaction observed a few hours after sun exposure, is not triggered by the invasion of the skin by any foreign substance, but by a reaction of the skin's immune system to the direct damage caused by the UV light to some of the essential components of the cells such as cell membrane and DNA. Interestingly, this excessive reaction of the innate immune system, occurs at a time when the acquired immune activity is subdued, due to the migration of antigen-processing cells such as Langerhans cells out of the skin to the lymph nodes.[165] Consequently, despite the virulent inflammatory response induced by the damage generated to the cells upon UV light exposure, the skin is at that time defenseless against an invasion by bacteria or other toxic substances.[166] Clearly, exposure to UV light, triggers simultaneously an overreaction of the immune system in the form of an inflammatory reaction and a significant reduction of the skin immune function, due to the disappearance of the Langerhans cells from the skin itself.

One of the most fundamental reasons behind the overreaction of the skin immune system to an antigen, to UV light exposure, or to an oxidative stress such as exposure to ozone, can be traced to the deficiency of the barrier function of the stratum corneum.[167] If this function is compromised either locally because of the disorganization of the multi-lamellar structure of the extra-cellular lipids, or generally because of the thinness of the stratum corneum, one will expect to see a significant increase in the penetration of molecules through the stratum corneum. This will result in either the activation of the immune response by antigens, or the induction of a cascade of free radical reactions by oxidants that will damage skin cells, leading to the development of an inflammatory reaction. Interestingly, one of the consequences of the development of these localized inflammations is the hyper-proliferation of epidermal keratinocytes, leading to hyperkeratosis, which on its own is the main cause for the deficiency of the stratum corneum barrier function. In such a condition, this self perpetuating process will lead to a constant stream of inflammatory reactions in the skin leading to accelerated premature aging.[168]

Many laboratories over the past decade have developed new technologies to maintain certain equilibrium in the activity of the immune system during and after the exposure to the environment. The topical application of mixtures of anti-oxidants and non-steroidal anti-inflammatory drugs has been shown to significantly reduce the magnitude of the inflammatory reaction resulting from the exposure to environmental stress conditions. Similarly,

new technologies have been developed to prevent UV induced immunosuppression, in order to maintain the proper level of immuno surveillance in the skin.

The most relevant consequence of an inflammatory reaction in the skin, is the occurrence of oxidative reactions induced by the various free radicals released by the immune cells such as mast cells, neutrophils and macrophages.[169] As discussed in detail at the beginning of this chapter, oxidative stress plays a major role in the intrinsic as well as extrinsic aging process, and therefore, it is important to control the inflammatory reactions which occur as result of an excessive activity of the immune system.

Another indirect result of the development of an inflammatory reaction is the activation of a series of proteolytic enzymes deeper in the dermis which will not only target the elimination of bacteria, but in addition will create significant damage to the proteins of the extracellular matrix such as the collagens and elastin.[170] The manifestation of this inflammation-induced degradation of key components of the dermis will not become apparent for a while, as the production of fresh collagen and elastin supersedes the destruction caused by the activity of the inflammation-induced proteases. However, as the skin ages, the "replenishment" and reorganization of fresh collagen and elastin diminish markedly, and will not be able to compensate for the inflammation-induced degradation of the extracellular matrix.

The physical manifestation of the inflammatory reactions we have described so far are quite visible and are expressed in the form of redness, swelling, stinging and burning. These reactions occur occasionally only after exposure to a significant stress. The chronic cumulative exposure to very low doses of UV or other oxidative stresses has been shown to generate what is called subclinical inflammation; that is, a constant, invisible series of reactions similar to those occurring after a visible inflammation, but which cannot be perceived even by a trained observer. The consequence of this quasi constant level of sub-clinical inflammation is a continuous degradation and disorganized remodeling of the ECM, as well as the accumulation of additional oxidative damage in the skin leading to accelerated premature aging.[171]

Although the importance of the damage induced by short term exposure to a high level of UV light cannot be underestimated, it becomes evident that the effect of low dose chronic exposure to sun, pollution, and other oxidative stresses conditions may be more devastating over the long run, and have a significant impact on the evolution of the aging process. Therefore,

it becomes necessary to develop technologies that will control constantly these low intensity inflammatory reactions, by developing topically applied cosmetic products which will be used on a daily basis (moisturizers, liquid foundations), and not just products designed to protect the skin from the occasional high intensity UV exposure (beach products).

10.3.2 Technologies to Control Inflammation

The increased awareness in the dermatological community of the role played by inflammatory reactions in the course of the skin aging process has led to the development of a multitude of technologies, mostly based on the control of specific inflammatory pathways to reduce the impact of this process on skin cells. However, before describing the different anti-inflammatory therapies available today, it is important to review first an alternative and relatively successful technology to prevent the development of inflammatory reactions on the skin. This approach aims to strengthen, and in some cases to reactivate the barrier properties of the stratum corneum.

Such repair benefits can be achieved in two different ways, first by applying on the skin surface a blend of lipids similar in composition to the lipids found between the cells of the stratum corneum, or/and by activating the natural differentiation process of the keratinocytes into corneocytes.

10.3.3 Restoration of Skin's Barrier Function

The first technology is currently being used in a variety of cosmetic products mostly to increase skin moisturization, as improving the barrier property will slow down the trans epidermal water loss (TEWL), resulting in a longer retention of the water inside the skin especially in the stratum corneum.[172] The composition and ratio of the lipids which form the multilamellar structure that separates the corneocytes in the stratum corneum is well known, and consists of a mixture of ceramides, fatty acids, and cholesterol. Results indicate that the topical application of an emulsion containing a mixture of these three lipids used in equal concentration will significantly restore the barrier properties of the stratum corneum.[173,174]

The second technology utilizes the activation of the cellular differentiation process in order to increase the production of the epidermal lipids by the keratinocyte. One of the most important steps occurring at the time of the transformation of a keratinocyte in the living epidermis into a corneocyte

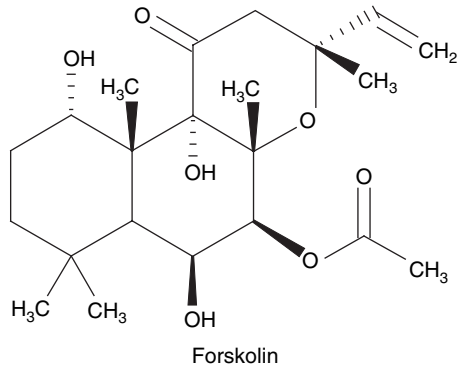


Figure 10.8 Chemical structure of Forskolin (MW 410.5), a diterpene extracted from *Coleus barbatus*.

in the stratum corneum is the synthesis and the release of these lipids into the extra-cellular space. These lipids form a very well defined multi-lamellar structure which will be responsible for the barrier properties of the skin.[175] The activation of this cellular differentiation process can be achieved by exposing the keratinocyte to forskolin, a molecule extracted from *Coleus barbatus*, resulting in an increased production of epidermal lipids by the epidermal cells.[176]

The advantage of these technologies is to slow down and even prevent the penetration of toxic, allergenic, or oxidative substances into the skin through the stratum corneum.[177] As a result, it will provide a very rapid benefit in reducing the likelihood to trigger skin irritation and inflammation. We have observed in our laboratory, that the regular usage of products containing these technologies significantly reduces the skin reactivity to irritants and sensitizers of individuals with what is claimed "sensitive skin." Consequently, this barrier strengthening technology, should be incorporated systematically in every moisturizer and anti-aging cosmetic product, as it provides significant skin protection benefits, and therefore will result in a significant reduction of the skin "reactivity" to the environment in the form of a reduction of inflammatory reactions.

10.3.4 Control of the Inflammatory Pathways: The Multi-Prong Approach

Once an irritant, a toxic molecule or a bacterium has penetrated through the stratum corneum barrier into the living layers of the epidermis and the

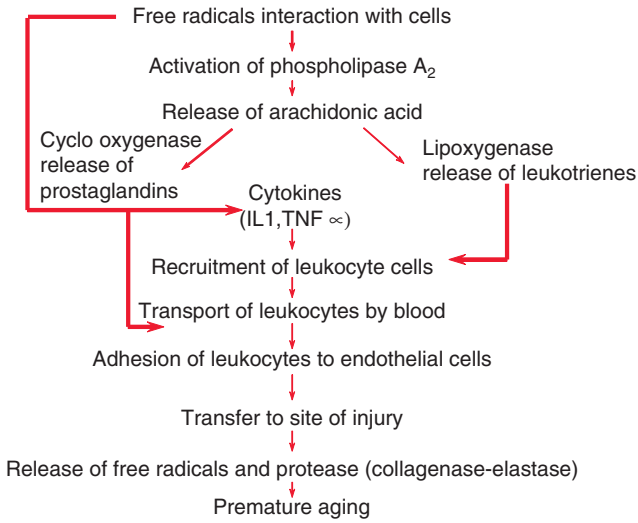


Figure 10.9 Cellular inflammatory pathways induced by oxidative stress.

dermis, it will trigger an immune reaction via the antigen presentation function of the Langerhans cells leading to the recruitment of T-cells.

Alternatively, the damage caused to a cell by a free radical will trigger a succession of inflammatory reactions, leading ultimately to the activation of matrix metalloproteases (MMPs) and the resulting premature degradation and disorganization of the dermal ECM.

A simplified scheme of this cascade of inflammatory reactions is shown in Figure 10.9, which describes the various inflammatory pathways activated once a cell has been exposed to an oxidative stress.

From the complexity of these pathways, one cannot expect to control inflammatory reactions by using antioxidants alone in order to prevent the activation of the first enzyme of the cascade (phospholipase A_2), with the hope by controlling this first step, a modulation of the rest of the inflammation cascade will be achieved. Similarly, one cannot expect to effectively control the course of the inflammatory reactions by just intervening in one of the pathway described in Figure 10.9, as even the most efficient control of the initial activation of phospholipase A_2 or cyclooxygenase, will not prevent the release of interleukins and tissue necrosis factor, leading to the recruitment and the activation of T cells. Clearly, a multi-prong

approach has to be taken to control each branch of the inflammatory pathway if one wants to reduce significantly the progress of the inflammation in the skin, and hence reduce its impact on the premature accelerated aging process.

Over the past ten years, the research leading to the development of new and more effective anti-inflammatory ingredients has led to the evaluation of the efficacy of many different plants which were known in folkloric medicine to soothe and calm irritated skin. New assays have been developed to measure the activity in controlling specifically each of the inflammatory reactions described in Figure 10.9, which allows for the thorough screening of new active ingredients extracted from various plants with known anti-irritant properties.

10.3.4.1 Control of Phospholipase A_2 Activity

Starting with the control of the very first step of the cellular inflammatory reaction pathways, one needs to inhibit the phospholipase A_2 activity. This should in turn control the release of arachidonic acid into the extracellular environment, and therefore modulate the course of the inflammatory reactions.[178] Many plant extracts and synthetic molecules have been shown to control the activity of phospholipase A_2. Our studies have indicated that pomegranate extract is one of the most efficacious, with an EC50 of about 100 ug/ml, ranking this ingredient as one of the most potent phospholipase A_2 inhibitors we ever tested.[179] This extract is obtained from the fruit of *Punica granatum* L., belonging to the Punicaceae family growing in the Mediterranean area. This fruit extract is rich in ellagic acid, a dilactone and potent inhibitor of the mutagenecity of various carcinogenic substances, providing protection against chromosome damage. In addition to its anti-inflammatory activity, ellagic acid has been shown to be a potent antioxidant.

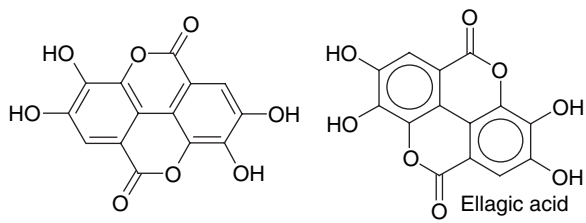


Figure 10.10 Chemical structure of ellagic acid (mw: 302), a polyphenol anti-oxidant.

10.3.4.2 Control of the Cyclooxygenase Pathway

The second step of the inflammatory reactions is the activation of the enzyme cyclooxygenase 2. This enzyme activates the production of various prostaglandins which play an essential role in the development of the inflammatory reactions.[180,181] Prostaglandin E_2, for example, increases blood infiltration via the dilation of the blood vessels and microcapillaries to facilitate the transport of the various immune cells from the lymph nodes to the site of injury. This process generates edema and redness. This enzyme's activity is responsible for the sunburn reaction observed a few hours after exposure to UV radiation. Extensive research has been conducted in the area of cyclooxygenase 2 inhibition, as it is a key to control this very important step of the inflammatory reactions, and new inhibitors are discovered on a regular basis by the pharmaceutical industry.

Many plant extracts have been found to be potent inhibitors of cyclooxygenase. In this review we will focus on the activity of resveratrol, (3,4′-5-trihydroxystilbene), a molecule commonly extracted from red grapes, as well as the root of *Polygonum cuspidanum*.[181]

Studies show that resveratrol is one of the most potent cyclooxygenase 2 inhibitors with an EC 50 of 30 ug/ml. Such activity has been demonstrated to be the underlying reason behind the anti-carcinogenic effects attributed to resveratrol, as it has been shown to control the initiation, promotion and progression steps of tumor development. Recent work links this effect to the ability of this compound to control the nuclear transcription factor (NFkB) as well as the activator protein 1 (AP1) which are linked to the initiation step of cancer.[182]

More recent work conducted by the group of Dr. Sinclair at M.I.T., reports very promising results showing the increase of Sirt1 expression in mammalian cells treated with resveratrol. As the activation of this deacetylase enzyme has been linked to an increase of cellular life span, resveratrol will

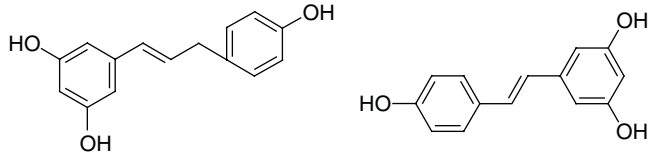


Figure 10.11 Chemical structure of resveratrol (mw = 228), a phytoalexin produced by various plants.

likely attract a lot of attention in the future, since it has been shown to exhibit more significant effects than simply its anti-inflammatory properties.[183]

10.3.4.3 Control of the Lipoxygenase Pathway

Parallel to the cyclooxygenase-induced release of prostaglandins, another category of enzymes called lipoxygenases have been shown to be a key step in the progression of an inflammatory reaction. This family of enzymes is responsible for the release of leukotrienes. Leukotrienes, because of their chemo-attraction properties for leukocytes, play an essential role in the development of the inflammatory reaction, as they will attract these immune cells to the site of injury for a more complete elimination of the bacteria or the toxic substance which has invaded the skin.[184] Again, the area of lipoxygenase inhibition has been the center of massive research by the pharmaceutical industry. An example is the molecule nordihydroguaiaretic acid (NDGA), which shows activity of reducing the release of leukotrienes.[185]

Nordihydroguaiaretic acid, better known initially for its anti-oxidant properties, has been demonstrated recently to be a very strong lipoxygenase inhibitor, with an EC 50 = 2.87 uM, resulting in a significant modulation of leukotriene activation. As an indirect consequence, NDGA has been shown to inhibit significantly the activity of various MMPs, and therefore to protect the dermal extracellular matrix from premature degradation.[186]

The modulation of the recruitment of leukocytes from the lymph nodes is not induced just by the release of leukotrienes alone. Proteins called cytokines are released directly by various skin cells, activating directly the whole process of recruitment and transport of leukocytes in the blood. Cytokines are a group of small secreted proteins which mediate and regulate immunity and inflammation. Interleukins are a class of cytokines that by definition are produced by one leukocyte and act on other leukocytes.

Interleukin-1 (IL-1) is one of the first described cytokines and is also known as lymphocyte activating factor and endogenous pyrogen. IL-1 is secreted by a wide variety of cells including monocytes, Langerhans cells, fibroblasts and keratinocytes. It is synthesized and released by these cells in response to an inflammatory stimuli. IL-1 has a wide range of biological activities on many different cell types, including B cells, T cells, and monocytes. In addition, they will increase the expression of cell adhesion molecules on endothelial cells which will facilitate the extravasations of white blood cells. These

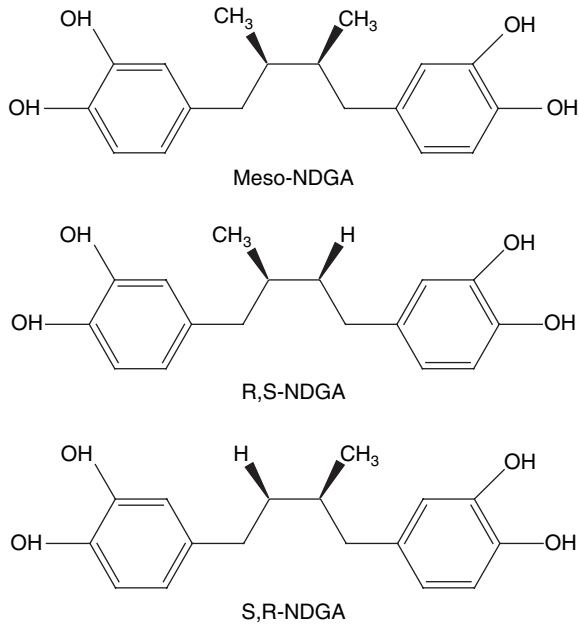


Figure 10.12 Chemical structure of nordihydroguaiaretic acid (mw = 302.3).

interleukins will also stimulate the growth and action of immune cells.[187] It is therefore essential to control the release of these inflammatory cytokines to minimize the triggering of the skin immune reaction each time the skin is exposed to UV light even at sub-erythemal doses.

10.3.4.4 Control of Chemotaxis and Cellular Adhesion

The inflammatory response is characterized in part by local vasodilation and the accumulation of white blood cells at the site of insult. White blood cells, in particular, macrophages and neutrophils, accumulate at the site of "insult" via a process known as chemotaxis. Chemotaxis is the directed movement of cells up a concentration gradient toward a site of inflammation. The concentration gradient that it follows is known as a class of molecules known as chemoattractants. Chemokines are a special class of cytokines that induce cell directed movement (chemotaxis). Chemokines such as interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1) are released from cells at the site of injury. The leukotrienes belong to a class of molecules (eicosanoids) generated from free arachidonic acid via the 5-LOX enzyme. Leukotriene B4 (LTB4) is a potent chemoattractant for

neutrophils. In addition, LTB4 will elicit the adhesion of white blood cells to the endothelial cells that form the walls of capillaries.

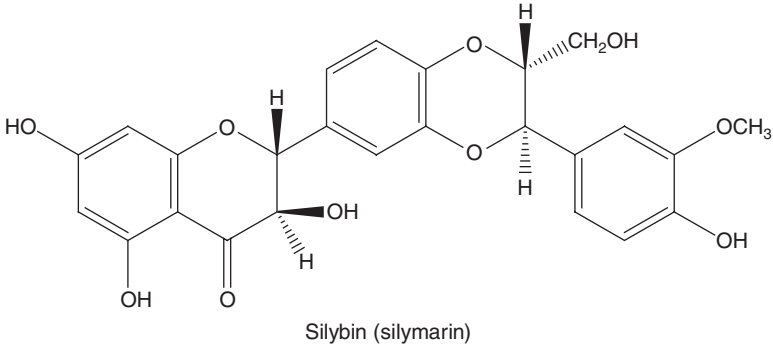
The control of this series of events is achieved first and foremost by the release of prostaglandins which, as we described previously are responsible for the increase in blood flow and as a consequence for the transport of the immune cells (neutrophils, macrophages) to the site of damage in the skin. Various plant-derived compounds have been shown to counteract the chemoattraction process, and as a result to reduce the severity of the inflammatory reaction.[188]

Examples of a few plant derived compounds such as caffeine, sucrose, and a class of sugars called Sialyl-Lewis, have been shown to reduce the infiltration of leukocytes into the skin. More specifically, 3′-Sialyl lactose has been shown to inhibit cell adhesion, and is thought to act by binding to the Sialyl-Lewis X which is expressed on the cell membrane of neutrophils.[189]

10.3.4.5 Control of MMPs

Lymphocytes or other immune cells (i.e., mast cells, neutrophils, macrophages) which have been activated to eliminate a foreign cell (bacteria), or the damage resulting from an oxidative stress, are surrounded by a dense network of collagen and elastin fibers which hinder their progression toward the site of injury closer to the skin surface. The displacement of these immune cells within the dermal and epidermal layers is made possible by the *controlled* breakdown of collagen and elastin fiber network. This process is the result of the activity of various enzymes called metalloproteases which digest these proteins in a very controlled manner to allow for the better migration of the immune cells to the site of injury.[190] This process generates over time a significant degradation of the ECM, leading to a deficiency of the dermal structure.

As we age, the reduction of collagen and elastin production together with this premature degradation of the dermal structure leads to a loss of the skin cohesiveness leading to sagging as well as a loss of elasticity. Although such a process is necessary for the proper development of a skin immune reaction, its impact on the skin structure is significant, especially in a condition of low dose chronic inflammation where a continuous degradation of the dermal structure is taking place.[191] As in the case of the previous phases of the inflammatory reactions, technologies have been developed, mostly by the pharmaceutical industry to control the amplitude and the frequencies of those enzymatic reactions.



Silybin (silymarin)

Figure 10.13 Chemical structure of silymarin (mw = 482.4) a polyphenolic flavonoid.

We have selected examples of various plant extracts with well documented activity in controlling the most relevant metallo-proteases namely, collagenase, elastase, and finally hyaluronidase.

The control of collagenase activity can be achieved via treatment with an extract of *Siegesbeckia orientalis*. This extract, rich in darutosides has been shown to specifically inhibit collagenase activity with an EC50 of 56 ug/ml. The control of elastase activity is achieved by activity of ursolic acid found in *Centaurium erythraea*. Again, the high specific activity of this extract (EC50 = 10 ug/ml) allows for the specific protection of the elastin in the dermis, and as a result prolongs the more desirable property of the skin (i.e., elasticity and bounce).

Finally, it is possible to control the degradation of the glycosaminoglycans which play a key role in maintaining skin plumpness and elasticity and improve the evenness of skin texture due to their ability to bind a large amount of water in the dermis. Hyaluronic acid is especially important in that regard, and needs to be protected from premature degradation by the enzyme hyaluronidase. Silymarin, a flavonoid extracted from *Silybum marianum* (milk thistle), has been shown in vitro to inhibit by 92 percent the enzyme hyaluronidase, when used at a 0.001 percent concentration.

10.3.5 Summary

The foregoing discussion explains in detail the reasons for the limitations of control of inflammatory reactions in the skin by using these ingredients in an isolated manner. Only a carefully designed blend of these anti-inflammatory

ingredients will provide an efficient control of the inflammatory reactions induced by exposure to different environmental stresses. Obviously, there are many other anti-inflammatory actives which can be used together to control all the different pathways we have described. Our selection criteria was based first on the activity of each of these molecules to control specifically the different pathways, as well as the ease to incorporate these molecules into a cosmetically acceptable formula.

Results obtained recently in our labs seem to indicate that such combination of molecules will significantly reduce the intensity of an inflammatory reaction induced either by an irritant, balsam of Peru, or by exposure to UV light. In fact, the reduction of redness measured after treatment with a similar blend of anti-inflammatory substances, was significantly better than what was observed after treatment with a commercial product containing 1 percent hydrocortisone. The obvious advantage of this multi-prong approach to the control of inflammation is that it can be used liberally without any safety or regulatory restrictions all around the world. Independently from the efficacy of this multi-prong anti-inflammatory technology in alleviating the redness and the edema associated with an inflammation, this type of approach will provide significant protection from the damage caused by exposure to the environment.

We believe that the most important impact resulting from the regular use of a cosmetic product containing a similar blend of anti-inflammatory agents is to reduce significantly the premature degradation of the ECM in the dermis caused by the cumulative effect of the chronic sub-clinical inflammatory reactions generated by the exposure to environmental pollution. For this reason, we believe that such anti-inflammatory approaches should be used not only in products designed to control the redness and edema associated with severe inflammatory reactions, but more importantly should be incorporated in daily moisturizers and sun protection products to provide more consistent protection. The regular use of anti-inflammatory blends like the one described in this chapter, is certainly the safest way to provide consistent protection of the skin from *all* the damages generated by exposure to oxidative environmental pollution.

In addition to the obvious benefits generated by a product which reduces the redness and swelling caused by an inflammatory reaction, the most important consequence of the regular treatment with this anti-inflammatory technology is in its anti-aging benefits. Clearly, the succession of events which take place from the very beginning of the reaction when the antigen,

the bacteria or even the UV rays get in contact with the skin, to the very last event where the skin cells are bombarded by the free radicals generated by the immune skin cells, leads to severe damage of both the intra- and extra-cellular structure of the skin leading to premature aging.

Obviously, each inflammatory episode on its own will have fairly limited impact on the skin structure, but if we consider that the skin is constantly exposed to low doses of such stress, we can accept that, over the long run it will age prematurely because of the cumulative damage generated by these low intensity, chronic inflammatory reactions. Under such conditions, because of the frequency of exposure of the skin to these damaging inflammatory situations, it is imperative to use cosmetic products which contain blends of anti-inflammatory components similar to what we have described in this chapter. Only the regular usage of such products on a daily basis will provide the necessary protection which will over the long term reduce the impact of the premature aging process on the skin.

10.4 The Extracellular and Skin Aging

The ECM which is part of the dermis provides the fundamental structure of the skin. It is a highly organized, extracellular network of collagens, elastin, glycoproteins, and proteoglycans that provides physical structure but is also biologically active.[192,193] The ECM is generated by fibroblast skin cells and then interacts with them, thereby regulating the development, regeneration, and normal turnover of the skin. The major component of the dermis at 70–80 percent dry weight is collagen. The proteinaceous fibers of collagen consist of a regular assembly of different, structurally-related collagen subunits that are oriented for strength and resiliency. Necessary remodeling and repair of the collagen matrix is regulated through a balance of synthesis and degradation by specific MMPs, enzymes that are also controlled by tissue inhibitors of metalloproteinases (TIMPs).[194] Elastin, another protein that is part of the matrix, is a highly resilient protein that is dependent upon extensive crosslinking to maintain its function. Elastin associates with additional microfibrils to create elastic fibers within the ECM that are anchored in the basement membrane and thus can achieve elasticity and memory within the skin. The collagen and elastin fibrils are embedded within a third aspect of the ECM, namely the extrafibrillar matrix. This matrix consists of glycoproteins and proteoglycans as well as free glycosaminoglycans like hyaluronic acid that are not protein-bound. These molecules also play a role in the development and remodeling of the skin, but one of their most

prominent features is that they bind and hold water in the skin, providing a hydrated milieu for normal physiological function.

Synthesis of collagen and elastin decreases with age and, at the same time, the MMPs that degrade these proteins are up-regulated in fibroblasts and keratinocytes, resulting in deficiency in collagen and elastin relative to younger skin. Senescent cells also express less TIMP-1, creating a further shift toward catabolic activity in the dermis.[195] UV irradiation potently induces the transcription of MMPs within hours of exposure,[196] compounding the disturbance of the ECM. This age-related shift in the biochemical balance toward net loss of key components compromises the structural and functional integrity of the ECM.[197] Collagen fibrils are irregular and disorganized manifesting in such changes as loss of skin elasticity and wrinkles. This state is further exacerbated in photoaged skin, with characteristic "elastotic" matter, a dysfunctional association of elastin, microfibrillar protein, proteoglycans and hyaluronic acid, accumulating in the dermis.[193] Inflammation, UV-induced or otherwise, leads to infiltration of neutrophils, themselves a source of matrix-degrading enzymes like elastase, MMP-1 and MMP-9.[198] The inflammatory cytokine, IL-1α, has been found to stimulate activity of MMP-9 in human skin by downregulating TIMP-1.[199] Agents that can slow or reverse the age-related shift toward degradation of the ECM clearly have potential to reduce visible signs of aging.

10.4.1 Antioxidants and the ECM

ROS appear to play a fundamental role in the regulation of MMPs,[200] and antioxidants such as the glutathione precursor, *N*-acetyl-cysteine, catalase, tretinoin, and resveratrol all reduce expression of MMPs. Phenolic phytochemicals covering a range of structural classes have been found to inhibit human leukocyte elastase as well as the gelatinases, MMP-2 and MMP-9.[201] An active compound derived from *Pothomorphe umbellate* root, 4-nerolidylcatechol, inhibited MMP-9 in mouse skin, although the extract proved more active than the pure compound.[202] The major green tea catechin, EGCG, reduced UV-A-stimulated degradation of the ECM upon topical application to an artificial skin model, lowering gelatinase levels and enhancing expression of TIMP-1.[203] An aqueous extract of pomegranate peel was studied in human skin organ cultures and found to inhibit fibroblast MMP-1 and to stimulate type 1 procollagen synthesis.[204] Anthocyanin or OPC-enriched fractions of blueberry fruits (*Vaccinium angustifolium*) down-regulated MMP-2 and 9 and increased activity of TIMP-1 and 2 in a human prostate cancer line.[205] The anthocyanins worked via inhibition of a

protein kinase C-MAP kinase cascade while the activity of the OPC fraction was not PKC-dependent. A partially purified phenolic glycoside from peony roots, paeoniflorin, was reported to protect human keratinocytes as well as hairless mouse skin from UV-B-induced DNA damage as detected *in vitro* by the comet assay.[206,207] A subsequent 8-week clinical trial with 0.5% paeoniflorin resulted in a significant decrease in wrinkles. Ellagic acid and tannic acid were shown to inhibit the proteolytic degradation of elastin in dermal fibroblasts and to stimulate elastogenesis.[208] Chalcones, flavonols, isoflavans and lignans were also reported to display potent anti-MMP-1 activity in fibroblasts.[209–211] Decreased MMP-1 expression in aged human fibroblasts by α-tocopherol treatment was consistent with inhibition of PKC α activity, an enzyme whose expression and activity characteristically increases with age.[212]

10.4.2 Hormones and the ECM

The profound decrease in endogenous estrogens after menopause is associated with reduction in skin thickness, moisture, and collagen levels.[213] The increased risk of breast cancer and other diseases with systemic hormone replacement therapy precludes the use of systemic estrogens to address skin aging; therefore, topical application of estrogen is being explored as a potential alternative. 17β-Estradiol has been shown to inhibit proMMP-1 at the protein and mRNA levels in human dermal fibroblasts.[214] Furthermore, topical application of 0.01 percent 17β-estradiol to the skin of elderly female subjects 3 times a week for 2 weeks was found to significantly decrease MMP-1 protein levels and to increase expression of type 1 procollagen mRNA, tropoelastin and fibrillin-1 compared to vehicle-treated control.[215] Increases in elastic fibers, keratinocyte proliferation and epidermal thickness were also attributed to 17β-estradiol in this study.

Phytoestrogens are plant compounds that have a generally weak effect on the mammalian estrogen receptor. Isoflavones from soy beans *(Glycine max*) and red clover flowers (*Trifolium pratense*) have been found to decrease menopausal symptoms and osteoporosis when taken internally.[216,217] A recent study demonstrated that topical application of 20–40 mg/day of red clover isoflavones (RCI) significantly reduced degenerative skin changes secondary to ovariectomy (Ovx) in rats.[218] Skin structure in RCI-treated Ovx rats was well-organized with normal vascularity, thickness, and keratinization compared to control Ovx rats. In addition, collagen levels were higher in treated rats, and elastin fibers and collagen bundles were well developed.

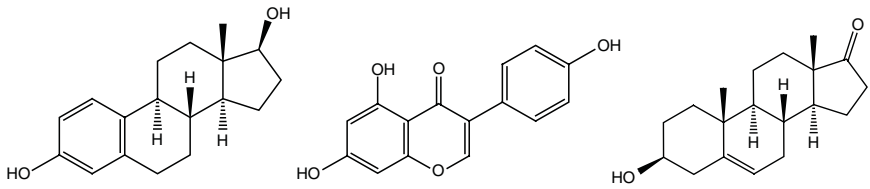


Figure 10.14 Structural comparison of the hormones estradiol and DHEA with genistein.

Another steroidal hormone that is known to decrease with age is dehydroepiandrosterone (DHEA). In cultured human dermal fibroblasts, DHEA reduced MMP-1 protein and increased both type 1 procollagen and TIMP-1.[219] A subsequent clinical investigation confirmed the *in vitro* results; topical DHEA (5 percent solution, 3 times a week for 4 weeks) was found to induce a significant increase in procollagen 1 mRNA and protein as well as TIMP-1 protein, while inhibiting expression of MMP-1 mRNA and protein in aged skin compared to a vehicle control.[219]

10.4.3 Other Secondary Metabolites that Affect the ECM

The Tibetan multi-herbal formula PADMA 28 was found to stimulate type I procollagen levels while decreasing MMP-1 in human skin organ cultures. This retinoid-type dermal response was not accompanied by epidermal hyperplasia or skin irritation.[220]

Ethanol extracts of soybeans were found to enhance the synthesis of collagen type I in primary dermal fibroblasts prepared from mature adults. The isoflavone, genistein, also stimulated collagen synthesis in this model, but it was not found to account for all of the activity of the extract.[221]

The iridoid glycoside, aucubin, from *Eucommia ulmoides* reduced both MMP-1 levels in human skin fibroblasts and expression of MMP-1 mRNA.[222]

Triterpenoid phytochemicals have been reported to exert positive effects on the ECM. The plant *Centella asiatica* has long been employed in Ayurvedic medicine to heal wounds and diseases of the skin. A major active triterpene saponin component, asiaticoside, demonstrated antiinflammatory activity and induced the synthesis of glycosaminoglycans as

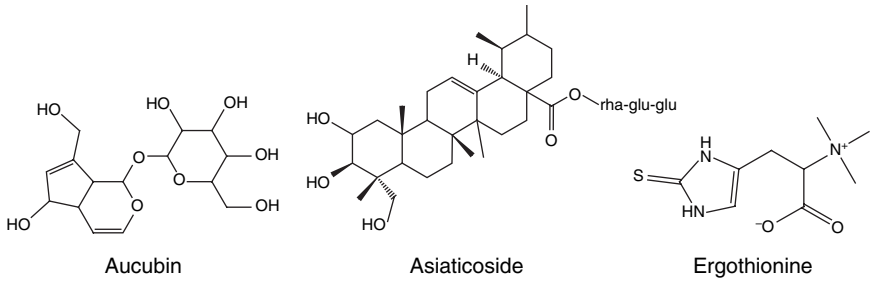


Figure 10.15 Some secondary plant metabolites that affect the ECM.

well as type I collagen.[223] A triterpene-containing extract of *Styrax japonica* also significantly reduced MMP-1 and enhanced type 1 collagen levels in primary human skin fibroblasts.[224]

There is also some evidence that amino acids, carbohydrates, and lipids can protect the ECM. The sulfur-containing amino acid, ergothioneine, is a fungal metabolite that is acquired through the diet. It was shown to suppress both MMP-1 protein and mRNA in human dermal fibroblasts after UV-A irradiation as well as reducing TNF-α expression.[225] Another fungal product, a proteoglycan exopolysaccharide from the mushroom, *Grifola frondosa*, produced a potent, dose-dependent reduction in the expression of MMP-1 in UV-A irradiated human dermal fibroblasts.[226] The highly unsaturated fatty acid, eicosapentaenoic acid (EPA) has been found to reduce UV-induced MMP-1 expression in human dermal fibroblasts. Topical application of EPA to human skin inhibited UV-induced epidermal thickening, collagen decrease, COX-2 expression and activation of MAP kinases JNK and p38. In addition, EPA increased expression of collagen, tropoelastin and fibrillin-1 in human skin.[227]

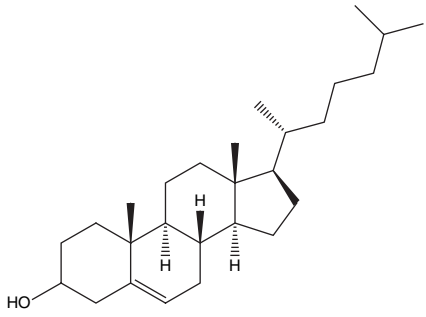
10.5 Skin Barrier Integrity and Aging

Lipids are key elements in the maintenance of the skin as an effective barrier. The stratum corneum is comprised of keratin-rich corneocytes surrounded by lamellar bilayers of intercellular lipids including ceramides, sphingolipids, cholesterol and cholesterol esters, and free fatty acids. These lipids constitute the major permeability barrier of the skin and are therefore essential for proper hydration by protecting against excessive moisture loss. The skin barrier functions quite well in aged skin, however, barrier repair after compromise has been shown to be delayed.[228] The distribution

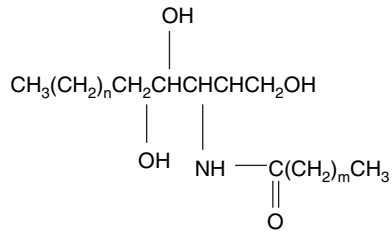
pattern of barrier lipids remains consistent with age, but total lipid content is significantly decreased in aged versus young human subjects.[228,229] Cholesterol synthesis decreases with age, resulting in an elevated cholesterol sulfate/cholesterol ratio that impedes desquamation and may lead to rough and scaly skin.[230] Ceramidase, the enzyme that cleaves ceramides is upregulated in aging skin, thus ceramide and sphingolipid levels in general are decreased.[231] The synthesis of glycosaminoglycans including hyaluronic acid also declines with age.[232] These findings, as well as reduction in sebum production, account in part for the fact that older skin is characteristically dry. This scenario presents an opportunity for replenishment of aging skin through application of natural lipids and glycosaminoglycans and their precursors, as well as agents that stimulate them, whether from plant or animal sources.

Topical application of cholesterol has been found to accelerate barrier recovery in aged mouse and human skin (Thiele *et al.* 2006).[9] Some investigators have reported that skin-lipid identical blends of fatty acids, ceramides, and cholesterol are superior to individual lipids in repairing the barrier in aged mice and in humans after treatment with irritants like SLS.[173,174] In both chronologically aged mouse and human skin, the application of cholesterol-dominant, physiological lipids was shown to enhance barrier recovery as monitored by TEWL after tape stripping.[174] Others did not find a significant clinical difference between emollient preparations containing physiologic lipids and other treatments such as petrolatum.[233] Loden and Andersson[234] showed that topically applied canola oil and its sterol-rich fraction reduced SLS-induced irritation and substantially lowered TEWL in human subjects, while application of other lipids resulted in no benefit. Highly lipid-rich moisturizers have even been found to increase the perturbation of the skin barrier and render skin more susceptible to irritation.[235] Topical application of the natural cholesterol precursor, mevalonic acid, to the skin of aged mice reduced the cholesterol sulfate/cholesterol ratio and enhanced desquamation.[230]

Hyaluronic acid itself can be applied topically to help heal the skin and preserve hydration, although its very high molecular weight prevents penetration through the stratum corneum.[236] In addition, *N*-acetyl glucosamine has been shown to increase hyaluronic acid synthesis significantly in human keratinocytes at concentrations of 3 mM.[237] A soy extract was shown to significantly enhance the synthesis of hyaluronic acid in primary dermal fibroblasts in culture from older (age 50–67) adults.[221] The authors also studied the effect of topical application of the extract on the skin of human volunteers (age 55 ± 6 years) twice daily for 2 weeks. The papillae



Cholesterol



Ceramide 3

Figure 10.16 Important skin barrier lipids.

index (density of papillae at the dermal-epidermal junction) as determined *in vivo* was found to increase significantly by 21 percent in the soy-treated skin vs the placebo control indicating a reversal of the age-associated flattening of the dermal-epidermal junction.

Glycerol is an endogenous humectant that is also often used as a cosmetic ingredient (derived from the saponification of triglycerides from plants or animals) to increase skin moisture.[238] Glycerol has also been shown to act *in vitro* as a molecular chaperone analogous to heat shock proteins in refolding heat-denatured luciferase.[239]

Pomegranate seed oil (PSO) was shown to increase keratinocyte proliferation in cultured cells. Further study of PSO-treated human skin in organ culture confirmed that keratinocyte stimulation led to normally differentiated cells and a mild thickening of the epidermis.[204]

Psychological stress has been shown to disrupt skin permeability barrier homeostasis in mice, and exposure to natural, sedative odorants prevented the stress-induced delay of barrier recovery subsequent to tape stripping. A study with human subjects who were not specifically stressed confirmed an improvement in skin barrier repair after inhalation of sedative odorants such as terpinyl alcohol and phenylethyl alcohol.[240] Similarly, application of the inhibitory neurotransmitter, γ-amino butyric acid (GABA) or other GABA receptor agonists were demonstrated to enhance barrier recovery in hairless mice.[241] A number of plant extracts including valerian, skullcap and ginseng are thought to act as GABA agonists.[242–244]

10.6 DNA Damage and Repair in Aging

Aging can be defined as the accumulation of damage.[245] DNA damage in cells, can lead to accumulation of damage and therefore accelerate the rate of aging of the skin, mainly of the ECM. A cell with DNA that has been damaged, for instance by ultraviolet radiation, stops the synthesis of RNA and proteins,[246] (with the exception of those related to a small set of UV-inducible genes, e.g. the heat shock genes)[247,248] until DNA is repaired. In the course of DNA repair, calcium is mobilized and DNases are activated.[249] This activation is necessary to perform the excision step of the damage, but can lead to excessive nicking (damage) of nuclear DNA. Nicked DNA is a trigger to activate poly (ADP-ribose)-polymerase.[250] This enzyme, also called PARP, helps to initiate the DNA repair process by catalyzing the degradation of the electron transporter nicotinamide adenosine dinucleotide (NAD) into free ADP-ribose and subsequently

- the binding of ADP-ribose to the histones
- the binding of ADP-ribose to the histone-bound ADP-ribose
- the formation of the next ADP-ribose = ADP-ribose bond

resulting in the formation of large, ADP-ribose containing, polymers endowed with highly negative charges.

It is commonly accepted that by binding highly charged polyanions to the histones, PARP helps unwind the chromatin and renders the damaged DNA accessible to DNA-repair enzymes for the excision of damaged nucleotides. The downside of this process is that, while helping DNA-repair, PARP uses up the NAD pool.[251] When NAD is consumed, glycolysis is inhibited and ATP is no longer synthesized.[252] Without ATP, membrane potential is impaired and the cell undergoes zeiosis (blebbing) which can eventually result in apoptosis or necrosis.[253]

The physiological and macroscopic consequences of this cellular phenomenon (DNA damage) are easily understood when the process is considered by keeping in mind the micro-inflammatory model of skin aging.[254] The research leading to the conception of the micro-inflammatory model of skin aging was prompted by the understanding of the relevant role played by mediators of the inflammatory response in wound-healing and in post-UV-repair. In the course of such research, it was noticed that all of the commonly accepted accelerators of the aging process shared as a common feature the capability to induce the synthesis of ICAM-1 in the endothelium.

This meant that all the factors recognized as being able to accelerate aging had the capability to trigger a self-maintained inflammatory response. When cells with damaged DNA undergo necrosis, they release diffusible pro-inflammatory signals which trigger a cascade of autacoids and cytokines which provoke the synthesis of I-CAM 1 in the vascular endothelium.

Upon synthesis of ICAM-1 in the endothelium, circulating monocytes and macrophages roll over and secrete hydrogen peroxide to perform diapedesis across the endothelium lining the vascular wall. Once in the dermis, the immune cells secrete lytic enzymes and ROS, to fray a path in the dermis. They follow chemotactic signals to reach and remove damaged skin cells. The degradation of cells to be removed is performed via the release of peroxides. Under these conditions, it is very likely that nearby resident cells, such as fibroblasts or keratinocytes, will be damaged by the free radicals. When this happens, the damage can trigger the cascade of arachidonic acid and secrete prostaglandins and leukotrienes, which diffuse toward the next resident mast cells. Upon binding these mediators on specific receptors, the resident mast cells release histamine and TNF-α which in turn stimulate the release of P-selectins and the neo-synthesis of ICAM-1 in endothelial cells. The cycle is therefore closed, and the micro-inflammatory status is maintained.

When the DNA of a cell is damaged (for example, by UV-radiation), an inflammatory infiltrate is observed in the epidermis.[255] This is accompanied by many types of damage to the ECM, to resident cells and to vessel walls provoked by the free radicals and lytic enzymes, which are released in the course of the inflammatory response, consequent to the diffusion of cytokines produced via the arachidonic acid cascade. The micro-inflammatory model of skin aging emphasizes that DNA damage can have as a dire consequence, the accumulation of damage in the connective tissue and of the ECM. Histological and visible consequences of this model have been verified.[256–258] The recognition that post-UV-repair and wound-healing share ECM remodeling as a common feature has allowed one to understand why blood vessels are located deeper down in aged skin then they are in young skin. The sagging of the dermis is the consequence of a modified ECM and is accompanied by an overall increase of the surface of the skin, particularly of the face. Muscular and nervous actions maintain that increased surface to cover a skull, which is constant in volume, and thus generate the appearance of wrinkles, a visible sign of aging. The increase of skin surface and the reduction of body volume can be invoked to explain the observation that, notwithstanding a nearly constant rate of turnover of the keratinocytes

through the life span, the thickness of the epidermis is diminished with aging. This is more the consequence of the stretching of the skin than the consequence of a modification of the turnover rate of the keratinocytes. Indeed the turnover of the keratinocytes does not change much with aging and this is witnessed by the fact that the thickness of the stratum corneum does not change with aging.[259]

Is it possible to reduce the rate of aging by helping the cells of the skin to repair their DNA? Recent reports suggest that the answer may be yes. Several technologies have been set up in recent years to affect the DNA repair of the keratinocytes in human skin, *in vivo*. The most direct approach has been to isolate DNA repair enzymes and encapsulate them into specific liposomes.[260] Upon topical application of creams containing these liposomes after exposure to ultraviolet radiation, phenomena known to be induced by ultraviolet radiation, such as immune-depression, were hindered. From these observations it was concluded that the DNA repair enzymes contained in the liposomes had reached the epidermal cells responsible for the immune response and repaired their DNA. Another approach has been to encapsulate NAD into liposomes to deliver NAD to cells depleted of NAD because of exposure to ultraviolet radiation. When these liposomes were applied to the epidermis after exposure to simulated solar radiation, the erythema which is generally provoked by ultraviolet was not observed.[261] From this observation it was concluded that the administration of NAD had helped restore the NAD pool, the synthesis of ATP and the repair of cellular DNA.

Additional evidence to provide proof of principle that natural products can repair damaged DNA comes from the marine cyanobacteria, *Anacystis nidulans*. This organism elicits a photolyase enzyme which binds to pyrimidine dimers and then converts them back to their original monomeric form when exposed to blue light. Topical application of liposomes containing this photolyase to human skin that had been exposed to UV-B-irradiation demonstrated that the UV-induced dimers were reduced by 40–45 percent upon exposure to blue light.[262] These results indicate that the lyase works efficiently to repair human skin's DNA subsequent to UV-induced damage.

Direct or indirect damage of DNA is a major contributor to the aging process, and DNA repair might become less efficient with age; thus the ability to repair DNA is paramount in reducing the effects of aging.

10.7 Summary

Cosmetic formulations have been based on botanical ingredients since ancient times, and botanical and natural extracts maintain a major role in contemporary cosmetics. Present means of treating aging skin have become more technological and more invasive; however, natural products including botanicals are still relevant and can be highly efficacious. Scientific research continues to corroborate traditional uses of many plants for skin benefits, and to elucidate biochemical mechanisms of action for a growing number of phytochemicals. Additional clinical trials will be necessary to optimize the application of natural ingredients for cosmetics, but scientific substantiation for the safety and efficacy of a host of botanical extracts and compounds for treating aging skin is evident, with the continued potential of many more.

Only a small fraction of those plants that have been traditionally used for health, beauty or longevity have been scientifically investigated for bioactivity. Certainly not all of those would yield potent or practical application as cosmetic ingredients, but it is intriguing that phytochemicals as commonplace as tannins and flavonoids have such potent activity. It is highly unlikely that science will ever identify all of the active compounds in a given plant, but it is equally unlikely that we will stop finding new activities in plants that have relevance to healthier aging. The main barrier to continued discovery may indeed be the accelerating loss of biodiversity on the planet. It will not be sufficient to harvest this bounty to develop excellent plant-based ingredients and products, but also to play an active role in sustaining the plant kingdom for future generations.

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11

Approaches to the Development of Cosmetic Products to Counter the Effects of Skin Aging

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11.1 Introduction

Aging is defined as the progressive deterioration of physiological functions in organisms, eventually leading to senescence and death. As deterioration can be slowed down by various means of intervention, anti-aging can be defined as anything that slows down the progressive deterioration. As far as the cosmetic industry and aesthetic surgery practices are concerned, the primary target of anti-aging intervention is the human face, although improvement of appearance of the body in general are also in the forefront of today's anti-aging strategies. This chapter will focus mainly on the treatments for face, as facial beauty and appearance is the most significant and recognizable yardstick of youth and aging in human societies (1).

11.2 Aging Changes in the Face

A series of drawings in a paper by Larrabee and Caro (2) accurately conveys changes in specific facial lines and wrinkles that characterize transformation from young to very old. Less dramatic, yet quite telling, are the aging changes that one sees from a portrait of three individuals of a family that spans three generations (Figure 11.1). These changes are brought about by intrinsic (chronological) aging, including peri- and post-menopausal changes in women (3), as well as extrinsic causes of aging like sun exposure, smoking, lifestyle, stress, and pollution (4,5). One of the most important anti-aging advances made in the last two decades is delineation, at physiological and molecular levels, of the basis of key extrinsic factors. This leads to formulation of appropriate strategies to protect, prevent and treat these maladies. Much credit goes to the investigative dermatologists and basic scientists who unraveled the true anti-aging benefits of retinoids (known previously as morphogens to the developmental biologists), and dissected the molecular targets in skin and pathways underlying its actions (6,7). Retinoids bind to retinoic acid receptors (RARs) and retinoid X receptors (RXRs), members of the superfamily of nuclear receptors, which in turn activate the retinoid-sensitive genes and bring about gene transcription, either up- or down-regulating the production of these gene products. Expression of RARs is also influenced by aging. For example, intrinsic aging of skin *in vivo* is accompanied by significant increase in RAR alpha, while other isoforms do not show changes (8). RAR alpha gene induces expression of matrix metalloproteases (MMPs) that play an active role in remodeling, via breakdown of matrix components, of the dermis in aged skin. Watson *et al.* (8) found that retinoic acid diminishes the RAR alpha

Figure 11.1

expression, and returns the MMP levels to basal levels. While retinoic acid is regulated as a drug, retinol (vitamin A) and its esters can be used as cosmetic ingredients that have proven benefits for improved appearance of skin. The early and crucial findings of retinoid effects had opened the floodgates for the search for truly effective anti-aging molecules. A fusion of cosmetic and pharmaceutic research efforts ensued (leading to actives named as cosmeceuticals); to be followed by the nutraceutical and biotechnological efforts, use of devices to augment delivery of actives, and spawning a whole new industry of bioengineering methods and instruments to measure, analyze, and quantify the effects of anti-aging products. It is indeed staggering to attempt a review of the multitude of anti-aging strategies employed, or to provide a complete list of the targets that are currently being investigated, not to mention the companies (ranging from the multi-billion dollar global leaders to the smaller, yet innovative, firms), scientists, and publications in peer-reviewed journals and posters at dermatology and cosmetic science meetings worldwide. In this overview, we attempt to provide an outline of the key structural components and targets thereof in the skin, along with the active classes of ingredients used in treating the aging face, realizing fully well the limitations of such an approach.

11.3 Targets in Skin: The Surface Matters (Stratum Corneum, SC)

The outermost layer of skin, the SC, is a 10 μm thick layer, with 10–18 cell layers (depending on the anatomic location) of corneocytes or terminally differentiated keratinocytes (Figure 11.2). The loose basket-weave appearance of SC in light microscopy is an artifact of histological preparations. Electron microscopy has shown a distinct two-compartment arrangement of the SC where individual corneocytes are embedded in a matrix of lipids, resembling a brick and mortar arrangement (9). As the skin's barrier layer, the SC is the first target of any cosmetic treatment by virtue of the following: this is the perceived and perceiving surface of skin (dry skin manifests here), the substrate on which every cream, lotion, or device is applied, and the barrier to the penetration of actives. Abnormal cohesion of the corneocytes leads to dry skin appearance, affecting the way light reflects off the SC, altering both the texture and feel of the skin. Optimal hydration of SC is the key to plasticize keratin, a structural protein. Hence the most instant anti-aging effect is provided by an excellent moisturizer that optimizes the plasticity of SC, and strengthens the barrier lipids (mortar) of the SC extracellular spaces that lock in moisture in individual corneocytes (bricks). Water, humectants, lipids, and amino acid components of the natural moisturizing factors (NMF) of SC are effective moisturizers. Glycerol,

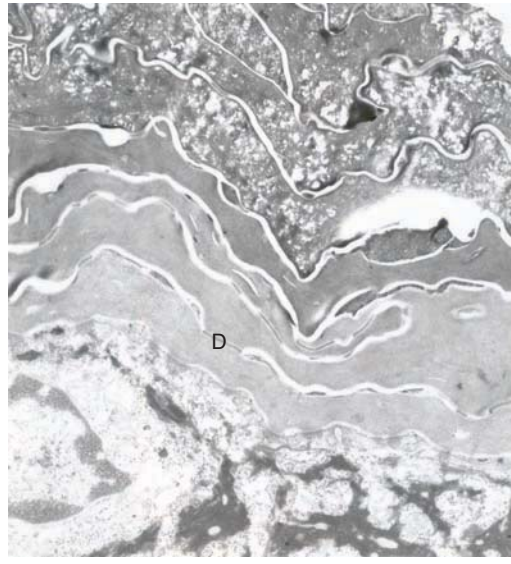

Figure 11.2

one of the oldest humectants used by the industry, is now known to affect a variety of functions such as improving skin barrier repair (10), functions of aquaporins, the water channels of epithelia (11), compensating for the lack of sebum (12), as well as influencing the rate of desquammation (13). Clinical use of glycolic acid (an alpha hydroxy acid, AHA) to treat extremely dry pathological conditions and improve skin appearance by accelerated desquamation (14) lead to a breakthrough invention—functional levels of AHAs, such as glycolic, lactic, tartaric, and maleic acids, which are highly effective anti-aging actives (15,16). Besides improving appearance via smoothening out the surface, AHAs are functional in increasing the dermal glycosaminoglycan (GAG) contents, and strengthening the skin barrier (17,18). AHAs may initiate the cell signaling cascade via cytokines that are known to be released from corneocytes (19). Beta hydroxy acids like salicylic acid have also been used like AHAs to chemically exfoliate the SC, and have been recognized as a treatment for enlarged pores, acne as well as oily skin conditions, which affect the SC at the other end of the spectrum—unsightly and shiny appearance.

SC is also the target for compounds to improve the skin barrier, as barrier defects lead to sensitive skin or atopic dermatitis that potentially triggers inflammatory events (20,21). Inflammation is a leading cause of atopic disorders which accelerate aging changes of the skin. Hence the SC barrier lipids (cholesterol/ceramides/free fatty acids in a roughly equimolar ratio) are of crucial significance to skin health, and are considered a "tail that wags the dog" (22). Several strategies are employed to improve the barrier and maintain skin health and youthful appearance, including physiological lipid blends (23), vegetal sterols, glycosphingolipids (24), hyaluronic acid (25), and vitamin C (which up-regulates endogenous lipid production by keratinocytes), as well as improve epidermal proliferation and differentiation (26). Specific fatty acids are also known to activate the peroxisome proliferator activated receptors, nuclear hormone receptors that induce gene transcription in conjunction with retinoid receptors, stimulating epidermal differentiation, and barrier properties of the SC (27,28). As all these strategies contribute to the cohesion and integrity of the corneocytes of SC, such an approach has been famously termed as "corneotherapy" by Kligman (29) and is in the first line of anti-aging treatment.

11.4 The Nucleated Layers of Epidermis

Often termed as the viable epidermis, it spans the three layers of epidermis underneath the SC, which are called stratum basale (Figure 11.3), stratum

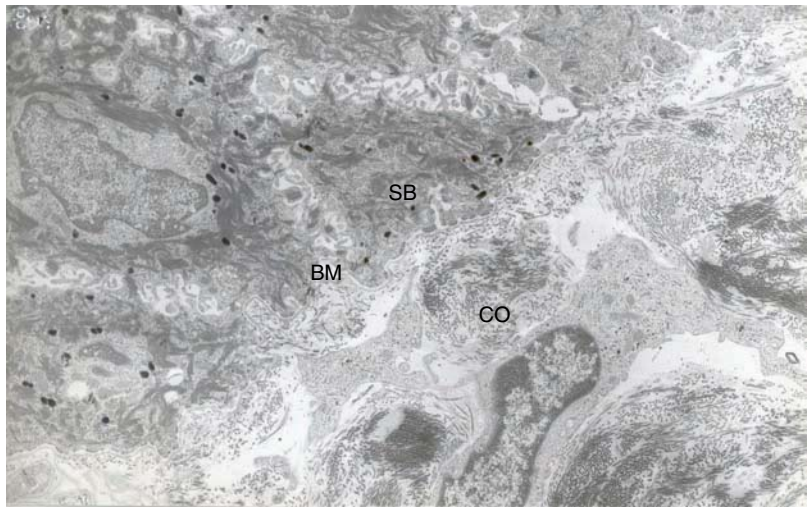

Figure 11.3

spinosum and stratum granulosum. The viable epidermis is what produces epidermal keratin, NMF and the barrier lipids, proliferates to heal the wounds (following laser resurfacing, cosmetic peels, etc.), and replaces the corneocytes that are lost by desquamation. Cells of this layer also transport water and glycerol through the aquaporins, receive and transfer melanin from the melanocytes for photoprotection, house the antigen presenting langerhans (sentinel) cells, produce anti-microbial peptides, and secrete a variety of chemokines, growth factors, etc. for cellular communication within the epidermis as well as with dermal cells (fibroblasts, mast cells). This layer stimulates production of the dermal matrix, or when appropriate, its degradation. Being avascular, transport of nutrients within this layer is conducted by diffusion through intercellular fluids, once they have passed the selective barrier of the basement membrane separating epidermis from the vascular dermis. Sensory nerve fibers do extend into the epidermal compartment, and secrete trophic neuropeptides that influence keratinocyte physiology, as well as play some roles in dysfunctions associated with sensitive skin. Each and every aspect of this tissue and its functions are potential targets for anti-aging intervention, and a multitude of approaches have been used to achieve these results.

Intrinsic aging leads to a decrease in keratinocyte cell proliferation and thinning of the epidermis, as seen in histologic comparison between sun-protected skin from young and aged (30). Other changes in aged skin include flattening of dermal–epidermal junction (DEJ), loss of dermal

papillae, loss in dermal matrix proteins, and disorganization of the fibrous network. An association between increased oxidative stress and intrinsic aging in general has been highlighted (31), and it is possible that chronologically aged epidermal cells have higher oxidative stress than epidermal cells of younger individuals. Strategies to counter oxidative stress or otherwise improve cell proliferation and subsequent increase in viable epidermal thickness, (measurable with histology), have been employed. They are achievable with several actives such as AHAs, BHAs, retinoic acid/retinol, vitamin C, and several phytochemicals such as pomegranate seed oil (32). Application of large scale gene expression analysis, such as sequential analysis of gene expression (SAGE) technology, has provided valuable insight into the differential expression patterns of genes in aged skin. Decreased expression of c-fos and IKBA (inhibitor of NF-kappa B, a well known gene regulator) in aged skin correlates with reduced sensitivity to mitotic stimuli, and increased expression of NFkB, respectively (30). However, c-fos and c-jun (components of AP-1 or Activator Protein 1) can be reactivated in cultured fibroblasts from old donors (33), an indication that it is biologically possible to reactivate genes that are down-regulated in aging.

Ingredients with known medicinal or health promoting effects are tested in cultured human skin cells for their potential to increase or decrease transcription of such genes, and if devoid of any potential risks associated with topical use, are selected for further investigations and, if viable, eventual use in cosmetics. For epidermal cells, actives that enhance differentiation, synthesis of barrier lipids, anti-oxidant enzymes, energy production, cellular nutrition, aquaporins, and cellular communication are currently being identified using gene expression analysis, and successfully brought to market. On the other hand, over-activation of AP-1 by UV radiation has been found to induce over-production of Matrix Metalloproteinases by the epidermis, causing aging changes via degradation of the dermal matrix (34). Inhibitors of MMPs, mostly botanically derived, have often been used as part of an anti-aging strategy. Retinoids, including retinol, prevents the over stimulation of AP-1 by UV, one of the hallmarks of its anti-aging effects (34,35).

Another site of aging within the epidermis is DNA, because of the damage caused by UV radiation (36,37). UVB is absorbed by the double bond in pyrimidine bases in DNA, opening the bond so they can react with adjacent pyrimidine bases, resulting in a tight four member ring. These genetic lesions in DNA are corrected quickly by a cellular process termed

"nucleotide excision repair" by DNA repair enzymes. DNA repair enzymes from algae have been reported to be effective (36) and several actives and delivery systems targeted to this endpoint have been tested *in vivo* showing positive results (38,39). Prevention of UV-induced pyramidine dimer formation in epidermis by green tea polyphenols, in addition to anti-inflammatory and antioxidant functions of this popular cosmetic ingredient, has also been reported (40,41).

UV radiation also leads to lipid peroxidation and generation of reactive oxygen species, which have been postulated as leading to mitochondrial damage and aging (42). A multitude of antioxidants, both enzymatic and non-enzymatic, such as superoxide dismutase, catalase, glutathione oxidase, ascorbic acid and their esters, vitamin E, and alpha lipoic acid have also been employed, often with documented efficacy in *in vitro* and *in vivo* test conditions. Another theory of aging, the telomere shortening hypothesis of aging, follows the oxidative stress theory. Telomeres, located at the ends of chromosomes, shorten with subsequent cell divisions, and when the telomeric DNA reaches a critically short length, it leads to cell cycle arrest and senescence (43), observed in human cells during the aging process (44). Introducing telomerase, an enzyme that repairs telomere damage, into cells, has been shown to extend the life span of human cells (45). An extract of the fruit of Terminalia chibula, which shows significant inhibition of oxidative stress as well as the age-dependant shortening of the telomeric DNA in cultured cells, has potential as an anti-aging ingredient (46).

One of the most visible signs of photoaging is pigmentary changes, such as focal hyper-pigmentation or uneven pigmentation of the facial skin. Among Asians and other darker phototypes (47), dyspigmentation is a more common denominator of aging than wrinkles are, until the middle of the fourth decade of life (48). Skin lighteners are highly popular in Asia and Latin America, and provide a desirable anti-aging benefit by decreasing the appearance of uneven pigmentation. Skin lightening strategies have traditionally utilized hydroquinone, but this active has fallen out of favor due to safety and regulatory issues in several countries (Japan, European Union). The classical strategy is to use plant-derived tyrosinase inhibitors to reduce the activity of tyrosinase, the crucial enzyme in the biochemical pathway of melanin synthesis. However, the use of plant derived tyrosinase inhibitors (bearberry extract, mulberry extract, kojic acid, etc.) alone as skin lightening agents is no longer considered adequate, due to market trends and consumer demands for increased efficacy. Hence, a whole slew of new ingredients, such as cococin, thiodipropionic acid (49), endothelin

antagonists (which block keratinocyte-melanocyte interaction for increased pigment production and transfer to epidermis), protease inhibitors from soy (50), peptides, melanocyte stimulating hormone antagonists, and small interference RNAs that silence the messenger RNA for tyrosinase (51) have appeared in the cosmetic field. Again, use of sunscreens as a general anti-aging (skin lightening in darker phototypes) strategy has gained much ground around the world. The increasing use of UVA blockers, along with traditional UVB blockers, by consumers attests to the high level of consumer awareness of extrinsic aging and the role of UVA radiation in dermal damage.

As to the changes in immune sentinel cells, the number or activity of Langerhans cells in the epidermal compartment is known to decline somewhat in chronological aging (52), and especially so in the photo-aged skin (53). Products aimed at boosting the skin's immune function are claimed in a few anti-aging products but, by and large, the immunocompetence of skin is not a widely addressed facet of anti-aging cosmetics.

11.5 Dermal–Epidermal Junction (DEJ) or Basement Membrane Zone (BMZ)

The BMZ was originally identified and defined by histology, and is a 0.5–1.0 μm thick band situated between the epidermis and dermis, that stains positively for periodic acid-Schiff stain. Subsequent ultrastructural studies helped identify multiple structural components in the BMZ. The major function of the BMZ is to anchor the epidermis to the dermis, as mutations in its components cause heritable, blistering skin diseases. The different regions of BMZ are (1) hemidesmosomes (connect to the epidermis); (2) the lamina lucida (situated under the hemidesmosomes) above the next layer, lamina densa) appears electron dense and has fine anchoring fibers connecting the hemidesmosomes to lamina densa. It is composed of laminins, which are heterodimers of various combinations of alpha, beta, and gamma submits, secreted by the keratinocytes; (3) the lamina densa, named due to its electron dense appearance, is 35–45 nm thick, and is composed mainly of type IV collagen, perlecan (heparan sulfate proteoglycan), and possibly laminin; (4) the sub-lamina densa, located below the lamina densa, the fibrillar structures connecting lamina densa to dermal plaque-like structures, termed anchoring fibrils, are composed mainly of type VII collagen, secreted both by keratinocytes and fibroblasts (54). A detailed electron microscopic study revealed that most anchoring fibrils originate

and terminate in the lamina densa (i.e., they do not extend perpendicularly into the dermis, but form individual semicircular loops that constitute a network of anchoring fibrils) (55). In addition to facilitating adherence of epidermis to dermis, BMZ also functions in structural support, regulation of permeability of substances from dermis to epidermis, and in embryonic differentiation (56).

The DEJ is altered in aging skin, and is often seen as "duplicated" in photodamaged skin (Figure 11.4). Flattening of the DEJ is seen both in sun-protected and sun-exposed skin of the elderly (57), but in younger individuals, photoaged skin shows much more prominent flattening of the DEJ than sun-protected sites. Epidermal and/or dermal cell derived MMPs damage the BMZ in photoaging, and the protective effects of MMP inhibitors have been documented in skin equivalents partially mimicking the photoaging process (58). Strengthening the DEJ, correcting deficiencies in its composition and function (such as enhancing production of laminins and collagens IV and VII), can be expected to improve the overall integrity of skin, stimulate cell-to-cell communication and epidermal-dermal interactions

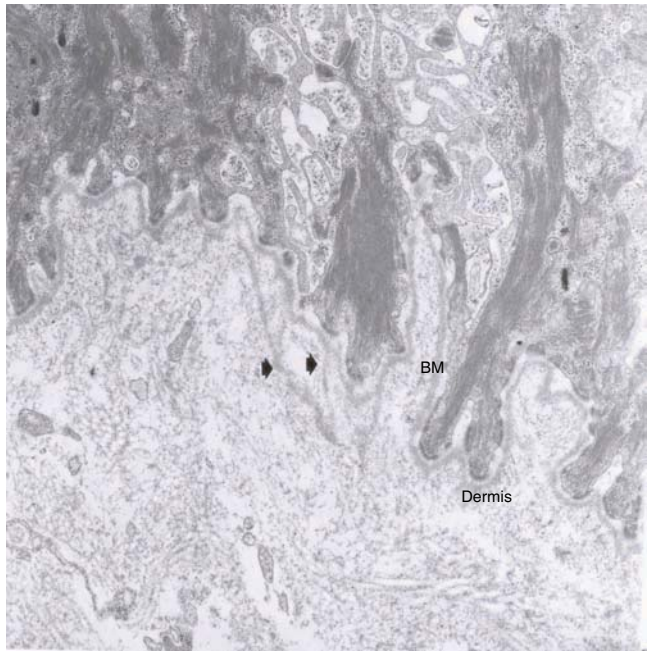

Figure 11.4

and signaling; all of which have positive implications for anti-aging strategies. Cosmetic ingredients used for these benefits include peptides, synthetic compounds and botanicals, including plankton and algae derived extracts.

11.6 The Dermis

Dermis, the structural foundation of skin, accounts for about 90 percent of its weight. The primary cell type in the dermis are fibroblasts, which produce the extracellular structural proteins, collagen, elastin (Figure 11.5), as well as GAGs, the major water holding components of the dermis. Together, these components form the extracellular matrix (ECM), once thought to be an inert compartment of skin, providing a structural foundation. This view of the dermis (and the ECM) has now been completely discarded (59), and the significant physical, chemical and biological roles of these components continue to be unraveled. Today, there is no debate as

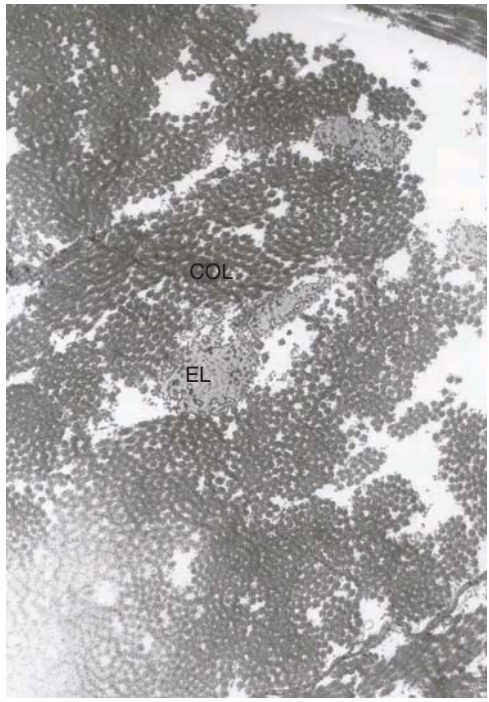

Figure 11.5

to the fact that true anti-aging benefits can be achieved by impacting the dermal matrix, with its abundant blood and lymph vessels, sensory and trophic neuronal components, mast cells, dendritic cells that act as sentinels of the immune system, sweat glands, and pilo-sebaceous follicles that extend into the dermis; as well as adipocytes that synthesize and store fat, impacting the skin appearance, but in addition secrete a slew of cytokines and hormones that have vast implication to human health, metabolic syndrome, obesity, and longevity.

11.7 Fibroblasts

Fibroblasts are well recognized as central to the production and remodeling of the dermal components, and are also crucial for regulation of epidermal morphogenesis (60) (Figure 11.6). Human fibroblasts are one of the major cell types widely used for *in vitro* tests to assess toxicology of various compounds. Additionally, efficacy of hormones, retinoids, and phytochemicals in providing anti-aging benefits (such as synthesis of elastin, collagen, and inhibition of MMPs) continues to be evaluated by several methods to find expression of activated genes (61). There is an abundance of published papers, as well as posters that are presented yearly at the annual meetings of the American Academy of Dermatology and the Society for Investigative Dermatology. Recent findings that fibroblasts from different anatomic locations may have differences (62,63) in their physiology is not totally unexpected, as the epidermal morphogenesis and differentiation in different body areas such as lips, palm, and sole depend on local epithelial-mesenchymal interactions. Differences in fibroblast physiology from fetal and adult skin also underlie the scarless wound healing properties of fetal skin (64). Fibroblasts *in vitro* have a finite replicative lifespan, and hence have been the model to study cell senescence, and often an inverse relationship between replicative capacity and donor age has been claimed. Notable decline in efficiency of oxidative phosphorylation capacity of fibroblasts (i.e., mitochondrial protein synthesis, respiration, and coupling of respiration to ATP synthesis, starting from the fourth decade of life) has been reported (65). However, studies on growth and responsiveness of fibroblasts from Centenarians showed that there is no direct correlation between *in vivo* aging and *in vitro* proliferative capacity of human fibroblasts, at least at the individual level (66).

Fibroblast functions encompass production and degradation of hyaluronic acid and a variety of other matrix molecules, collagen, and elastin, as well

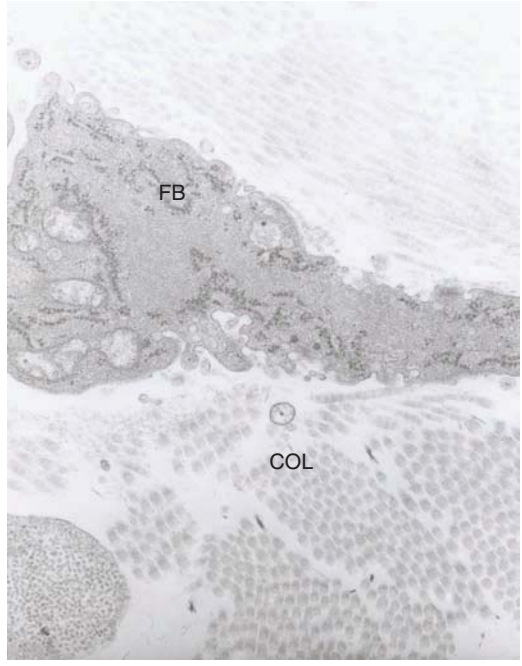

Figure 11.6

as wound healing. The degradation of matrix via production of MMPs by fibroblasts has been a target for anti-aging. With intrinsic aging, collagen levels are reduced and MMP secretions increased in sun-protected skin, compared with young skin (34,67). Several peptides, phytochemicals (32), lipids such as eicosapentaenoic acid (68), and a variety of synthetic molecules have shown inhibitory effects on MMP production by fibroblasts *in vitro*. Additionally, topical vitamins, especially ascorbic acid (69,70) and its derivatives such as cholesteryl ascorbyl phosphate (71), minerals and botanical agents also stimulate collagen production and modulating effects of chronological and photoaging (72). Ascorbic acid acts as a free radical scavenger in tissues, is a cofactor needed for synthesis of collagen, as well as enzymes such a prolyl hydroxylase and lysyl hydroxylase (essential for stabilizing and cross-linking of collagen molecules, respectively). A synergistic effect of ascorbic acid and retinol in improving epidermal photodamage has also been documented (73).

Elastin, the crucial matrix protein which imparts resilience to skin, is one of the components irrevocably altered by photodamage (74). Elastin fibers,

although quantitatively much less prominent than collagen, have a significant role in skin appearance and resilience—as seen in inherited deficiencies such as the cutis laxa—a rare inherited or acquired disorder where the skin becomes inelastic and hangs loosely in folds. Histology of cutis laxa skin shows altered elastin morphology, with fragmentation of elastin or loss of elastin fibers, and cultured fibroblasts from patients showed 2–3 fold increase in elastase (enzyme that degrades elastin) production, and decreased lysyl oxidase (75). In contrast, solar elastosis, which characterizes photodamaged skin, shows an overall increase in elastin content; however, the elastic fibers have lost the characteristic candelabra-like structure, and appear as large clumps—an analogy would be the difference between individual rubber bands versus a clump of rubber bands that have been coalesced by heating. The elastotic material also shows strong immunoreactivity for MMP-1 and MMP-2, the major dermal proteases that degrade collagen, elastin proteoglycans and fibronectin (76). Solar elastosis is considered as irreparable, and with the loss of elasticity, the integrity of the DEJ-dermal connection is weakened, resulting in a flattening of the DEJ, a hallmark of photoaged skin. However, there are indications that new elastic fibers are formed in skin after procedures such as laser resurfacing (77), and via topical treatment with certain active ingredients such as pomegranate extract. As inflammatory cells and their secretions, such as neutrophil elastases, play a significant role in destruction of elastic fibers, anti-inflammatory actives such as curcuminoids, bisabolol, silymarin, pomegranate, and a slew of flavanoids have also been employed in treating and preventing elastotic damage. Topical application of human growth factors derived from bioengineered tissue cultures have also been found to increase collagen content of the dermis, as well as viable epidermal thickening (78).

Another notable dermal aging change involves glycation of the proteins and formation of advanced glycation end products (AGE), with high degree of cross-linking of collagen and elastin. Proteins with low rate of turnover (i.e., long lived proteins such as collagen and elastin) show aging changes that include glycation, altering their physical and physiological properties such as mechanical and tensile strength, stiffness, and interactions with cells and matrix. Detailed reviews on this topic are available (79,80). It is clear, though, that visible signs of aging are especially prominent in tissues and organs such as the skin (and arteries, joints, and lens of the eye) that are rich in such long lived proteins. An accelerated aging of skin in the diabetic state lead researchers to examine the role of non-enzymatic glycosylation (glycation) in aging of the dermal matrix. Glycation involves the initial condensation of a sugar aldehyde or ketone with a free amino

group of protein, with the rapid formation of a Schiff base, which can undergo rearrangement to form a more stable Amadori product. Further reactions of intermediate products form fluorescent, pigmented adducts and cross-links, AGE such as pentosidines, considered as molecular markers of protein aging (79). The sallow appearance of aging skin is often considered as being the result of AGE in the dermal matrix. Glycation is known to alter elastin and the proteoglycans of the extracellular matrix as well. In an investigation of human skin, Jeanmaire *et al*. (81) found a positive correlation between intrinsic aging and dermal glycation, which generally manifests itself in the third decade of life, and increases rapidly afterwards. They consider glycation of dermal components an important and early component of intrinsic aging. Further, they found that in a 29-year-old, the AGEs were only 1.34 percent of dermis in photo-protected skin areas, whereas in sun-exposed skin, it reached about 28 percent. Additionally, this study revealed that AGE accumulation in photodamage in upper dermis was mainly found in the elastotic tissue.

Investigations such as those of Jeanmaire *et al.* (81) have also helped develop experimental models for evaluating potential anti-glycation compounds for their ability to block glucose-induced AGE production *in vitro*. Compounds that show efficacy in such tests would be ideal candidates to prevent or decrease skin glycation both during intrinsic aging as well as photoaging. Apart from prevention of glycation, more recent efforts have been focused on trying to break the cross-links that have already been formed in aging collagen and elastin, from a biomedical perspective of treating hardening of arteries (82) by developing chemical compounds that actively break the cross-links (83). These are exciting new anti-aging actives for the cosmetic industry.

Skin microcirculation is also known to be affected by aging (84), as the dermal papillary loops are significantly reduced in old skin compared with young skin. Li *et al*. (85) have used some of the bio-engineering techniques that are currently available for non-invasive evaluation of cutaneous functions to examine age related changes in microcirculation. They found that the capillary loops in dermal papillae decrease, but the sub papillary plexus increase with age. Such changes are expected to result in a reduced nutrient supply to the epidermis, and result in thinner epidermis and a flattened DEJ, both making the skin more transparent and fragile. Subsequently, the sub papillary vascular plexus become more visible, as is observed in spider veins. Modulation of angiogenesis, via factors such as

thrombospondin 1 (a recognized inhibitor) have been under investigation in recent years (86,87).

11.8 Role of Inflammation in Aging

Besides the obvious inflammatory events in chronically sun exposed skin that lead to elastosis, the role of sub-clinical inflammation in aging has also been an area of interest (88). The term inflamm-aging has found its way into cosmetic industry lexicon (89) and considers the cumulative effects of such changes as functional defects in microcirculation (as in leaky spider web-like superficial capillaries), to release of inflammatory cytokines from the barrier compromised epidermis, and release of neuropeptides such as substance P and calcineurin that could be underlying causes for development of sensitive skin. Neuro-cosmetics, or treatments to soothe and calm the skin, are a focus today for anti-aging strategies. Phytochemicals with anti-inflammatory benefits, such as curcuminoid or its derivatives (tetrahydroxy curcumin), have been shown to down-regulate a battery of inflammatory cytokines *in vitro*, as well as reduce erythema and inflammation *in vivo* (following patch testing of irritants or UV exposure), and hence are used effectively in anti-aging skin care products. The list of such ingredients is growing, and has been supplemented by synthetic molecules with known cyclooxygenase 2 or lipoxygenase inhibitory actions.

11.9 The Neuronal Component

As the interface between the outside world and our bodies, the skin fulfills major regulatory and sensory functions. Signal detection, feed-back loops, and autocrine/paracrine modulation are part of the intricate cutaneous communication.

Two languages are spoken within the skin. The first is classical neurotransmission. It consists of neural and trophic input provided by the cutaneous nerves to all skin cells, sensory receptors and appendages. This language also has a reciprocal modulatory relationship with the immune system. Neuro-peptides and hormones are responsible for the translation of sensory information and the regulation of local inflammatory, immune, and survival processes. Neuroreceptors (meissner, merkel, and other corpuscles) are limited to the dermis, but free nerve endings invade all layers of the cutaneous integument.

The second language is the more traditional receptor signaling between skin cells. Cutaneous cells are endowed with many of the same receptors and secretory capabilities as their ontological partners, the neurons. However, rarely is a skin-derived neuropeptide entrusted with the same function in skin and in the nervous system. For example, many neuropeptides or neurotransmitters that serve a modulatory role in the nervous system are used purely for cell turn-over purposes in the skin.

Because of their trophic and cell-cell interaction roles, nerve-derived substances actively participate in homeostatic mechanisms that modulate the effects of cutaneous aging. In turn, denervation results in thinning of the skin that is similar to the effects of intrinsic aging.

Sensory fibers have been shown to be responsible for increased microvascular permeability, a component of the vascular inflammatory response (90). The term neurogenic inflammation is now well recognized (91), and several in-depth studies on the role of nerves and neuropeptides in skin function and dysfunctions have been published (91,92). Neuropeptides (NP) are produced by the neurones, and they act as transmitters or modulators of neuronal functions. Following peripheral release, NPs are metabolized by endopeptidases of the target tissue. Several different neuropeptides, such as Substance P (SP), calcitonin gene-related peptide (cGRP), neurokinin A (NKA), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), etc. co-exist with the classical neurotransmitters, such as acetylcholine and norepinephrine. Using immunohistochemical techniques, various NP-containing cutaneous nerve terminals have been identified within the dermis, as well as extending into the epidermis. Putative connections between the dendritic cells and epidermal nerve fibers have also been documented (93). Among the known NP activities in the skin are vasodynamic responses leading to cutaneous inflammation, as well as those brought about by induction of mast cell degranulation (SP, VIP, somatostatin, neurotensin/NT). CGRP release leads to a prolonged erythema, not mediated by histamin release or degranulation of mast cells. NPs, such as SP, enhance proliferative response of T lymphocytes to mitogens and haptans; as well as stimulate chemotaxis of lymphocytes, neutrophils and monocytes, and release of inflammatory cytokines by several cell types present in skin. The reader is referred to reviews by Fantini *et al*. (91) and Roosterman *et al*. (92) for more detailed information on neuropeptides and skin reactivity, including sensitive skin that can lead to early skin damage and aging changes. Hence "neurocosmetics" with functional ingredients (natural or synthetic), designed to alleviate or neutralize the undesirable effects of

neuropeptides, have stimulated interest in the past decade. We can expect this to be a growing area of anti-aging skin care, as striking similarities between the epidermal cells and neurons (sharing a common neuro-ectodermal origin) continue to be discovered (92).

11.10 Impact of Lifestyle on Aging

Realization about the impact of lifestyle on how old one looks can be attributed to the coming of age of the baby boomer generation. A lifestyle of sunbathing and tanning led to premature appearance of lines and wrinkles (not to mention the more serious skin cancers), and kindled the surge in use of sun protection. The role of sun protection in anti-aging strategies cannot be over emphasized. As there are several excellent and recent reviews on this topic (5), we will focus on some of the other lifestyle issues such as smoking, stress and unhealthy dietary habits.

It is well recognized that people look older in the face when there are more than the expected number and severity of wrinkles, and a resultant lack of skin elasticity (94). Smokers, in general, have been known to look older than their chronologic age (95–97). Stress, be it environmental or psychological, has a huge impact on skin, as is evident from several publications dealing with effects of stress on skin barrier repair (98,99). Recent findings on the shortening of telomeres in individuals under chronic stress (100) lend further credence to what the cosmetic industry has long been trying to educate the customer about. Stress-proofing the skin, to some limited degree, can be achieved by induction of stress proteins. Various phytochemicals and extracts (such as golden root, ginseng, and neem), as well as plant adaptogens that have been used for centuries in Asian and Indian traditional medicines, induce the stress protein expression in skin cells *in vitro*. Here we are seeing a fusion of Eastern philosophy and Western science—a truly holistic approach towards wellness and longevity. In an attempt to determine the physiological factors associated with looking older than the actual age, Bulpitt *et al.* (101) found a correlation with total cholesterol and hemoglobin in men, and erythrocyte sedimentation rate and bilirubin in women. This interesting sex-related difference needs further in-depth evaluation.

Longevity is also on the cutting edge of anti-aging medicine today, as the original research on Sirtuins (102,103) has appeared in the popular science magazines and even in the press, creating a buzz. Sirtuins are gene products

mediating the effects of caloric restriction that underlie longevity of organisms as diverse as yeast, worms and mammals. Caloric restriction counters metabolic syndromes that encompass obesity, insulin resistance and diabetes; all of which are significant health challenges not restricted any more to the affluent Western societies. As caloric restriction for humans is near impossible to practice, caloric restriction mimics, which activate sirtuins, are touted to be the answer to these maladies (104). *In vitro* and *ex vivo* studies have suggested that application of cosmetic ingredients such as resveratrol, yeast extracts, and peptides that mimic SIR1 or SIR2 have potential for extending the healthy life span of skin cells (105). Such ingredients have been introduced in some of the pioneering skincare products in 2007.

11.11 Summary

In summary, anti-aging cosmetic products are being developed with the most sophisticated cell and molecular biology techniques, including gene arrays, SAGE, and proteomics (106) in scientific laboratories the world over, using isolated skin cells (such as fibroblasts, keratinocytes and melanocytes) to pinpoint the beneficial effects of active ingredients. Such studies are followed by testing on three-dimensional skin equivalents, which alleviates the need for animal testing (a practice abandoned by most of the industry). An array of non-invasive techniques utilizing instrumentation to measure a wide array of skin responses, parameters, and physiological functions has aided the cosmetic scientist and the dermatologist in quantifying the biological effects for claim substantiation of anti-aging products.

True anti-aging efforts should have a holistic approach, addressing not only skin care, but nutrition, wellness (physical and emotional), balanced lifestyle, and stress management. The cosmetic industry is embracing such an approach, and with increasing consumer awareness industry investments in research and development efforts to meet this need, anti-aging is no more just a buzzword.

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12

The Design and Development of Anti-Aging Formulations

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12.1 Introduction

12.1.1 Background

Forever youth, or the concept of maintaining a youthful condition and appearance has been the desire of mankind for ages. In the ancient times, women used flour to make their skin appear white and non-shiny. Cleopatra used black charcoal for her eyebrows, and the kings of France made their cheeks rosy.

Recently developed advancements in medicine and science allow humans to live longer than any other time in past history. These demographic changes have a large impact on social structure, especially in respect to health and care management. This change encompasses significant economic benefits among nations that will support and demand to extend and beautify their peoples' lives.

Pharmaceutical companies are focusing on providing medication of different forms to address different disorders. The medical community is doing wonders putting bones together, reassembling fractures, addressing sexual malfunctions, and almost producing babies in test tubes. These wonders are also driven by economical advantages. But does it really support the elderly population? Can we search for a miracle pill that can prevent or reduce the risk of stroke, dementia, heart disease, cancer, Alzheimer, osteoporosis, or arthritis? Can we grow older without having to worry about any of the old age diseases or even getting them in a milder form? Can we look in the mirror again without having the urgent need to see a cosmetic surgeon? Can we grow older and not look older in a decent way?

This chapter will present an up to date illustration of skin aging and available cosmetic approaches in today's market place. The "look better—feel younger" approach will have an immediate visual effect on old people skin, with long term benefits on their state of mind. This may be a new approach to cope with aging that can very well indeed slow down the aging process, by extending the brain power and increasing well being and wealth.

Wealth? Yes! Just imagine what a great deal of social impact these old, good looking ladies and guys would have. It is well known that globalization made excellent improvements in humans' lives. Today everything is available cheaper and faster than ever imagined; we don't need to wait

years for a new videogame; it will come out next holiday season from across the ocean. But globalization is also a threat to people's job security. Large and small companies have consumed all their possible cheap labor countries, although the cheap labor exists in their own environment. This is a new, inexpensive labor that is not yet acknowledged, and it is based on the labor generated by retired population. The economy should be structured in such a way that one should be able to pay a retiree the same amount of money as a full time employee gets elsewhere. This should certainly happen in the near future, when older people will look good grace to the new preventive care program.

While in recent years much attention is drawn towards both prevention and repair of signs of aging, the future should focus more on the preventive approach. The anti-aging skin treatment should start at earlier ages so elderly people will look younger and will be able to maintain their productivity in the society. They will be the ones starting to use moisturizers and sunscreens, taking vitamins, exercising, at the time when there isn't a real need to adopt a "maintenance" program. In addition, they need a long term skin care program termed preventive skin care. The new system ideally should start when someone turns thirty and it should be designed to address the upcoming aging process. This is not to be confused with a miraculous pill expected to magically slow down the aging. Also, it isn't at all an invasive surgical process. It looks more like a treatment, close to preventive care, the same as going to the doctor or dentist for a regular check up, as a routine part of a life style.

We're now looking to extend and elevate the quality of life in which an individual has high levels of physical and mental capacity; we're changing the old age into a useful period, not only at the individual and family level, but also at the social level. The correlation between one's appearance, its mental condition and social attitude, is well known even if not completely scientifically documented.

Over the past decade, a substantial number of studies have been published on aging; they attempted to answer the everlasting questions about the reasons for aging, and to what extend will we be able to limit the degeneration of body functions and to control ones appearance.

The global consumer skin care market in 2006, was valued at $58 billion, of which $6 billion for body care, $30 billion for face products, sun care $4.0 billion, bath and shower $15, hand care $1.1 billion. It was also

confirmed that global sales of anti-aging products are now at $9.8 billion, a 108.5 percent increase since 1997.[1]

A special attention should be drawn to the outstanding contribution of a Romanian doctor, Professor Ana Aslan who coined the term gerontology, the science of the aging process and its treatment. Ana Aslan is renowned for her essential contribution to gerontological research as well as for having patterned the best geriatric treatment influencing the aging process. She was the first scientist to rule out the fatalistic approach to aging, and scientifically addressed the needs of elderly people, with intensive treatments. She provided a new way in gerontology with the prevention and treatment of aging, with results that improved many people's lives. She founded the National Institute of Gerontology and Geriatrics in Bucharest, which adapted an original therapy for regeneration and stimulation of vital functions of the human body, known under the name of Ana Aslan Therapy. A great number of people came to her clinic for treatment, hoping to gain their vitality. Dr. Aslan never accepted aging in a passive way; she knew how important a healthy skin and young appearance for a good disposition. She then dedicated all her creative energy to find ways to preserve physical condition through cosmetic and medical treatment.

Recently, as more knowledge has been gained with the help of gerontology, skin aging and all the aspects of getting "the older look" should be now approached methodically, the same way medicine is approaching curing diseases.

12.1.2 Skin Aging: Physiology

Skin aging reflects chronological genetic changes in the body and is also termed intrinsic aging, where cells renewal slows down (laxity). Getting older is a mechanism governed by an internal clock in which the cells are subject to influence of genetic factors. As a result they stop dividing and enter into senescence. Supposedly, all cells enter a state of senescence after a number of predetermined cell divisions. With age, more senescent keratinocytes and fibroblasts accumulate in the skin, accelerating the aging process. Communication between cells is altered, tissue becomes rigid, and wrinkles appear.

Aging can also be the result of environmental stress factors such as climate and pollution. Aging which is governed by external factors is referred to as extrinsic aging. UV rays generate free radicals which can damage collagen

and elastin network. As a result, the cell renewal process is disrupted and can produce hyper pigmentation, rough and wrinkled skin. Dehydration and impaired barrier functions lead to loss of moisture in the deeper dermis, with a stratum corneum dry appearance.

Aging is reflected directly by the way we look and indirectly by the way we act. The first impression one makes is his appearance; we estimate his or her age by the appearance of the skin. We need to learn more about the aging phenomenon, and look closely particularly at the nature of the changes occurring in human skin, in order to understand it and to develop approaches to change it.

The skin has three main layers: the epidermis, the dermis and the subcutis. The Epidermis is the outer most layer of skin, composed of stratified keratinized epithelium and living keratinocytes in its live levels. The dermis consists of a complex fibrous matrix that contains mucopolysacharide gel, in which blood vessels, lymphatic channels, nerves, glandular ducts, and hair follicles are embedded. The structural components of the dermis are collagen and elastin (elastic protein), that form the matrix which is also composed of glycosaminoglycans (GAGs), and water. Collagen imparts the tensile strength to skin, while elastin maintains normal skin tension. This layer is also the site of the primary water retention molecule, hyaluronic acid (HA), which is located inside the collagen fibers network. This compacted matrix structure resists compression forces providing smoothness to skin. HA has a high water binding capability, being able to bind up to 5000 times its weight in water.[2] Therefore, it plays an important water regulating role within the matrix. With age, the ability to synthesize HA decreases. Dermal HA accounts for the majority of HA in skin, but the most dramatic changes observed in senescent skin are occurring in the epidermal HA. In senile skin, HA is still present in the dermis, while HA of the epidermis has disappeared entirely.[3] The third layer, the subcutis, is predominantly populated by *adipocytes*.

Visually, there are four main skin problems caused by aging which may be potential targets for improvements; either through cosmetic surgery or topical cosmetic applications via semisolid creams or formulations such as serums:

- Visible fine lines and wrinkles
- Dehydration with a rough texture
- Loss of skin radiance, with appearance of age spots
- Loss of skin tonicity, thinning of the epidermis

As skin ages, there are fundamental changes in skin's basic components. Significant depletion of HA mainly generates a decrease in hydration, which implies reduced skin elasticity and collapse of collagen network. The consequences are wrinkling, thinning and dryness of skin.

In mature skin, microcirculation is slower. The transport of key nutrients and vitamins is slowed down, leading to reduced efficiency of biochemical reactions needed to support skin integrity. The skin barrier capacity is affected. Also, dermal–epidermal junction is less flattened and has fewer connections and as a result the epidermis is more prone to be damaged by friction and slower to repair after an insult.

Young skin is characterized by firm and elastic properties, supported by strong tissue, named the connective tissue. In young skin, strong collagen fibers are produced in sufficient amounts to impart tensile strength to skin and the connective tissue. The way the collagen fibers are packed and organized is equally important in creating necessary tissue strength. Young skin is able to produce large amounts of procollagen1 (the precursor of collagen), and to balance the production of the enzyme Matrix Metalloprotease1 (MMP1), required to degrade proteins. The production and chemical transformation of Collagen1 is only one parameter among many, involved in the creation of a youthful skin appearance. Post-translational changes of Procollagen, the precursor of collagen, must happen in order to form a three dimensional structure which will form the mature collagen. The process involves a variety of enzymes, of which Procollagen-lysine 2-oxoglutarate 5-dioxygenase (PLOD) is the most important. It has an important role in anchoring the collagen fibers to the extracellular matrix, and increasing the tensile strength.[4]

Aged skin is characterized by low levels of mature collagen. The production of Collagen1 in aged skin is reduced, while the enzyme MMP1 production is upregulated. Also, PLOD levels, the enzyme involved with post collagen modification are reduced; the balance between anabolism and catabolism is changing.

Youthful skin appearance is also characterized by an increase of small, intermediate proteins which anchor collagen fibers to the extracellular matrix. All these processes are factors contributing to maintaining a young skin appearance.

Another major role in maintaining a youthful look is attributed by the lipids in epidermal layer of stratum corneum, called the intercellular lipids.

Although being the major route for skin penetration, they seal the gaps between corneocytes and ensure its moisture-retaining properties. The lipid seal also provides resistance against penetration of external agents. Ceramides are the major lipid components of the stratum corneum; they belong to the large family of sphingolipids. In aged skin, the ability of the skin to repair itself after insult is affected, and topical application of ceramides containing products was shown to induce repair and replenish the levels of stratum corneum lipids.

12.2 Active Ingredients Used in Anti-Aging Products

The next paragraph will focus on reviewing a variety of active ingredients currently available to use in skin care products, and understanding their mode of action. Are they effective, and if so to what extend?

This section will delve deeper into the understanding of possible mechanisms of action of few ingredients, while focusing on different anti-aging targets. The examples below do not list all the ingredients available in today's anti-aging market, but will introduce ingredients that have shown activity when applied topically and illustrate various ingredient categories by their action.

With the market being flooded with an enormous number of actives aimed to target the anti-aging segment, one would believe that the consumer is expected to know at least basic chemistry and be very well versed in knowing the physiology of aging. Since clearly additional knowledge and education are required, we could rather see slightly disoriented consumer which has difficulty to pick and choose the right product. At the end, one buys a product, but isn't sure that this was the appropriate one. And if isn't, is there one existing? The conclusion is that the consumer is exposed to many product choices with little or no solid information regarding product claims or enough explanatory reasons to make sure that the purchase is the right choice for her needs. In today's market it is not quite clear if the purchased product truly addresses one's initial desire. It is no secret that the most successful skin care items in current market are the ones that claim benefits related to true ingredients activity along with expected performance.

We can assume that the right product will be the one providing rejuvenation similar to *restylane* injection, to a *botox* treatment, or a surgical procedure. Is there such a product in the market? Although some of the claims do

point out for such similarities, it may take a long way to match the dermatological procedures performance. A more honorable approach to this is to educate the consumers and give them sufficient explanation of ingredients, actives or of what FDA consider over the counter drug items that can be found listed on the package.

One way to generally classify anti-aging ingredients can be done by dividing them into six main categories:

- Moisturizing, plumping
- Cell renewal/repair
- Anti-oxidants
- MMPs inhibition—to address dermis network degradation
- Collagen synthesis, skin elasticity and firmness
- Photoaging prevention

Some of the ingredients may have multiple actions, for example Retinol, a non-cosmetic prescribed medication, best known for its action of reducing fine lines and wrinkles, and stimulating cell growth and metabolism.

The list below is organized in a chronological order, where the first few ingredients came out in the 70s and the last on the list are the newest in the market.

Vitamin A—retinol, well known for its influence on skin cell differentiation, improvements in skin appearance and its action of smoothing fine lines and wrinkles. It is also known in cosmetics as the anti–age vitamin, representing the golden standard among active ingredients. Vitamin A has been used in cosmetics under different forms, and its effects on the biology and biochemistry of the skin are well documented.[5] It is an antioxidant, it acts on skin by favoring the correct cell metabolism, and it is essential for correct function of epithelial cells. It has been shown to stimulate overall metabolic activity by increasing collagen and GAG synthesis, both *in vivo* and *in vitro*, with action on dry and rough skin. It affects the production of human growth factor, which favors cell multiplication, along with tissue regeneration. It is perceived as the epidermis protector, with direct action on protecting epithelial tissues as a physical barrier from infections. Its activity starts at as low as 200 international units per ml in topical applications.

It should be noted that Retinol is different from retinoic acid and its derivatives (i.e., trans-retinoic acid or retinoic acid[6] (Retin A). Retinol and

retinoic acid are related but distinctly different. Retinol and other forms of vitamin A, such as retinal and retinyl palmitate, exhibit a milder effect on the skin. These compounds first need to be converted by specific enzymes into the active metabolite, retinoic acid.[7]

In theory, someone should be able to apply retinol to the skin, expect a conversion to retinoic acid and hope for benefits to show. In reality, the conversion rate is quite low and varies between individuals. Also, when exposed to air either during storage or during use, most of the retinol may be oxidized or degraded even before becoming available for conversion to retinoic acid in the skin.

Many anti-wrinkle and skin rejuvenation products contain retinol. Its activity provides divers anti-aging benefits, such as reducing fine lines and wrinkles, promoting a more youthful texture and appearance to skin. It also helps skin cells growth, keeping the structural integrity. It also accelerates growth of epithelial tissue, normalizing dry and rough skin appearance.

Vitamin E—alpha tocopherol is known for its antioxidant properties. It is a free radical scavenger, combating reactive oxygen species that can cause damage to lipids, proteins and nucleic acids inside the cells, endangering tissue integrity. It reduces phospholipids peroxidation, thus preventing cross-linking of collagen by the oxidation-breakdown products, mainly malondialdehyde. Vitamin E is also known as an emollient, to improve skin feel and texture. Application of 5–8 percent tocopherol has been reported to reduce skin roughness and wrinkle depth. When used in skin care products, vitamin E can protect skin from UV light, due to antioxidant properties thus, reducing the appearance of fine lines and wrinkles, delaying the progression of aging,[8] and preventing skin cancer development.

Vitamin C—ascorbic acid is an important antioxidant that participates in many cellular activities. Same as vitamin E, it combats the reactive oxygen species that can cause damage to lipids, proteins and nucleic acids inside cells. It works synergistically with vitamin E, when its anti-oxidant effect is enhanced. Free radicals are effectively suppressed, thus enabling vitamin C to act as photoprotective compound. Ascorbic acid is also known for inhibiting the enzyme tyrosinase,[9] which is the regulator of melanin pigment production.

Vitamin C, the protective corrector, is essential for the formation and well function of collagen to ensure a firm skin. Oxidative processes in the skin are commonly accepted as major causes for skin aging, and they are generally involved in the intrinsic/chronologic aging of skin. The use of Ascorbic Acid in cosmetic or dermatological products is limited due to its instability and self oxidation in aqueous solutions, and also its non predictable penetration into the skin. Scientists have looked for ascorbic acid derivatives that function similar to that of ascorbic acid, with better chemical stability and comparable percutaneous penetration.[10] As a result, magnesium L-Ascorbyl phosphate and magnesium ascorbate have been introduced in the market. It is hypothesized that they are able to undergo hydrolysis in the skin, while penetrating it as free vitamin C which can exert its functions.

Vitamin C, specifically its stable derivatives, are mainly used in cosmetic formulations to protect against free radical damage due to UV radiation, for skin brightening, lightening and prevention of age spots formation.[11]

Panthenol—the beautifier, is known as pro-vitamin B5, included in the 1984 listing of OTC drugs published by the FDA. It is assumed that panthenol is hypothetically metabolized in the skin to pantothenic acid. Present in all living cells, pantothenic acid is the major constituent of Coenzyme A which is needed in most of the energy releasing reaction in the human body. Known for its revitalizing and conditioning effects in the hair and skin, panthenol has a humectant—like properties that promote moisture absorption. Panthenol is used as a hair conditioner and in cosmetics as an *emollient*, commonly at concentrations between 0.1 and 1.0 percent. It is used by the pharmaceutical industry in ointments, creams (typically at 2 percent) for the treatment of various minor skin disorders. As an emollient and moisturizer, it makes dry skin softer and more elastic, has anti-inflammatory effects, and soothes irritated skin.

Hyaluronic acid (HA) is a carbohydrate, chemically classified as a glycosaminoglycan, naturally occurring throughout the human body in the connective tissue, epithelial and neural tissues (GAG). It is one of the main components of the extracellular matrix, and plays a key role contributing to cell proliferation and migration. It can be several thousand sugars long, and when it is not bound to other molecules it binds to water, giving it stiff, Jello-like texture quality. HA, has a very unique property; it can hold up to 5000 times its own weigh in water.[12] Its main function in the body is to

bind water and lubricate moveable parts of the body. Due to this property, HA is found in almost every cell in the body (i.e., bones and cartilages, tendons and ligaments, scalp tissue and hair follicles, eyeball, skin, and lips) at high concentrations.

In the skin, HA is found together with collagen, and they are both vital to maintain the skin's layers and structure. Roughly about 50 percent of the entire HA in our body is found in the skin, in the deep underlying dermal area, as well as in visible epidermal top layers. Young skin is smooth and elastic because it contains high concentration of HA, which will keep skin healthy. With age, the skin looses the ability to maintain the same concentration and HA molecular weight, as a result, skin becomes drier, without the necessary moisture to hydrate together with collagen, and therefore wrinkles are formed.

In early 2004 HA was approved by the FDA as an injection for filling soft tissues defects, under the trade name of Restylane. The treatment approved in the US, improves wrinkles appearance for aesthetic purposes. This procedure can be only done by dermatologists.

Used in topical applications, HA was proven to improve skin conditions, increasing its moisture content, bringing a healthy appearance.

AHA's—alpha hydroxy acids are a class of chemical compounds that consist of a carboxylic acid substituted with a hydroxy group on the adjacent carbon. They may be either naturally occurring or synthetic. AHAs are well-known for their use in the cosmetics industry. They are often found in products claiming to reduce wrinkles or the signs of aging, and improve the overall look and feel of the skin. They have been applied on face as fruit compresses since ancient times. α-hydroxy acids in fruits like grapes (tartaric acid), lemons and other citrus fruits (citric acid), apples (malic acid), almonds and apricots (mandelic acid), are the actives that may contribute to skin rejuvenation.

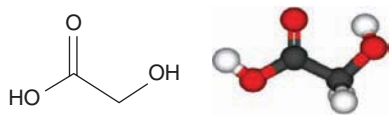


Figure 12.1 AHA: glycolic acid ($C_2H_4O_3$) 2-hydroxyethanoic acid.

AHAs are known to induce skin exfoliation, peeling and cause skin irritation, particularly at their effective higher concentration and lower pH. However, their action is associated with increase of skin smoothness and disappearance of lines and wrinkles. The most known compounds of this family are glycolic and lactic acids.[13, 14] Glycolic acid (or hydroxyacetic acid) is the smallest α-hydroxy acid. It appears in the form of a colorless, odorless, and hydroscopic crystalline solid that is highly soluble in water and other solvents. Glycolic acid is associated with sugar-crops and is isolated from sugarcane, sugar beets, pineapple, cantaloupe, and unripe grapes.

BHAs—beta hydroxy acids are a group of acids often found in flowering plants and herbs. Most common is salicylic acid, believed to dissolve dead skin cells to leave a smooth, even surface. They are similar to alpha hydroxy acids. The main difference between them is that alpha hydroxy acids attach the hydroxyl group to the alpha site of the molecule, while beta hydroxy acids attach it to the beta site.

In terms of physical properties, the main chemical difference between alpha hydroxy acids and beta hydroxy acid is the difference in their solubility. Alpha hydroxy acids are water soluble, while beta hydroxy acid are lipophilic—oil soluble. This means that beta hydroxy acid is more probable to penetrate into the pore which contains sebum and exfoliate the dead skin cells that are built up inside the pore. Beta hydroxy acids appear to be less irritating than alpha hydroxy acid despite their penetration properties.

ARGIRELINE® (acetyl hexapeptide-3) is a unique peptide composed of three amino acids: glutamic acid, methionine, and arginine. It has been shown to be effective against the development of skin wrinkling, specifically designed to fight against expression wrinkles. As described before, increased wrinkles with aging are naturally occurring overtime due to histological or physiological factors. They are secondary factors that can

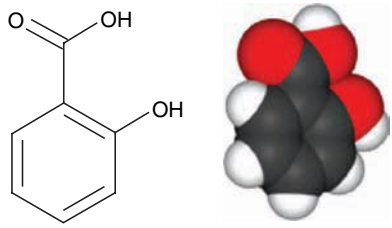


Figure 12.2 BHA: salicylic acid ($C_7H_6O_3$) 2-hydroxybenzoic acid.

produce characteristic folds or creases of the face, either during sleep or repeated facial movements and they are caused by the contraction of the muscle of facial expression. As part of cosmetic benefits, ARGIRELINE® reduces the depth of these wrinkles, especially in the forehead and around the eyes areas. The mechanism is related to the inhibition in the formation of SNARE complex[15] and catecholamines inhibition. SNARE (SNAp Receptor) is a complex formed by various proteins, including SNAP-25 (SyNaptosomal Associated Protein), with an important role in the neurotransmitter release. If SNARE complex is slightly destabilized, the neurotransmitters can't act efficiently and therefore the muscle contraction is attenuated. ARGIRELINE® is a designed peptide which mimics N-terminal end of SNAP-25, which competes with SNAP-25 for a position in the SNARE complex. As a result, SNARE complex is slightly destabilized, and muscle contraction is diminished, preventing the formation of lines and wrinkles.

Botox™ is a trade name given to a product derived from the Botulinum Toxin, a neurotoxin protein produced by the bacterium Clostridium Botulinum; one of the most poisonous naturally occurring toxins in the world. Doctors later found that a weakened form of this powerful toxin could be used in small amounts to treat certain medical conditions. Botox™ was approved by the US Food and Drug Administration as a treatment for problems with the eye muscles. It was later observed that a side effect of Botox™ usage was a slight reduction in wrinkles in the areas around the eyes where eye muscles had been treated. Botox™ is injected underneath the skin and into the muscle where it temporarily paralyzes the muscle. This may result in a lessening of the appearance of lines and wrinkles that can last up to 2–4 months.

Therefore, ARGIRELINE® is a safer and milder alternative to Botulinum Toxin, topically targeting the same wrinkle formation process using a different mechanism.

Matrixyl™ 3000 (glycerin, water, butylene glycol, carbomer, polysorbate 20, palmitoyl oligopeptide, palmitoyl tetrapeptide-7). This is an anti-wrinkle and skin lifting ingredient. Exemplified peptides include N-Palmitoyl-Pro-Arg, N-Palmitoyl-Thr-Lys-Pro-Arg, N-Palmitoyl-Arg-Lys-Pro-Arg, and N-Palmitoyl-Gly-Gln-Pro-Arg. While combinations of various peptides are possible, the patent applied for this product does not specifically identify a certain combination of a tripeptide and a tetrapeptide.[16] Matrixyl™ 3000 contains *matrikines*, which are messengers of

cutaneous restructuring and repair, to maintain skin's youthful appearance. The matrikines activate the synthesis of extracellular matrix micromolecules, providing a visible anti-wrinkle efficacy. *In vitro* tests with fibroblasts incubated with Matrixyl™ 3000 showed a significant ability to stimulate synthesis of extracellular matrix components. *In vivo* studies showed visible and measurable wrinkle reduction demonstrated by wrinkles density, average depth, volume, and roughness (via profilometry and photography techniques). The study was done on two panels of 23 volunteers, aged 39 to 74, which applied a cream containing 3 percent Matrixyl™ 3000 to one side of their face against a placebo on the other side. The study conducted over fifty-six days, and wrinkles reduction and lifting efficacy was assessed by profilometry and photography as compared to baseline. The results showed significant reduction in wrinkles for all studied parameters.

Dermaxyl™ (C12-15 alkyl benzoate, tribehenin, ceramide2, PEG10 rapeseed sterol, palmitoyl oligopeptide) is a matrikine (the matrix peptide VGVAPG) and ceramide 2 combination. *In vitro* studies showed that the matrikines components of Dermaxyl™ help attract cells to sites that need repair and contributes to reconstruction of the dermis. Ceramide 2 helps strengthen and repair the skin barrier, improving cell cohesion, enhancing water retention and reducing skin dryness.

In vivo studies conducted on a pigmented cosmetic product (make up), showed significant results in reducing the volume of main wrinkle on average by 13.7 percent, depth of main wrinkle on average by 10.1 percent, surface occupied by deep wrinkles reduced on average by 40.3 percent and the surface occupied by medium wrinkle on average by 24.5 percent, according to clinical study assessed by profilometry and photography.

ActiMatrix™ M (mucor miehei extract, butylene glycol) is a protease derived peptide from mushrooms which used in topical applications to help reducing the appearance of aged skin. It has been demonstrated *in vitro*, that ActiMatrix™ increases the expression of Collagen1 and decreases the expression of MMP1, up regulating the *PLOD*, the enzyme responsible for collagen post translational modification. It is known that collagen 1 has to be coupled with post-translational modifications, as well as anchoring proteins to attach fibers to the extracellular matrix to produce strong, covalently cross-linked tridimensional mature collagen with increased tensile strength, which is the sign of a young skin appearance.[17] ActiMatrix™ has been shown *in vitro* to increase Collagen1 and decrease the activity of MMP1enzyme and also upregulate *PLOD*.

In vitro experiments were followed by *in vivo* testing in order to assess skin moisture content, skin firmness, and measure superficial facial lines. For each test, a topical composition comprising an extract of miehei was applied to twelve female subjects aged 40–60, with normal or normal to dry skin. The results showed a significant skin moisture increase, increase in skin firmness and respectively overall improvements of the appearance of superficial lines and skin smoothness.

Tegosphere®VitA(acrylates-dimethylaminoethyl methacrylate copolymer and aluminum tristearate-retinol-polysorbate 20) is a relatively stable form of retinol achieved through an innovative encapsulation technology. Retinol is known as being difficult to work with in its pure form, due to heat, light and oxygen sensitivity. It also causes skin irritation; therefore protection and a slow release mechanism would be favorable.

This non-conventional encapsulation technology is designed to release Retinol when triggered by the change in pH, usually in contact with the skin. Made of 85 percent pH sensitive polymer, dimethylaminoethyl methacrylate copolymer, 5 percent retinol (2.5 percent pure all-trans retinol), and 10 percent aluminum tristearate. The ingredient should be used in formulations with a pH equal or greater than six. It offers a variety of advantages compared to other encapsulation techniques, such as minimal diffusion of the active ingredient out of capsules, very good thermodynamic stability,[18] controlled release and delivery of active ingredient after topical application, also oxygen and UV stability; small particle size (average 20 microns) and improved skin penetration of the active make Tegosphere®VitA, an effective active ingredient compared with pure retinol. To demonstrate improve stability of Tegosphere®VitA over the pure form of retinol, several stability tests were performed, detecting the amount of retinol with HPLC. After 1 hour exposure under UV light (6-fold accelerated compared with the natural UV radiation), pure retinol was detected at 20 percent versus the encapsulated form which retained 70 percent of retinol. A similar quantification was done on oil in water (O/W) formulation containing pure retinol and the encapsulation form. Similar results were obtained.

CoQ10 (ubiquinone) is one of the most popular anti-aging ingredients. Coenzyme Q_{10} is found naturally in all forms of animal life, present in higher concentration in the mitochondrial membranes; it is essential to generate Adenosine triphosphate energy for the body. Since the level of Coenzyme Q_{10} declines with age, topical application may assist to help

balance the antioxidant level in skin. Co Q10 is a co-enzyme antioxidant, which stimulates other enzymes actions. Among its health benefits, it is believed to help prevent damage to collagen and elastin production, an activity that has made it valuable for use in anti-aging formulations.

Until 2002 CoQ10, was regulated as a pharmaceutical in Japan. Since its release for use in cosmetics, the personal care industry has shown increased interest, leading to reduced stocks available internationally. The vast majority of the world's CoQ10 is produced by four manufacturers in Japan, using difficult and quite costly processes that in most cases are based on fermentation.

HELIOMODULINE® (*Gossypium hirsutum* (cotton) extract) it is claimed to provide DNA repair through the stimulation of XCP protein which is essential to the initial phase of repair, in the Nucleotide Excision Repair System of genome lesions. Excessive exposure to UV results in a number of clinical signs in over exposed zones, the skin becomes slack and deep wrinkles can form. Heliomoduline® is made of low molecular weight peptides from cottonseed, that boost the skin repair to accelerate the elimination of DNA damage, reducing the risks of inflammation and erythema and limiting cell aging of photo exposed skin. XCP favors the elimination of cyclobutane pyrimide dimmers (CPD), thus protecting the cell from the mutagenic effects of UV radiation.

Tested at 2 percent level *in vitro* in human fibroblasts irradiated with UVA, Heliomoduline® significantly stimulated the expression of XCP[10] (by 91 percent). Due to its ability to repair and protect the skin, this ingredient may be used in anti-aging, sunscreen and after sun products.

LONGEVICELL® (Water, hydrolyzed myrtus communis leaf extract) is an anti-aging active for mature skin, developed from myrtle plant, a scented white flower from a Mediterranean shrub. LONGEVICELL® acts on cell longevity, to slow down the expression of the senescence factors and increasing the lifetime of cells. It can be regarded as a cell regenerator and maintains tissue longevity, by limiting the degeneration of skin tissues and erasing the signs of aging.

Naturally the capacity of a normal, well-nourished cell to keep dividing is limited. When a cell stops dividing but remains metabolically active for a time and gradually fades away, it is said that it entered senescence. Apoptosis, programmed cell death is a scenario that can be initiated by

senescence, in which healthy cells act decisively to "commit suicide." When senescence and programmed cell death occur at the right time and location, they are an integral part of the life and death of a normal organism. When they occur inappropriately or in acceleration, developmental abnormalities or disease can result.

When fibroblasts enter senescence, *SIRT-1* synthesis decreases and the cell is exposed to the anti-aging. SIRT-1 is known to be the universal proteins for longevity. LONGEVICELL® stimulates the synthesis SIRT-1, and also controls intercellular communication by acting on caveolins, the proteins that enable a good communication between cells. Tested at 1 percent on senescent human fibroblasts, LONGEVICELL® was also found to inhibit the glycation, cross linkage reaction of collagen1 by 40 percent, thus preventing dermal tissues from becoming rigid, and loose its flexibility.

In vivo tests have shown that LONGEVICELL® has a wrinkle reduction effect. Tested in a formula at 4 percent in an emulsion versus placebo, it demonstrated reduction of total number of wrinkles (–30 percent, $P \leq 0.0009$), total wrinkled surface (–39 percent, $P \leq 0.0006$) and in the total length of wrinkles (–36 percent, $P \leq 0.0004$). LONGEVICELL® is claimed to offer the skin a new youth.[18]

ORSIRTINE (butylene glycol, oryza extract) is a botanical extract of rice, patented anti-aging active ingredient.[19] It is a sirtuin activating complex that has been specifically designed to address skin aging through SIRT activation. It activates *SIRT1* expression in human skin, increasing cellular longevity. It also increases skin repair and protection. Sirtuins are a class of enzymes that are involved in cellular metabolism through the selection of gene expression DNA–RNA-protein.

In vitro testing showed SIRT1 increase in cultured cells, increased cell longevity by decreasing senescence associated with an increase of beta-galactosidase, and finally an increase in skin protection by reducing oxidative DNA damage and UV-induced DNA damage.

Biopeptide CL (glyceryl polymethacrylate, propylene glycol, palmitoyl oligopeptide) that was demonstrated to stimulate the collagen and glycosaminoglycan synthesis, reinforces the epidermis, and diminishes wrinkles. *In vitro* test on human fibroblasts showed an improvement in collagen synthesis by +350 percent, compared to untreated cells. *In vivo* studies conducted using ultrasound technique showed a reinforcement of

the epidermis with increasing the skin's thickness by 4 percent. When used in a topical application at 3 percent level, it was proven to have an anti-wrinkle effect; the lengths of deep wrinkles, depth of wrinkle, and skin roughness were all improved.[20]

Radiance CR (sodium ascorbyl phosphate, biotin, manitol) is a water soluble vitamin based powder, made out of stable vitamin C derivative and *coenzyme R*. *In vivo* studies showed an improvement of skin roughness, reduced fine lines and wrinkles by 13 µm after two months and 25µm after three months of application. It can be used in formulations with a pH around 7. It has also been proven to fade the appearance of age spots.

ALL_Q™ plus (ubiquinone, tocopheryl acetate) is composed of Coenzyme Q_{10} and vitamin E, the most important lipophilic antioxidants for skin. They are naturally present in the skin, with higher levels in the stratum corneum layer. Studies by Quinn *et al*.[21] have shown that both lipid antioxidants are part of a cycle. Vitamin E is oxidized to its tocopherol radical which can by reduced by Ubiquinol to regenerate Tocopherol. The combination of Coenzyme Q_{10} and vitamin E is thought to exhibit a defense antioxidant mechanism.[22] Environmental aggressors such as UV light may generate free radicals, and they have been identified as major inducers of premature aging. *In vivo* studies of a product containing 2 percent ALL-Q™ plus, showed a significant inhibition of UV light induced oxidation compared to placebo. *In vivo* placebo controlled study showed that the appearance of visible wrinkles around the eye area was significantly reduced by 27 percent with a cream containing 0.3 percent Coenzyme Q_{10}.

RIGIN™ (water, glycerin, steareth-20, palmitoyl tetrapeptide-7) is a lipopeptide, with a sequence of Palmitoyl-Gly-Gln-Pro-Arg in hydroglycolic solution. It controls the secretion of Interleukin 6, a cytokine that is involved in mechanisms related to the generation of signs of aging, delaying the effects of premature aging. Clinical studies done on seventeen volunteers have shown significant improvement in skin elasticity and firmness. *In vivo* testing has also shown a significant improvement in skin hydration and skin smoothness.[23]

In vitro tests on keratinocytes culture showed a mechanism that is similar to that of dehydroepiandrosterone (DHEA), known as the youth hormone.

IBR-Dormin® (water, narcissus tazetta bulb extract) is an extract from the bulbs of the narcissus plant, suggesting a newer technology approach to anti-aging. Dormancy is the phenomenon found in perennial plants and certain fruits. It constitutes a special condition in which cell growth functions are slowed down and cell proliferation is subdued. Environmental stress such as UV light, pollution, cold weather, enhances cell proliferation and leads to premature aging.

IBR-Dormin® was demonstrated to slow down cell proliferation to allow longer cell maturation cycle and providing better skin protection against environment stress factors. Studies using a cream containing 1.5 percent IBR-Dormin® applied on skin, showed a significant increase in skin resistance, a decrease in sensitivity, increase in protection, decrease in fatigue, increase in suppleness, and decrease in fine line.

LaraCare A200 (galactoarabinan) is a highly functional natural polysaccharide, by the name of Galactoarabinan, extracted from Larch tree. It is a natural polymer, linked with sugar units consisting of galactose and arabinose. It is a very high purity ingredient and displays a synthetic like consistency, with a narrow peak molecular weight distribution. It is a unique ingredient in a sense that it provides moisture control, and reduces TEWL. Used in a topical application together with AHA's, it promotes exfoliation without irritation, which could improve skin superficial fine lines. It also provides skin tightening, with SPF enhancement.

In vivo testing showed that LaraCare A200 effectively reduced the TEWL at 1, 2 and 4 hours after a single application of a lotion containing LaraCare A200 at 2 percent. The enhancement of skin exfoliation without irritation was also studied by adding 2 and 5 percent LaraCare A200, to an 8 percent Lactic Acid formulation. By the day 4, reading showed a cumulative increase in exfoliation of 16.42 percent and 20.46 percent respectively, without irritation. The exfoliation was measured using by visual scoring of skin cells collected on D-Squame discs, at the end of twenty-four hours period.[24]

DECORINYL™ (water, lecithin, tripeptide-10 citrulline, carbomer, triethanolamine, phenoxyethanol, caprylyl glycol) is a tetrapeptide which mimics the sequences of decorin that specifically binds to collagen fibrils. DECORINYL™ has proved to regulate *fibrillogenesis*, control collagen fibril growth and increase skin suppleness, due to a better cohesion

of collagen fibers which provides higher resiliency and improves skin appearance.

DECORINYL™ makes up for the reduction in functional *decorin* as skin ages. It binds to collagen fibrils and regulates collagen fibrillogenesis, also enhancing collagen fibril stability. It was also demonstrated to enhance uniformity of fibril diameter and the regular spacing of collagen fibrils, maintaining tissue shape and providing skin suppleness. It was shown to provide a high and sustained moisturizing profile.

Green tea polyphenols are antioxidants with antibacterial activity from a tea shrub, Camellia sinensis. Green tea contains polyphenolic compounds also known as epicatechins, which are antioxidant in nature. Studies have shown that green tea extract possesses anti-inflammatory activity.

Understanding the molecular mechanisms of these effects of green tea is a subject of investigation in many laboratories. Treatment of green tea polyphenols to skin has been shown to modulate the biochemical pathways involved in inflammatory responses, cell proliferation as well as ultraviolet light-induced inflammatory markers of skin inflammation in various animal models. The protective effects of green tea treatment on human skin are not well understood.[25]

Based on existing literature showing beneficial effects of green tea on mouse skin model many pharmaceutical and cosmetic companies are supplementing their skin care products with green tea extracts, despite the unclear photoprotective effects of green tea polyphenols to skin. Green tea contains caffeine, one of the reasons why the extracts are often used to help reduce inflammation caused by AHA in dermal abrasive creams.

RENOVAGE™ (caprylic/capric triglyceride, teprenone) has been demonstrated to exhibit a significant activity on functional and structural signs of aging. Functional activity addresses hydration, barrier function, and pigmentation, while structural action refers to firmness, fine lines and wrinkles, pore size, *erythrosis*.

Renovage™ was tested on skin cell culture *in vitro* for function loss, cell resistance, self defense, and wound healing capacity. The results indicated that cell senescence was delayed by three months, meaning that the cell life span was increased by one-third.

Also, *in vivo* studies with a cream formulated with 3 percent **Renovage™** showed remanent skin moisture, reduced TEWL, decreased UV generated sunspots, significant improvement in firmness, tone and skin elasticity. Also, skin appeared more regular, smoother, and younger with pore size reduction and reduction in skin redness.

12.3 The Development of Anti-Aging Finished Products

12.3.1 Starting at the Beginning

Our industry went through major changes in the past decade and there seems to be a consensus about the fact that nothing today seems to look as it looked ten years ago. The dynamism is what characterizes many of professions today, and the formulation chemists working in a cosmetic environment is particularly facing an uncommon dilemma: to create or not create a new formula and in what timeframe. Who will be the first to the market that will gain market shares and feel the success?

With this in mind, the developmental process of any type of product can be anywhere between a routine job to a challenging obsession for the respective professionals.

With increased cometition in packaging options, availability of new delivery systems, and advancement in the technologically driven ingredients, one may say that today's formulation chemist has a new horizon to gravitate in. However, the situation is much more complex. Contract manufacturers, packaging and fragrance houses with satellite centers around the world, practically compete for the best product idea. But who needs ideas today? There is a constant debate between marketers in terms of novel ideas and finished goods with the fastest delivery time.

In today's world, research and development departments need to work faster to deliver a product that can be part of many others waiting to be commercialized by a factory. Factory flexibility is one of the factors that determine if the product will be developed internally or will be contracted out.

The cosmetic formulators of today are very different than any other scientists. While scientists may have tools, books, and protocols to follow, the cosmetic formulator needs creativity and luck. This discipline combines science and engineering skills with strong rules on one hand, with art and

creativity with no rules on the other hand. If the concept is right and the execution impeccable, someone will have the chance to be a hero.

Bench chemists should be respected for their curiosity and search for new ways of using ingredients, and especially be recognized for the creative effort made by them to live in symbiosis with the marketing departments. These can be demanding and dynamic relationship.

I will focus below on an example of the development of an anti-aging make up product for mass market. The objective was to develop efficacious decorative cosmetic product that is a pigmented emulsion but and feels, acts, and provides skin care benefits to the consumer. This idea in itself may sound as a paradox. Skin care products are mainly based on providing benefits of the actives to be targeted to different skin sites, while decorative cosmetics are by definition products that are applied to be maintained on the surface of the skin to impart color to skin's appearance.

We assume that skin especially face, which represents a quite large area of application for a decorative cosmetic product, will be the targeted area for penetration of actives. Skin delivery and penetration is a well studied topic, with different testing approaches *in vitro* and *in vivo*. Recently, most suppliers of active ingredients choose to conduct both types of tests to the formulator chemist; this represents an important tool for the formulator because it provides understanding that may shorten the development time, and also eases the selection process of the desired active ingredients. Despite the difficulty that one faces in measuring the accurate concentration of actives at the site of action, most recently almost every new active ingredient presented to the chemist provides such testing information *in vitro* and *in vivo*, as a complete package. *In vivo* testing is generally conducted with sample formulations containing certain amounts of the active.

For a cosmetic chemist, stratum corneum is the most important part of the epidermis, as it represents the targeted surface. It is known to act as a diffusion barrier for chemicals entering the skin. Due to their nature and the extremely efficient way of packing the stratum corneum's lipids, the skin permeability is very low. Based on the law of diffusion (Fick's law) where the rate of penetration is dependent on the active concentration, partition coefficient, diffusivity, and the length of the diffusion pathway of a specific molecule, one can calculate the flux of chemicals penetrating the skin. The polarity of the ingredient penetrating the skin is an important factor, as it relates to the lipophilic nature of stratum corneum. The viable

epidermis is hydrophilic; thus, there is high water concentration gradient between the two layers. The concentration of the active chemical at the top layer should be high, while its concentration in the deeper layers should be maintained at a lower level to allow the flow based on the concentration gradient difference and assure penetration. Delivery is generally defined as the process by which the right chemical entity (active ingredient), reaches the right site at the right concentration, in a defined period of time, usually long enough to allow the active to function.

In vitro testing of skin penetration of various actives is the most relevant of the methods for studying skin penetration. Penetrants or actives need to exhibit certain physicochemical properties, to enables their penetration. Some key features are long time understood by scientists studying drug delivery through epidermal layers, and they are:

- Low molecular weight of the active ingredient
- Good solubility both in water and lipids
- Solvent choice to elevate solubility
- Choice of emulsifying system—to establish an optimum surface tension
- Formula (base) composition
- No binding or accumulation in the stratum corneum

The last feature is important in respect to color products containing active ingredients. Decorative cosmetics need to provide aesthetics and sensorial features when applied. The product application is one of the key product attributes: it has to be easy to spread it on face, and should dry in a short amount of time. The wet sensation is only temporarily acceptable. This may be the reason for customer to purchase the product. Typical cosmetics are based on mixtures of various additives, fillers, and pigments that are purposely incorporated in the formulations to achieve or to improve tactile, olfactory, or visual impact. Actives are more likely to penetrate from systems that are lightweight formulations, or when made out from vehicles with very limited aesthetic properties. All these challenges should justify the lengthy time of development of an active emulsion.

RENOVAGE™ The idea behind a functional make-up product was to combine the long term effectiveness of the formula with the immediate beautifying attributes. There have been numerous studies on delivering actives in personal care and skin care formulations. Not too many studies have been conducted to address the delivery of actives in make up formulation. Until

recently, make up products were strictly identified product functionality, that is the color application, and not to comfort or treat the skin.

12.3.2 Product Design

With today's stringent regulations in the European Community and US for safety and evidence for testing, claiming cosmetic activity such as anti-aging, moisturizing, skin elasticity, and visible percentage of skin improvements, makes it important for the cosmetic chemist to understand and draw from the beginning the right methodology by which the benefits and product claims are substantiated. Figure 12.3 represents a cascade of a standard process development from the first stage of marketing request to commercialization. It presents three main parts, each outlines specific department involvement: marketing (red), R&D (green) and manufacturing (blue). All three departments function completely independent, however during this process the groups are collaborating with each other.

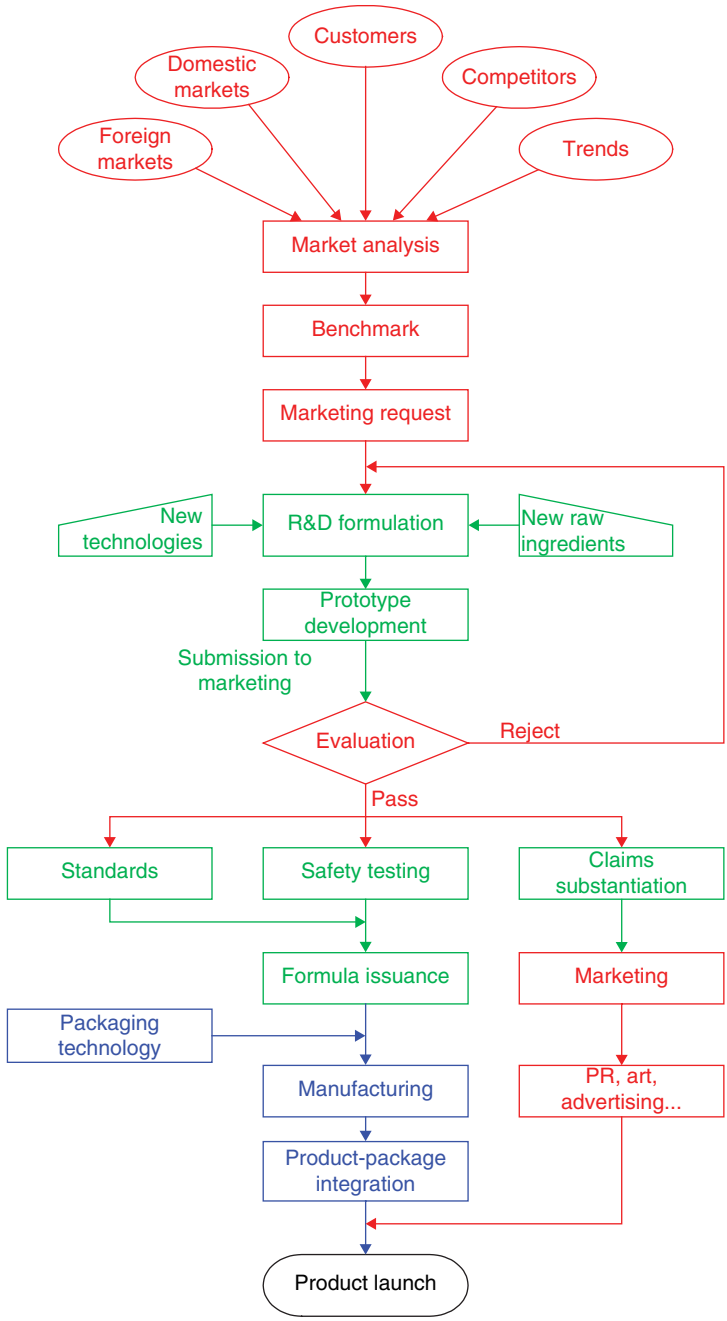
The key of success resides in the connectivity and communication between the three groups.

In this chapter's specific example, marketing department submits a request for a liquid make up development for mass market distribution. Marketing requests that the development will focus on bringing to market the following claims:

- The product should be a liquid foundation
- The makeup should exhibit anti-aging benefits
- The product should provide
- immediate firming and lifting action
- The product keeps the aesthetic attributes of a liquid foundation
- The product texture should be new(er) generation regarding the touch and feel
- The makeup should impart immediate, visible make up finish combined with long term caring benefits

12.3.3 Formulation

The type of the formulation chosen is based on marketing requests. The example below was designed to fit into the anti-age segment for women between thirty-five and forty-five years of age. Additional restriction such



Firure 12.3 Standard process development from marketing request to product commercialization.

as a low cost formula base was imposed, in order to allow incorporation of higher end beautifying agents and active ingredients that are generally higher priced. It should be noted that the base should meet the consumer's aesthetics expectations and at the same time should pass several general criteria for leave-on topical products on skin, such as the following.

- Meeting safety criteria such as non-irritating
- Meet the rheological attributes for correct pump dispensability
- Meet the aesthetic expectations of consumers

After screening a variety of optional bases and eliminating the ones that did not meet the criteria, we identified a formula which type was chosen from a family of silicone in water emulsions, known to provide an elegant feel, along with long wearing benefits. The emulsifying system was based on a standard PEG/PPG Dimethicone. This represented at that time the best approach, considering the product rheology and dispensing through a pump, meeting cost parameters. It allowed the incorporation of additional ingredients with a pleasant touch, in order to achieve a good formula aesthetics. It also allowed incorporating of state of high end ingredients and the most up to date technological anti-aging ingredients that allow us to achieve product efficacy.

An example for a formula that can serve as the base for development:

1.	Iron oxides	3.5
	TiO_2	6.0
	Talc	1.5
	Cyclopentasiloxane and cyclohexasiloxane	10.0
2.	Cethyl PEG/PPG-10/1 dimethicone	3.0
	Bis-PEG/PPG-14/14 dimethicone	1.0
	Ethylhexyl methoxicinnamte	5.0
	Hydrogenated polyisobutene	2.0
	Phenyl trimethicone	2.5
	Cyclopentasiloxane and cyclohexasiloxane	9.0
3.	Propylene glycol	3.0
	Glycerine	2.0
	Water, DI	49.0
	NaCl	1.0
4.	Preservative	q.s.
	Fragrance	q.s.

On this back bone, additional additives and actives may now be incorporated to create an aesthetic and appealing texture and improved performance.

During the development, this formula needed to undergo several adjustments to meet marketing requests for product texture, application, and wear. With these continuous changes, the product base viscosity needed to remain within the same pattern to allow a correct pump delivery. This process involved a substantial exchange of opinions between marketing and product development teams in order to come up with the desired product parameters, to keep the appropriate product coverage avoiding a masking appearance on application, to have the correct play timeon application, and finally to obtain an appealing make up result. The formula texture plays an important role, since it creates the initial impact to the customer. With continuous use, the product should also create a need for the customer to have this product and use it continuously; therefore, once the product makes the customer to desire and purchase it, the first impact of the product has been created. The secondary goal is to meet the consumer satisfaction with the continuous use of the product. In other words, the product should meet the consumer expectations in terms of performance. Parameters such as ease of application, sensorial and olfactive pleasure at application, wear expectations, and ultimately beautifying benefits should all be outstanding. If the product claims are positioned under the anti-aging umbrella, the consumer should feel like using a skin care product, and the makeup should provide the ultimate care and the improvements matching the claims listed on the pack. Once the secondary goal is achieved, it will provoke a repetitive purchase which will make the product a real success. The role of marketing and product development teams was achieved.

One of the challenges a formulator has when first starts to design a formula, is to be aware of the fact that its cost will meet the target. Depending on the cost, the formulator has a multitude of choices between expensive or moderate raw ingredients that will be added to the formula as fillers or for other specific purpose to improve skin's aesthetic appearance.

In this particular case, *talc extra*, a finely divided mineral, was chosen to be added. It controls oil absorption providing a matte look; it is also the ingredient used as additive in shade matching. The formulator had a choice of adding a blend of various spherical powders such as silica, boron nitrate, to achieve an improved skin appearance.

Methyl methacrylate crosspolymer is a hybrid powder composed of spheres bound to mica by weak electrical charges. This allows the particles to

rotate between the surface of mica and skin, providing a lubricious feel to the skin.

Silica and calcium *aluminum borosilicate* in a form of hollow glass bead. They are light weight particle, which appear nearly invisible. Light can pass and diffuse through these particles without dulling the appearance of the color. During the diffusion, some of the emitted rays can create a scattering of visible light. As a result, a soft focus effect will be created. This effect contributes to the overall luminosity on the face, and may reduce the appearance of fine lines, providing a more even tone to the skin.

As mentioned earlier in the formulation discussions, the vehicles and the polarity of active ingredients play an important role in the delivery to the skin and in overcoming the stratum corneum as a barrier; ultimately, these parameters influence the delivery of the actives and their efficacy. A make-up formulation needs to include pigments in the form of iron oxides and titanium dioxide, to impart similar color to the skin tone. However, while providing the necessary product aesthetics to the these ingredients may impart certain negative effects to the formulation base. The ability to absorb fatty substances such as oils, esters, semisolids from the formula base or from the skin surface may lead to changes in formula aesthetics and negative sensorial implication on application and wear.

There are methods to counteract the negative drying effects of a makeup formulation such as lipsticks, foundations, and mascaras. One of the methods will reduce the amount of absorbing materials used, for example, materials from the class of silicones or other materials with reduced wetting properties. For example, dimethicones are widely used in color cosmetics as vehicles for pigments to prevent drying the skin as a result of the presence of pigments. Silicones also provide good spreadability on skin, which allows the creation of a uniform and even film on skin. A more modern approach to obtain an elegant and comfortable to wear product is to reduce the pigment/base interaction via pigment coating. The coating is generally used to enhance various properties of the ingredient such as oil or water repellency, stiffness, or roughness. Coatings can impart hydrophilic or lipophilic properties and can be made of either a single or a double layer. When coated, the pigment dryness can be reduced and becomes more compatible with the skin; wear is also improved.

In the discussed formulation, an amino acid coating treatment was used. This is a unique coating, where the pigment particles are treated with a vegetable-based glutamate. This technology allows for improved skin compatibility, providing long wearing, and a moist, dewy finish. These specialized pigments are easy to disperse and perform well in silicone formulations. In addition, this exclusive treatment ensures a uniform and natural appearance on skin, providing long wear.

Powerful humectants such as propylene glycol and glycerin have been added to provide moisturizing properties,[26] cushioning and comfortable wear.

When tested, the product demonstrated an SPF 20 *in vivo*, providing protection against UV and against harmful free radicals, and therefore aging prevention properties. The addition of anti-aging and anti-stress ingredients that protects and rejuvenates the skin such as ceramide 2 and palmitoyl oligopeptide (Dermaxyl™) work in combination to smooth wrinkles and repair the cutaneous barrier. Ceramides are a special class of sphingolipids, and they are known to be an important component in the outermost layer of the skin, the stratum corneum. They play an essential role in maintaining and repairing the lipid barrier and help the water retention capacity of skin.[19] Ceramide 2 allows the skin to maintain its elasticity, maintaining its moisture, probably by maintaining the lipid barrier of stratum corneum and therefore reducing the TEWL.[27] Palmitoyl oligopeptide is a short sequenced peptide derived from elastin. This peptide stimulates fibroblasts and activates extracellular matrix turnover reducing wrinkle size and depth making skin look smoother and more youthful.

Wild thyme and lupine extracts (*Gatuline lifting*) are thought to work synergistically to impart a tightening effect to the skin while when used on a long term basis, firms and reduces the pores size of the skin. This has been demonstrated in *in-vivo* comparative sensory analysis which revealed an effect of the cutaneous relief of skin and a "tensing" effect measured through skin print implementation and observation under both confocal and scanning electron microscopes. A stabilized form of vitamin C ester (*AA-2G*) that promotes synthesis of collagen was used. It also brightens and evens out skin tone while capturing free radicals.

A uniquely engineered polysaccharide (*Fucogel 1000 BC*) was added to the formula. It imparts a silky feel to the skin, showing a high moisturizing effect. Fucogel is built up from three monosaccharides: fructose, galactose, and galacturonic acid. This gel is obtained from the bio-technical

fermentation of soy/maize. It creates a protective, hydrating film on the skin's surface.

The three main benefits of this unique film are:

1. The polysaccharide macromolecules that act as natural humectants promoting the retention of moisture in the skin.
2. Protection of the skin from penetration of substances that can cause skin irritation, due the film forming properties; it is also rich in fructose, therefore can be used in hypo-allergenic lines.
3. Its components are naturally present in the epidermis, therefore Fucogel is well compatible with the skin, and it is evenly distributed, non-greasy, leaving a pleasant feel of freshness sensation to the skin.

Vitamin liposomes ACE is a blend of vitamins A, C and E, incorporated into a liposome delivery system to improve stability and bio-availability at the sub-epidermal level. Anti-aging benefits for the skin from vitamin A, C, and E are well documented in the literature. Their combined presence at the sub-epidermal site becomes an impetus to the metabolic process which may slow down during aging. By using this complex it is claimed that skin's metabolism is re-energized thus resulting in the formation of new skin cells. The salient features of these individual vitamins are described below.

Vitamin C provides the following benefits to the skin:

1. Provides strong anti-oxidant activity.
2. Helps brighten the overall appearance of the skin, by inhibiting.
3. Prevention of damage caused by ultra violet radiation.
4. Stimulation of collagen synthesis.

Vitamin A promotes the elimination of dead skin cells which contribute to the reduction in dryness and roughness of skin resulting in the smoothing skin appearance.

Vitamin E acetate is a strong *in-vivo* anti-oxidant that protects the membranes of cells from damage caused by free radicals.

To summarize the technology approach that led to product design, compounds were incorporated for the following rationale:

- Continuous hydration from a polysaccharide, promoting *skin moisturization*.
- Powerful moisturizers to keep the *skin moist and fully replenish*.
- Antioxidant complex of natural extracts and vitamin C and E to help *protect against the stress caused by free radicals*.
- Combination of peptide and ceramides, known to help *repair epidermal structure*.
- Exclusive treated pigments make *skin look brighter*, with a younger, dewy finish.
- Unique combination of optical light diffusing powders, that visibly *smooth out imperfections*.
- Natural extracts (bearberry, wild thyme, and lupine), to *tighten and firm the skin*.
- Liposome vitamins A, C, and E known to help reduce the *signs of aging*.
- Exclusive, *in situ* B-Liftox complex, to help *restore and regenerate the skin*.

12.3.4 Product Attributes

Upon product application, this make up was shown to leave a comfortable, natural finish on skin. The skin feels soft and natural with even coverage. This foundation has long wearing properties, suitable for all skin types. The makeup demonstrates visible cosmetic benefits, improving skin appearance combined with a distressing and relaxed expression. As a result, the skin is visibly firmer, with a perfectly healthy and radiant look. Fine lines and wrinkles are visibly reduced, while skin is softly plumped and refined. This make up provides immediate visual effects, combined with impressive long term results, bringing back tonality and healthy looking appearance. The product therefore exhibits the following benefits:

- Elegant, prestigious texture.
- Combines lifting and distressing action on skin.
- Skin is softly plumped and refine.
- Fine lines and wrinkles are visibly reduced.
- Powered with antioxidants, fruit extracts and vitamins.

- Maintaining optimal moisture balance and skin comfort.
- Toning effect for a guaranteed healthy looking skin.
- Luxury, ultra hydrating and moisturizing foundation.
- Even coverage, while leaving the skin soft and natural looking.
- All day wear, suitable for all skin types.
- Comfortable wear, with skin feeling fully replenished.
- With superior results in skin elasticity and tonicity.
- Available in seven shades, light to medium coverage.
- SPF 20.
- Natural, healthy looking skin.
- Fresh dewy finish to the skin.

12.3.5 Product Testing

The foundation was clinically tested on 30 female volunteers with dry skin (corneometer below 60 RU), on the face and on one randomized side of inner forearm. One side was treated with formulation and the other side remained untreated. The study was designed to provide balanced placement of fifteen volunteers on the right and fifteen on left side. The panelists' age ranged between thirty and fifty years. The product was applied for six weeks, twice daily. Readings were taken before the test, at three weeks and six weeks. All measurements were conducted under standard conditions, in a bio climatized room and hold constant at the trial time.

Measurements were taken with various apparatus in order to validate the product attributes.

- Hydration, moisture content, humidity of skin by Corneometer CM820® (Courage & Khazaka, D).
- Contact free profilometry of skin surface, by Quantimet 970® and Quantimet 600®, together with Polyvar Microscope/ Cambridge instruments , Bensheim) for smoothness and fold count.
- Biomechanical properties by Cutometer SEM 575® (Courage & Khazaka, Colone, D) skin elasticity, firming, tension.
- Microcirculation by Laser Doppler Perriflux Instrument (Perimed KB, Sweden) by Image Scanner for Perfusion for blood flow profile/erythrocytes, and by Laser Pointer for vasomotion of blood vessels, interpretation of calming or irritating effects.

- Visual analog scale (VAS), subjective assessment on a "VAS" 0–10 cm.
- Dermal inspection by palpation and scoring.
- Consumer test done on 240 panelists.

Clinical test results demonstrated that the product exhibits the following attributes:

- Increase in skin moisturization by 52 percent after three weeks and by 67 percent after six weeks.
- 82 percent of women felt that the product made their skin firmer in as little as 12 days.
- Increase in smoothness by 50 percent.
- Decrease in fine lines ad wrinkles by 40 percent in three weeks.
- Reduction in depth of wrinkles in three weeks by 50 percent.
- Increase in firmness by 23 percent.
- Increase in elasticity by 21 percent in three weeks and by 34 percent after 6 weeks.
- Skin lifting action increased by 18 percent after 3 weeks; increased 36 percent after six weeks.
- Increase in blood flow/microcirculation by 97 percent in three weeks, and by 137 percent after six weeks.
- Calming effect—decrease in vasomotion by 21 percent.
- Dermatologist tested.
- SPF 20.

Consumer's opinion and the purchase intent are essential parameters in a consumer test study and it revealed the following results:

- 82 percent of the women felt that the product made their skin firmer in as little as 12 days
- look up to eight years younger in only 12 days
- 86 percent of the women felt that the product de-stressed their skin in as little as 12 days
- 82 percent of women felt that this foundation made them look years younger

These significant clinical and extensive consumer testing results performed with the product demonstrates its uniqueness. These results were achieved via a careful, well thought designed product development with a smart use

of active ingredients already available, in conjunction with exclusive ingredients created in collaboration with suppliers.

The technology used in formulating this color product is comparable to the top selling skin care products. This foundation is clinically proven to function as an anti-age foundation, on parity with skin care products.

12.4 A Promising Future

With over eighty-two million, healthier than ever, baby boomers having at their disposal (and showing a willingness to spend) an unprecedented amount of wealth, the potential market for anti-aging skin products are truly enormous. However, the market is also wary of unfulfilled promises. For a company to succeed its products must not only catch the eye of impulse buyers but also perform as advertised. As we demonstrated in this chapter there are reasons to believe that using current and upcoming technologies our consumers' expectations can finally be met and maybe even exceeded.

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PART 4
TESTING METHODOLOGIES

13

In Vitro Methods to Screen Materials for Anti-aging Effects

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13.1 Introduction

The skin represents the body's most observable organ system and, although its main functions include protection, thermal regulation, and sensory detection, its visible appearance also plays an important role in human social interactions. Therefore, while aging affects the entire body, the signs of aging are often first noted in the skin. These signs include fine lines and wrinkles, alterations in skin pigmentation, and a thinner appearance of the skin due to epidermal and dermal atrophy. Although these signs of aging are accompanied by a decline in skin function, it is perhaps their impact on the physical attractiveness of the skin that is the main driving force behind the multibillion dollar US market for over the counter topical anti-aging products (1).

As companies compete for their part in this ever expanding anti-aging marketplace, it becomes vital to test new and existing materials for their anti-aging effects. In light of ever increasing scrutiny by both consumers and regulatory agencies, the testing of anti-aging products is essential in order to ensure that the product will succeed in the marketplace by being safe, effective and to substantiate advertising claims. In order to design an appropriate test for a product, it is necessary to first understand the basic mechanisms associated with the aging process. Many of the effects of aging have been well characterized at both the cellular and molecular level, and traditionally have been attributed to two possible factors. The first factor is intrinsic aging, a time dependent decline in biological functions which occurs independently of environmental factors. Intrinsic aging is primarily of cellular origin where time dependent changes that occur on a molecular and cellular level can limit the lifespan of an organism (2). The second source is extrinsic aging, occurring due to the exposure of the skin to environmental factors which can lead to oxidative damage. While the primary contributor to extrinsic aging of the skin is ultraviolet radiation, other environmental pollutants such as cigarette smoke and ozone can contribute to induce premature skin aging effects (3). Some mechanisms of

aging are difficult to classify as purely intrinsic or extrinsic. For example, free radicals can be generated by UV radiation or by naturally occurring intracellular metabolic pathways and have been shown to be another contributor to cellular aging (4). While these free radicals are clearly generated within the cell and may be classified as intrinsic in nature, they are inducing aging effects in a manner that is identical to the extrinsic factors of ultraviolet radiation or ozone.

Regardless of the classification as intrinsic or extrinsic, many of the mechanisms associated with aging can be replicated *in vitro*. Human skin cells or skin tissue models can be grown in a laboratory under carefully controlled conditions such that certain aspects of aging can be observed. This does provide a means to study the mechanisms involved in the aging process, but also provides a valuable set of experimental models with which one can screen compounds for anti-aging effects. This chapter will discuss some of those aging related mechanisms and approaches that can be replicated and studied *in vitro*. A primary focus will be given to aging related mechanisms that have been shown in the literature to respond to compounds that could be used as ingredients in cosmetic or personal care products.

13.2 Senescence

13.2.1 Cellular Senescence

Research into the mechanisms responsible for aging has progressed dramatically. Near the turn of the last century, Weismann (5) had theorized that an organism's lifespan was limited by a finite number of somatic cell divisions. Weismann speculated that cells could only go through a finite number of cell divisions and as tissues were damaged over the lifetime of an organism, the tissues would reach a point where cell renewal would cease resulting in the eventual death of the organism. Although at the time Weismann's work was widely criticized as inaccurate, by the mid 1900s work by Swim and Parker (6) and Swim alone (7), had shown that the observation that cells grown in culture could only undergo a limited number of cell divisions was a common observation in many experiments. Both scientists concluded that the limited number of cell divisions did not occur to artifacts in culturing technique but due to a true aging effect. Additional work by Hayflick *et al*. (8) further concluded that the limited number of cell divisions observed in cultured cells, approximately fifty population doublings for fibroblasts, was a characteristic of all normal diploid cells. Collectively, this work introduced the serial cultivation of

diploid cells until the cells are no longer capable of cell division, a state referred to as cellular senescence, as one of the first and simplest *in vitro* models for studying mammalian aging.

13.2.2 Replicative Senescence

Cellular senescence is characterized primarily by growth arrest with serial culturing. As cells are grown in culture over time, the percentage of replicating cells within the population gradually decreases while the percentage of cells in growth arrest increases by a corresponding amount. However, while the cells are replicatively inactive they are still metabolically active and can live in this senescent state for at least one year in culture (9). Since the cells are mainly showing a lack of replicative ability, this model of aging is referred to as replicative senescence. Replicatively, senescent keratinocytes and fibroblasts have been observed to accumulate in the skin with aging Dimri *et al.* (10), and it has been theorized that a very small population of these senescent cells could dramatically impact the structure and function of the skin (11).

One of the main experimental measurements in replicative senescence is to quantify the number of times the cultured cells go through the process of cell division. This is accomplished by counting the number of times the population of cultured cells doubles in number. Every time the number of cells doubles, it is referred to as a population doubling. The number of population doublings can be determined by using the following equation:

$$\text{Log}_2(\text{final cell count/initial cell count})$$

In this equation, the initial cell count is the number of cells used to establish the culture while the final cell count is the number of cells at the time of subculturing. The number of population doublings can then be plotted against the time in culture to generate a growth curve. While the number of population doublings through which different types of cells can progress depends upon many factors and is highly variable (12), it is possible to use changes in the number of population doublings within a single given cell type to screen compounds for anti-aging effects. Using this measurement, Wang *et al.* (13) demonstrated that human fibroblasts cultured with various herbal extracts were able to undergo 15–20 more population doublings than control treated fibroblasts. Similarly, Atamna *et al.* (14) showed that when human fibroblasts were cultured with *N*-t-butyl hydroxylamine, which is a hydroxyl radical scavenger (anti-oxidant), they were able to

undergo 17–20 more population doublings in culture than untreated fibroblasts. Thus, it is possible to use changes in the number of population doublings until replicative senescence is reached as a method to screen materials for anti-aging effects.

The mechanism behind replicative senescence was first attributed to a loss of telomeres (15,16). Telomeres are repeating DNA sequences (TTAGGG) at each end of linear chromosomes which function to assist DNA polymerase in replicating the 5' end of the lagging strand during DNA synthesis. They also function to cap the end of the chromosome such that it does not appear to be a double strand break. In normal human cells, these telomere repeats can extend for 10,000–20,000 nucleotides at both ends of the linear chromosome. Due to the chemistry associated with DNA replication, a cell can lose 50–100 nucleotides from each of its telomeres with each round of DNA replication and for many cell types once the telomere repeats are shortened to a critical length, the cells will no longer replicate (17). In certain cell types telomere length can be restored after each cell division through the activity of telomerase, an enzyme which extends telomere repeats on each end of the chromosome. In the skin, dermal fibroblasts have been observed to lack appreciable telomerase activity (18), while keratinocytes in the basal layer of the epidermis have been observed to have a minimal level of telomerase activity (19). With serial culturing, both epidermal keratinocytes and dermal fibroblasts demonstrate a progressive decrease in telomere length, and this decrease has been associated with a loss of replicative ability (18,20). The decrease in telomere length observed with serial culturing *in vitro* mirrors observations made on declining telomere length in both cell types with chronological aging of human skin *in vivo* (21).

Telomere length can be measured using a technique called Southern Blotting (22). Genomic DNA is first isolated from either the cells or skin tissues of interest. The genomic DNA is then digested using restriction enzymes that recognize common sequences found in most of the DNA, but not found in the region of the telomere repeats. This results in the breakdown of most of the genomic DNA, leaving only terminal restriction fragments containing intact telomere repeats. These fragments can then be denatured such that they are single strands rather than double stranded, and resolved using gel electrophoresis which will separate the terminal restriction fragments based on the length of the telomere repeat. The fragments are then transferred from the gel and adsorbed onto the surface of a nylon membrane. This process allows the fragments to be easily probed,

in this case with a DNA strand that is complimentary to the telomere repeats. Typically, the complimentary DNA probes are either radiolabeled for autoradiography or labeled to facilitate their detection via chemiluminescence. For autoradiography, the membrane is placed next to a piece of x-ray film for a period of time. The radiation emitted by the radioisotopes in the DNA probes will be detected by the film, and once the film is developed the telomere restriction fragments can be visualized and compared to a set of standards to determine their length. The process for chemiluminescence is similar to autoradiography, except that the DNA probe is labeled using biotin. The biotinylated DNA probe is then incubated with avidin, a glycoprotein with a strong affinity for biotin, which has been linked to a detection enzyme such as horseradish peroxidase. When a suitable substrate is added, the horseradish peroxidase enzyme will generate a light, or chemiluminescent, signal. This signal can also be detected using film. Once the film is developed and the length of the telomere fragments can be visualized and compared to a set of DNA standards of known length.

Telomerase activity can be measured using variations of the telomere repeat amplification protocol (TRAP) (23). For the basic TRAP assay, cell lysates or tissue homogenates are used as the source of telomerase. Samples of these materials are combined with a synthetic oligonucleotide substrate. Telomerase will add telomere repeats to this substrate, increasing its length by multiples of six nucleotides (one telomere repeat). These lengthened oligonucleotides can then be amplified using polymerase chain reaction (PCR) based methods, and the amount of each amplified oligonucelotide will be proportional to its original amount. Once the PCR products are amplified, they can be resolved using gel electrophoresis. The lengthened oligonucleotides can be visualized by staining with ethidium bromide, and the procedure should generate a regular, ladder like pattern of oligonucleotide fragments in each sample lane, where the staining intensity of the fragments in each column is proportional to the initial telomerase activity in the sample.

Using these measurement techniques, Min *et al.* (24) have shown that when epidermal keratinocytes are serially cultured in the presence of retinoic acid, telomerase activity can be increased and telomere length can be maintained through a greater number of population doublings resulting in a highly significant delay in replicative senescence. In addition, work by Yokoo *et al.* (25) demonstrated that when keratinocytes are serially cultured in the presence of vitamin C derivatives or very low levels of hydrogen peroxide (as a mild oxidative stress), they could maintain telomere

length through a greater number of population doublings and delay replicative senescence without any increase in telomerase activity. These studies demonstrate that measurements of telomere length or telomerase activity can be used to screen compounds for anti-aging effects.

While telomere shorting is one factor in replicative senescence, additional contributing factors have also emerged including an increase in the expression and/or activity of the following key aging related proteins: p53, p21, and $p16^{ink4a}$ (26–28). The names for p53, p21 and $p16^{ink4a}$ are derived from the fact that they are proteins (p) and their approximate molecular weights (53, 21, and 16 kD, respectively). When active, p53 functions as a transcription factor and as it binds to DNA it can induce the expression of several aging related genes, including the cyclin dependent kinase (CDK) inhibitor p21 (29). CDKs control the progression through the cell cycle and their inhibition prevents the cell cycle from making the transition from the G1 phase to the S phase. This blockage of the cell cycle prevents cell division leaving the cell in a growth arrested (senescent) state. In cultured cells, p53 activity progressively increase with serial culturing, accompanied by an increase in p21 expression, and these increases have been observed to correspond with a decline in the number of replicating cells (30). Enhanced p53 activity and p21 expression can be stimulated by a number of factors, including DNA damage in the form of double strand breaks, oxidative DNA damage or by critically short telomeres (31,2).

$p16^{ink4a}$ is another CDK inhibitor whose expression increases with aging (32). In cultured cells, p21 expression has been observed to precede $p16^{ink4a}$ expression; however while $p16^{ink4a}$ expression continues to increase until the cell population reaches total growth arrest, p21 expression will begin to decline prior to the culture reaching total growth arrest (95). This has led some to suggest that the initial increase in p53 activity and p21 expression are required to start cells on the path to senescence, after which it is $p16^{ink4a}$ that is required to maintain cells in the senescent state (34). Recently, it has been established that $p16^{ink4a}$ expression increases in both the epidermal and dermal layers of human skin with chronological aging *in vivo* (35), suggesting that this CDK inhibitor may be a good marker of the aging process.

The use of p53, p21, and $p16^{ink4a}$ expression to screen compounds for anti-aging effects offers a convenient alternative to serial culturing until growth arrest. With serial culturing it can take months to reach a point at which all

of the cells within the population are in replicative senescence. Long term cultures are very labor intensive to maintain, and also have a continual risk of becoming contaminated with microbial infection leading to the loss of the culture before the aging process is complete. However, since p53 activity and p21 and $p16^{ink4a}$ expression gradually increase over the duration of the culture process, it may not be necessary to wait for complete growth arrest to determine if a material is having an anti-aging effect.

Changes in p53, p21, and $p16^{ink4a}$ expression can be measured using standard Western blotting or enzyme linked immunosorbent assay (ELISA) based methods. With Western blotting, protein samples are first separated by molecular weight using gel electrophoresis and then blotted onto a membrane, which acts as a solid support. For ELISA based applications, protein samples are normally added to microtiter plates whose wells have been coated with a capture antibody specific to the protein of interest. The wells are then washed to remove any unbound protein leaving the target protein bound to the solid support of the microtiter plate. For both Western blotting methods and ELISA based methods, after the samples have been bound to the solid support they are probed using primary antibodies that recognize the protein of interest. The primary antibodies are then detected using a secondary antibody that is coupled to an enzyme that either generates a chemiluminescent or colorimetric signal in proportion to the amount of the target protein. With Western blotting, the chemiluminescent signal is detected using film, and quantified using densitometric analysis. For ELISA based methods, chemiluminescent signals can be measured using a luminometer, while colorimetric signals can be measured spectrophotometrically.

Using protein based measurements, Kang *et al*. (36) found that it is possible to delay the aging associated increase in p53 and p21 expression. They observed that when fibroblasts are serially cultured in the presence of nicotinamide (vitamin B3), there was a decrease in the rate of p53 and p21 induction with aging when compared to untreated cells. In addition, when the fibroblasts were treated with nicotinamide, they were able to undergo more population doublings than the untreated cells.

13.2.3 Stress-Induced Premature Senescence

In addition to the serial culturing of cells, replicative senescence can also be induced by exposing cells to non-lethal oxidative insults in the form of

hydrogen peroxide or ultraviolet radiation (37,38). When senescence is induced in this manner it is referred to as stress-induced premature senescence (SIPS) (39). Similar to replicative senescence, SIPS results in growth arrest and can produce senescent-like changes in cell morphology. In addition, both SIPS and replicative senescence are associated with an induction of p53, p21, and $p16^{ink4a}$ (40).

It is also important to note that SIPS and replicative senescence are both associated with the induction of senescence associated-β-galactosidase (SA-β-gal) activity. β-galactosidase is an enzyme that removes β-linked terminal galactosyl residues from substrates such as glycoproteins and glycosaminoglycans. This enzyme functions optimally at a pH of 4, and is normally found within the acidic environment of the lysosome. However, in contrast to lysosomal galactosidase, SA-β-gal is optimally active at pH 6. SA-β-gal has become a useful marker for senescence since its expression increases as cells are taken through serial culture passages until they reach replicative senescence (10), or in response to SIPS (31,41). Aging associated increases in SA-β-gal have also been observed in the skin *in vivo* (10), further supporting its use as an *in vitro* marker for aging. SA-β-gal activity can be detected both in cell culture and in histology sections from *in vivo* skin tissue through the use of 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-gal). In cultured cells or tissue sections that have been briefly fixed and then maintained at a pH of 6, cells containing SA-β-gal activity will convert the X-gal into an insoluble blue-green chromagen. After staining, the percentage of the cells staining positive can then be determined.

Due to the similarity between SIPS and replicative senescence, SIPS has become a useful model for studying the role of oxidative damage in the aging process, and to also screen materials for anti-aging effects. Satoh *et al.* (42) used the SIPS model to investigate the anti-aging effects of an extract of Kangen-Karyu, a traditional Chinese medicine composed of extracts derived from the following plant sources: peony root, cnidium rhizome, saf flower, cyperus rhizome, saussurea root, and Salvia miltiorrhiza root. When cultured human fibroblasts were treated with this mixture, a subsequent exposure to a concentration of hydrogen peroxide normally capable of inducing SIPS was unable to induce changes in the cell morphology, induce SA-β-gal expression, or induce growth arrest. Additionally, Ho *et al.* (43), used a SIPS model to support the anti-aging effects of aucubin, an iridoid glycoside derived from the leaves of the plant *aucuba japonica*, by demonstrating that pretreatment with the compound could prevent

UVB induced SA-β-gal expression in fibroblasts. As a screening method, SIPS based experiments offer an alternative to aging based experimental methods using replicative senescence. Again, while replicative senescence can take several months to achieve, SIPS can be induced within a few days. In fact, a screening approach using reduced p21 and p16 expression, along with reduced SA-β-gal expression in response to SIPS was used by Sklavounou *et al.* (44) to identify antioxidants for clinical applications.

13.2.4 Genetic Diseases Associated with Early Senescence

Certain genetic diseases have been associated with accelerated aging. Collectively, these diseases as a group have been referred to as Progeria, based on the Greek word for accelerated aging. While this group of diseases includes Werner's syndrome, Cockayne's syndrome, Bloom's syndrome and xeroderma pigmentosum, the term Progeria is more specifically intended to refer to Hutchinson-Gilford Progeria syndrome. Cells obtained from these individuals and grown in culture have been valuable in studying the aging process. For example, Hutchinson-Gilford Progeria has been found to be associated with a mutation in the LMNA gene which encodes the lamin A protein (45), while mutations in WRN and BLM, genes, which encode DNA helicases, have been found to be a contributing factor in Werner's and Bloom's syndromes, respectively (46). Both Cockayne's syndrome and xeroderma pigmentosum have been found to be associated with defects in various DNA repair mechanisms (47).

While the specific defects associated with accelerated aging diseases can clearly induce premature aging, their contribution to the normal aging process leading to cellular senescence is not yet well defined. Interestingly, when dermal fibroblasts obtained from individuals with Werner's syndrome are compared with dermal fibroblasts from aged individuals using a DNA microarray containing over 6000 human genes, they were found to have an expression pattern that was 91 percent similar (48). However, even with this much similarity in gene expression, due to the residual differences in gene expression it is possible that compounds which are effective in preventing early senescence in cells obtained from individuals with accelerated aging diseases may not have the same efficacy in preventing senescence in cells obtained from normal individuals. Therefore, while these accelerated aging diseases provide insightful models of the aging process, the use of cells or tissues obtained from these individuals may not be the best models to substantiate anti-aging claims.

13.3 Functional Changes with Aging

While cellular senescence can represent one of the final outcomes for aging cells, there are other functional changes that can progressively occur in the skin over our lifetime. In general, many of the *in vivo* cellular characteristics observed in the skin of elderly individuals are maintained when their cells are obtained for culture. With respect to the dermis, fibroblasts cultured from elderly individuals obtained from areas that were not exposed to the sun (chronologically aged) display a reduced rate of type I and type III collagen production (49), reduced elastin synthesis (50), reduced hyaluronic acid production (51) and elevated matrix metalloproteinase-1 (MMP-1) production (52). Epidermal cells also display functional changes with aging. These changes in keratinocytes cell function include a reduced rate of proliferation, which is associated with an increased rate of apoptosis (53), and an increased release of inflammatory mediators (54). In addition, with aging there is a general trend towards overall skin hypopigmentation in light skinned individuals due to a loss of active melanocytes, with an accompanying increase in areas of focal hyperpigmentation (age spots) (55). Many of these functional changes that occur with chronological aging also occur in response to exposure to ultraviolet radiation or other environmental factors such as ozone or tobacco smoke (56). When exposed to UV light, dermal fibroblasts increase the production of MMP-1 and elastase (57). UV exposure also reduces the production of type I collagen (58).

In vitro, aging related changes in skin cell function can be measured using either cells cultured in a monolayer, or skin tissue equivalent models. The skin tissue equivalent models can be generated in a variety of forms. The simplest equivalents use just keratinocytes which have been grown in cell culture inserts and develop a three dimensional architecture, complete with a basal, spinous, granular, and a cornified layer complete with corneocytes and a stratum corneum. This basic model can be modified to include melanocytes in the epidermal layer for pigmentation studies, or with an underlying dermal layer composed of fibroblasts seeded into a collagen gel, or a gel composed of a mixture of extracellular matrix (ECM) proteins. With these models, the stratum corneum of the epidermal layer is exposed to air, while the lower portion of the skin tissue is in contact with cell culture media. The air exposed stratum corneum of the skin tissue equivalent allows for the topical application of materials such that raw materials or fully finished formulations can be tested. Since the lower portion of the tissues is in contact with the culture media, any mediators released by the tissues during the course of an experiment, such as cytokines, ECM components,

nitric oxide, or matrix metalloproteinases (MMPs) can accumulate in the media and be measured. Both the culture of cells in a monolayer and the skin tissue equivalents have been useful models for studying aging related changes in skin function.

13.3.1 Changes in ECM Peptide Production

Fibroblasts are the main source of the ECM proteins, which includes type I collagen and elastin. A mature type I collagen fibril is composed of two $\alpha 1$ subunits and one $\alpha 2$ subunit. The collagen subunits are synthesized within the fibroblast as propeptides, and after the subunits come together a short sequence of amino acids are cleaved off of both the carboxy terminal and amino terminal ends of the propeptides to form a mature collagen fibril. After the ends are cleaved off, both the mature collagen fibril and the terminal amino acid fragments are released into the extracellular environment. In cultured fibroblasts or in tissue equivalent models with a dermal layer, both the mature collagen fibrils and the carboxy and amino terminal fragments of the collagen propeptides accumulate in the cell culture media and can be measured using ELISA based methods, or by directly blotting media samples onto nitrocellulose or polyvinylidene difluoride membranes and using traditional immunoblotting detection methods. Alternatively, changes in collagen production can also be measured at the level of transcription through the use of Northern Analysis or DNA microarrays to measure changes in mRNA expression.

Following changes in mRNA expression, Lee *et al.* (59) demonstrated that including a ginseng root extract in cell culture, an increase in collagen release in cultured fibroblasts was observed, and suggested that the same extract could have anti-wrinkle effects when applied topically *in vivo*. Additional work by Sudel *et al.* (51) demonstrated that soy extracts demonstrated increase in collagen production in cultured fibroblasts, and were also effective when used *in vivo* to rejuvenate the structure of mature human skin. Both of these studies illustrate the use of increasing baseline collagen production in cultured fibroblasts as a means to demonstrate anti-aging effects. Using a slightly different fibroblast model, work by Yan *et al.* (60) demonstrated that treatment with 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol), a membrane permeable radical scavenger, was able to prevent the decline in type I collagen production that occurs after UV exposure, and concluded that this compound would be an effective anti-photoaging compound.

Collagen production depends in part on heat shock protein 47 (HSP47). HSP47 is a chaperone protein which is localized in the rough endoplasmic reticulum of dermal fibroblasts and functions to specifically assist the folding of procollagen into its proper three-dimensional conformation (61). HSP47 expression has been shown to decline with chronological aging *in vivo*, and also with replicative senescence and SIPS *in vitro* (62). Interestingly, this aging related or SIPS induced decline in HSP47 could be reversed when cultured fibroblasts were treated with a polyphenol rich extract of the Golden Willow plant (Salix Alba) (62).

Elastin is the main component of a network of extracellular elastic fibers that gives the skin the ability to recoil after a transient stretch. Elastin is released by fibroblasts in a soluble form into the extracellular space where it is then cross-linked to other elastin proteins to form an extensive network of insoluble fibers and sheets. Again, with cultured fibroblasts or in tissue equivalent models with a dermal layer, changes in elastin production can be determined by measuring soluble elastin in the cell culture medium or by measuring changes in elastin mRNA.

Using cultured fibroblasts, Cenizo *et al.* (63) screened a library of commonly used cosmetic active ingredients and observed that one of the ingredients, a dill extract, was able to stimulate elastin production. Within the same study, it was also observed that when the dill extract was topically applied to skin tissue equivalents composed of either an epidermal and dermal layer or just a dermal layer alone, the extract could stimulate an increase in elastin production within these models as well. In this study, the increase in elastin production occurred without an increase in mRNA production, suggesting that the reason for the increase in elastin synthesis was due to post translational mechanisms or due to reduced elastin degradation. This observation serves as a reminder that functional changes may occur without a corresponding change in gene expression. This is an important fact to keep in mind, especially when using high throughput methods such as DNA microarrays that rely on changes in gene expression as their measurement to screen compounds for anti-aging effects.

Other ECM proteins that have been shown to be altered in aged skin may also be used as markers for screening anti-aging compounds utilizing approaches similar to the ones described for collagen and elastin. These markers include fibulin-5 (64), laminin (65), and fibrillin-1 (66).

13.3.2 Changes in MMP-1 Production

MMP-1 is a zinc and calcium dependent endopeptidase that is produced and released from both dermal fibroblasts and keratinocytes and functions to break down collagens located in the ECM. MMP-1 is synthesized and released in an inactive proenyzme form with an approximate molecular weight of 55 kD. *In vivo*, MMP-1 is activated via proteolytic cleavage, resulting in the release of the active form of MMP-1 with an approximate molecular weight of 24 kD. In cultured fibroblasts or in skin tissue equivalent models, both active and inactive MMP-1 accumulate in the culture media. Traditional assays for MMP-1 have used zymographic techniques (67), which are based on the standard sodium dodecyl sulfate-polyacrylimide gel electrophoresis (SDS-PAGE) technique. For zymography, the samples to be screened for MMP-1 activity are prepared in a standard SDS-PAGE buffer; however, in contrast to the usual SDS-PAGE procedure, the samples are not denatured by boiling, nor are any reducing agents added to the buffer (2-mercaptoethanol or dithiothreitol are normally added to reduce disulfide bonds in proteins). A special acrylimide gel which includes a MMP-1 substrate that copolymerizes within the gel, called a zymogram, is prepared. The samples are then loaded into the gel and resolved using standard electrophoresis techniques. After the electrophoresis, the gel is incubated in unbuffered Triton X-100, to remove the SDS, which will interfere with the MMP-1 activity, and then incubated in a second buffer which will optimize MMP-1 activity. Although the MMP-1 is for all intensive purposes immobilized in the gel it is still active and will digest any of the substrate in the local area. After a period of time the zymogram is then stained with a dye, such as Coomassie Blue, which will strongly stain the entire gel except for the areas where the substrate has been digested. In the areas where the substrate has been digested, the staining will be lighter in color, such that the staining intensity of the gel will be inversely proportional to the amount of MMP-1 activity. The staining intensity can be measured using densitometric analysis to provide a semi-quantitative measurement of MMP-1 activity in each sample.

While zymographic techniques can provide a good index of MMP activity, they are labor intensive and can only evaluate a small number of samples in a single run. However new fluorescence-based ELISA methods are available which have the advantage of being more applicable to high-throughput screening (68). Briefly, for the fluorescent ELISA method the wells of a microtiter plate are coated with antibodies specific for MMP-1. Cell culture media samples containing MMP-1 (both active and/or inactive

forms of the enzyme) are then added to the wells, and after a period of incubation the wells are washed leaving only the bound MMP-1. If desired, inactive MMP-1 can be activated at this point by adding p-aminophenylmercuric acetate to the sample, which will cleave the inactive MMP-1 into the active form. To quantify the active MMP-1 a fluorogenic substrate linked to a quencher molecule is then added and the active MMP-1 present will cleave the peptide linkage between the fluorophore and the quencher molecule. This cleavage will eliminate the quencher molecules ability to inhibit the fluorescent signal of the fluorophore allowing a fluorescent signal that will be proportional to the amount of active MMP-1 present. Since the fluorescence based methods to measure MMP-1 activity is better suited to high-throughput analysis, it is easier to include a set of MMP-1 standards of known levels of activity. Using these standards it is possible to calculate the actual quantity of active MMP-1 in unknown samples. It should be noted that in both zymographic techniques and fluorescence-based ELISA methods, if the MMP-1 is in an inactive form it will not be detected.

Measurements of MMP-1 activity have been used most often to show anti-photoaging effects of compounds by either preventing or reversing changes induced by UV light, and several types of compounds have been shown to be effective. Lee *et al.* (69) demonstrated that the topical application of epigallocatechin-3-gallate or retinoic acid to a full thickness skin tissue model prior to UVA irradiation could prevent an increase in MMP-1 expression and activity. Moon *et al.* (70) observed that treatment with 2′,4′,7-trihydroxyisoflavone also prevented UV induced increases in MMP-1 activity in cultured fibroblasts. Modulation of MMP-1 activity in cultured fibroblasts in response to UVA to promote anti-photoaging effects has also been demonstrated using plant extracts (71,72).

13.3.3 Changes in Pigmentation

Aging is often associated with alterations in skin pigmentation, with one of the most noticeable alterations being age spots, or areas of focal hyperpigmentation (55). Skin pigmentation is controlled through an interaction between melanocytes and keratinocytes in the epidermal layer of the skin. For skin pigmentation, melanin is produced through the process of melanogenesis within specialized organelles in the melanocytes called melanosomes. Melanosomes are similar in structure to lysosomes, and contain the enzymes and other components that are necessary for the synthesis

of melanin. Melanin can be made from L-tyrosine, with phenylalanine that has been enzymatically changed into L-tyrosine via phenylalanine hydroxylase (73) or from derivatives of other amino acids such as tryptophan. Melanin synthesis starts with the conversion of L-tyrosine to dihydroxyphenylalanine (DOPA), and the subsequent conversion of DOPA to DOPA quinone. Both of these reactions are catalyzed by the enzyme tyrosinase (also called tyrosine oxidase and DOPA oxidase), and tyrosinase activity is considered to be one of the rate limiting steps in melanin production. Therefore, materials which can inhibit tyrosinase activity can be useful in preventing the aging associated accumulation of focal hyperpigmentation areas on the skin.

Tyrosinase inhibition assays can take many forms. One of the simplest *in vitro* methods involves the use of purified tyrosinase enzyme derived from mushrooms such as Agaricus Bisporus, or of tyrosinase derived from lysates of human melanocytes grown in culture. When the purified tyrosinase enzyme or the melanocyte lysate is mixed with DOPA it will result in the formation of DOPAquinone, which can be measured spectrophotometrically. Since the rate of DOPAquinone formation is proportional to the amount of tyrosinase activity, if tyrosinase inhibitors are added to the reaction then there will be a decrease in the amount of DOPAquinone formed over time, resulting in a spectrophotometric reading that is lower than non-inhibited tyrosinase samples (control). Although tyrosinase inhibition models are very simple, they can be quite effective. Using the mushroom based tyrosinase *in vitro* model, Boissy *et al.* (74) were able to show that deoxyarbutin was a potent inhibitor of tyrosinase, and were then able to follow up with *in vivo* clinical studies further showing the efficacy of this compound as a skin brightening agent.

In addition to tyrosinase inhibition assays, compounds can be screened to determine if they have an effect on skin pigmentation using cultured melanocytes, co-cultures of melanocytes and keratinocytes, or using skin tissue equivalents containing melanocytes as the models. With cell culture based models, compounds are added directly to the media for a pre-determined period of time, after which the cells are collected, lysed, and the melanin is extracted using solvents. The extracted melanin can then be measured spectrophotometrically. Skin tissue equivalent models containing melanocytes can be used in a similar manner to the culture models. However, with tissue models test materials can be applied topically, rather than having to be mixed into the water based culture media. Therefore, using this method one can facilitate the testing of lipophilic compounds. Typically, treatment

of the tissue models is conducted over a period of 7 to 21 days to allow for an appreciable amount of melanin to accumulate within the tissue such that the effectiveness of the various test compounds can be better determined. After the treatment period, the tissues are typically homogenized and further broken down with detergents and proteases to release the melanin. The melanin is then extracted with the use of organic solvents and quantified spectrophotometrically.

Both cell culture and skin tissue equivalent models have been used effectively to screen compounds for skin brightening effects. For example, Zhong *et al*. (75) used both melanocyte culture and melanocyte-keratinocyte co-culture to investigate the de-pigmenting effects of various Chinese herbs. In addition, work by Yoon *et al.* (76) demonstrates the use of skin tissue equivalents to screen skin brightening agents. It should be noted, however, that while these models can be used effectively for screening purposes, the measurement of melanin alone with these models may not be adequate to confirm that these materials are effective skin brightening agents. Since exposure of the cell cultures or skin tissue equivalents to cytotoxic materials can also result in a decrease in melanin production, in this case due to cell death and not due to a specific effect on the melanogenic pathway, it is advisable to obtain an index of cell or skin tissue viability in addition to the measurement of melanin content. With both models, cell viability can be qualitatively determined via microscopic examination. Healthy melanocytes display a normal, dendritic morphology, while melanocytes exposed to a cytotoxic material will have a rounded up and non-dendritic morphology. Quantitative measurements of cell culture or skin tissue viability can be achieved using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. With the assay, water soluble MTT is added to the culture media and can be taken up into living cells. Once inside the cell it is converted by mitochondrial enzymes into an insoluble formazan with a strong purple color. This conversion of MTT to formazan only occurs in viable cells, and so the intensity of purple color formation will be proportional to the number of living cells. The insoluble formazan can be extracted using dimethyl sulfoxide or isoproply alcohol and quantified spectrophotometrically.

13.4 Aging Related Intracellular Changes

In response to elements within the cells environment, or even within the cell itself, damage occurs to all of the cells components. Most commonly

this damage is induced by reactive oxygen species (ROS), and depending upon the severity of the insult, damaged intracellular components can be repaired. One of the most sensitive intracellular targets to ROS damage is DNA, both genomic and mitochondrial. There are numerous repair pathways that are dedicated to maintaining the integrity of the information encoded within the cellular DNA. Yet, despite these repair mechanisms, aging is associated with a gradual accumulation of DNA damage and once the damage reaches a critical level the cell responds by either entering into senescence or, if the damage is severe enough, beginning the process of apoptosis. Therefore, compounds which can either prevent or reduce the rate of intracellular damage, or aid in the cellular repair process may exert an anti-aging effect by promoting cell survival and delaying cellular senescence. This next section will discuss this issue in greater detail.

13.4.1 Aging Related Changes in Cellular DNA

In addition to the aging related decline in telomere length, aging is also associated with an increase in the amount of oxidative DNA damage. One of the most common forms of oxidative DNA damage is the hydroxyl radical mediated conversion of guanine residues into 8-oxo-7,8-dihydro-2′deoxyguanine (8-oxo-dG) (77). If not repaired this DNA damage can result in a change in the nucleic acid sequence, specifically a G/C to A/T mutation, when the DNA is replicated. The amount of 8-oxo-dG present within the cellular DNA has been observed to increase both *in vivo* with human aging (78) and *in vitro* either with cells cultured to replicative senescence (37) or with cultured cells subjected to treatments known to induce SIPS (79).

8-oxo-dG can be measured in genomic DNA using blotting based techniques. For this method, genomic DNA recovered from cultured cells or skin tissue equivalents is boiled to separate the two complimentary strands of DNA into single strands, and the samples are blotted onto a nylon membrane. The DNA is then cross-linked to the membrane by baking, which ensures that the DNA is immobilized and will not come off of the membrane during subsequent processing. The membrane is then probed using an antibody which recognizes 8-oxo-dG. Since the DNA is now single stranded, the 8-oxo-dG residues will be exposed and able to interact with the antibody, rather than hidden within the center of the DNA double helix. The antibody bound to the DNA can in turn be recognized and bound by a second antibody which is coupled to a light-generating chemiluminescent

system and can be detected in a manner similar to the method described for measuring telomere length.

8-oxo-dG can also be measured using competitive ELISA based measurements. For the ELISA measurement, aliquots containing genomic DNA are typically digested with a nuclease to form free nucleotides. These free nucleotide samples are added to wells in a 96-well plate that are already coated with 8-oxo-dG, and an antibody which recognizes 8-oxo-dG is then added. There is a competition between any 8-oxo-dG in the free nucleotide sample and the 8-oxo-dG coating the well with respect to binding the antibody. After a period of time, the wells are washed and a second antibody coupled to a detection system is added to the well. In this case, the detection system is again most likely a peroxidase enzyme, only this time the substrate used will generate a colored end product that can be measured spectrophotometrically using a plate reader. With this assay system, the color formation is inversely proportional to the amount of 8-oxo-dG in the sample. If there is a large amount of 8-oxo-dG present in the sample, more of the antibody will bind to it rather than the 8-oxo-dG bound to the wells, resulting in less of the secondary antibody/detection system binding an hence less color formation. The ELISA based methods can provide relatively quantitative data since a set of known 8-oxo-dG standards can be included.

Since oxidative DNA damage has been shown to increase with aging, treatments which reduce the formation of oxidative DNA damage may have anti-aging effects. Using 8-oxo-dG as a marker for oxidative DNA damage, Pelle *et al*. (80) demonstrated that when normal human epidermal keratinocytes are pretreated with the hydroxyl radical scavenger mannitol, the amount of oxidative DNA damage induced by UVB was significantly decreased. In addition, in cultured cells the formation of 8-oxo-dG in response to other forms of oxidative stressors such as lipid peroxides or hydrogen peroxide has been shown to be preventable by pretreatment with either esculin, a coumarin glycoside (81), or casuarinin, an extract from the bark of Terminalia Arjuna (82).

Genomic rearrangement is another type of DNA damage that has been shown to increase with chronological aging and has also been proposed as a causative factor for the aging process (83). Genomic rearrangement primarily occurs when DNA double strand breaks are improperly repaired via non-homologous end joining (NHEJ) methods. With NHEJ methods, the two broken ends of the DNA fragment are reattached to each other in

a random manner which can result in an inversion of the DNA sequence if the broken section is attached backward, or a translocation/deletion mutation if the broken DNA fragment is reattached to the wrong chromosome (84). Thus if the strand break occurs within a gene sequence and it is incorrectly repaired then the interrupted gene can no longer be expressed. Depending upon the function of the interrupted gene the loss of the expression could have many possible end results, ranging from no effect to the cell, a fatal effect to the cell, or it could result in the transformation of the cell to a cancerous phenotype. In human skin *in vivo* and cultured cells *in vitro*, double strand breaks can be induced by exposure to UV radiation (85,86).

In vitro, DNA strand breaks can be detected through the use of the "Comet" assay, also known as single-cell gel electrophoresis. With this method, cells are grown in culture and exposed to a treatment that induces DNA strand breaks, such as UV irradiation. The cells are then collected and embedded in a thin layer of agarose, typically on the surface of a microscope slide. The cells are then broken open through the use of detergents and salts, leaving the DNA immobilized in the agarose gel. The DNA is denatured for a period of time in an alkaline solution, and subjected to electrophoresis. Since DNA has a net negative charge to it, it will move in response to an electrical field. Yet the large, intact nuclear DNA strands will have limited mobility in the agarose gel. However, DNA containing strand breaks, and hence cut into smaller strand lengths, can migrate more freely through the gel. After a period of time the electrophoresis is stopped and the gel is stained to visualize the DNA. When stained and examined microscopically the differences between intact DNA and DNA with strand breaks can be recognized by their morphology. DNA which does not contain any strand breaks will have a round, globular appearance that has roughly the same shape and appearance of the cell nucleus. In contrast, DNA that has double strand breaks will have the appearance of a comet. The main nuclear DNA will look like the comet itself, while the DNA that migrated during the electrophoresis due to presence of strand breaks will make up the tail of the comet. For quantitative purposes, the staining intensity of the comet and tail can be measured, as can their respective lengths, to provide an index of DNA strand breaks.

Since the ability of skin cells to efficiently repair double strand breaks decreases as the cells become senescent (87), compounds that can prevent double strand breaks can help prevent genomic rearrangements and thus have anti-aging effects. Using the comet assay to measure DNA strand breaks, Lee *et al.* (88) observed that when human keratinocytes were cultured with

paeoniflorin, a compound isolated from the roots of the plant Paeoniae lactiflora, the amount of DNA damage after UVB exposure was significantly reduced. This study also demonstrated that paeoniflorin was effective at reducing facial wrinkles *in vivo*, further supporting its anti-aging effect. Additional compounds have also been observed to be effective at preventing DNA strand breaks *in vitro*, including (–)-epigallocatechin gallate (89), ellagic acid (90), and lycopene (30).

13.4.2 Aging Related Changes in the Mitochondria

Mitochondrial theories of aging are based on the work by Harman (91,92), and propose that damage to mitochondrial DNA (mtDNA) may be a causative factor in the aging process. While the primary role of the mitochondria is ATP production, which occurs through the process of oxidative phosphorylation, as a byproduct of oxidative phosphorylation the mitochondria generates small amounts of ROS, such as superoxide and hydrogen peroxide. It is the generation of these ROS that form the basis for the mitochondrial based theory for aging.

Like the cell nucleus, the mitochondria also contain DNA. This short section of mtDNA is circular, in contrast to the linear structure of the genomic DNA, and makes up only 3 percent of the cells genetic material (93). Of the 1500 polypeptides required for mitochondrial function the mtDNA only encodes for 13 of them (94), however these 13 polypeptides contribute to some of the key enzyme systems responsible for the electron transport chain and ATP synthesis including NADPH dehydrogenase, cytochrome c oxidase, cytochrome B, and ATP synthase (95). Since this circular strand of mtDNA is located within the mitochondria, it is extremely susceptible to oxidative damage by the ROS generated as a byproduct of oxidative phosphorylation. Damage to the mtDNA limits the ability of the mitochondria to effectively produce the key polypeptides that are encoded within the mtDNA sequence. As a result, the components of the electron transport chain do not function as efficiently and electrons moving through the electron transport chain are less capable of producing ATP and more likely to contribute to an enhanced ROS formation (96). This enhanced ROS formation in turn can elicit further mtDNA damage, which activates a viscous cycle of decreasing mitochondrial efficiency and increasing ROS formation. The effects of this loss in mitochondrial efficiency have global implications within the cell since it results in reduced ATP availability and an increase in the probability for oxidative damage to the genomic DNA due to the increased presence of ROS.

This mitochondrial based aging process can be potentially accelerated in the skin, since in addition to the ROS species formed within the mitochondria skin cells face the additional oxidative insults of UV radiation. Exposure of cultured keratinocytes (97) or fibroblasts (98), to UV light can induce mtDNA mutations and is an excellent model for photo-aging.

MtDNA damage can be determined directly by examining the 16,000 base pairs mtDNA sequence for base deletions. The most common aging associated mtDNA deletion observed in skin cells occurs between bases 8483 and 13459, and is referred to as the 4977 deletion (99). This deletion is normally detected using PCR based methods. For this method, mitochondrial DNA is extracted from cells and incubated with DNA primers that will specifically amplify certain regions of the mtDNA genome. In the case of the 4977 deletion, the two primers used will usually correspond with the mtDNA sequences that range just outside of the deleted region, namely between bases 8,100 to 8,200 and between bases 13,500 to 13,700. In mitochondria with intact DNA, under optimal PCR conditions these sets of primers will generate DNA fragments that are between 5,300 and 5,600 base pairs in length. However, in mtDNA where the 4977 deletion has occurred, these PCR primers will generate DNA fragments that are only 300 to 600 base pairs in length. Once the samples of mitochondrial DNA are amplified via PCR, the DNA fragments produced can be separated by size using gel electrophoresis and visualized by staining the gel. Using measurements of the 4977 mtDNA deletion, Eicker *et al.* (98) found that beta-carotene could be effective in preventing UV induced mtDNA damage in cultured human fibroblasts, suggesting that this material could have anti-photoaging effects.

As indicated, mtDNA mutations will lead to functional changes within the mitochondria. One of the ways that mitochondrial function can be assessed is to measure the mitochondrial membrane potential. The mitochondrial membrane potential is generated by the electron transport chain. As electrons travel through the different components of the transport chain, the energy from this flow of electrons is captured by several polypeptide complexes and used to pump protons from the mitochondrial matrix to the intermitochondrial membrane space. This creates a membrane potential across the inner mitochondrial membrane, since the intermembrane space with the accumulated protons becomes positively charged while the mitochondrial matrix side of the membrane which is losing protons takes on a relative negative charge. This mitochondrial membrane potential not only provides the energy for ATP synthesis, but is also needed so that the mitochondria can import the mitochondrial polypeptides that are encoded by

nuclear genes rather than mitochondrial genes. Therefore, any alteration in mitochondrial membrane potential will not only disrupt ATP synthesis, but can also further reduce the efficiency of mitochondrial function by disrupting the import of polypeptides that are essential to mitochondrial function.

In vitro, the mitochondrial membrane potential can be measured through the use of special fluorescent dyes, such as 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol-carbocyanin iodide (JC-1) (100). When added to cultured cells, JC-1 is selectively taken up into cellular mitochondria, and its rate of accumulation within the mitochondria depends upon the mitochondrial membrane potential. With low membrane potentials, the amount of JC-1 uptake is minimal, however at high membrane potentials JC-1 accumulation within the mitochondria increases. With high amounts of accumulation JC-1 will shift from a monomer form, which emits green fluorescence (excitation 510 nm, emission 527 nm), to a "J-aggregate" form, which emits red fluorescence (excitation 485–585 nm, emission 590 nm). By measuring the ratio of red fluorescence intensity (high membrane potential) to green fluorescence intensity (low membrane potential), an overall estimate of cellular mitochondrial membrane potential can be determined.

Using this method, two groups have observed that the organic acid creatine can help preserve mitochondrial membrane potential with aging. Lenz *et al.* (101), observed that when aged individuals were topically treated with creatine, epidermal cells that were isolated from the treated individuals, placed in cell culture and exposed to UVA did not have any declines in mitochondrial membrane potential when compared to epidermal cells of untreated individuals. Additional work by Berneburg *et al.* (102), demonstrated that when creatine is directly added to keratinocyte cell cultures, the cultured cells did not undergo any decline in mitochondrial membrane potential when exposed to UVA irradiation. This study also demonstrated that creatine supplementation could prevent mtDNA deletions in response to UVA irradiation as well.

13.5 Anti-oxidant Assays

13.5.1 Reactive Oxygen Species and the Aging Process

ROS play a role as a causative factor in many of the theories of aging. ROS have been observed to affect by several ways: (1) cause oxidative damage to both genomic and mtDNA; (2) induce markers of cellular senescence such as p53, p21, $p16^{ink4a}$, and SA β-gal activity; and (3) reduce the replicative ability of cultured cells. Since many of the aging associated effects

of ROS can be countered with antioxidant materials, there is a great deal of interest in screening compounds for antioxidant activity. However, since there are multiple forms of ROS, and since there are also many different types of materials that can act as antioxidant, there is not a single standard assay that is used to screen materials for antioxidant capabilities. There are many different types of assays, some of which have a very broad application and can be utilized to test different types of antioxidants, while other assays are better suited to specific types of materials.

13.5.2 Ferric Reducing Ability of Plasma (FRAP) Method

The FRAP method was first developed by Benzie and Strain (103) to determine the antioxidant capacity of plasma, and has since been modified for use with plant extracts (104). The mechanism of the assay depends entirely on the ability of the test compound to transfer a single electron to reduce ferric 2,4,6-tripyridyl-s-triazine (TPTZ) to ferrous TPTZ, which has an intense blue color and can be measured spectrophotometrically. The assay is conducted at pH 3.6 to maintain the solubility of the iron in solvent. One of the limitations of this assay is that it does not react with materials that act via hydrogen donation to quench radicals (i.e., thiols and proteins) (105).

13.5.3 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2-2′-Azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) Methods

The DPPH methodology was developed by Brand-Williams *et al.* (106), and makes use of the stable free radical DPPH, which has a strong purple color that can be measured spectrophotometrically. In the presence of compounds that are capable of either transferring an electron or donating hydrogen, the DPPH will become discolored. In the literature, the change in DPPH absorbance after the addition of a test material is often used as an index of the antioxidant capacity of the material (107). Although the DPPH method is widely used, it does have some limitations. The radical portion of the molecule is a nitrogen atom located at the center of the structure. While this centralized location is freely accessible to small molecules, larger molecules may have limited access to the radical portion due to steric hindrances (108). In addition, materials like carotenoids also have a strong absorbance at the same wavelength as DPPH, which will interfere with the assay (16).

The ABTS assay, developed by Miller *et al*. (109), is similar to the DPPH method in that they both use a strongly colored stable radical compound.

In contrast to the DPPH method, in which the radical form of DPPH is already generated and commercially available, the ABTS must be oxidized into its radical cation form at the beginning of each assay. This is commonly accomplished by incubating ABTS with metmyoglobin and hydrogen peroxide (110). The reactions are then carried out in a manner similar to the DPPH assay in that the ability of test materials to induce the discoloration of the ABTS cations is measured spectrophotometrically. ABTS has an advantage over other antioxidant systems in that it is freely soluble in both organic and aqueous solvents so it can be used to screen both hydrophilic and lipophilic compounds.

13.5.4 2′,7′-Dichlorodihydrofluorescein (DCF) and cis-Parinaric Acid (CPA) Methods

DCF is often used as a fluorescent indicator of ROS formation in both simple biochemical antioxidant assays and in cell culture based antioxidant assays. DCF can be oxidized by peroxynitrite, hydroxyl radicals, and peroxidase to generate a highly fluorescent end product. When used in cell culture based assays it is used in a diacetate form (DCF-DA) (14). In this form, the DCF-DA can penetrate the cell membrane and once inside of the cell, intracellular esterase enzymes will cleave off the acetate groups trapping the DCF within the cell. The cells can then be exposed to conditions which generate ROS, such as UV irradiation. Since the dye does not impact cell viability, ROS formation can then be tracked in real time by making periodic fluorescent measurements of the cells with a fluorometer. This not only allows for measurements of the immediate effects of oxidative damage, but it also allows for continued measurements as the cells recover. When screening test materials with this assay, if the materials have antioxidant properties, then they will reduce the amount of DCF fluorescence in response to the ROS insult.

CPA can also be used in cell culture based assays to screen antioxidant materials. CPA is a naturally occurring polyunsaturated fatty acid and is highly fluorescent in its natural state. It is susceptible to peroxyl radicals, and when it interacts with peroxyl radicals its fluorescence becomes quenched. Due to this sensitivity to peroxyl radicals, CPA is often used in assays to screen materials for their ability to prevent lipid peroxidation (111). With cell culture based assays, CPA can be added to the culture media and will be taken up by the cells and incorporated into the cellular membranes. After washing away any unincorporated CPA, the cells can be exposed to peroxyl radicals and the subsequent decline in CPA fluorescence can be

measured using a fluorometer. When screening test materials with this assay, if the materials have antioxidant properties they will help maintain the amount of CPA fluorescence in response to the peroxyl insult.

13.6 Final Remarks

This chapter has described several key *in vitro* methods which can be used to screen materials for anti-aging effects; however, many other methodologies are available. While the methods discussed in this chapter are specifically used to screen compounds for anti-aging effects and also provide some information on the mechanism of action of effective compounds, a review of scientific literature will reveal a variety of additional *in vitro* methods which can detect aging related changes (Table 13.1).

Table 13.1 ***In Vitro*** **Methods Which Can Detect Aging-Related Changes**

Aging Related Measurement	References Describing Methodology	References Using Methodology to Screen Materials
Replicative senescence	8–10, 12	13, 14
Telomere length	18, 20, 21	22
Telomerase activity	23	24, 25
p53, p21 or $p16^{ink4a}$	26–28, 32, 33, 35	36, 44
SIPS	37–40	42–44
SA-β-Gal activity	10, 31, 41	42–44
Collagen synthesis	49	51, 59, 60
Elastin synthesis	50	63
MMP-1 activity	52, 57, 68,	69, 70–72
Pigmentation changes	55	74, 76
8-Oxoguanine	80	80–82
DNA strand breaks	85, 86	30, 88–90
Mitochondrial DNA deletions	97, 98	98
Mitochondrial membrane potential	100	101, 102
FRAP assay	103, 104	104
DPPH assay	106	16, 107
ABTS assay	109	109
DCF assay	112	112
CPA assay	113	111

These additional *in vitro* methods can be readily adapted to screen materials for anti-aging effects and can also provide further insight into the mechanism behind a compound's anti-aging properties. Once effective anti-aging compounds have been identified and characterized through *in vitro* testing, they can proceed to the next phase of testing: *in vivo* testing.

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14

Clinical Testing to Uphold an Anti-aging Claim

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14.1 Introduction

A diverse and myriad number of clinical testing protocols and technologies are available to uphold a cosmetic product anti-aging claim. The specific protocol and technologies chosen may reflect time, cost, and to a certain extent, the proposed or known mode of action of a particular ingredient or product. As our knowledge of the aging process continues and as technological innovations in instrumentation improve, the number and types of testing is expected only to increase. Cognizant of this, this chapter attempts to summarize the clinical methods used in upholding a topical cosmetic product claim of "anti-aging," with the caveat that the reader beware. Novel methods and new instrumentation are continually being developed and improvements in existing methodologies will continue to add to the repertoire of available clinical methodologies.

Aging of human skin occurs via two independent yet related mechanisms—innate (chronological) aging and actinic or photoaging. Innate aging progresses from the changing of cellular functions which gradually result in such deficiencies as fine lines, skin thinning, and a decreased ability to repair wounds and heal the skin. Photoaging occurs following prolonged exposure to solar ultraviolet (UV) radiation and is associated with skin thickening, texture changes (roughness), coarse wrinkles, and mottled pigmentation. Both tried-and-true methodologies and new leading technologies that provide a reasonable basis upon which to substantiate an anti-aging claim for an ingredient or product are presented in this chapter.

While exogenous skin aging has been studied extensively, the pathway-mechanisms of endogenous skin aging remain far from elucidated. Some of the typical characteristics involved in upholding an anti-aging claim includes the use of demonstrating a reversal in such parameters as fine

wrinkles, skin dryness, sallowness, loss of elasticity, improvement in skin thickness, enhanced production of surface lipids, reduction of wrinkle depth, restoration of collagen fibers and increase of the collagen III/I ratio, and certain dermal receptors.

This chapter treats the anti-aging claim as one being utilized for cosmetic product substantiation and may or may not be reflective of international regulatory requirements needed for substantiation of a cosmetic claim that comes under scrutiny by an individual country authority.

14.2 Subjective Assessment

Subjective assessment of an anti-aging claim may take one of three forms: (1) consumer research; (2) comparator research; and (3) clinical trials. However, these studies, as all scientifically valid studies, should be conducted as adequate and well-controlled investigations that will withstand the review of, for example, FDA's substantial evidence review. Adequacy of a study is usually defined as "sufficient to establish effectiveness" and may require confirmatory evidence. In the strictest sense, these studies should be conducted as well-controlled, double blind placebo studies, using procedures generally accepted in the profession to yield accurate and reliable results.

Subjective studies carried out to uphold anti-aging claims are usually conducted on human volunteers who are given the test product (usually in a blinded fashion) and asked to evaluate certain attributes of the test product in a clinical setting or in take-home use conditions utilizing the test product's proposed label use instructions. User questionnaires are employed to assess the user panel's response to specific questions related to an anti-aging claim.

Double-blind studies are those in which neither party (subject nor clinician) know the product/placebo identity. Placebo-controlled studies use both the final formulated product (active) and placebo (without the active ingredient(s)). Crossover studies are those in which the same subjects receive product and placebo products at different times after a washout period.

These types of studies may also consider the specific features and benefits ("appearance", "look of", "beautify", "cleanse", "visibly reduces the look

of fine lines and wrinkles by 25 percent"), product comparisons, testimonials (name, data, original writing or recording) and endorsements (contract terms, user or prescriber, specialty or industry [e.g., dermatologist or entertainment personality]).

In the past many cosmetics claims were considered "puffery," i.e., "emotional" claims [feel better, sensuous, happy …] that were not considered to require substantiation. However both marketing research and technological methodologies have evolved so as to permit a more objective evaluation of these claims. These kinds of claim support studies are called subjective psychological cosmetic claim studies.

Implicit and explicit claims may be tested by the use of different mood questionnaires, as well as recording verbal responses and psycho-physiological responses such as sniff latency, respiration rate, facial corrugator electromyogram, and skin conductance. Attributes such as pleasantness, familiarity, and intensity of fragrance may also be measured. Several standardized quality of life surveys and questionnaires with visual analog scales are used to quantitatively evaluate improvements in emotions and psychological anxieties as well as functioning scales used to assess social relationships.

14.3 Visual Assessment by Trained Evaluators

The ability of a test product to affect a reduction in the appearance of fine lines/wrinkles in a group of volunteers during and after a specified use regimen which usually involves a similar application scheme to that suggested in its label instructions or during normal product use is a commonly employed technique in clinical testing. Good results can be obtained by using trained evaluators to visually measure a pre- and post-treatment response (1). Most of the time such studies also include a skin compatibility or mildness (safety) component that evaluates the ability of the test product, or lack thereof, in producing skin irritation.

A visual assessment claim study most appropriately is conducted as a double-blinded efficacy study, whereby both the study population and the trained evaluator(s) involved in the study are not aware of the product being used. The application of the test product occurs per the label instructions of the test product and may involve an immediate response to, or a much longer claim, for instance, up to ninety days. Subjective responses

may be gathered and trained clinical evaluators or a dermatologist may conduct evaluations throughout the study (e.g., Week 2, 4, 8). The subjective responses may use a visual assessment score for grading or responses to specific questions as the basis for determination of an effect. The clinical grading is compared to the baseline evaluations and statistical significance of a response may be obtained for the test population.

The routine subjective home-use study is often expanded to include appropriate skin bioengineering techniques to objectively correlate an anti-aging affect with subjective test population responses.

The visual assessment study may also utilize a comparator product, or one or both of a positive and/or negative control product. These methods usually increase the sensitivity of a study and are especially useful and necessary when seeking a comparative test product claim. Such methodology, however, is usually not applied not only because of the increased costs involved but because cosmetic claims are so involved with subjective results that being able to control for environmental and subjective changes in hormonal status, psychological changes and various other phenomenon are usually outside the realm of a cosmetic use study.

14.4 Non-invasive Objective Testing Methodologies

Standardization is a key issue in the successful application of using almost any instrumental method to define an anti-aging affect for a cosmetic product. Standardization must consider the following parameters:

- The environment—room temperature, relative humidity, light sources, air circulation;
- The instrument—zero setting, calibration, probe properties, and positioning;
- The subject(s)—age, sex, race, skin site, hormonal or diurnal rhythm, skin type, skin site cleansing (or non-cleansing), medications; and
- The test product—stability, light exposure, dilution, amount per surface unit, frequency of application, mode of delivery.

The consideration of these factors is the fundamental basis in designing a well-defined protocol and providing for a study that is reproducible and most able to withstand scrutiny.

14.4.1 Profilometric Analysis of Silicone Replicas for Fine Lines and Wrinkles

Silicone replicas for evaluating fine lines and wrinkles has been one of the most acknowledged and may be the oldest method to objectively analyze for lines and wrinkles such as around the crow's feet area (2). It is widely used for substantiation of an anti-aging and anti-wrinkling product claim. A silicon replica is taken of the chosen test site, before test product application and at the test conclusion, or at various designated time intervals during a test products' use. The silicon replica represents a negative of the skin. A typical skin replica is shown in Figure 14.1.

Once the skin replica dries, a light source directed at a twenty-five degree angle from the plane of the replica is used (Figure 14.2) to capture luminance along a set of ten equal length lines and the reflected light is captured with a high resolution camera mounted vertically to the silicon replica.

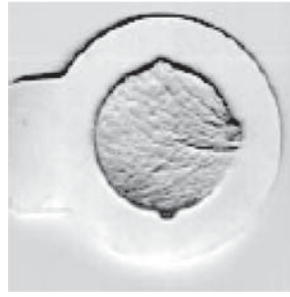

Figure 14.1 Skin replica of the crow's feet area. (Adapted from http://www.cuderm.com/bionet/reppacket.pdf)

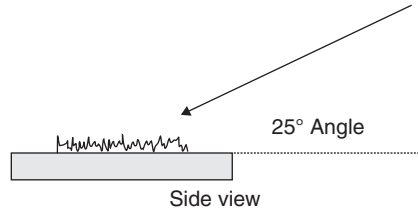

Figure 14.2 Typical configuration of light source for silica replica analysis.

Software algorithms analyze the length and depth of the wrinkles ingrained on the replica. Pretreatment and post-treatment values are compared. Various parameters may be measured including:

- Rz—the average maximum difference in luminance that defines skin roughness;
- Ra—the average optical roughness;
- FSpace—indicating fine line spacing (mm);
- FNum—number of fine line markers (per mm);
- Num Wrinkles—the total number of features detected in the ten bands of sub areas used to calculate spacing and breadth.

As skin lines and creases disappear: the FSpace increases; the FNum decreases; the spacing of lines increases; the breadth of wrinkles decreases as wrinkles become shallower; shadows decrease; and Num Wrinkles generally decrease with smoothing of the skin.

14.4.2 Cell Turnover: Corneosurfametry and Squamometry

The corneocyte size is a good indication of the epidermal turnover rate, i.e., the regenerative capacity of the skin. The faster the turnover, the smaller the size of the horny cells (corneocytes). In humans there is a clear relationship to the corneocyte area size of skin and aging. An almost linear increase exists from birth to old age on the parts of the body exposed to little or no UV radiation.

Minimally invasive methods have been used to harvest the superficial layers of skin (stratum corneum) which when compared to baseline (pretreatment) versus post-treatment regimes provide an objective and quantitative record of a topically applied products' effect on stratum corneum. The methods include gentle rubbing of the skin surface and collection of the rubbed-off skin. Stripping with adhesive tape has been successful in the hands of some researchers (3,4). But perhaps one of the most reliable techniques has been the use of commercially available self-adhesive coated discs (D-squame® Cuderm Corps., Dallas, Texas, USA) that are applied to the skin under calibrated pressure for a defined period of time. The corneocyte aggregates are removed with the adhesive discs and the degree of scaling is measured related to an anti-aging treatment effect.

Cyanoacrylate skin surface stripping has also proven to be particularly suitable for the evaluation of the renewal dynamics of the stratum corneum.

A high-bond glue is used to strip the skin surface onto a polyethylene slide which can then be examined microscopically (5). All of these types of skin surface samples may be evaluated directly or stained with appropriate dyes or evaluated visually, microscopically, or by image analysis so the efficacy of a cosmetic product in reversing or affecting an-anti-aging skin surface effect can be assessed.

Monitoring dansyl chloride fluorescence has long been used to evaluate the rate of epidermal cell renewal (6). The study is usually performed with a group of subjects who are pre-treated with a test product on one fore-arm site and with a positive and/or negative control on another site. After the pre-treatment, dansyl chloride is applied to both the treated and untreated test product sites. The fluorescence of dansyl chloride is monitored usually for a 2–4 week period during which time the test product is continued to be applied to the appropriate site usually twice daily. An anti-aging topical product that enhances cell renewal is associated with a faster fluorescence disappearance of the dansyl chloride compared to a negative control site.

14.4.3 Hydration/Humidity of Skin

Hydration or water content of the outer layer of the skin (stratum corneum) has been correlated with the intrinsic aging process and menopause. It has been postulated that a decrease in hydrophilic glycoaminglycans leads to a direct reduction in water content (7).

The amount of moisture in the horny layer can be determined by one or more instruments which operate by various principles. The Corneometer works by the capacitance principle in which a place builds up an electrical field at its edges. Properties of this electrical field change in accordance with the water content of the environment through which it moves. The plate capacitor is separated from the skin by an extremely thin film which prevents ion-containing skin surface water from causing a short circuit between the capacitor plates. The measurements should take place in a constant temperature environment, largely because the measurement can be influenced by the activity of the sweat glands.

Measuring the before treatment and after treatment electrical properties of the skin (whether it be by capacitance, impedence, or conductance) may determine the moisture content of the skin in relation to a skin product.

14.4.4 Skin Fatigue/Elasticity (Cutometer/Elastometer/ Ballistometer)

Wrinkling and sagging (firmness) of skin during aging is physiologically associated with diminished elasticity, which can be attributed to increased fibroblast-derived elastase activity. The assessment of the mechanical properties as measured through the tensile function of skin has been used as a physiological characteristic in determining skin aging and the effect of a topical product on the effectiveness of producing an anti-aging effect (9). From a histological point of view, it is accepted that the mechanical characteristics of human skin result from the composite contribution of connective tissue, dermis, hypodermis, and epidermis.

A decrease in skin elasticity has been related to aging with older skin showing greater fatigue than young skin. Measurements for skin elasticity may be made with a non-invasive suction meter (cutometer, elastometer, and/or the ballistometer). The measuring principle of these devices is based on measuring skin suction and elongation. The cutometer, for example, generates negative pressure, which can be varied. The skin area to be measured is drawn into the aperture of the probe due to negative pressure. The suction and release times are specified and set before starting the measurements. The depth of penetration (deformation) of the skin into the probe (usually 2 mm wide) is measured optically by a measuring system consisting of a light emitter and a light acceptor. Two opposing glass prisms transmit the light from emitter to acceptor. The light ratio changes proportionally to the psenetration depth of the skin. Analysis of the recorded measurement curves makes it possible to determine the elasticity and plastic characteristics of the skin.

Young skin shows a high degree of elasticity and loses its shape only gradually and regains its original state after the end of the suction procedure. Skin which is healthy, supple, and adequately moist will have a higher elasticity than a dry, rough skin.

Using these principles a set of measurements which allows the quantification of skin elasticity characteristics is gathered. Some of the more widely measured parameters are:

- Skin suppleness (Δ ue)—if immediate extensibility increase, the skin is more supple.
- Skin tension (Δ uf)—if total extensibility decreases, the skin is tighter.

- Skin Firmness (Δ ur/Uf)—if the immediate retraction/total elongation ratio comes close to one, the skin is firmer.
- Skin tonicity (Δ X)—if residual distention decrease, the skin is more tonic.
- Skin fatigability [Δ (X1–X)]—if the slope of the curve between the first and fifth aspiration decreases, skin is less fatigable.

Measurements are usually made on the temporal region and volar forearm. This non-invasive method can be applied for objective and quantitative investigation of age-related changes in skin fatigue and evaluation of the effects of cosmetic anti-aging topical products.

14.4.5 Transepidermal Water Loss Measurements (TEWL)

The measurement of transepidermal water loss or skin surface vapor loss is a good indicator of the integrity of the skin barrier function which inherently refers to the skin's ability to retain moisture. An increase in the TEWL indicates an impaired barrier function. Altered skin barrier and TEWL have been shown to be correlated to skin aging (8,10,11). TEWL response to irritation has also been shown to be increased in post-menopausal females as compared to pre-menopausal females (12).

There are a range of instruments, most of which provide results in g/m^2/hour, which measure TEWL. Numerous variables can affect TEWL measurements, including inherent subject factors, as well as environmental and instrumental differences (12–17).

14.4.6 Skin Roughness

Skin surface topography is dependent upon the body site and age of the subject. Skin surface aging has been correlated with skin surface waviness, and skin furrow profiles in skin surface topographies of the face and forearm (18). Numerous tools have evolved over time to measure the human skin surface topography *in vivo* and non-invasively, starting from visual assessment to highly sophisticated optical measuring tools.

Visual evaluation of skin roughness or skin creepiness has long been a good standard for evaluating skin roughness by a trained evaluator.

Silicone replicas are used to evaluate both skin roughness, the ability of a product to affect a rejuvenation of the skin surface by eliminating or

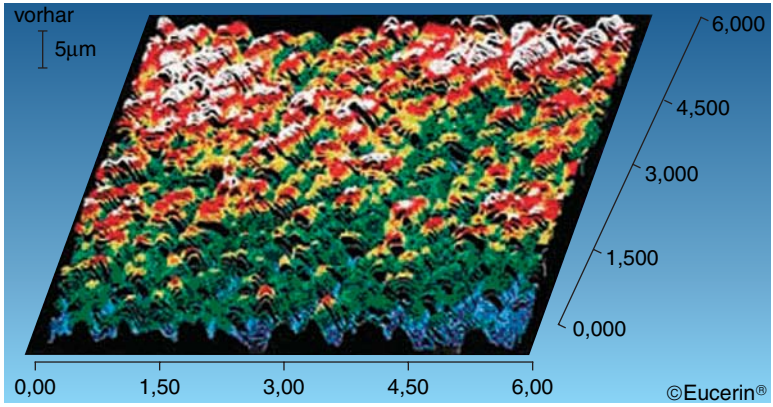

Figure 14.3 Skin replica depiction of skin roughness. Red color depicts greater skin roughness with blue coloration, the least skin roughness. (Adapted from http://www.eucerin.co.uk/product_info/methods.html)

improving its surface roughness and to measure fine lines/wrinkles. An exact replica of the skin's surface profile is made using a silicone/dental-impression material from which the measurements of skin roughness are taken and described, according to a set of standards.

The skin replica technology was further enhanced with the aid of imaging analysis in which the replica surface is reproduced in a 3D format. Thus the various surface parameters, such as roughness could be measured and compared to each other, before and after treatment. A typical 3D scan of skin surface is depicted in Figure 14.3—color changes depict skin roughness variation: warm colors (red, yellow) show large depths of wrinkles and cold colors (green, blue) small wrinkle depths.

14.4.7 Digital Photographic Methods

A vast improvement on the silicone replica methodology has occurred with the use of highly sophisticated, totally non-invasive, digital photographic systems that can be used to determine skin roughness, texture and fine lines/wrinkles for anti-aging cosmetic products. Several marketed stand-alone systems incorporating photographic technology have been successfully produced. One such system, phaseshift rapid *in vivo* measurement of skin (PRIMOS) uses an optical acquisition and associated software to create a 3-D profile of the skin. A typical color coded

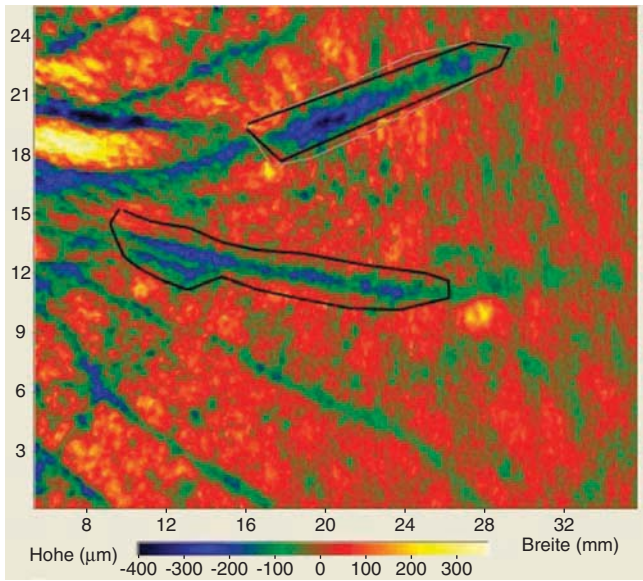

Figure 14.4 Typical crow's feet area wrinkles. Blue represents the deepest lines, green shallower lines and red relatively smooth skin. (Adapted from http://www.simitecno.it/GFM/Produktkatalog_E_Version_1.0.pdf)

analysis of the peri-orbital crow's feet area for eye area wrinkles is presented in Figure 14.4.

The evaluation functionality of this system includes skin roughness and skin line measurements and graphical and numerical measuring results. Another, perhaps unique functionality is its ability to evaluate not only skin surface topography but also provide a validated estimate measurement of the depth of fine lines/wrinkles.

A similar system with greater functionality for skin surface topography and skin color evaluation is the VISIA™ CR (Canfield Scientific, Fairfield NJ) imaging system which provides high resolution photography in a well controlled subject capture box. Proprietary software allows the user to measure skin texture affects without impinging upon the skin surface and provides real-time analysis to measure an agent's ability to affect a change. This system provides real-life images of the skin under various lighting parameters (visible, cross-polarized, parallel-polarized and UV fluorescence lighting) that are able to differentiate skin surface reflief (wrinkles, fine lines), pores, skin texture, skin color, and

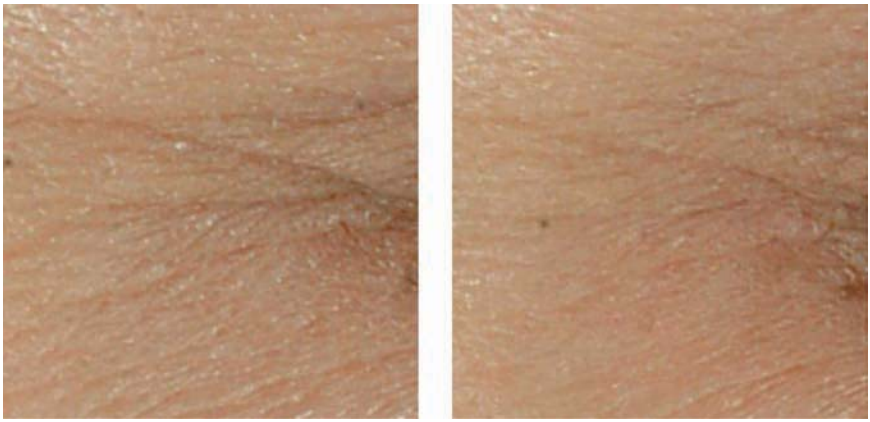

Figure 14.5 Pre-Treatment and post-treatment image of the "crow's feet" area using VISIA™ CR imaging. (Source: Essex Testing Clinic Inc., Verona, NJ, 2007)

UV spots. All of these parameters may be used for anti-aging and skin renewal product substantiation. A typical image of a pre- and post-treatment anti-aging product effect captured with the VISIA™ CR is shown in Figure 14.5.

14.4.8 Skin Color Effects

A growing wealth of research has related skin color changes to ageing. Numerous testing rationales may be used to document such changes, ranging from the simple subjective and/or trained evaluator measurement to using very sophisticated objective imaging techniques. The skin color changes include dark under-eye circles, skin dullness and yellowness (sallowness), uneven skin tones, lack of luminance, skin browning, skin darkening, age spots, and UV skin color changes. Recently age-related increases in mean intensity and surface reflected light, and darker and more yellowish skin color along with decreased translucency have been reported in age-related studies (19).

Effects of anti-aging products can be documented in well controlled pre- and post-treatment studies either by subjective questionnaires, and or trained visual evaluator grading the before and after appearance, usually using a range-scale of color, with chromameter measurements and with highly sophisticated imaging techniques, such as VISIA™ CR.

14.4.8.1 Chromameter Analysis

Measurement of the skin color is usually conducted using an instrument such as a chromameter. A chromameter converts colors into a digital code:

- L*—for clarity (from dark to light). If the L* parameter is high, the skin color is light.
- a*—for the green to red spectrum. As a* increases, the redness of skin increases.
- b*—for the blue to yellow spectrum. As b* decreases, the blueness (whiteness) of skin becomes greater.

These parameters may be used to calculate the individual typological angle (ITA) which defines the degree of skin pigmentation. The higher the L parameter and the ITA, the lighter the skin pigmentation.

In anti-aging studies the subject test population should be chosen to reflect the user market profile and should also be screened for a prior history of its use of whitening products. Baseline, post-use, and intermittent analyses may be conducted to document skin color changes. Anti-aging substantiation studies of skin color changes are usually conducted with baseline (pre-treatment) and post-treatment evaluations, and depending upon the formulations tested may be very fast (hours to 1–2 weeks) or very slow 4–8–12 weeks) depending on the specific formulation. In general when comparing young to older subjects, blueness (b*) and redness (a*) increase with age and lightness (L*) decreases with age.

Although both the chromameter and the VISIA™ CR use the same basic technology of L*a*b* to measure skin color changes, recent studies conducted at Essex Testing Clinic, Inc., have shown a consistent and significantly increased sensitivity of the VISIA™ CR to measure skin color changes when compared to the Chromameter (data on file at Essex Testing Clinic Inc, Verona NJ.).

14.4.8.2 Pigmentation analysis

Pigmentation specific to photodamage was originally determined by subjective assessment or trained clinical evaluation. With the augment of the chromameter and now digital imaging techniques incorporating color analysis, the standard of the industry has become one of the later of these techniques. The chromameter as described in Section 14.4.8 remains a useful tool in monitoring the L* and b* component of skin. As skin

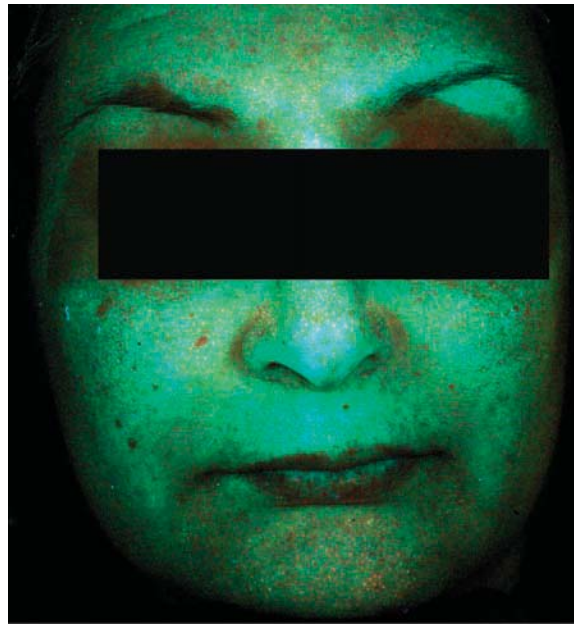

Figure 14.6 Pre-treatment UV VISIA™ CR image. (Source: Essex Testing Clinic, Inc., Verona, NJ)

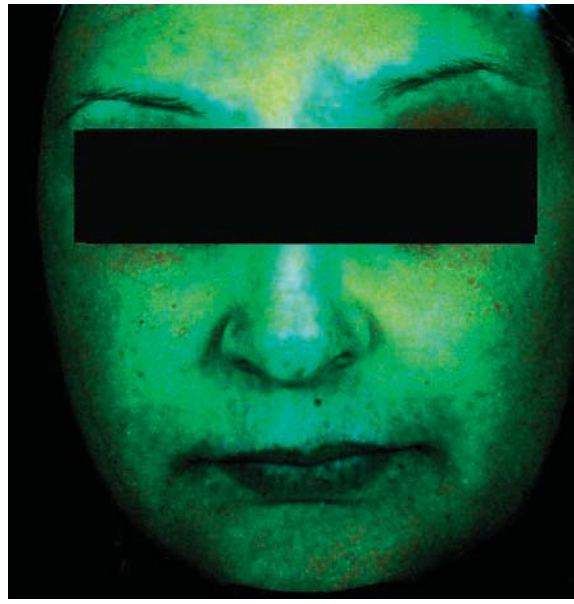

Figure 14.7 Post-treatment UV pigmentation VISIA™ CR image. (Source: Essex Testing Clinic, Inc., Verona, NJ)

pigmentation decreases the skin becomes lighter (the L* and b* component increase). But a much more powerful tool using the same basis of analysis as the chromameter is the VISIA™ CR which provides inherent algorithms for analysis of such skin parameters as: tone, color (L*, a*, b*), luminance, change in skin color (delta e), brown spots, etc. in a non-invasive mode while allowing visualization of the skin area.

The following figures show representative images of a photodamaged face subject pre- and post-antiaging cosmetic product application. Figure 14.6 shows the UV pigmentation images at baseline and Figure 14.7 shows the post-treatment UV pigmentation images after a two week treatment with an effective skin pigmentation treatment regime. The images are analyzed for UV spot density (Figures 14.8 and 14.9) and the statistical significance of the anti-aging response is obtained.

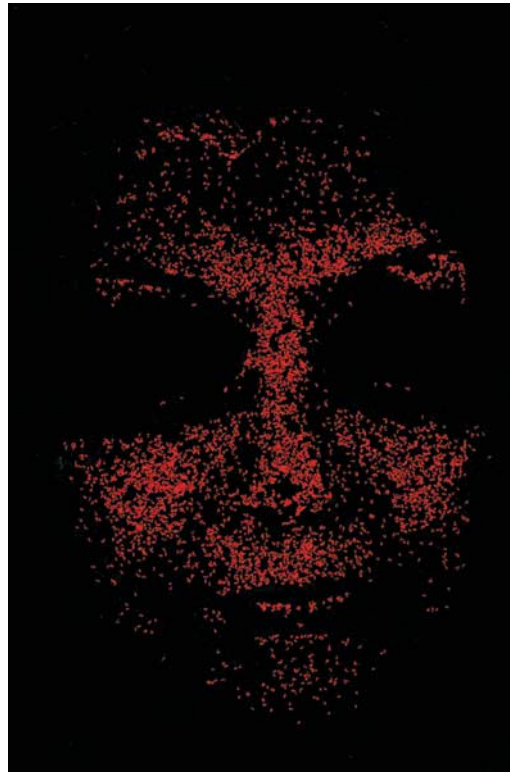

Figure 14.8 Baseline density analysis of UV pigmentation spots. (Source: Essex Testing Clinic, Inc., Verona, NJ)

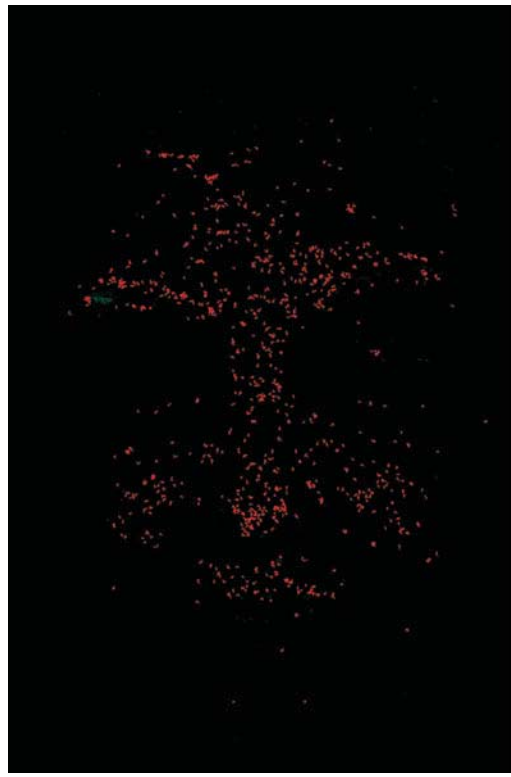

Figure 14.9 Post-treatment density analysis of UV pigmentation spots. (Source: Essex Testing Clinic, Inc., Verona, NJ)

14.4.9 Skin Surface Lipids—Squamametry

The amount of skin surface lipids, which mostly originates from sebum varies according to skin region and depends on the density of the sebaceous glands and on their level of activity. Skin surface lipid peaks in puberty and then continually declines with age.

Skin surface lipids may be quantified by measuring the amount of lipid on a certain are of skin. Several systems are available for measuring sebum. The original systems consisted of an opaque film which soaked up the sebum. The film becomes more or less transparent depending on the amount of sebum. The transparency is measured against a standard and expressed as a numerical value. Based upon this principle there are now systems that provide continuous numerical measurements of the lipid density captured on specific film paper.

14.4.10 Ultrasound Imaging

Ultrasound uses high frequency sound waves (frequency higher than 20 MHz) to create an image of the skin and its immediate substrate. A high frequency signal emission strikes the skin and an echo is sent back to a capture mechanism which translates the response into a two dimensional representation of the skin tissues.

Ultrasound imaging has been used to measure the thickness of the epidermis, the dermis, subcutaneous fat layer, and collagen layer. Quantification of the epidermal and/or dermal response captured by ultrasound provides substantiation of many qualitative anti-aging claims, including collagen and elastin effects, microcirculation effects, skin thickness effects and in some cases, skin penetration effects. Skin thickness studies have shown that normal dorsal skin is thicker than volar forearm skin. This proportion remains constant until the seventh decade of life, diminishing thereafter (20). This concept has been useful in designing anti-aging studies, especially when a post sixth to seventh decade of life subject population is being targeted. Skin ultrasound measurements are captured for a subject test population at baseline and various times during product test usage. These values are compared and analyzed and the effect of a product on skin thickness may be established.

A typical ultrasound image of skin is provided in Figure 14.10.

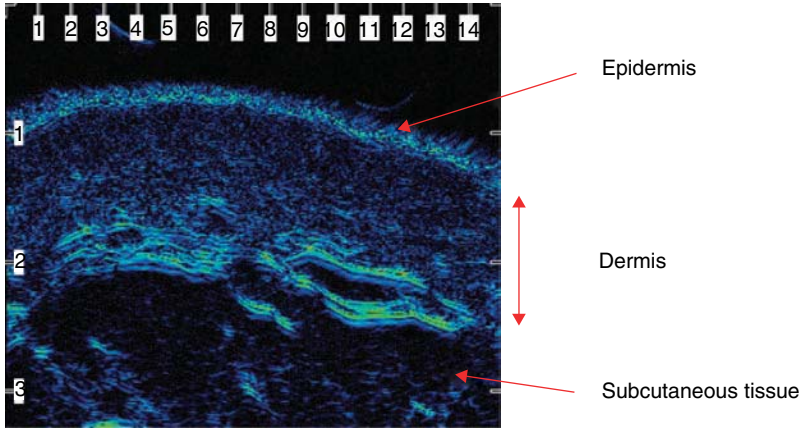

Figure 14.10 Epidermal and dermal components visualized with ultrasound imaging. (Adapted from http://www.longportinc.com)

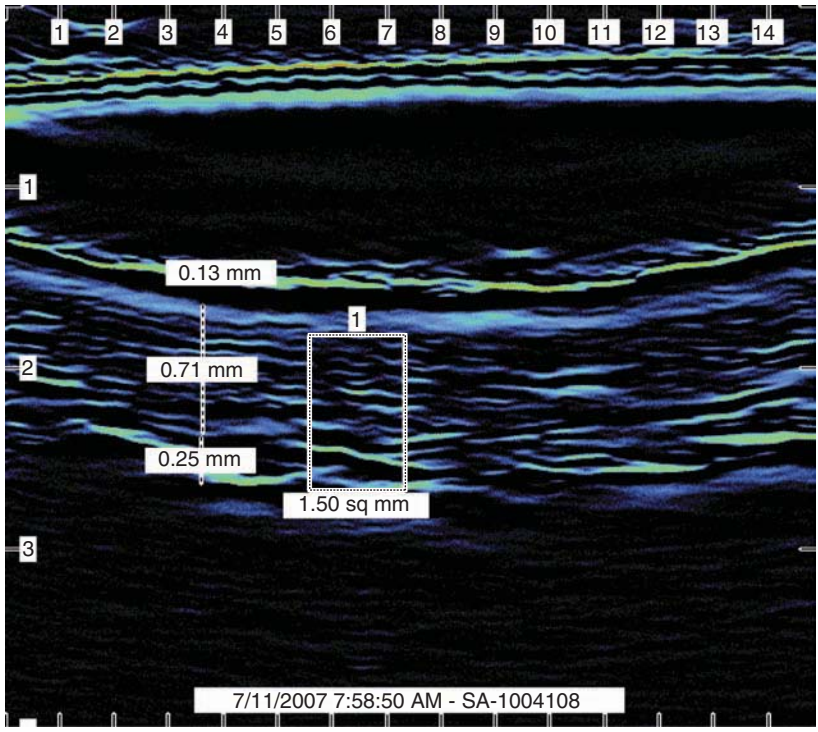

Figure 14.11 Ultrasound image of the skin of a lower leg scan before treatment. (Source: Essex Testing Clinic, Inc., Verona, NJ)

Ultrasound imaging has been used to substantiate anti-aging claims (21). The echogenic ratio between the upper and lower dermis has been shown to be a useful objective estimate of photoaging (22). The subepidermal low echo-genic band associated with elastosis increases with age and becomes less dense. Although differences in skin site have been shown to occur, even on the face, elder subjects show an increase in skin thickness and overall echogencity compared to younger subjects in all facial sites except for the infraorbital regions (23). Differences in the echogenic response of photodamaged and non-photodamaged skin have also been reported (24).

Figure 14.11 (baseline) and Figure 14.12 (post two-week treatment) present ultrasound images of a subjects leg skin with an anti-aging product showing the effectiveness of a two week treatment period.

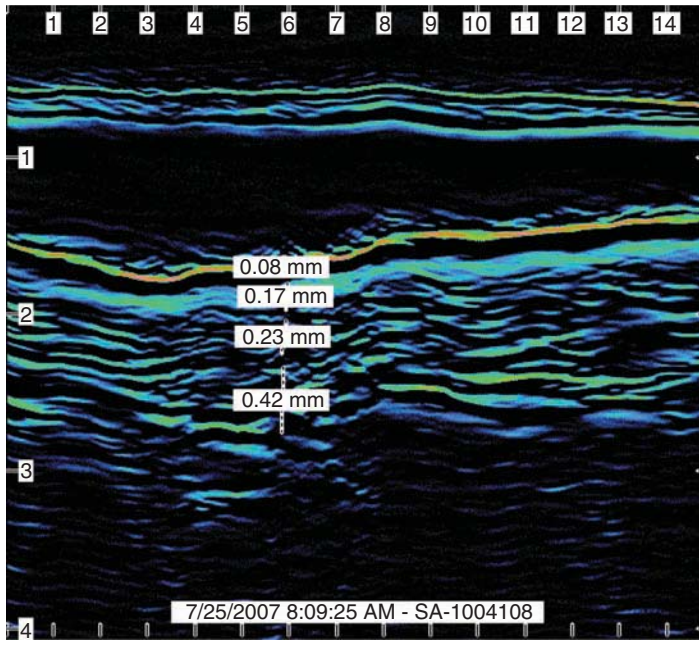

Figure 14.12 Ultrasound image of the lower leg skin 2-weeks after treatment with an effective skin anti-aging product showing an increase in epidermal thickness and density of dermal elastin/collagen fibers. (Source: Essex Testing Clinic, Inc., Verona, NJ)

14.4.11 Microvasculature—Laser Doppler Profilometry

The use of laser doppler flowmetery to document cosmetic anti-aging effects is not widespread. The instrumentation started out as very large machinery has migrated to very portable desk size and may find a greater use in the future. Several studies have reported that the microvasculature of skin undergoes characteristic changes with aging when the skin site is controlled (25). Capillary loops in the dermal papillae decrease and subpapillary plexus increase with age (26). Significant decreases in cutaneous vascular reactivity have also been described following menopause. The density of capillary loops in an older group of women (test population aged 20–74) decreased significantly by 40–70 percent compared with the youngest group whereas vascular length increased with age, but blood flow increased with age (27).

14.4.12 Skin Autofluorescence

In vivo skin inherently autofluoresces with the absorption of light by chromophores such as melanin and hemoglobin. Several researchers have been able to characterize the changes in skin fluorescence with intrinsic ageing and photoaging beginning with animal models and then with human skin (28–33). Using this technology, several inherent skin fluorescence peaks have been identified. The 375 nm skin autofluorescence peak has been used as a biologic marker of skin aging *in vivo* and the increase in tryptophan fluorescence (297 nm) has been correlated with photoaging effects. Dramatic changes in photo damaged vs. non-photo damaged skin can be visualized with commercially available fiber optic spectrofluorometry. This technology can be successfully used to characterize the effects of anti-aging products on skin, but demographic characteristics need to be tightly controlled as age, skin type, and other inherent factors may influence the results. Figures 14.13 and 14.14 show the vast differences in the skin autofluorescence peaks observed with photo-damaged and non-photodamaged forearm skin sites.

The characterization of skin autofluorescence before and after treatment with an efficacious anti-aging product can be used to monitor a cosmetic products' efficacy.

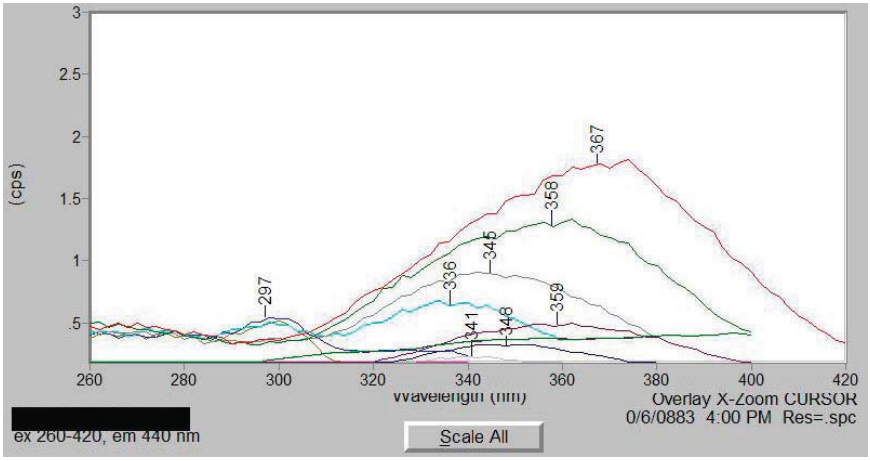

Figure 14.13 Non-photodamaged skin site: Minimal tryptophan peak at 297 nm. (Source: Essex Testing Clinic, Inc., Verona, NJ)

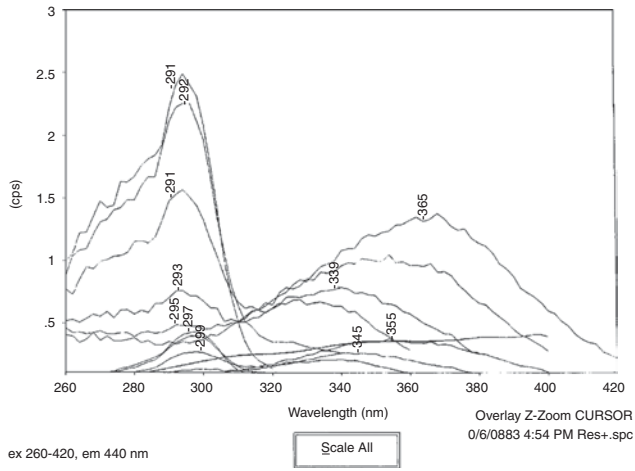

Figure 14.14 Photodamaged skin site: Increased tryptophan signal at 297 nm. (Source: Essex Testing Clinic, Inc., Verona, NJ)

Another recent use of skin autofluorescence has made using *in vivo* multiphoton laser tomography (34). A negative relationship between the excited autofluorescence of collagen and elastin with age were observed. This type of technology holds promise for future characterization of skin aging cosmetic products effectiveness.

14.4.13 Emerging Spectroscopic Methods

Various novel spectrophotometric techniques are beginning to be applied to the study of skin anti-aging effects. They include B-scan Ultrasound, Magnetic Resonance Imaging, Confocal Microscopy, Optical Coherent Tomography, and Raman spectroscopy. Each of these methods utilizes a different set of physical instrumental principles and each may provide a sensitive tool to assess changes in skin with anti-aging products. Most of these methods are considered to be research tools not employed for routine claim substantiation of anti-aging claims. It is, however, likely that with time they may become routine methods of investigation.

Perhaps one of the most popular methods is Raman spectroscopy that allows *in vivo* demonstrations of skin thickness and quantification of the effectiveness of skin care composition. The major advantages of Raman Spectroscopy are the non-destructive nature of the technique, virtually no need for sample preparation, and the ability to track an effect by skin depth.

Confocal optics relate to the illumination of a sample with a diffraction limited spot such that the illuminating spot is imaged on an ideally point-like detector, the point-like detector being realized with an adjustable pinhole called the confocal hole in front of the real detector (entrance slit). Spatially offset capture of the laser beam has increased the detection ability of the instrumentation. Changes in skin lipids, natural moisturizing factor, and water content are being studied, but as of the current time, few studies have related these new and powerful tools to direct correlation with anti-aging dermal products or the normal aging phenomenon of the skin. Raman spectroscopy has been used to measure melanin and hemoglobin content changes related to aging, decreases in skin thickness, changes in natural moisturizing factor, anti-oxidant effects, and density of melanocytes (35–38). With greater use and decreased instrumental costs, this technology may provide extremely broad and sensitive non-invasive monitoring of anti-aging cellular physiological effects in the skin.

14.5 Minimally Invasive Technique—Punch biopsies

The use of punch biopsies, a minimally invasive method, has proven useful in supporting anti-aging claims for products that affect not only the dermal surface topography but deeper intracellular physiologic parameters such as skin thickness, collagen, and elastin. For histologic investigation, a 1- to 4-mm biopsy sample is usually taken before test product application and at an adjacent site after product treatment.

Epidermal thickening without alteration of the stratum corneum has been reported with effective photodamaged skin treatments (39). Staining for antibodies has shown that anti-aging product treatment is correlated with decreases in IL-1b, IL-6, and matrix metalloproteinase-1 (MMP-1) and an increase in collagen I (40–42). Staining with anti-collagen1 antibodies has demonstrated an increase in density while staining with anti-MMP-1 has shown a significant reduction (43).

14.5.1 Anti-Oxidative Processes and Free Radical Studies

One theory of aging is that aging cells become more susceptible to free radical (oxidative) damage and decreased cutaneous immune function. Most of the studies providing evidence of specific mechanisms of anti-aging processes through anti-oxidative mechanisms have occurred in human skin *in vitro* systems. But there is a growing base of evidence being generated with human skin *in vivo*. Perhaps one of the most robust studies

to date is that of McDaniel *et al*. (39) who utilized standard design techniques with enzymatic analysis of punch biopsies to substantiate photo anti-aging claims. Immuno-fluorescence staining revealed a decrease in the presence of inflammatory cytokines, IL-1b, IL-6 (known to stimulate collagenase), and MMP-1 and an increase in collagen I for the tested products along with improvements in the standard parameters associated with anti-aging.

Other interesting examples of perhaps less conclusive studies include antioxidative studies showing enzymatic and nonenzymatic antioxidant activity in the epidermis and dermis of human skin during aging and photoaging; direct correlation with increased catalase function in the epidermis of photoaged and naturally aged skin; significantly increased activity of glutathione reductase in naturally aged epidermis; decreased levels of alpha-tocophoryl in the epidermis of photoaged and aged skin; and lower levels of ascorbic acid in both epidermis and dermis of photoaged and aged skin (40–44). The ability of a product to quell enhanced production of reactive-3-oxygen species has also been associated with age-related changes (43). ERK1 and JNK2 activity and cJun protein in skin supernatants have been assayed for enzymatic activity and related to photoaging effects (45,46). H_2O_2 quantification recycling assays have been used to show changes with anti-aging product use (41). Total RNA, determined by using various commercially available kits, has been used to relate product use to anti-aging affects (47,48).

The sensitivity of these effects provides a new area for further research into the specificity of physiological changes in skin aging and the potential effects of anti-aging products.

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PART 5
REGULATORY ASPECTS

15

Cosmetic Anti-aging Formulations—International Regulatory Aspects

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15.1 Introduction

Where science and art meet in the human race's centuries-old quest for youth and beauty is where in modern times the regulator necessarily steps in. It is where art, indeed is "made tongue-tied by authority,"[1] at least, up to a point. Most countries in the world today regulate cosmetics

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in some way to protect consumers. In some, this regulation is extensive and prescriptive, in others more flexible, but the overall trend is to protect consumer health through regulatory restrictions on the manufacture, composition, and sale of cosmetics. The growing popularity of anti-aging products has attracted attention to a number of regulatory issues, such as whether these products ought to be regulated as cosmetics or drugs as well as the challenges of how to advertise a product to the consumer using artistic superlatives without venturing beyond scientific reality. The trend toward increased and more restrictive regulation places growing emphasis on manufacturer responsibility for ensuring that products are safe for their intended use and that claims are adequately substantiated to safeguard industry's freedom to develop better and more effective products.

This chapter will provide an overview of the regulatory systems for cosmetics of the United States, the European Union, Japan, and a number of other countries; the development and interpretation of the definition of a cosmetic; and an overview of the current status and interpretation of anti-aging claims.

15.2 Regulatory Systems of Major Markets

15.2.1 United States

In the United States cosmetics are regulated by the Food and Drug Administration (FDA)[2] under the federal Food, Drug, and Cosmetics Act (FDCA)[3] and the Fair Packaging and Labeling Act (FPLA).[4] According to the FDCA a product may be regulated as a drug, a cosmetic, or both a drug and a cosmetic,[5] depending on the claims made about the intended use of the product. Cosmetic products are additionally regulated as consumer products and any claims made with regard to product efficacy to the extent such claims may affect a consumer's choice whether to purchase a product or not, are regulated by the Federal Trade Commission (FTC) under the authority of the Federal Trade Commission Act (FTCA).[6] The issues, therefore, that may affect anti-aging products under the regulatory system of the United States may be complex and involve more than one regulatory agency.

Cosmetics are defined by the FDCA as "articles intended to be rubbed, poured, sprinkled, or sprayed on or introduced into, or otherwise applied

to the human body or any part thereof for cleansing, beautifying, promoting attractiveness, or altering the appearance."

A drug is defined in part as an "article intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animal; and…articles (other than food) intended to affect the structure or any function of the body of man or other animals."

An anti-aging product, therefore, may fall under either one or both definitions, not on the basis of its composition, but according to the intended use that the manufacturer establishes for the product. That is, a product that claims to alter the appearance of the skin, but not at the same time to alter its structure and function, would considered a cosmetic, while a product that claims to induce a change in the structure or function of the body would be regulated as a drug.[7] Under the FDCA, a product that makes both types of claims would be considered both a cosmetic and a drug.

This system of classification, however, in the context of the FDCA, does not make the product's composition irrelevant. Even though most claims made with regard to the product apply mainly to the classification of the product, the mere mention of certain recognizable drug actives on the product label are considered implied drug claims.[8] Furthermore, the FDCA addresses substances that should not be added to a product or claims that may not be made by prohibiting the sale of adulterated and misbranded products. The adulteration provision protects consumers by prohibiting the sale of products that contain any substance that is injurious to health, while the misbranding provision prohibits the sale of any product that contains any false and misleading statements associated with it.

Thus, an anti-aging product would be considered a cosmetic if it claims to only alter the appearance of the skin. As a cosmetic, it must be safe for its intended use to comply with the adulteration provision of the FDCA and its labeling may not contain any statements that are false or misleading. On the other hand, a product that claims to change the structure or function of the skin, either permanently or temporarily, would most likely be considered a drug. Like a cosmetic, a drug may not contain any false or misleading statements on its labeling; however, a drug must not only be safe for its intended use, but it must also be proven to be effective for its intended use and be subjected to pre-market approval[9] to demonstrate through detailed clinical studies its safety and efficacy.

Therefore, from a practical point of view, and presuming that safety has been substantiated, the manufacturers of anti-aging products are confronted with a dilemma—if the product is to be represented as a cosmetic, no claims may be made about any active ingredients that may penetrate the skin; if a physiological effect is claimed, on the other hand, the manufacturer would be faced with a lengthy and costly New Drug Application (NDA)[10] process or a possible enforcement action by the FDA.

The FDCA cosmetic and drug definitions have been subject to interpretation by the courts on a number of occasions. The principal cases that dealt with the distinction between a drug and a cosmetic have involved anti-wrinkle products, which make them especially interesting. These are the Line Away,[11] Sudden Change,[12] and Magic Secret[13] cases. All three involved a similar type of product: a lotion consisting of albumen and water. The mode of use is to apply the product directly on the skin of the face and allow it to dry. Upon drying the product would form a film which would cause a tightening of the surface of the skin and thus make it smoother for a number of hours.[14]

While the products involved in these cases had similar compositions, the claims varied. Line Away was said to "visibly smooth out fatigue lines, laugh lines, worry lines, frown lines, tiny age lines, and crows feet, while discouraging new lines from forming." Sudden Change was advertised as a "facelift without surgery." Both products had a number of additional similar claims. In both of these cases the court decided that the products, on the basis of the claims associated with them, were drugs because they were intended to affect the structure and function of the body and would therefore be subject to the rules for new drugs. The court in each case noted that consumers, including the "ignorant, unthinking, and credulous" consumer, would believe that the product would induce either permanent or temporary change in the structure and function of the skin.

On the other hand, the court in Magic Secret found claims that the product "smoothes away wrinkles in minutes," "can last as long as 8 hours…," and causes "astringent sensation" would lead consumers, including the "ignorant, unthinking, and credulous" to believe that the product would only change the appearance of the skin and not its structure or function and ruled that Magic Secret is not a drug under the provisions of the FDCA.

There are numerous examples of warning letters that the FDA has issued over the years with regard to products that make anti-aging claims, where

the agency goes into great detail of which claims are considered drug claims and that any products associated with them would have to be subjected to the NDA process. Similarly, products imported into the United States making these claims are denied entry into the United States, if they are represented otherwise as cosmetics.

It is important to note here that the cases described above concerned products that in fact did not alter the structure or function of the skin in any substantive way, but only claimed to do so. The court in each of these cases was concerned with the consumer's perception of such claim. Both the FDA and FTC now use the reasonable person standard, which raises the threshold at which the consumer would be presumed to be deceived from the much lower "ignorant, unthinking, and credulous" person standard. That is, courts would decide how a consumer might perceive a given statement and whether the statement can be considered deceptive on the basis of what a hypothetical "reasonable" person in the same or similar situation might perceive, not what a consumer with specialized knowledge would think. Similarly, a reasonable consumer is not a person who might be described as gullible, even though in many cases courts do take into consideration the "ignorant, unthinking, and credulous" consumer as in the aforementioned cases. Nevertheless, as newer, more complex and effective compounds and products are developed that involve active substances or complexes that do indeed change the structure or function of the skin well beyond its mere surface, that do in fact eliminate wrinkles and make the skin not only appear younger, but truly rejuvenate it, manufacturers will be able to make claims to that effect. At this point however, they would also have to face the reality that such products are more likely to be regulated in the United States as drugs, and be prepared to invest in the clinical studies for safety and efficacy substantiation under the NDA process required for drugs under the FDCA.

15.2.2 European Union

The European Union[15] regulates cosmetic products under the Cosmetics Directive[16] which was adopted in 1976 as a harmonization measure of the national laws and regulations of the EU Member States and has as an objective "the establishment and functioning of the internal market."[17] The Articles of the EU Cosmetics Directive have been amended seven times and its annexes have been modified by approximately annual adaptations to technical progress. Over the years, the EU Cosmetics Directive has

developed into an important system for regulating cosmetic products, which has been adopted by many countries around the world.

Although the Ell Cosmetics Directive requires that manufacturers keep records and make part of the product information package[18] of "proof of the effect claimed for the cosmetic product, where justified by the nature of the effect or product"[19] the directive does not specify any methods to be used or standards to be met. There are industry guidelines,[20] which outline a number of tests, including consumer self-evaluation tests, tests on human volunteers under controlled parameters, instrumentation tests, and others. Thus, manufacturers have a great deal of discretion in determining the extent and nature of efficacy substantiation for anti-aging claims.

Anti-aging formulations are classified in the EU as cosmetics. According to the EU Cosmetics Directive, a cosmetic product is "any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odors and/or protecting them or keeping them in good condition." The preamble to the EU Cosmetics Directive helps define the scope of this definition by noting that "products containing substances or preparations intended to be ingested, inhaled, injected, or implanted in the human body do not come under the field of cosmetics."

The definition of a medicinal product is "(a) Any substance or combination of substances presented as having properties for treating or preventing disease in human beings; or (b) Any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis."[21] The preamble of the aforementioned amending directive further clarifies that "Where a product comes clearly under the definition of other product categories, in particular food, food supplements, medical devices, biocides or cosmetics, this Directive should not apply." Therefore, anti-aging formulations are not likely to be reclassified as drugs even if certain restoring correcting and modifying physiological functions are attributed to the product because anti-aging products have traditionally been regarded in the EU by both consumers and regulators as cosmetics. In fact, the EU Cosmetics Directive includes

anti-wrinkle products in Annex I, illustrative list by category of cosmetic products.

Accordingly, most enforcement action in the EU with regard to anti-aging products has centered on truth in advertising issues and level of scrutiny rather than product classification. Anti-aging claims are generally reviewed to determine the extent they can be considered objectively true, consumer's perception of these claims, and the likelihood these claims can be considered to be misleading, while taking into consideration the cultural diversity that exists in the European Union. Thus, the European Court of Justice (ECJ) has established a general Community standard for evaluating claims, while leaving it to the national courts to apply the standard on a case by case basis in light of consumers' national and regional characteristics.

For example, in Lauder v. Lancaster[22] the German national court referred a question whether community law precludes the application of national law on unfair competition where national law imposes a much stricter standard for review than Community law. The case concerned a facial cream named Monteil Firming Action Lifting Extreme Creme and the claim under scrutiny was the word "lifting." According to German law[23] a word used in advertising may be prohibited from being used with regard to a product under certain circumstances and furthermore, German case law has established that a prohibition is in order if 10 percent to 15 percent at least of potential consumers may be misled.[24]

In this case, it is presumed that the cream does not provide the same effect as a surgical "lift" and the issue is whether a consumer would actually think that it did and therefore be induced to buy the product. This would give the company who uses such a claim an unfair advantage. Most consumers (reasonable persons) would know that this is advertising puffery and do not expect to get the equivalent of a surgical lift from a cream, but a certain part of the population may believe that they would (like the "ignorant, unthinking, and credulous" consumer of the Line Away, Sudden Change, and Magic Secret cases in the United States). Under German law, the courts would be required to extend the protection even to that part of the population. The ECJ however, adopted a standard to be applied at the Community level where "it is necessary to take into account the presumed expectations of an average consumer who is reasonably well informed and circumspect" in order to determine whether a statement is misleading.[25] Because of this decision of the ECJ, all national courts of the EU Member States would have to apply it, so that companies would not have an unfair

advantage in some countries as would be the case if different standards are used.

Because the EU definition of a cosmetic allows for some structure and function effects, it is unlikely that anti-aging formulations would be regulated as drugs in the EU, or that it would be necessary to do so as newer and more effective formulations are developed. Any questionable claims associated with a product's labeling and advertising are likely to be evaluated by the national courts using the community standard of a reasonable consumer described above, while taking into consideration the national and regional characteristics of each individual case.

15.2.3 Japan

Japan has a long history of regulating cosmetics and drugs, including an intermediate category of products called quasi-drugs. Japan's regulatory system is unique in that the classification leaves considerable discretion to the regulator and that product safety, not only for the intended use, but in general, plays a determining role.

Under Article 2 of the *Pharmaceutical Affairs Law*,[26] the term cosmetic applies to "items (other than quasi-drugs) intended to be used by means of rubbing, sprinkling, or by similar application to the human body for cleaning, beautifying, promoting attractiveness, and altering the appearance of the human body, and for keeping the skin and hair healthy, provided that the action of the article on the human body is mild."

The term drug (or pharmaceutical) product in the law applies to items recognized in the Japanese Pharmacopoeia, items (other than quasi-drugs) which are intended for use in diagnosis, cure or prevention of disease in man or animals, and which are not equipment or instruments (including dental materials and medical supplies and sanitary materials); and finally, items (other than quasi-drugs and cosmetics) which are intended to affect the structure or function of the body of man or animals, and which are not equipment or instruments.

Japan's third classification for products is known as "quasi-drugs." Quasi-drugs by definition must have only a mild effect on the body, but are neither intended for the diagnosis, prevention or treatment of disease, nor to affect the structure or function of the body. Unlike cosmetics, however, quasi-drugs have a "definite purpose of use."

There is considerable difference from a practical point of view whether a product is classified as a cosmetic or a quasi-drug. While no pre-market approval is required for cosmetics that comply with the regulations in effect, quasi-drugs are subject to licensing on a case by case basis where all active and inactive ingredients, in addition to any claims associated with the product, are subject to pre-market approval by the authorities.

Cosmetics may make one or more of fifty-five specifically listed cosmetic claims.[27] Claims for skin-care products in general are limited to:

- Clean the skin (by removing dirt).
- Prevent pimples and heat rash (by cleaning) (face cleaning products).
- Condition the skin.
- Condition skin texture.
- Keep the skin healthy.
- Prevent skin chapping.
- Astringent for the skin.
- Moisturize the skin.
- Give and maintain skin moisture and oil.
- Maintain skin elasticity.
- Protect the skin.
- Prevent dry skin.
- Make the skin soft.
- Make the skin supple.
- Give the skin luster.
- Make the skin smooth.
- Prevent sunburn.
- Prevent sunburn induced spots and freckles.

Products that are safe in most situations; that is, not only for the intended use, and also fall under the definition of a cosmetic are currently limited to these claims. For this reason, most anti-aging formulations that claim any other effects would more likely to be classified as quasi-drugs under Japan's regulatory system and be subjected to a case-by-case licensing procedure before such products may be placed on the market in Japan.

15.2.4 Canada

The Food and Drugs Act,[28] Section 2, provides the definition of a **cosmetic** as "any substance or mixture of substances manufactured, sold or represented

for use in cleansing, improving or altering the complexion, skin, hair or teeth, and include deodorants and perfumes…"

A drug is defined in The Food and Drugs Act, Section 2 as "any substance or mixture of substances manufactured, sold or represented for use in (a) the diagnosis, treatment, mitigation or prevention of a disease…, (b) restoring, correcting or modifying organic functions in human beings or animals, or (c) disinfection in premises in which food is manufactured, prepared or kept." A product that claims to provide a therapeutic benefit would be required to be registered as a drug or a natural health product (NHP) (see below) and follow the applicable regulations. Drugs that are like cosmetics are regulated under category IV monographs.

In 2004 Canada introduced a third category of products. A natural health product under the NHP regulations "means a substance set out in Schedule 1 or a combination of substances in which all the medicinal ingredients are substances set out in Schedule 1, a homeopathic medicine or a traditional medicine, that is manufactured, sold or represented for use in: the diagnosis, treatment, mitigation or prevention of a disease, disorder or abnormal physical state or its symptoms in humans; restoring or correcting organic functions in humans; or modifying organic functions in humans, such as modifying those functions in a manner that maintains or promotes health."

"Schedule 1" is appended to the regulations and contains a list of NHP substances as follows: a plant or a plant material, an alga, a bacterium, a fungus, or a non-human animal material; an extract or isolate of a substance described above, the primary molecular structure of which is identical to that which it had prior to its extraction or isolation; any of the following vitamins: biotin, folate, niacin, pantothenic acid, riboflavin, thiamine, vitamin A, vitamin B_6, vitamin B_{12}, vitamin C, vitamin D, vitamin E; an amino acid, essential fatty acid or a synthetic duplicate thereof; a mineral; and a probiotic. Thus, cosmetic-like drugs that contain only mineral or natural-based ingredients are now regulated under the NHP regulations. If the product contains any non-natural (most synthetic organic compounds) active ingredients, either alone or in combination with "natural" actives, they continue to be regulated as drugs. Note that the definition of "natural" in this case is a legal one, that is, some of these substances may in reality be manufactured synthetically, but are considered natural under this definition.

Advertising and allowed claims are under greater regulatory control in Canada than in the United States. Pre-clearance by Advertising Standards Canada (ASC) is optional for broadcast advertising of cosmetics and required for broadcast and print advertising of drugs and NHPs.

Additionally, ASC and Health Canada publish the *Guidelines for Cosmetic Advertising and Labeling Claims*, 2006.[29] These guidelines cover such items as conformity with the appropriate legislation, claim substantiation, competitive advertising, and acceptable and unacceptable claims.

There is a recognized category of anti-winkle and anti-aging products. Allowed claims are:

- Covers up age spots;
- Hides age spots;
- Feel younger;
- Look younger;
- Moisturizes aging skin;
- Smoothes wrinkles (from an appearance perspective);
- Reduces the appearance of aging;
- Helps prevent signs/the look of aging (visibility);
- Reduces the appearance of age spots;
- Reduces the appearance of skin blotches;
- Covers/conceals;
- Anti-wrinkle cream/anti-wrinkle moisturizer (when qualified in a cosmetic sense);
- Anti-wrinkle/anti-aging (when qualified in a cosmetic sense);
- Slows appearance/the look of aging (visibility);
- Slows signs/the look of aging (visibility);
- Reverses the signs/look of aging (visibility);
- Face rejuvenator (when qualified in a cosmetic sense, rejuvenates look);
- Wrinkles appear/look reduced;
- Fight the look of wrinkles;
- Skin appears/looks visibly younger;
- Reduces the look of puffiness/dark circles;

Qualified in a "cosmetic sense" refers to claims that what is altered is the appearance of wrinkles or other signs of aging and not imply that there have been any structural or physiological changes.

Accordingly, some unapproved claims are as follows:

- Prevents aging;
- Eliminates aging;
- Stops aging;
- Reduces aging;
- Slows aging;
- Reverses aging;
- Prevents new spots from appearing/eliminates age spots;
- Anti-wrinkle (unqualified);
- Anti-aging (unqualified);
- Slows appearance/onset of aging (i.e., development of aging);
- Any reference to action at cellular level (living);
- Stimulates circulation;
- Collagen, elastin, skin enzyme synthesis/replenishment/stimulation;
- Prevents the onset/emergence of wrinkles/the return of wrinkles;
- Wrinkles are reduced;
- Rejuvenates skin (unqualified);
- Provides the effect of a medical/surgical procedure;
- Reduces puffiness/dark circles.

"Unqualified" in the cosmetic sense means that the claim is not made in the context of changing the appearance only.

Although there is some similarity in the regulatory principles between Canada and the United States, there are some important differences. In general, a product, even if it falls under more than one definition, would be regulated as a cosmetic, a drug or an NHP depending on which characteristics and claims predominate. Composition, as noted above, is considerably more important for classification in Canada than in the United States; however, claims are just as important in product classification. As in the United States, as more effective anti-aging formulations are developed, they are more likely to be regulated as drugs.

15.2.5 Mexico

Under the Regulations for Health Control of Products and Services of August 9, 1999,[30] cosmetics are: "perfumery and beauty products that, through suitable technical means, can modify the natural odor of the body

and maintain and improve its aesthetics." Additionally, "perfumery and beauty products are those intended for application directly to the skin, its adnexa and appendages, the purpose of which is to beautify, improve the appearance, and maintain the cleanliness and attractiveness of persons."

A distinctive feature of Mexican regulations is the category of cosmetic treatment products which are defined as "perfumery and beauty products intended to mitigate or prevent deficiencies or alterations of the function of the skin, or which modify the structure of the skin." Anti-aging products are considered cosmetic treatment products and in spite of the different classification these products are regulated as cosmetics.

15.2.6 South Korea

South Korea is another country that takes into consideration the existence and growing development of anti-aging products.

Under the *Cosmetics Law* of 1999, cosmetics are articles with mild action on the human body for the purpose of cleaning, beautifying, adding to the attractiveness, altering the appearance, or keeping and promoting the skin or hair in good condition.

Additionally, the Cosmetics Law provides a definition of functional cosmetics as articles which fall under the definition of cosmetics as above, and additionally fall under one of the following categories listed and are designated such by Decree of the Ministry of Health: (a) Cosmetics designed to whiten the skin; (b) Cosmetics designed for smoothing out skin wrinkles; (c) Cosmetics designed to protect the skin from UV rays of the sun or to develop natural-looking tanning of the skin. Such functional cosmetics are subject to greater scrutiny and require pre-market registration and approval of both ingredients and claims before they may be placed on the South Korean market.

15.3 Conclusion

In most countries of the world anti-aging products are still regulated as cosmetics. Regulatory concern centers on their safety and claim substantiation, but considering the very low health risk most cosmetic products represent for consumers, they have a rather low priority with regulators. Regulatory hurdles that must be overcome may vary from country to country

depending on whether pre-market approval and registration are required, or whether greater responsibility is placed on the manufacturer so that enforcement may occur after a product is placed on the market. In all cases, however, even though the standard of judicial review or level of enforcement may vary, there are two regulatory principles applicable to anti-aging products that are present in all regulatory systems. These are: (1) the product must be safe for its intended use; and (2) any claims made with regard to the product must be substantiated. Nevertheless, it is important to note here that regardless of the level of regulatory control, and even if cosmetic products were not regulated at all, close adherence to these principals is the ethical responsibility of every manufacturer of cosmetic products. This way industry, consumers, and even regulators can continue to benefit from the greater freedom to create newer and more effective products so long as art is not "made tongue-tied by authority" any more than is absolutely necessary.

Notes

[1]William Shakespeare, Sonnet LXVI.
[2]The FDA is an agency within the US Department of Health and Human Services.
[3]21 U.S.C. section 301 et seq.
[4]15 U.S.C. 1451 et seq.
[5]This is a distinctive feature of the legal system in the United States. Under other systems a product is regulated usually as either a cosmetic or a drug, although most regulators do acknowledge the existence of "borderline" products, that is, products that do not belong to a single category.
[6]15 U.S.C. 42 et seq.
[7]*Supra* note 5.
[8]For example, the FDA has noted that: "The use of the word ''hormone'' in the text of the labeling or in the ingredient statement is an implied drug claim." 21 CFR 310 530 (a).
[9]FDCA, 21 U.S.C 355, section 505. A new drug may not be legally marketed in the United States without prior approval from the Food and Drug Administration (FDA).
[10]Id.
[11]United States v. An Article … "Line Away," 415 F.2d 369 (3d Cir. 1969) (*hereinafter* Line Away).
[12]United States v. An Article … "Sudden Change," 288 F. Supp. 29 (EDNY 1968 reversed, 409 F.2d 734) (*hereinafter* Sudden Change).
[13]United States v. Article … "Magic Secret," 331 F. Supp. 912 (D. Md. 1971) (*hereinafter* Magic Secret).
[14]The mechanism of the product's effect is described in Magic Secret:

> "As the film contracts, its adhesive qualities cause the valley of the wrinkle to be drawn up to the level of the normal surface of the skin. As the film contracts, its adhesive qualities cause the valley of the wrinkle to be drawn

up to the level of the normal surface of the skin. When, after a period of hours, the amount of unevaporated liquid in the film is reduced below the quantity required to maintain surface tension, the film breaks down into its component solids and liquid, permitting the skin to return to a wrinkled configuration. Application of a small amount of moisture to the residual solids reforms the film, which then resumes its wrinkle-smoothing function until the evaporation process is repeated," 331 F. Supp. 912 at 915.

[15]The European Union is currently composed of the following 27 member states: Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Ireland, Hungary, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Romania, Portugal, Slovakia, Slovenia. Spain, Sweden, and the United Kingdom.

[16]Council Directive 76/768/EEC of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products, 1976 O.J. (L 262) 169, *hereinafter* EU Cosmetics Directive.

[17]CONSOLIDATED VERSION OF THE TREATY ESTABLISHING THE EUROPEAN COMMUNITY, O.J. (C 325) 33 (2002) (*hereinafter* EC Treaty), Article 95.

[18]Product Information Package (PIP), also Product Information Requirement (PIR), Technical Information File (TIF), or Dossier, is a requirement introduced by the Sixth Amendment of the Cosmetics Directive, Article 7a, which includes the qualitative and quantitative formula, physical, chemical, and microbiological specifications of the raw materials, method of manufacture, safety assessment of finished product, data on undesirable effects on human health, and proof of effect as well as the name, address, and qualifications of the person responsible for the safety assessment. Additional requirements were introduced by the Seventh Amendment: the qualitative and quantitative (limited to substances covered by the Dangerous Substances Directive 548/67/EEC) composition of the product and existing data on undesirable effects and information on any animal testing relating to development or safety evaluation of product or ingredients including any animal testing conducted to meet requirements of third countries. This information must be made available, upon request, to the Competent Authorities of the Member State where this information is kept.

[19]EU Cosmetics Directive, Article 7a (1) (g).

[20]Guidance for the Evaluation of the Efficacy of a Cosmetic Product, Colipa (The European Cosmetic, Toiletry and Perfumery Association), 1995.

[21]Directive 2001/83/EC on the Community code relating to medicinal products for human use, *as amended* by Directive 2004/27/EC of the European Parliament and of the Council of 31 March 2004.

[22]Estee Lauder Cosmetics GmbH & Co. OHG v. Lancaster Group GmbH, Case C-220/98, Judgment of the Court (Fifth Chamber of 13 January 2000), reference for a preliminary ruling: Landgericht Köln-Germany. ECR 2000 page I-00117. *See also* Opinion of Mr Advocate General Fennelly delivered on 16 September 1999, Case C-220/98, ECR 2000 page I-00117.

[23]Gesetz gegen den unlauteren Wettbewerb (Law against Unfair Competition; 'the UWG') of 7 June 1909. *See also* Lebensmittel-und Bedarfsgegenständegesetz (Federal Law on Foodstuffs and Consumer Items) of 15 August 1974 ('the LMBG'), para 27(1).

[24]See Lauder v. Lancaster, Judgment of the Court, para 17.

[25]*See* opinion of Mr Advocate General Fennelly deliverd on 16 September 1999, Case C220/98, ECR 2000, page I-00117, where he notes that "German unfair-competition law should abandon the attempt, which is as stupid as it is pointless, to seek to protect practically the last "simpleton" (Trottel") from the danger of being misled by advertising."

[26]Pharmaceutical Affairs Law (Law No. 145) published on August 10, 1960, as amended.

[27]Notification No. 1339, Ministry of Health, Labour and Welfare, December 28, 2000.

[28]Food and Drugs Act, R.S.C. ch. F-27 (1985) (Can.) as amended.

[29]Available at Health Canada's website; http://www.hc-sc.gc.ca/cps-spc/alt_formats/hecs-sesc/pdf/legislation/pol/cosmet/guidelines-ld_e.pdf (last visited on March 19, 2008).

[30]*Reglamento de Control Sanitario de Productos y Servicios* (Regulation on Health Control of Goods and Services), Official Journal, August 9, 1999.

16

Strategic Regulatory Planning—Key to Success in Anti-aging Cosmetic Product Development

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16.1 Introduction

16.1.1 Bombarded by Claims in the Pursuit of Beauty and Health

"*New, powerful anti-aging skin care*"
"*Aging is reversible and optional*"
"*Breakthrough herbaceutical*"
"*Regenerate damaged skin*"
"*Penetrates deeply into the layers of the skin*"
"*Dramatically reduce wrinkles, fine lines*"
"*Erase wrinkles and boost collagen synthesis within ten days*"
"*Anti-inflammatory and healing for sun-damaged skin*"
"*Stimulates cellular metabolism within days*"

A significant amount of media coverage has been devoted to the topic of anti-aging in recent years. Googling the keywords "fountain of youth" brings up more than 1.6 million references, most of them about products and services to turn back the clock, while only a fraction of them actually focuses on the story of Ponce De Leon's search of the Fountain of Youth in the 1500s. Mankind's quest for eternal beauty, health, and youth started at the age of dawn. The first archeological evidence of cosmetic use dates back to about 4000 BC in ancient Egypt. It was the ancient Egyptians who coined the expression "cleanliness is next to godliness." Cosmetic use was an integral part of their hygiene and health. Botanicals such as thyme, marjoram, chamomile, lavender, lily, peppermint, rosemary, cedar, and rose were frequently mixed with oils to formulate perfumes for personal and ritualistic purposes. In fact, the inventive ancient Egyptians had enjoyed a wide spectrum of personal care products that could probably still satisfy the demand of any modern day consumers: wrinkle creams and stretch mark removers, scar camouflage, deodorants, color cosmetics, face cream, perfumes, fragrances, and body oils, just to name a few.

16.1.2 Current Anti-aging Market Trend

Apparently baby boomers' obsession to remain forever young is not new. But appearing young at all times by arresting or reversing the "unnecessary" aging process is the modern men's new ideal. Along with the human genome

mapping project comes the hope that is both thrilling and tempting to all—that the scientists might discover the specific gene expression to terminate or even reverse the aging process. If popular culture has its way, it would have us stop viewing youth as a transitory phase of life and think of aging as optional. Consumers embrace these promises for which they spend lavishly and willingly. According to a market research report published in February 2005 by BCC Research, baby boomers' unprecedented purchasing power, coupled with the youth-dominated cultural shift and modern technological advancement, have fueled a rapidly growing US anti-aging industry that exceeded $45.5 billion in 2004, $7.7 billion of which was spent on appearance products alone. The annual growth is expected to continue at a stunning rate of 9.5 percent and the market is expected to reach $72 billion by 2009.[1] Another market trend report published by the Packaged Facts in 2005 stated that sales of cosmeceuticals in the US would reach $12.4 billion in 2004 (of which, $6.4 billion is for the skin care products) and continues to grow to over $16 billion by 2010. The new product trends would center on "anti-aging everything" with a strong demand for botanicals.[2] The annual global spending on over the counter (OTC) cosmetics and cosmeceuticals to enhance appearance is at an even more staggering number of $230 billion.[3] The frenzied marketing activities prompted the United States Senate Special Committee on Aging to hold a public hearing on September 10, 2001, entitled "Hearing on Swindlers, Hucksters and Snake Oil Salesmen: The Hype and Hope of Marketing Anti-aging Products to Seniors."

It is indeed true that we have entered an "anti-aging everything" era. The bright future outlook of the business is motivated and fueled by the baby boomers' relentless lust for eternal youth and further enabled via the seemingly endless supply of new scientific discoveries of the aging process, more effective delivery systems for topical skin care products, and exotic new ingredients often touted as the next best "something-ceutical." Notably, one branch of medical practice most enthusiastically embraced by the baby boomers is the "anti-aging medicine" where some medical practitioners claimed that human life expectancy could be dramatically increased through certain chemical and/or lifestyle interventions including caloric restriction, genetic manipulation, anti-oxidants and hormone treatments, stem cell replacement therapies, etc. Buoyed by these claims, new products allegedly incorporating the benefit of such advanced medical interventions arrive at the storefront at an increasingly alarming rate. Amidst the frantic pace of the anti-aging product and market evolution, even a veteran formulator can be uncertain as to what constitutes the best practice to better, more efficient product development for successful global marketing.

This chapter attempts to provide a comprehensive strategy and tools for product developers and formulators by outlining the most important factors and their complicated inter-relationship for sound business decision making.

16.2 Making Sense of Global Regulatory Product Classification

16.2.1 Drugs or Cosmetics

16.2.1.1 Legal Definitions of Drugs and Cosmetics in the US

In the United States, the regulatory requirements for cosmetics and drugs are established by the Federal Food, Drug & Cosmetic Act (FD&C Act), enacted in 1938 and enforced by the Food and Drug Administration (FDA). The act defines cosmetics as "articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body … for cleansing, beautifying, promoting attractiveness, or altering the appearance."[4]

Drugs are defined by the FD&C Act as:

> "*(A) articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease … and*
>
> *(B) articles (other than food) intended to affect the structure or any function of the body of man or other animals.*"[5]

16.2.1.2 Distinction between Drugs and Cosmetics in the US

The biggest distinction between a drug and a cosmetic in the US regulatory environment is that a cosmetic is not allowed to possess any physiological activity as to affect the structure and function of the body. In other words, articles intended to be used externally to improve attractiveness or appearance are regulated as cosmetics. But when the intended use is considered to have the potential to change the structure or function of the body, the product will be regulated as a drug. Essentially, cosmetic effects are supposed to be only superficial, while drug effects are physiological in nature. The "superficial effect" and "not affecting the structure and function" clauses are two tests most often used by FDA to differentiate a drug from

a cosmetic in terms of its proper regulatory product classification.[6] This distinction between a drug and a cosmetic forms the basis to determine how and to what extent the FD&C Act will be implemented to each respective product classification.

Based on a policy established by the Seventy-fourth Congress in 1935, products are classified by the act based on their intended use. A product's intended use may be established through its direct advertising or product claims. However, FDA advises that consumer perception and expectation of the product also constitutes the basis for determining the intended use, be it through direct advertisement or indirectly implied promotional messages.[7,8] The following examples demonstrate product claims and ingredient listing:

Example 1: Product claims stated in the labels, advertising material in the media and on the Internet, and any other relevant promotional materials. The distinction between a drug and a cosmetic is based on whether it claims to affect the structure or function of the body. FDA considers it a drug claim if the statement indicates the product will affect the body in some physiological way, even if the effect is only temporary.[9] A shampoo intended for cleaning is regarded as a cosmetic, but a shampoo advertised for preventing or treating dandruff is a drug. A deodorizing body spray is a cosmetic, but the same product claiming an antiperspirant property is considered a drug. A moisturizer that smoothes and softens the skin is a cosmetic, but it legally becomes a drug if the same product claims to penetrate to the inner layer of the epidermis to repair damage, retard aging, stimulate cellular growth, or erase wrinkles.

Example 2: Ingredients used and how they are identified. For any retail cosmetic product intended for home use, if it contains ingredients that are commonly regarded as "drugs" (e.g., recognized to have therapeutic benefits), or if the product is represented in such a way as to imply health or nutrient benefits, the product is considered and regulated as a drug even if no such claims are explicitly made. Therefore, a cosmetic product containing acetaminophen is regarded by the FDA as a drug, even when no claims are made regarding its analgesic or anti-inflammatory properties. Another example is the hormone cream. FDA considers it false and misleading if the word "hormone" appears in the product labeling or its cosmetic ingredient declaration statement, as it implies drug effectiveness. This is especially so because all hormone ingredients used for cosmetics must not contain biological activity.

However, simply incorporating an active ingredient in a cosmetic product does not automatically move it to the drug category. The product only becomes a drug when the chosen active ingredient is well known for its therapeutic uses and when its name identified in the labeling leads the users to expect therapeutic effect. For the case of vitamins, FDA stipulates that the manufacturers should use the proper chemical names in the ingredient label so as not to imply any intended nutrient or health benefit and mislead the consumers. Doing so would make the products misbranded cosmetics. To maintain their cosmetic classification, the proper chemical names such as tocopheryl acetate (instead of vitamin E acetate), ascorbyl palmitate (instead of vitamin C palmitate) should be used in the ingredient statement. However, as long as the manufacturers do not make therapeutic claims and mislead the consumers into associating the products with health benefits, the manufacturers are allowed to list vitamins by their common names on the principal display panel of the package.[10,11]

16.2.1.3 Navigating the Unharmonized Global Cosmetic Regulatory Environment

The global definition of a therapeutic drug is quite similar, most agreeing that a drug is intended for the diagnosis, cure, mitigation, treatment, or prevention of diseases via some means of physiological action. But when it comes to cosmetics, differences exist in the major world markets including Australia, US, EU, Canada, and Japan.

In Australia, a cosmetic is defined as "a substance or preparation intended for placement in contact with any external part of the human body, including the mucous membrane of the oral cavity, and the teeth, with a view to altering the odours of the body; or changing its appearance; or cleansing it; or maintaining it in good condition; or perfuming it; or protecting it." Similar to the classification standard practiced in the US, a cosmetic product will be considered a therapeutic good if it intends to treat, alleviate, or prevent disease; or claims to affect the structure or functions of the human body or have therapeutic effects; or contains ingredients possessing therapeutic effects.[12] Two major factors are used to differentiate cosmetics from the therapeutic product—the composition of the product, and the proposed use and claims of the product. According to the third edition of Guidelines for Cosmetic Claims published by the Therapeutic Goods Administration on May 9, 1997, cosmetics may not make therapeutic claims unless they are listed in the Australian Register of Therapeutic Goods.

The Canadian Food & Drugs Act defines a cosmetic as "any substance or mixture of substances, manufactured, sold or represented for use in cleansing, improving or altering the complexion, skin, hair or teeth and includes deodorants and perfumes." Similar to the US FDA, Health Canada further stipulates that claims of physiological effect are not allowed for cosmetics. A cosmetic product making a therapeutic claim (e.g., to prevent or treat disease) will be classified as a drug under the Food and Drugs Act and a drug identification number (DIN) will be required.

The Japanese Pharmaceutical Affairs Law regulates all pharmaceuticals, quasi-drugs, cosmetics and medical devices in Japan. Its definition of a cosmetic is stated as "a substance with mild effect on the human body which is intended to be put on the human body for the purpose of cleansing, beautifying, enhancing the attraction, changing the appearance, or maintaining the skin or the hair healthy." This definition allows the cosmetics to have a mild effect on the human body.

In the European Union, the seventh amendment of the Cosmetic Directive (original directive 76/768/EEC) defines a cosmetic as "any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips, and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or keeping them in good condition." The directive further instructs in Article 7a,1(g) that a proof of effect claimed for the cosmetic product be kept readily accessible to the competent authorities of the member state. Based on this definition, one can expect the cosmetic products to show some "effect."

In terms of the breadth of definition and its interpretation, there are two camps. The US and Canada adopt the narrower definition while the EU and Japan take the broader view. In addition, the US places a substantially less amount of restrictions on ingredients and demands less safety testing requirement. Canada defines cosmetics in a similar fashion as the US but it imposes a far more extensive list of restrictions and prohibitions on ingredient selection. In contrast, the EU represents the other end of the spectrum; a broader definition accompanied by heavier control over the ingredients and product safety through various positive lists, restricted and

prohibited lists, and specific safety testing and data requirements. Japan has a similar definition as the EU but has a much narrower list of product categories. In addition, Japan provides a quasi-drug product category that imposes more requirements than it does for cosmetics but not up to the level required for drugs. The Japanese quasi-drug category includes hair dyes, acne prevention products, skin and oral disinfectants, bath preparations, and medicated cosmetics such as anti-dandruff shampoos and rinses, preparations to prevent minor dermatological disorders including rashes, frostbites, chaps, cracks, and pimples.

Even though the product classification standard may differ, the regulatory requirements for cosmetics are quite similar for these major markets in terms of demanding the manufacturer to carry the full responsibility of product safety, not requiring pre-market product approval and registration, establishing cosmetic-specific good manufacturing practice (GMP) industrial guidelines and imposing no restrictions on sales distribution channels.

16.2.1.4 Borderline Products in the Global Regulatory Environment

Borderline products are those that might fit in more than one product category due to overlapping regulatory definitions of different product categories within one country/region, or due to varying product classification standards by different countries/regions. For instance, a sunscreen product containing UV filters, regardless of what type, is considered an OTC drug in the USA. The situation in Canada is a little complicated since the introduction of the Natural Health Products Directorate (NHPD) in 2004. Under the NHPD, some products previously regulated as drugs under the Therapeutic Product Directorate (e.g., the Non-Prescription Drugs Category IV Monograph Products) are reclassified as natural health products (NHP). A sunscreen product may be regulated as an NHP or a drug, depending on the type of UV blockers used.[13] However, this type of product is regulated as a cosmetic in Japan and the EU as long as they adhere to specific ingredient control rules. Ordinance No. 331 of the Japanese Ministry of Health & Welfare and Annex VII of the EU Cosmetic Directive each provides a list of permitted UV filters for use in cosmetics. The following table lists a few examples of these borderline products:

Table 16.2.1.4.1 Examples of Product Classification in Major World Markets

Product	USA	Canada	EU	Japan
Anti-acne lotion	OTC drug & cosmetic	Category IV monograph product (Drug or NHP)	Medicinal product	Quasi-drug
Anti-caries toothpaste	OTC drug & cosmetic	Category IV monograph product (NHP)	Cosmetic	Quasi-drug
Anti-perspirant	OTC drug & cosmetic	Category IV monograph product (NHP)	Cosmetic	Quasi-drug
Hair dye	Cosmetic	Cosmetic	Cosmetic	Quasi-drug
Lipstick	Cosmetic	Cosmetic	Cosmetic	Cosmetic
Sunscreen	OTC drug & cosmetic	Category IV monograph product (drug or NHP)	Cosmetic (compliant with annex VII ingredients rule)	Cosmetic (compliant with ordinance #331 ingredients rule)

Source: Risk & Policy Analysts Limited, London, 2004; a summary of NHP/DRUG classification of TPD Category IV Labeling Standards Ingredients, Health Canada; Australian Government Department of Health & Ageing Therapeutic Goods Administration/ NICNAS, 2005.

Cosmeceuticals are another example of borderline products. The term, cosmeceuticals, was believed to have originated in the 1960s when Raymond E. Reed, then vice president of The Toni Co., published a paper entitled "The Definition of 'Cosmecutical" in the *Journal of the Society of Cosmetic Chemists.*[14] The term was made famous by Dr. Kligman later at a meeting for the Society of Cosmetic Chemists.[15] It has been widely used by the cosmetic industry to refer to cosmetics that also possess drug-like effects. Anti-aging products are among the fastest growing segment of the skin care market, many of them are claimed as cosmeceuticals, able to deliver rejuvenation benefits far beyond skin moisturization or merely covering up wrinkles. Countless new products spring up everyday to claim that they have incorporated the newest science in a bottle to not just retard

aging, but make it entirely optional. Consumers are bombarded with ads claiming enhanced cellular turnover rate, DNA repair, molecular energy renewal at the mitochondria level, and collagen synthesis stimulation, etc. These scientifically sounding claims share one common theme—the products are represented in such a way as to suggest that they do not simply offer "superficial" cosmetic benefits but actually provide meaningful age-reversing or—arresting effects.

However, USFDA flatly refuses to recognize the cosmeceuticals as a valid product class nor does it plan to include it as a sub category of cosmetics and warned it will apply the same "structure and function" standard to determine the classification of anti-aging products. If a product is intended for cosmetic use but its claims also suggest physiological (drug-like) properties, the product will be subjected to both drug and cosmetic regulations.[16] In a policy letter issued in 1987 to cosmetic companies, FDA stated that claims to have "an effect within the epidermis as the basis for a temporary beneficial effect on wrinkles, lines, or fine lines" are "unacceptable" drug claims. Words that describe a product to "retard," "counteract," or "control" aging, or to "rejuvenate," "repair," or "renew" the skin are considered drug claims. However, FDA would not object to claims that "products will temporarily improve the appearance of … outward signs of aging." Such claims are accepted by the FDA as cosmetic claims.[17] FDA's stance is further illustrated by the 1989 court case of *Estee Lauder v. United States* (727 F. Supp. 1).[18] It is acceptable to advertise the anti-wrinkle cream that "diminishes the appearance of fine lines" as a cosmetic, but when it claims to "remove fine lines" or to "reverse the aging process", it becomes an OTC drug. Under an inter-center agreement, both the Center for Drug Evaluation and Research (CDER) and Center for Food Safety and Applied Nutrition (CFSAN) may initiate regulatory actions against products that purport to be cosmetics but meet the statutory definition of a drug.[19]

Health Canada takes a similar stance on cosmeceuticals as the USFDA. It does not recognize it as a legitimate product category. The product will be regulated either as a cosmetic or a drug depending on the claims it makes and/or the composition of the product. A table of acceptable cosmetic claims is provided in the Guidelines for Cosmetic Manufacturers, Distributors and Importers posted in the official website of Health Canada:[20]

Table 16.2.1.4.2 Canadian Cosmetic Claims

Cosmetic	Acceptable Claim	Unacceptable Claim
Moisturizer	Softens skin	Heals skin
Contour cream	Reduces the look of cellulite	Lose inches; slims/slimming
Acne-prone skin product	Removes oil	Stops acne
Mouthwash	Helps eliminate odor-causing bacteria	Kills odor-causing germs
Fragrance	Soothes	Causes hormonal attraction
Anti-aging/anti-wrinkle product	Helps prevent the look of aging	Eliminates wrinkles

The Australian National Coordinating Committee on Therapeutic Goods gave the following examples as industrial guidance for anti-aging cosmetic product claims:[21]

- Acceptable Wording for Cosmetics:
  - Cover up or hide age spot or blemishes, dark pigmented areas.
  - Feel younger or look younger.
  - Helps prevent or reduce or slow the signs or appearance of aging.
  - Moisturize aging skin.
  - Smooth wrinkles.
- Unacceptable Wording for Cosmetics unless Sufficiently Modified to Provide a Cosmetic Implication:
  - Anti-aging.
  - Temporarily reduces depth of wrinkles by moisturization.
- Unacceptable Wording for a Cosmetic (but not necessarily acceptable for a drug):
  - Eliminates or prevents or stops or reduces or slows or reverses aging, wrinkles, premature aging, or aging process.
  - Any references to fading age spots or de-pigmentation, skin bleaching, etc.

In the EU, anti-aging products were among the thirty-two "borderline cosmetics" listed by the Council of Europe Publishing in 2000.[22] These

products are not adequately covered by the EU Cosmetic Directive and can be regulated by each different member state differently as consumer products or cosmetics or even drugs, depending on the claims and ingredient used, essentially creating a regulatory and marketing complication for the industry.

16.3 Relevant Global Regulations Governing Personal Care Products

Most personal skin care products fall into the cosmetic product category by design. The reason is several-fold:

- Most products are intended for cosmetic use as defined by the regulations;
- Less complicated regulatory framework and requirements;
- Relative ease in product development in terms of cost and time and more reliance on voluntary industry self-regulating responsibilities.

This chapter is written with a focus on the US cosmetic regulation while also attempting to provide the readers with a regulatory framework for comparing and contrasting of the US drug regulation to other cosmetic regulations in the major global markets.

16.3.1 Brief Overview of US Drug Regulations

In the United States, prescription drug products require pre-market approval from the FDA and are generally regulated under the jurisdiction of FDA's Center of Drug Evaluation & Review. In general, before a new drug can be allowed to be tested in any human clinical setting, it would have gone through extensive preclinical research as well as a substantial amount of preclinical toxicological and pharmacological testing that can take four to eight years to collect the information necessary for new drug approval. Drug companies are required to register both the establishments and products with FDA in compliance with 21 CFR 207. Prior to entering the clinical testing phase, the drug sponsor must submit the investigational new drug (IND) application to FDA for approval to proceed. IND requires an extensive submission of preclinical data for FDA to conduct a thorough review of all pertinent medical, chemical, pharmacological, toxicological, and statistical data. When the approval is given, clinical testing can commence.

Once the safety and effectiveness of the drug is established through the clinical testing, the drug sponsor may submit the new drug application (NDA) to request FDA approval to market the new drug in interstate commerce. FDA sets further instructions and limitations on how the product should be packaged, labeled, advertised, and promoted. Promotion of unapproved investigational products is usually prohibited unless it falls well within the limited areas granted for pre-approval promotion. CDER sets very strict rules for mandatory post-approval surveillance including reporting of changes and adverse event reporting, and current good manufacturing practices (cGMP).[23,24] The entire process from initial preclinical research to the final market approval can take decades and millions of dollars of investment.

Non-prescription OTC drugs fall under the jurisdiction of FDA's Division of Over-the-Counter Drug Products. In general, there are two ways to market OTC drug products. The first is the NDA path similar to the prescription new drug approval process described above: a pre-approval application is required, each application is product-specific and confidential, clinical studies may be necessary and post-approval maintenance (such as strict reporting requirements for adverse effects) is mandatory. When approved, the manufacturer obtains the individual license to market and may also enjoy marketing exclusivity. The second path is marketing under the OTC drug monograph process which is active ingredient-specific, but not product-specific. This is a simpler and more cost-effective process as pre-approval is not required and clinical studies may not be necessary. However, there is no marketing exclusivity as the final monograph is open to the public; the labeling is essentially the same for all similar products.

In summary, a US drug product needs to satisfy all of the following regulatory requirements:

- Mandatory NDA approval or compliance with the OTC monograph.
- User fees usually required.
- Mandatory registration for drug establishments and products.
- Extensive preclinical and clinical testing to prove both safety and effectiveness.
- Mandatory cGMP with strict rules of compliance. Failure to comply will cause a drug to be deemed adulterated.
- Specific labeling and advertising requirements, such as OTC-specific "Drug Facts" labeling, as described in 21 CFR 201.63,

or a combination OTC drug/cosmetic labeling including the Drug Facts panel and the prescribed order of listing of the active and inactive ingredients.
- Strict and mandatory post-market maintenance requirements.

16.3.2 Overview of US Cosmetic Regulations

The Division of colors and Cosmetics within FDA's CFSAN is the regulatory authority for cosmetic products. Except for color additives which must be pre-approved, FDA does not require pre-market approval of cosmetics. The registration of cosmetic establishments and ingredient information, though expected, is a voluntary program called voluntary cosmetic registration program (VCRP) described in 21CFR 710 and 721. It is not an FDA requirement. FDA does not have the authority to require safety testing of cosmetics by their manufacturers. However, FDA strongly urges the manufacturers to establish the safety of their products via appropriate safety testing. FDA requires a warning statement on the label if the safety of the product has not been adequately substantiated. The Center for Food Safety and Applied Nutrition office maintains a post-marketing Adverse Events Reporting System called CAERS. CAERS is used by the FDA to notify companies of any reported illness or injury associated with the use of their products. FDA intends to use CAERS to formulate post-marketing policies regarding cosmetic products. Two main provisions of the FD&C Act that apply to cosmetics are adulteration and misbranding. The adulteration provision addresses the composition of the product, how the product is manufactured, stored and shipped, and the product container.[25] There are four conditions where a cosmetic may be considered adulterated:

- The product contains a potentially harmful substance (in the product itself or the container) that may cause injuries to the consumers upon normal use;
- The product contains filth;
- The product is contaminated with filth due to unsanitary manufacturing or storing conditions;
- The product contains a non approved color additive.

The provision of misbranding focuses on the representation of the product.[26] Reasons to cause a product to be considered misbranded could be any of the following:

- The labeling is deemed false or misleading (including what is said and what is not revealed).

- Required labeling information and its appropriate placement is not included in product labeling.
- The container is filled or represented in a manner FDA considered deceptive.
- Improper packaging and labeling of color additives.

However, FDA bears the burden of proof. If FDA intends to classify a cosmetic as adulterated and remove it from the market place, it must first prove in a court of law that the product may be injurious to users. FDA can consider a cosmetic misbranded if it determines that the labeling is false and misleading or does not bear the correct, required information and warning statements. FDA has no authority to order product recalls, but it can request the company to do so and recommend recall strategies. Once the company initiates the recall, FDA may actively monitor its progress.

The US cosmetic regulations can be summarized below:[27–28]

- FDA can only take post-market enforcement actions. There are no pre-market approval requirements for either the product or the ingredients, except for color additives.
- There are no user fees requirements.
- VCRP is strongly encouraged by the FDA but not required.
- There is no mandatory safety testing requirement. The manufacturer is fully responsible to ensure the safety of each ingredient and finished product.
- GMP is strongly encouraged but not a regulatory requirement.
- FDA oversees cosmetic labeling. Cosmetic labeling requirements are listed in the table below. These are very specific requirements governing many labeling design details including the product identity display, the placement of the name and place of business, appropriate ingredient listing, an accurate statement of the net quantity of contents, appropriate directions for safe use, and appropriate warning statements when deemed necessary. Labeling is defined to include all written, printed, or graphic material that appears on the products, containers, packaging inserts, and any material accompanying the product. So effectively, any promotional material and statements including those appear on the Internet, product catalogs and flyers are considered cosmetic product labeling. These requirements are regulated under two main regulations:

the FD&C Act and the Fair Packaging & Labeling Act (FPLA). FDA's main enforcement focus for cosmetic labeling is on "misbranding." Incorporating drug claims in the cosmetic labeling causes the product to be considered misbranded and will lead to FDA enforcement action.

Table 16.3.2.1 US Cosmetic Labeling Requirements

	Outer Container	**Inner Container**	**Relevant Regulation**
Product identity statement	Required in the principal display panel	Not required	FPLA
Net quantity of contents	Required in the principal display panel	Required	FPLA FD&C Act
Name & place of business	Required	Required	FPLA FD&C Act
Ingredient declaration	Required	Not required	FPLA
Warning statements	Required	Required	FD&C Act
Direction for use	Not required	Not required	FD&C Act

- Cosmetic consumer product advertising is regulated by the following mechanisms:
  - Federal law, such as the Federal Trade Commission Act (FTC Act) enforced by the Federal Trade Commission (FTC). The FTC Act prohibits unfair or deceptive practices to influence the consumers.[29] It sets the definition of false advertising and prohibits false advertising for cosmetics.[30,31] The unfair or deceptive advertising the FTC targets includes both printed and broadcasted matters such as those appearing in the newspapers, magazines, television and the Internet.
  - State law and local enforcement such as the federal Mail Fraud Act that is actually enforced by the office of the Attorney General of each individual state.[32]

- Voluntary industry framework: the National Advertisement Division (NAD) of the Council of Better Business Bureaus acts as the industry's independent self-regulatory body to monitor the truth and accuracy of national advertising for both broadcast (including the Internet) and printed matters. The NAD works in conjunction with the National Advertising Review Board (NARB): the NAD investigates complaints and determines the appropriate substantiation needed for advertised claims. If an advertiser is not satisfied with the NAD decision, it can appeal to the NARB for reconsideration.[33]
- Private judicial actions under the Lanham Act targeting false and misleading advertising. The injured parties can bring private suits and seek monetary or other compensations for the resulting injuries.[34]

- Cosmetics are currently grouped into thirteen product categories by the FDA. Cosmeceuticals are not a FDA-recognized cosmetic or drug product classification. The official FDA cosmetic product category codes and description are given below:

Table 16.3.2.2 USFDA Cosmetic Product Categories

Code	Category Description	Product Examples
01	**Baby Products**	Baby Shampoos Lotions, Oils, Powders, and Creams Other Baby Products
02	**Bath preparations**	Bath Oils, Tablets, and Salts Bubble Bath Bath Capsules Other Bath Preparations
03	**Eye makeup preparations**	Eyebrow Pencil Eyeliner Eye Shadow Eye Lotion Eye Makeup Remover Mascara Other Eye Makeup Preparations

Code	Category Description	Product Examples
04	**Fragrance preparations**	Cologne and Toilet Waters Perfumes Powders (dusting and talcum, excluding aftershave talc) Sachets Other Fragrance Preparations
05	**Hair preparations (non-coloring)**	Hair Hair Spray (aerosol fixatives) Hair Straighteners Permanent Waves Rinses (non-coloring) Shampoos (non-coloring) Tonics, Dressings, and Other Hair Grooming Aids Wave Sets Other Hair Preparations
06	**Hair coloring preparations**	Hair Dyes and Colors (all types requiring caution statements and patch tests) Hair Tints Hair Rinses (coloring) Hair Shampoos (coloring) Hair Color Sprays (aerosol) Hair Lighteners with Color Hair Bleaches Other Hair Coloring Preparations
07	**Makeup preparations (not eye)**	Blushers (all types) Face Powders Foundations Leg and Body Paints Lipstick Makeup Bases Rouges Makeup Fixatives Other Makeup Preparations
08	**Manicuring preparations**	Basecoats and Undercoats Cuticle Softeners Nail Creams and Lotions Nail Extenders Nail Polish and Enamel Nail Polish and Enamel Removers Other Manicuring Preparations

(*Continued*)

Table 16.3.2.2 USFDA Cosmetic Product Categories (Continued)

Code	Category Description	Product Examples
09	**Oral Hygiene Products**	Dentifrices (aerosol, liquid, pastes, and powders) Mouthwashes and Breath Fresheners (liquids and sprays) Other Oral Hygiene Products
10	**Personal Cleanliness**	Bath Soaps and Detergents Deodorants (underarm) Douches Feminine Deodorants Other Personal Cleanliness Products
11	**Shaving Preparations**	Aftershave Lotion Beard Softeners Men's Talcum Pre-shave Lotions (all types) Shaving Cream (aerosol, brushless, and lather) Shaving Soap (cakes, sticks, etc.) Other Shaving Preparations
12	**Skin Care Preparations (creams, lotions, powders, and sprays)**	Cleansing (cold creams, cleansing lotions, liquids, and pads) Depilatories Face and Neck (excluding shaving preparations) Body and Hand (excluding shaving preparations) Foot Powders and Sprays Moisturizing Night Paste Masks (mud packs) Skin Fresheners Other Skin Care Preparations
13	**Suntan Preparations**	Suntan Gels, Creams, and Liquids Indoor Tanning Preparations Other Suntan Preparations

16.3.3 Comparison of Cosmetic Regulations in Major Global Markets[35–36]

16.3.3.1 Definition of Cosmetics and Corresponding Regulatory Agency

Country/ Region:	Regulatory Agency:	Definition of Cosmetics:	Regulatory Citation:
Canada	Health Canada	"*any substance or mixture of substances, manufactured, sold or represented for use in cleansing, improving or altering the complexion, skin, hair or teeth and includes deodorants and perfumes*"	Food and Drug Act[37]
European Union	Overarching directive to EU member states	"*any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odors and/ or protecting them or keeping them in good condition.*"	7th Amendment (Directive 2003/15/EC[38]) of the original Council Directive 76/768/EEC[39]
Japan	Ministry of Health, Labor, and Welfare	"*A substance with mild effect on the human body which is intended to be put on the human body for the purpose of cleansing, beautifying, enhancing the attraction, changing*	Pharmaceutical Affairs Law[40]

16.3.3.1 Definition of Cosmetics and Corresponding Regulatory Agency (Continued)

Country/ Region	Regulatory Agency	Definition of Cosmetics	Regulatory Citation
		the appearance, or maintaining the skin or the hair healthy."	
USA	Food and Drug Administration	"*articles intended to be rubbed, poured, sprxinkled, or sprayed on, introduced into, or otherwise applied to the human body…for cleansing, beautifying, promoting attractiveness, or altering the appearance*"	Food, Drug and Cosmetics Act[41]

16.3.3.2 Pre-Market Requirements

The global cosmetic legislative trend has shifted from pre-market product approval to in-market surveillance. The information necessary for pre-market notification, if required, has also been significantly reduced.

Country	Product Notification	Establishment Registration
Canada	Mandatory: ○ Product name & function ○ Quantitative or semi-quantitative ingredients list	Mandatory: ○ Manufacturer ○ Importer
European Union	○ Not required by EU ○ May be requested by individual member state	Mandatory
Japan	Mandatory: ○ Product name	Mandatory
USA	Voluntary	Voluntary

16.3.3.3 Testing Requirements

Country	Safety & Efficacy Data	Animal Testing
Canada	○ Manufacturer fully responsible for product safety ○ No prescribed specific tests ○ Safety must be proven upon request	Allowed
European Union	○ Manufacturer fully responsible for product safety ○ Testing guidelines established by the SCCNFP ○ Manufacturer must maintain a product information file (PIF), and ○ PIF must be available at all times upon request by competent authorities	Banned by the 7th Amendment
Japan	○ Manufacturer fully responsible for product safety ○ No prescribed specific tests ○ Rely on industrial guideline ○ Safety/efficacy proof must be available from the manufacturer	Allowed
USA	○ Manufacturer fully responsible for product safety ○ No prescribed specific tests ○ Rely on industrial guidelines ○ A mandatory warning statement stating "The safety this product has not been determined" must appear on the label if the manufacturer can not provide proof of product safety	Allowed

16.3.3.4 Ingredients Control Requirements

Country	Scientific Advisory	Positive & Negative Lists
Canada	Government officials	○ List of prohibited substances ○ The Cosmetic Ingredient "Hotlist"
European Union	SCCNFP	○ Annex II: Prohibited substances (negative list) ○ Annex III: Restricted substances (restricted list)

(*Continued*)

16.3.3.4 Ingredients Control Requirements (Continued)

Country	Scientific Advisory	Positive & Negative Lists
		○ Annex IV: Permitted cosmetic colorants (positive list) ○ Annex VI: Permitted cosmetic preservatives (positive list) ○ Annex VII: Permitted UV filters (positive list)
Japan	Government officials & the Cosmetic Advisory Committee	○ Prohibited substances (negative list) ○ Restricted substances (restricted list) ○ Permitted coloring agents (positive list) ○ Permitted cosmetic preservatives (positive list) ○ Permitted UV filters (positive list) ○ Ingredients of quasi-drugs
USA	CIR committee, a voluntary industrial expert committee	○ List of prohibited & restricted substances ○ List of approved color additives

16.3.3.5 GMP Requirements

Country	Source of Guidelines	Requirement
Canada	Industry guidelines	Voluntary
European Union	COLIPA & EU guidelines	Voluntary
Japan	Industry guidelines	Voluntary
USA	Industry guidelines	Voluntary

16.3.3.6 Labeling Requirements

Country	INCI Name	Quantity Labeling Sys	Expiration Date	Required Business Info
Canada	Mandatory	○ Metric system[a] mandatory ○ Non-metric systems allowed as supplementary	Not required	○ Name & address of mfg or dealer ○ Non-Canadian address is accepted

Country	INCI Name	Quantity Labeling Sys	Expiration Date	Required Business Info
European Union	Mandatory	○ Metric system mandatory ○ "Metric-only" labeling for all EU products on January 1, 2010 ○ Non-metric systems allowed as supplementary till 2009	○ Date of minimum durability required if ≤ 30 months ○ Period of opening required if > 30 months	○ Name & address of person placing the product on market
Japan	Japanese translation of INCI names required	○ Metric system mandatory	Required if shelf-life < 3 years	○ Person responsible for placing product on market
USA	Mandatory	○ Both metric and non-metric systems mandatory	Not required	○ Name & address of business marketing the product

[a]Metric system—The International System of Measuring units (SI), modified for use in the United States by the Secretary of Commerce. A complete list of SI units of the metric system can be found in Federal Standard 376A.

16.4 Future Regulatory Trends and Challenges

16.4.1 Non-governmental Organizations (NGOs) Becoming the Fifth Branch of Government

The World Bank defines NGOs in its Operational Directive 14.70 as "private organizations that pursue activities to relieve suffering, promote the interests of the poor, protect the environment, provide basic social services, or undertake community development." Even though the current data is not complete, the total number of international NGOs estimated by a 1995 UN report on global governance was at close to 30,000 and increasing. By definition, NGO is not founded or funded by states and is

not part of any government. Though not restricted by law, it is generally not for profit and dedicated mainly to social, economical, environmental, and cultural advocacy. Two defining principles of NGO are altruism and volunteerism. There are two main types of NGOs, operational NGOs and advocacy NGOs. The former seek to design, develop, and implement projects for social, economic, or environmental improvement. The latter are dedicated to influencing legislation or policy making via defending or promoting a certain cause, many of them are dubbed the "single issue" NGOs where the entire organization is dedicated to one specific cause such as human rights, animal rights and protection, or sustainable environment. NGOs usually have strong grassroots commitment and extensive field work expertise; they are also quite innovative and savvy in communicating to the general public and through the media. Through the grass-root connections, they speak directly to the consumers and often become the trend setters in raising important socioeconomic issues that force the change of the political atmosphere and lead into eventual legislative corrections. This section will briefly describe the advocacy NGOs and their increasingly important role and ability to influence global legislation.

In both the EU and the US, the legislative bodies have traditionally sought the input of business-interest NGOs and/or national trade organizations on proposed legislation. Such NGOs include the Cosmetic, Toiletry, and Fragrance Association (CTFA) of the United States, the European Cosmetic Toiletry and Perfumery Association (COLIPA), and the Cosmetic, Toiletry, and Perfumery Association (CTPA) of the United Kingdom. These organizations focus on industry-specific scientific, regulatory and legislative issues and engage in a constant dialogue with the government to help shape cosmetic-related legislation and policy.

In recent years, non-business NGOs have gained importance in sharing the influence. One recent example centers on the successful lobbying campaign launched by the animal rights advocacy groups that convinced the European Parliament to include the animal testing ban provisions in both the latest amendment of the Cosmetics Directive and the proposed chemical regulations, REACH. From the domestic front, the following table of recently proposed California chemical legislation and sponsors and/or supporters further illustrates the influence of NGOs in setting future legislative trends.

Table 16.4.1.1 Cosmetic-Related Californian Legislation

California Legislation	Relevant Provision in Proposed Bill Affecting Cosmetics	Examples of NGO Co-Sponsors or Supporters
AB 2012[42]	Prohibited the manufacturing, processing or distributing in California of any cosmetic or personal care product containing phthalates	• The National Environmental Trust • The Breast Cancer Fund • The Breast Cancer Action • The Environmental Working Group
AB 908[43]	Banned from cosmetics the use of one particular phthalate: Dibutyl phthalate	• The National Environmental Trust • The Breast Cancer Fund • Center for Environmental Health • Sierra Club – California Asian Americans for Civil rights and Equality • Environmental Justice Coalition for Water • Natural Resources Defense Council • Asian Health Services • Asian Pacific Environmental Network • Asian Communities for Reproductive Justice • Asian Immigrant Women Advocates • Association of Asian Pacific Community Health Organizations • California Nurses Association • Women's Foundation of California
SB 484[44]	Required cosmetic manufacturers to provide to State a list of its cosmetic products that contained any chemical ingredient identified as causing cancer	• The National Environmental Trust • The Breast Cancer Fund • The Breast Cancer Action • Center for Environmental Health • Environmental Justice Coalition for Water • Natural Resources Defense Council • Sierra Club – California Asian Americans for Civil rights and Equality

(*Continued*)

Table 16.4.1.1 Cosmetic-Related Californian Legislation (Continued)

California Legislation	Relevant Provision in Proposed Bill Affecting Cosmetics	Examples of NGO Co-Sponsors or Supporters
SB 484[44]	or reproductive toxicity, including non incidental ingredients contained in trace amounts	• Asian Communities for Reproductive Justice • Asian Health Services • Women's Foundation of California • California Women Lawyers • California Commission on the Status of Women • Environment California

16.4.2 Green Chemistry Legislative Trend

The Green Chemistry movement in the US originated in the 1960s following a series of increasingly disastrous environmental contamination by kepone, heavy metals, vinyl chloride, polychlorinated biphenyls, and chlorofluorocarbons. The Toxic Substances Control Act (TSCA) was enacted in 1976 as Congress took note of the public sentiment and recognized the "high-priority need for a program of testing and control of toxic substances."[45] Congress was particularly concerned about the lack of information regarding some widely used chemicals and wanted TSCA to provide an upstream protection mechanism against the introduction of any potentially dangerous new chemicals via a strong emphasis on "products" regulation, rather than waste control. Instead of focusing merely on toxic chemicals, via TSCA, Congress went one step further to grant EPA jurisdiction over *all* chemical substances and mixtures (excluding those specified under pesticide, food and drug or other similar federal regulations). For this, EPA was provided with substantial tools associated with regulating the manufacture (including importing), processing, distribution in commerce, use, or disposal of chemical substances. These tools include the inventory of chemical substances, new chemical review procedures, testing requirement of existing chemicals, protection against unreasonable risk, reporting and record-keeping requirements, import/export requirements, etc.[46] From the global standpoint, TSCA has many equivalent international counterparts, all aimed at similar goal of providing better protection of the public and environmental health through a thoroughly reviewed and controlled chemical regulatory system throughout the entire lifecycle of a product. The following table lists some of those international chemical regulations.

Table 16.4.2.1 International TSCA Counterparts

Country	Government Agency	Examples of International TSCA Counterparts & Regulatory Components
Australia	NOHSC	Industrial Chemicals Notification & Assessment Act 1989 (ICA) National Industrial Chemicals Notification & Assessment Scheme (NICNAS) Australian Inventory of Chemical Substances (AICS)
Canada	Environment Canada	Domestic Substance List (DSL) Export Control List Labeling Requirements beyond MSDS National Pollutant Release Inventory Non-Domestic Substance List (NDSL) New Substance Notification (NSN) Significant New Activity (SNA) Priority Substances List (PSL) Toxic Substances List
Europe	European Commission	Registration, Evaluation & Authorization of Chemicals (REACH)
Japan	METI & MHLW	Existing & New Chemical Substance Inventory (ENCS) Industrial Safety & Health Law Inventory (ISHL) New Chemical Substance Notification (NCSN)

In recent years, the Green Chemistry movement has continued to evolve to now include twelve principles that focus on pollution prevention and reduction and/or elimination of hazardous substances throughout the product lifecycle.[47] Contrasting to earlier practice, the regulatory agencies now encourage government—industry partnerships to jointly protect the health of the general public, instead of relying mainly on punitive enforcement actions against violations.

Through mass media coverage and community activism of many environmental NGOs, consumers are more informed of chemical usage in their daily life and demand a much higher level of protection via proactive chemical and product regulations. Although cosmetics do not fall within the jurisdiction of most of these international chemical regulations, recent trends indicate that the chemical legislative initiatives are often incorporated into laws and regulations that have impact on the cosmetic industry.

One good example is the consumer products volatile organic compounds (VOCs) regulations. VOCs can be found in almost all products people use daily. They are considered a major source of ozone, a hazardous air pollutant affecting the normal function of the lung in many healthy humans at ground level. In order to improve indoor air quality, California Air Resources Board (CARB) and several northeastern states of the Ozone Transport Commission are regulating the levels of VOCs of many consumer products (including many cosmetics). Personal care products targeted by CARB to face a higher VOCs limit include personal fragrances, aftershave/personal fragrances, nail coatings, temporary hair color sprays, astringents/toners, hand sanitizers, and hairsprays (both pumps and aerosols).[48,49]

The REACH chemical program represents the European answer to the harmonization of chemical regulations and the ultimate Green Chemistry legislative movement. The aim of REACH is to ensure a high level of protection of human health and the environment while enhancing the competitiveness of the EU chemicals industry. REACH is founded on the Precautionary Principle, with special attention to the intrinsic hazard of the chemicals. Four separate EU directives & regulation constitute the legal foundation of REACH:

- Directive 67/548/EEC (classification and labeling of dangerous substances).
- Directive 88/379/EEC; revised by Directive 1999/45/EC (classification and labeling of dangerous preparations).
- Regulation EEC 793/93 (evaluation and control of the risks of existing substances).
- Directive 76/769/EEC (restrictions on the marketing and use of certain dangerous substances and preparations).

REACH stands for registration, evaluation and authorization of chemicals; it creates one single regulatory system for all chemical substances within the EU member states. Before REACH, there were over forty separate chemical regulations in effect in the EU, each with a different set of requirements for chemical control. Once the harmonized REACH comes into effect on June 1, 2007, all chemical substances involved in commerce, unless exempt, are covered throughout the entire EU under one single chemicals policy. Anyone who produces, imports, or uses a regulated substance at one or more metric ton a year is regulated by REACH. Medical products, food, cosmetics, and pesticides are among those exemptions as they are regulated under other directives. However, provisions under

REACH could still have potential impact on cosmetic ingredients in terms of substance registration, down stream user-related activities, substance evaluation, restrictions (such as category I and II Carcinogenic, Mutagenic & Reproductive Toxins), R&D exemption, and environmental controls.[50–51]

Green Chemistry push also comes from the academia such as a 2006 report from the University of California, Berkeley, prepared for the California Senate Environmental Quality Committee, entitled "Green chemistry in California: A Framework for Leadership in Chemicals Policy and Innovation."[52] This report urges California legislature to adopt an EU approach to chemical policy not dissimilar to the future EU REACH program. The influence of non-business NGOs on future cosmetic and chemical legislation toward the "Green Chemistry" and "Precautionary Principle" direction, especially through the joint forces of the academic, environmental and the minority groups, is quite evidenced and can not be overlooked.

16.4.3 The Situation of Ever Creeping Anti-aging Product Claims and the Weakened FDA

FDA's main mission is to ensure cosmetic products are safe and properly labeled (differentiation between drug and cosmetic). Its regulatory authority comes from both the FD&C Act and the FPLA. As cosmetics increasingly take on the role of drugs (i.e., cosmeceuticals claims including anti-aging effect), a growing population is urging for increased FDA regulation of cosmetics to protect consumers from unsafe and/or untested products and from deceptive practice. Added to this confusing state is the creeping "organic" and/or "natural" product claims where there is currently no clear regulatory definition, nor is there a designated or collaborative competent authority to oversee the product regulation.[b] Some argue that creating a cosmeceuticals category would help FDA better safeguard consumer health by granting FDA authority to impose pre-market approval and more extensive safety testing requirements.

FDA's cosmetic regulatory branch has been seriously weakened in recent years due to significant budget constraints, limited resources and the need to place more priority over other high profile issues such as AIDS, bioterrorism against food, medical device, and prescription drug safety. FDA

[b]The USDA currently oversees the "organic" product claims and is trying to develop policy and enforcement rules.

must resort to allocating its resources to handle the most pressing and life-threatening public health issues. Cosmetics generally are not likely to cause serious adverse effects and traditionally have been relatively safer than food, drugs, and medical devices. As a result, the cosmetic industry has not experienced major FDA enforcement actions against extravagant anti-aging product claims.

However, recent warning letters issued to Basic Research LLC (January 20, 2005)[53] and University Medical Product USA Inc. (January 22, 2004)[54] may indicate a new trend in future FDA enforcement actions. In both cases, FDA stated that certain claims were considered structure/function claims and the cited products were not generally recognized as safe and effective for the intended use, causing the products to be considered as unapproved new drugs. These new drugs may not be marketed without prior FDA NDA approval. Cited by FDA for violation include some well-known products such as StriVectin-SD, FACE LIFT Collagen 5 products, FACE LIFT Daytime Advanced Retinol-A, Nighttime Advanced Retinol-A, Advanced Under Eye Therapy, Vitamin C Anti-Wrinkle Patch, etc. The following table lists some of the "structure/function" drug claims cited by FDA as inappropriate for cosmetic products.

Table 16.4.3.1 Cosmetic Products Cited for "Structure/Function" Drug Claims

Cited Product	Examples of Structure/Function Claims Cited by FDA
StriVectin-SD	• "Clinically Proven to Dramatically Reduce the Appearance of Existing Stretch Mark Length, Depth, Texture, and Discoloration" • "A stretch-mark reducing emulsion...to diminish fine lines, wrinkles and crow's feet." • "[S]uperior wrinkle-reducing properties of a patented oligo-peptide (called Pal-KTTKS)...on 'photo-aged skin'...[A] key ingredient in the StriVectin cream." • "[S]ignificant improvement' in wrinkle depth, length, wrinkle volume…" • "StriVectin-SD actually increases the synthesis of new collagen (StriVectin-SD increases collagen I synthesis by 117%, increases collagen IV synthesis by 357%, and increases glycosaminoglycan synthesis by 267%), making your skin; thicker and firmer."

Collagen5™	• "is proven to reduce deep wrinkles up to…70%" • "Stimulates your skin's own collagen building network" • "Reduces deep wrinkles from within the skin's surface…" • "Visible results that won't fade away…"
Vitamin C Anti-Wrinkle Patch™	• "Vitamin C helps reduce the effects of aging…by helping to strengthen collagen and elastin fibers" • "Clinical studies proved a 50% reduction in wrinkles…"

16.5 Successful Product Development Strategy

Apparently, the confusing and often contradicting global regulatory definitions and requirements can lead to a marketing nightmare, especially at a time when skin care product developers enjoy an unprecedented accessibility to new technologies and exotic ingredients that promise to help them formulate the best ever age defying products. In this highly regulated and inter-related modern world, a company can not measure its success simply via its ability to come up with the most scientifically advanced formula with measurable and effective skin care benefits. The biggest challenge currently faced by the cosmetic industry is how to walk the fine line considering the following:

- What we would like to claim for marketing advantages,
- What we can truthfully say from the scientific point of view,
- What the consumers will perceive as believable,
- What our competitors are claiming for their products, and
- What we are allowed to say under the regulations.

16.5.1 Advanced Marketing and Claims Substantiation Planning—Regulatory Affairs Meets Marketing and Product Positioning

Product developers and marketers have in their arsenal a diverse variety of market research tools to help them narrow down the playing field. They include market trend analyses, Internet consumer surveys, consumer habits and practices studies, preference surveys, and use tests. These studies have a proven track record in helping the company identify the most consumer appealing language that forms the best product selling strategy.

From the regulatory stand point, product claims and advertisement need to be truthful and not misleading. A 1983 FTC policy statement announced that substantiation needs to pass the "reasonable basis of support" requirement for both the express and implied claims. FTC uses a number of factors to determine the extent of reasonable basis for substantiation, including the type of product, type of claims made, the benefits of a truthful claim, the consequences of a false claim, the ease of developing substantiation, and what experts consider as adequate substantiation.[55]

It is crucial to obtain comprehensive prior understanding of global regulatory definitions, requirements and restrictions to ensure smooth sailing of any product marketing scheme. Obviously, additional tools need to be incorporated into the early phase of product development when product positioning has started the exploration effort and while initial marketing scheme is being scoped out. Regulatory analysis tools are useful in identifying the most feasible regulatory product classification path that is compatible with the overall marketing goal. The following is a list of some of the readily available regulatory analysis tools.

- FDA Cosmetic Handbook.
  http://www.cfsan.fda.gov/~dms/cos-hdb1.html
- Official governmental websites for pertinent regulatory affairs information:
  - Agreement on the ASEAN Harmonized Cosmetic Regulatory Scheme:
    http://www.thaicosmetic.org/documents/1agreement.pdf
  - Australia Regulation of Cosmetic Chemicals: Final Report and Recommendations 2005:
    http://www.nicnas.gov.au/Cosmetics/Regulation_Cosmetic_Chemicals_Final_Report_PDF.pdf
  - Canada Cosmetic Regulations:
    - Food & Drug Act:
      http://laws.justice.gc.ca/en/ShowFullDoc/cs/F-27//20070321/en?command=search&caller=SI&fragment=cosmetic&search_type=all&day=21&month=3&year=2007&search_domain=cs&showall=L&statuteyear=all&lengthannual=50&length=50
    - Other Cosmetic Topics:
      http://www.hc-sc.gc.ca/cps-spc/pubs/indust/cosmet_guide/act-loi_e.html

  - EU Cosmetic Directive:
    http://ec.europa.eu/enterprise/cosmetics/html/consolidated_dir.htm
  - Japan Pharmaceutical Affairs Law:
    http://www5.cao.go.jp/otodb/english/houseido/hou/lh_02070.html
  - Korea Food & Drug Administration:
    http://www.kfda.go.kr/
  - United Kingdom The Cosmetic Products (Safety) Regulations 2004:
    http://www.opsi.gov.uk/SI/si2004/20042152.htm
  - US Cosmetic Regulations:
    - Food, Drug & Cosmetic Act:
      http://www.fda.gov/opacom/laws/fdcact/fdctoc.htm
    - Other Cosmetic Topics:
      http://www.cfsan.fda.gov/~dms/cos-toc.html
  - Taiwan Statute for Control of Cosmetic Hygiene:
    http://www.doh.gov.tw/ufile/doc/200406_Statute%20for%20Control%20of%20Cosmetic%20Hygiene.doc
- CTFA International Cosmetic Legal & Regulatory Database
  http://www.ctfa-international.org/

From these websites, one can search for information on acceptable cosmetic claims, definition, and regulatory allowance for borderline products, as well as risk and benefit analyses of marketing borderline products in regulatory uncertainty.

16.5.2 Ingredient Selection Strategy for Anti-aging Products

16.5.2.1 Basic Product Development Principle

Through decades of scientific research and clinical studies, it has been established that there are two major contributors to aging: intrinsic factor such as genetically programmed chronological aging and extrinsic factors mainly due to environmental stressors such as photo damage, pollution, climate changes, and cigarette smoke. Intrinsic aging causes decrease in epidermal cell renewal rate, reduced skin barrier function, and dyschromic changes that lead to the appearance of fine wrinkles, mottled skin pigmentation, and freckles. It also causes the skin to become dry and thin. Chronic unprotected sun exposure is the most significant factor in extrinsic aging. Unlike intrinsic aging when the most adverse effect is mainly the appearance,

extrinsic aging can lead to severe adverse health consequences including photocarcinogenesis.[56] Topical application of UV blockers has long been established as the best practice of photoprotection. Properly formulated, topically applied antioxidants such as vitamins C and E, separately or synergistically combined had demonstrated their ability to provide protection against UV-induced damage.[57,58] Tretinoin (all-trans-retinoic acid, a derivative of vitamin A molecule) was the first FDA-approved medical treatment for photodamage caused by extrinsic aging. Numerous multicenter, double-blind trials have been conducted to investigate its anti-aging effectiveness on human skin. The research indicates that it can produce significant improvement following 4–6 months of daily use.[59] Now, evidence from years of *in vitro*, animal skin and clinical studies have shown that it is also effective against intrinsic aging such as fine lines and wrinkles by stimulating the synthesis of collagen and epidermal turnover rate.[60–61]

These exciting scientific discoveries play a major role in the current proliferation of anti-aging skin care products and services, as one cosmetic trade journal, HAPPI, proclaimed in one recent article, "… After decades of promise, hope in a jar has finally given way to science in a bottle."[62] However, a careful review of recently published data would also suggest that, in many cases, even though preliminary research studies suggest that some of these ingredients do exhibit properties that could lead to dermatological benefits, in theory, their actual effectiveness in delivering measurable clinical improvement that is also perceivable by naked eyes is still largely uncertain.[63] However, antioxidants such as vitamins, minerals, botanical extracts appear to be ingredients of choice for the future. The major trend for the future will continue to center on the prevention of premature aging such as treatment and conditioning products for de-pigmentation and skin (tone) brightening, and for smoothing wrinkles and fine lines. Formulators can choose from an endless supply of bio-active and multi-functional ingredients such as peptides, anti-oxidants, free radical scavengers, and alpha hydroxy acids. Many have shown initial success through *in vitro* studies or limited preliminary human testing but still require extensive well-controlled scientific studies to substantiate the initial findings. As cosmetic surgical procedures become more accepted into the mainstream, other futuristic ingredients for tomorrow's best seller, either as a stand alone product or as a post operation companion, are emerging:[64–65]

- Optical diffusion ingredients to blur and camouflage wrinkles and imperfections and give an immediate improvement of appearance.

- More UV protection including exotic botanical extracts and enzymes claiming to repair and/or protect DNA from photo damage.
- More free radical scavengers to protect skin from pollutants, ozone, pesticides and environmental stressors.
- Energy renewal starting from repairing DNA from within the mitochondria.

Today's formulators and product developers are fortunate to be presented with a vast selection of available and functional ingredients unprecedented in history. However, the basic principle of cosmetic product development remains the same even during our pursuit of profits:

- Be responsible
- Adhere to sound scientific discipline
- Make good and safe products
- Protect the safety of the consumers
- Comply with the law even when it is not being actively enforced; lack of FDA enforcement action is not and should never be a good policy for sound business practice

16.5.2.2 Considering Consumers Peace of Mind

A sound formulation strategy must figure in consumer's peace of mind. Consumers are constantly bombarded with new information on the products they use on a daily basis. NGO's community activism and public consumer education campaign further add to the information explosion. At times, scientific facts and laboratory findings are taken out of context, creating a false, negative impression of product safety among the audience who are not skilled in the art (and/or science). Witnessing the recent lack of official FDA actions while motivated by the global Green Chemistry movement, many consumer advocacy NGOs have taken it upon themselves to be the champion of cosmetic product safety. Front and center of the issue is the need to minimize or eliminate cruelty to animals during scientific research. The result is an overwhelming global acceptance of no animal testing for cosmetic products. At press time, the seventh amendment of the EU Cosmetic Directives officially includes the provision of animal testing ban for its member states. However, it does not mean manufacturers are exempt from conducting safety testing. Alternative testing is needed to ensure product safety. Another issue is the hot debate over controversial ingredients either scientifically proven or perceived by the

consumers to be harmful. Included in this list are preservatives such as formaldehyde, alleged endocrine disrupters such as parabens and substances referred to as carcinogenic, mutagenic or reproductive toxins, and persistent and bioaccumulative toxins.

16.5.2.3 Ensuring Product Safety

A thorough and well designed safety review is crucial to uphold the basic product development principle. Through decades of self discipline and regulation, the cosmetic industry has managed to provide safe products to the consumers. Many tools are available to assist the formulators in selecting safe ingredients. The CTFA sponsored Cosmetic Ingredient Review (CIR) of ingredient safety is one of the best-known tools to ensure the formulation of a safe product. This cosmetic ingredient safety review is conducted by a panel of scientific and medical experts. However, the development of new ingredients far outpaces the speed of review by the CIR committee. In addition to this useful database, formulators will need to rely on other resources as well.

CTFA's online International Cosmetic Legal & Regulatory Database, available through membership subscription, offers a wealth of global regulatory information on individual cosmetic ingredient. Another useful tool for the formulators also provided through the CTFA membership is the CTFA Online. Its Ingredient Database provides extensive listing of pertinent safety and regulatory reviews that are kept current including the most current CIR ingredient review status, global safety status of a particular ingredient in terms of whether it is included in various lists of substances that have an imposed prohibited use or restriction for the intended cosmetic product. Also included are summary reviews of its safety status regulated under various occupational and environmental statutes.

It is also important to obtain long-term toxicological and environmental profiles which are often only available through years of public use and monitoring. Clearly, safety is a continuous pursuit and no one single database is capable of providing all information required. Therefore, regular literature research of the peer reviewed scientific journals, attending scientific conferences, technical symposiums, and continuing education helps to secure the most current development in the quest of ingredient safety.

The following is an example of a broad-spectrum product safety evaluation scheme for a topical cosmetic skin care product. It is not intended as

an exhaustive check list. A product safety pre-evaluation planning session should be conducted with the participation of all stakeholders to determine the most appropriate safety testing scheme for each new product.

Example of Ingredient Safety Review:

Step 1:

a. Review vendor safety data as part of purchasing routine including all available chemical and physical properties such as source of raw material, synthetic and processing pathways, composition, impurities, batch to batch variability, solubility, thermal profiles, viscosity, potential chemical interactions and incompatibilities, etc.
b. Review published safety test studies such as CIR reports, medical and chemical literature
c. Review unpublished safety studies when available
d. Determine Intended Use, Route of Application, Concentration, and Exposure. Illustrated below is an example of check list:
   - Concentration upper limits in the finished product
   - Type of product:
     a. Rinse off
     b. Leave on, duration of time under normal use
   - The site of applications and the size of the area of exposure
   - Amount of product to be applied under normal use & the frequency of use
   - Potential for penetration via skin application (e.g., considering molecular weight partition coefficient)
   - Type of delivery systems and the presence of other ingredients
   - Potential for misuse and/or abuse

Step 2:

a. Select appropriate alternative testing protocols for animal testing when necessary
b. Determine and select additional safety testing when needed
c. Review additional safety testing data

Step 3: Go or No Go Decision

Example of Finished Product Safety Review:

Step 1:

a. Review formulation and safety data of related and similar products
b. Review similar competitive products
c. Review finished product definition, intended use, and potential of exposure
d. Review the presence of certain materials which may influence safety test design (e.g., fragrances, preservatives)

Step 2:

a. Select appropriate alternative protocols for animal testing when necessary
b. Determine and select additional safety testing, if needed
c. Review additional safety data

Step 3: Go or No Go Decision:

a. Go – end of pre-market safety review. Proceed to mfg and marketing
b. No Go:
   - Retest to confirm conflicting results
   - Reformulate product and restart the entire safety review process
   - Archive the project

16.5.2.4 Comprehensive Regulatory Lifecycle Management

Actions to be considered for a comprehensive regulatory lifecycle management for successful consumer product development are outlined below.

Table 16.5.2.4.1 Regulatory Lifecycle Management

Product Development Phase	Examples of Action Items
Discovery phase	Conduct preliminary regulatory feasibility analysis: • Identify current compliance status and schedule for full compliance • Align product, marketing and regulatory strategy

	Coordinate other regulatory affairs compliance issues including environmental, product safety, occupational safety, quality
Preclinical phase	Conduct initial ingredient and product safety review perform preliminary environmental, occupational safety & quality analysis
Clinical phase	Ensure the compliance of product safety and effectiveness initiate downstream environmental and occupational safety compliance propose downstream quality assurance compliance scheme
Regulatory submission phase	Examples of potentially required governmental submissions: FDA — Drugs: NDA/ANDA Biologics: PLA/ELA/BLA Devices: PMA/510(k) EPA — Air emission permits Waste water discharge permits
Commercialization Phase	Obtain approval of the following: • Environmental waste discharge/emission control permits • Required approvals from FDA/USDA/other agencies Complete all product safety reviews Complete all quality assurance & occupational safety protocols all product safety reviews complete
Marketing & post Marketing phase	Continue and monitor the environmental, occupational safety and quality performance Continue and monitor product related regulatory maintenance: • Product use/misuse monitoring • Product performance surveillance • Product safety monitoring & remediation • Advertising and promotion—labeling & misbranding monitoring

Today's consumer product companies face a tremendous task of bringing newer, more value-added products into the market within a much shorter time frame. Aging boomers' desire to remain forever young will continue to add fuel to the already feverish global growth of the anti-aging skin care product segment. Complex marketing strategies, global supply chain

distribution and product positioning often collide with last minute, unexpected regulatory restraints, causing costly delay in product launch. In extreme cases, the unforeseen and/or unresolved regulatory roadblocks could sink a profitable product line. This is rather unfortunate but avoidable. Incorporating a comprehensive and well orchestrated regulatory strategy and analysis during the early conceptualization phase is essential in overall product development success.

It is imperative that any successful implementation of a global marketing plan take into consideration different regional regulatory requirements for ingredient selection, product claims, advertisement, and promotional materials. To avoid a financially disastrous last-minute show stopper for any product launch, the best operatic rule for success is to scope out the marketing scheme during the early product development cycle and develop potential claims through careful examination of regulatory allowances and scientific support evidence, taking into consideration current consumer perception and awareness, and finally, followed up with a well designed product safety review and testing, both pre- and post-market.

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Glossary

Actinic—Referring to the ultraviolet (UV) rays from sunlight and UV lamps.

Activator protein 1 (AP1)—Transcription factor composed of dimers of proteins belonging to the c-Fos/c-Jun. Members of this family dimerize to upregulate transcription of a diverse range of genes involved in everything from proliferation and differentiation to defense against invasion and cell damage.

Acylceramide—An unusual linoleate-containing ceramide found in the stratum corneum. Acylceramide is important for the organization of lipids in the intercellular spaces of the stratum corneum and for permeability barrier function.

Acylglucosylceramide—An unusual linoleate-containing glycosphingolipid found in the noncornified layers of keratinizing epithelia. It is thought to be involved in the formation of lamellar granules and is the precursor of the acylceramide.

Adipocytes—Fat tissue cells that contain a high percentage of lipids. They can be found mainly in subcutis tissue, specifically abdominal or peritoneal tissue and under the skin.

AHA (alpha hydroxy acids)—A family of acids, often found in fruit, sour milk, sugar, and other products processed through bio-fermentation which, when applied to the skin, are believed to dissolve the glue-like lipids holding skin cells together in the stratum corneum. When tight connections are loosened, surface skin cells shed, revealing younger-looking, fresher cells.

AICS—Australian Inventory of Chemical Substances.

AIDS—Acquired immunodeficiency syndrome.

Allergen—An antigenic substance which is recognized by the immune system, and causes an allergic reaction.

Allergic sensitization—Over-expression of the antibody IgE on mast cells, causing higher reactivity to allergens

Amino acids—The building blocks of protein. A group of biological compounds containing nitrogen.

ANDA—Abbreviated New Drug Application.

Antihistamines—Drugs that inhibit allergy symptoms by blocking the actions of histamine at the H1 receptor.

Anti-oxidant—A substance which inhibits or prevents damage from free radicals.

AP-1—Activator protein-1, pro-inflammatory transcription factor.

APC—Antigen presenting cells.

Apoptosis—A type of programmed cell death (PCD). It is a process of deliberate life relinquishment by a cell in a multicellular organism.

Aquaporins—Integral membrane proteins that form pores in the membrane of biological cells. Members of this family are permeable to various molecules, from water to glycerol to urea.

Atopic (eczematous) dermatitis—A chronic or recurrent inflammation of the skin, for which there is genetic predisposition or a family history of allergic disorders. Induced upon exposure to food and inhaled allergens.

ATP—Adenosine triphosphate. The nearly universal source of chemical energy in biological systems.

Autacoids—A physiologically active substance (as serotonin, bradykinin, or angiotensin) produced by and acting within the body.

Autocrine signaling—A form of cell–cell biochemical crosstalk, in which the target cell and the signal-releasing cell are one.

Ayurvedic medicine—The ancient Hindu science of health and medicine.

Ballistometer—Instrumentation involving skin rebound technique for assessing intrinsic viscoelastic properis of a material.

Basal cell carcinoma—The most common form of skin cancer, a slow growing tumor that begins in the basal cell lining the deepest epidermal layers of the skin.

BBB—Better Business Bureaus.

Beta glucan—The active ingredient in oats. It is known to promote healing, stimulates collagen synthesis, promotes cellular turnover, and protects and moisturizes the skin.

BHA (beta hydroxy acids)—A group of acids, often found in flowering plants and herbs. Most common is salicylic acid, believed to dissolve dead skin cells to leave a smooth, even surface.

BLA—Biologics license application; part of the FDA-required biologics marketing applications.

Blistering—The formation of a fluid-filled vesicle after friction, heat, or sunburn or after exposure to a plant or chemical irritant.

Bloom syndrome—A type of premature aging disease caused by a defect in the BLM gene, which encodes an isoform of DNA helicase.

Botox—BOTOX® Cosmetic is a purified protein produced by the *Clostridium botulinum* bacterium, which reduces the contractions of the muscles that cause those frown lines between the brows to form over time.

Botox-botulinum toxin—A neurotoxin protein produced by bacteria named *Clostridium botulinum*. It is one of the most poisonous naturally occurring substances in the world. Though it is highly toxic, it is used in miniscule doses both to treat painful muscle spasms, and as a cosmetic treatment to reduce muscle movement and improving the facial expression.

Bp/yr (base pairs/year)—A measure of the rate at which telomere length changes.

B-scan ultrasound—Ultrasound method in which the amplitude of each returning signal controls the brightness (B) of the spot of reflection. So a single pulse of ultrasound passing into a series of tissues will give rise to a series of spots, with the brightness of the spots corresponding to the amplitude of the reflection from different layers.

CAERS—Adverse Events Reporting System, a post-marketing adverse event monitoring system maintained by the FDA.

CARB—California Air Resources Board.

Carboxylic acid—Simply, a compound present in living organisms or organic, non-living substances that contains one or more carboxyl groups (COOH).

Caspases—A group of proteases that mediate apoptosis.

CDER—Center for Drug Evaluation & Research, FDA.

Cell turnover—The rate at which cells are lost and/or regenerated through cell division.

Centenarians—People who live to 100 years or more.

Ceramide—A simple sphingolipid consisting of a fatty acid and long chain base connected through an amide linkage. Ceramides account for about 50% of the stratum corneum intercellular lipids and contribute to skin barrier properties.

CFR—Code of Federal Regulations.

CFSAN—Center for Food Safety & Applied Nutrition, FDA.

Chaperone protein—A class of proteins which assist other proteins fold into a proper conformation.

Chemoprevention—The application of specific chemical substances, many naturally occurring in foods, with the potential to prevent cancer initiation and to either slow or reverse the progression of premalignant lesions to invasive cancer.

Chromameter—Colorimeters are spectrophotomers analyzing the light reflected from surfaces.

CIR—Cosmetic ingredient review.

Claudication—Narrowing of blood arteries which can cause pain, limping, and lameness in the legs.

Cockayne syndrome—A type of premature aging disease caused by a defect in the ERCC6 and ERCC8 genes. These two genes encode proteins that are involved in repairing damaged DNA.

Co-enzyme Q-10—Enzyme activator and anti-oxidant. It is an essential component of the energy-producing machinery in the cells of the body. CoQ_{10} is also known as ubiquinone, and is found in most cells. High concentrations of CoQ_{10} are usually found in organs that require high energy, such as the heart.

Coenzyme R—It is also called biotin, belongs to the family of B-vitamins. Biotin plays and important part in energy metabolism and the production of various enzymes.

COLIPA—European Cosmetic Toiletry and Perfumery Association.

Collagen—Present in the dermis, provides the skin shape and structure, keeping it smooth and wrinkle-free when we are young, allowing wrinkles to form as the quality of collagen lessens with age. Structurally, it is a protein made of amino acids: alanine, orginine, glycine, hydroxyproline, lysine proline. Present in the skin, bone, ligaments and cartilage, makes up about 30 percent of total body protein.

Collagen 1—The main protein of connective tissue in skin.

Comet assay—A gel electrophoresis technique to detect DNA strand breakage *in vitro*.

Confocal microscopy—An optical imaging technique used to increase micrograph contrast and/or to reconstruct three-dimensional images by using a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane.

Consumption—A view of society that acknowledges that consumption of goods, services and symbols and symbols has become central. This is in contrast to an older view that sees production as central. Increasingly,

consumption is an element of personal identity, especially as portrayed in a distinctive "lifestyle."

Contact dermatitis—Inflammation of the skin, resulting from direct contact of an exogenous agent (allergen or irritant) with the surface of the skin.

Corneocyte—Flattened cells, with neither nucleus nor cytoplasmic organelles forming the top layer of skin cells. These cells are therefore dead, biologically speaking, but nevertheless remain active: the result of the final phase of keratinocyte differentiation, they are filled with keratin and other products such as lipids, fatty acids and ceramides.

Cornified envelope—A thick layer of cross-linked protein at the periphery of the cells of the stratum corneum. It confers physical and chemical resistance to the stratum corneum.

Cortisol—A steroid hormone produced by the adrenal cortex that is involved in the response to stress; it increases blood pressure, blood sugar levels, may cause infertility in women, and suppresses the immune system.

Cosmeceuticals—A term widely used by the cosmetic industry to refer to cosmetic products that also possess drug-like effects.

Covalently bound lipids—Lipids that are chemically attached. They are not free and cannot be extracted into organic solvents unless the chemical attachments are hydrolyzed.

cryoTEM—Transmission electron microscopy on specimens that are frozen rather than chemically fixed.

CTFA—Cosmetic, Toiletry, and Fragrance Association, USA.

CTPA—Cosmetic, Toiletry, and Perfumery Association, UK.

Cutis laxa—Also called elastolysis. A group of rare inherited connective tissue disorders in which the skin becomes inelastic and hangs loosely in folds.

Cutometer—Non-invasive suction skin elasticity meter.

Cyanoacrylate glue—The generic name for substances such as ethyl-2-cyanoacrylate, which is typically sold under trademarks like Superglue and Krazy Glue, and 2-octyl cyanoacrylate or n-butyl-cyanoacrylate, which are used in medical glues such as Dermabond and Traumaseal. Cyanoacrylate adhesives are sometimes known as instant adhesives.

Cyclooxygenase (COX-2)—An enzyme that mediates inflammation via production of prostaglandins.

Cytotoxic (CD8+) T Cells—A sub-group of lymphocytes, capable of inducing the death of cells that are virally infected or cancerous.

Dansyl chloride—A strongly fluorescent compound that will react with the terminal amino group of a protein.

Decorin—A leucine-rich protein substituted with one glycosaminoglycan chain.

Dermatophytosis—Fungal infection of the skin, caused by various fungi of the tinea strain. A contagious condition, also known as ringworm.

Desmosomes—Specialized proteinaceous junctions between adjacent cells that are localized on the lateral sides of plasma membranes. Function to facilitate cell-to-cell adhesion.

Desquamation—The process by which cells are sloughed off at the skin surface.

Digital image—A representation of a two-dimensional picture using a finite set of digital values (the so called pixel intensities).

Dihydrosphingosine—One of the common long-chain bases found in ceramides and other sphingolipids. Chemically, it consists of an 18-carbon aliphatic chain with hydroxyl groups on carbons 1 and 3 and an amino group on carbon 2.

DIN—Drug identification number.

DNase (deoxyribonuclease)—An enzyme that catalyzes the hydrolysis of DNA.

Downregulation—Decrease in expression at the protein or messenger RNA (mRNA) level.

DSL—Domestic substance list.

EC—European Commission.

Eicosanoids—Any of a class of compounds (such as the prostaglandins) derived from polyunsaturated fatty acids (such as arachidonic acid) and involved in cellular activity.

EINECS—European Inventory of Existing Commercial Chemical Substances.

ELA—Establishment License Application, part of the FDA-required biologics marketing applications.

Elastin—Highly elastic, hydrophobic protein fibers found in the Dermis, blood vessels and capillaries. Allows the skin to stretch and then snap back quickly, a quality that is progressively lost with aging.

Elastogenesis—Formation of elastic fibers.

Elastometer—Instrument to measure elasticity of the skin.

Elastosis (solar elastosis)—Elastosis is the breakdown of the elastic fibers in skin. Dermal or solar elastosis represents the accumulation of large quantities of elastotic materials in the skin in response to chronic UV irradiation. The exact chemical composition of the elastotic material is unknown.

ELINCS—European List of Notified (New) Chemical Substances.

Emollient—They are substances that soften and soothe the skin. Used to correct dryness and scaling of the skin, they are a key component in the formulation of lipstick, and other cosmetic products.

ENCS—Existing & New Chemical Substance Inventory.

Environment Canada—The Canadian government agency in charge of regulatory Compliance under the *Canadian Environmental Protection Act(CEPA) of 1999.*

Enzyme Linked Immunosorbent Assay (ELISA)—A generic term applied to assay methods that typically involve the adsorption of antibodies to a solid support (typically a ninety-six-well plate). Samples containing a target of interest are added to the wells, and the target of interest will be bound by the antibodies. The wells are washed and a second antibody which recognizes the protein of interest is added to the wells. This second antibody is normally coupled to an enzyme based detection system, which typically generates a colorimetric signal. This process can be used to determine if a given protein is present in a protein sample, and can also provide semiquantitative data on the relative amount of protein present when comparing cells or tissues undergoing different treatments.

Enzymes—Proteins that affect the speed at which biochemical reaction changes occur, usually speeding up an action. Thousands of different enzymes are produced in the body. The skin is the body's largest enzyme-producing organ.

EPA—Environmental Protection Agency.

Epidermolysis bullosa—A hereditary disease of the skin in which large blisters are produced by slight mechanical irritation. Several genetic variants exist ranging from mild to dystrophic.

ERK (extracellular signal regulated kinase)—ERK pathway mediates the anabolic response to growth factors.

ERK1—Extracellular signal-regulated kinases involved with skin inflammation.

Erythrosis—Histological signs of abnormal follicular keratinization.

EU—European Union.

Facial corrugator electromyogram—Measurement of electrical impulse response to brow muscle adductor moving the eyebrow downward and medially.

Fatty acid—A fat soluble acid, found naturally in the epidermis and in cosmetic products. It includes oleic, stearic, palmitic, and linoleic acids.

FD&C Act—Federal Food, Drug & Cosmetic Act.

FDA—Food and Drug Administration.

FG-NET Aging Database—A publicly image database containing face images showing a number of subjects at different ages. The database has been developed in an attempt to assist researchers who investigate the effects of aging on facial appearance. Dissemination information regarding the FG-NET Aging Database can be found at: http://fgnet.rsunit.com/.

Fibrillin—Chronic sun-exposed human skin shows increased expression of both elastin and fibrillin, another structural glycoprotein. Mutations in the fibrillin gene are responsible for some genetic diseases.

Fibrillogenesis—The development of fine fibrils normally present in collagen fibers of connective tissue.

Focal hyperpigmentation—Darker, well-defined skin area with increased melanin content.

FPLA—Fair Packaging & Labeling Act.

Free Radical—An atom or group of atoms that has at least one unpaired electron and is therefore unstable and highly reactive. In animal tissues, free radicals can damage cells and are believed to accelerate the progression of cancer, cardiovascular disease, and age-related diseases.

Free Radical Scavenger—An antioxidant that works by intercepting chemically-unstable radicals

FTC—Federal Trade Commission.

FTC Act—Federal Trade Commission Act.

Glucans—Polysaccharides with immune stimulating abilities; found on the cell walls of yeast, oat, barley, and other plants.

Gluconates—Copper, Manganese, and Zinc. Gluconates are used as dietary supplements and food additives. Copper is an important trace element for human nutrition, as it is a component of the powerful enzyme Superoxide Dismutase. Copper is also part of the many biophysical processes associated with wound healing. Manganese plays a vital role in the

antioxidant process of many body systems. Zinc is known to participate actively in the wound healing process and in acne treatment.

Glutathione—A tripeptide of the amino acids glycine, cystine, and glutamic acid occurring widely in plant and animal tissues and forming reduced and oxidized forms important in biological oxidation-reduction reactions.

Glycosaminoglycans (GAGs)—Carbohydrate family with a high affinity for water that is a very important component of all connective tissue.

GMP—Good Manufacture Practice.

Grenz zone—The zone of repair of photodamage in the dermis with replacement of damaged collagen and elastic fibers with normal collagen and elastic tissue.

Haematopoietic—It refers to blood cell origin.

Helper (CD4+) T Cells—A sub-group of lymphocytes which activate and direct the immune system towards efficient elimination of pathogens, but are incapable of cytotoxic activity.

Hemidesmosomes—Structures similar to desmosome but they joins a cell to basemant membrane and not to another adjacent cell.

Herpes—Infection arising from exposure to one of the viruses herpes simplex or herpes zoster. Typically manifesting in facial skin and genital areas.

High frequency image details—Regions in a digital image with significant variations in color/intensity.

Hormones—The body's chemical messengers; they stimulate or inhibit activities in the body, especially those involving growth, development, reproduction and other life processes. The skin is the largest hormone-producing organ of the body.

Hotlist—List of prohibited & restricted cosmetic ingredients, Canada.

HRT—Hormone Replacement Therapy.

Hutchinson-Gilford Progeria syndrome—A type of premature aging disease caused by a defect in the LMNA gene. This gene encodes a nuclear lamina protein, which acts as a scaffold to help organize DNA and RNA synthesis.

Hyaluronic acid (sodium hyaluronate)—Can be derived from potato, it regulates the level of hydration of skin. In cosmetic formulations, Hyaluronic Acid forms a moisturizing, non occlusive layer of moisture on the skin.

Hydrocortisone—An anti-inflammatory compound naturally produced by the adrenal glands and synthetically produced for use as a drug. Applied to the skin to cope with itching, redness, blistering and other signs of allergy. It is also called cortisol.

Hydroxy group—The chemical group that defines a hydroxy acid.

6-Hydroxysphingosine—One of the long-chain bases found in ceramides and other sphingolipids. Chemically it consists of an 18-carbon aliphatic chain with hydroxyl groups on carbons 1, 3 and 6, an amino group on carbon 2 and a double bond between carbons 4 and 5.

Hypothalamus—An important supervisory center in the brain, it regulates body temperature, blood pressure, heartbeat, metabolism of fats and carbohydrates, and sugar levels in the blood. Structurally, it is joined to the thalamus; the two work together to monitor the sleep-wake cycle. (www.britanica.com)

ICA—Industrial Chemicals Notification & Assessment Act.

I-CAM intercellular adhesion molecules (ICAMs)—Molecules that promote adhesion between cells.

IL-1b—Pro-inflammatory cytokine interleukin.

IL-6—Interleukin produced primarily by epidermal keratinocytes and involved with epidermal barrier repair.

INCI—International Dictionary of Cosmetic Ingredients.

IND—Investigational New Drug application.

Infraorbital—Lying under the eye.

Interleukin-10 (IL-10)—Is a cytokine that inhibits interferon secretion and thymocyte proliferation in the presence of other interleukins.

ISHL—Industrial Safety & Health Law Inventory (ISHL).

JNK—c-Jun Amino Terminal Kinase pathway mediates catabolic, oxidative stress response.

JNK2—Amino terminal kinase, thought to play role in nuclear signal transduction through its environmental stress activation and subsequent phosphorylation of the nuclear transcription factor p53.

510(k)—Also known as Premarket Notification (PMN) or 510(k). Stipulated in section 510(k) of the Food, Drug and Cosmetic Act, requiring medical manufacturers to register and notify FDA, at least ninety days in advance, when they intend to market a medical device in the interstate commerce of the United States.

Keratinocytes—The major type of cell in the epidermis. It makes keratin proteins.

Lacunae—Empty spaces or missing parts.

Lamellar granule—A small organelle unique to keratinizing epithelia. It secretes lipids and enzymes into the intercellular space. This is essential for formation of the permeability barrier.

Langerhans cells—Macrophages containing large granules. Local Langerhans cells on infected skin will take up and process microbial antigens to become fully-functional antigen-presenting cells.

Lentignes (senile lentignes)—Solar lentignes are circumscribed, small darkly pigmented macules that appear following exposure to UV radiation especially in the elderly. (cf. ephelides, or freckles, which appear predominantly on sun-exposed areas in children and young adults).

Linoleate—An ester of the fatty acid, linoleic acid. Linoleic acid is derived from vegetables or vegetable oils and is required in the human diet. It is found in the acylglucosylceramide that is thought to be involved in formation

of lamellar granules in the epidermis. It is also found in a related acylceramide that is thought to be required for the proper organization of lipids in the stratum corneum.

Lipid lamellae—Bilayer membranes.

Lipids—Found between epidermal cells and in cell membranes, these fatty substances make up a large family of ingredients and biological components that act as moisturizers, reduce moisture loss, restore skin's supple, flexible nature, and reinforce the skin's natural barrier protection.

5-Lipoxygenase (5-LOX)—An enzyme that mediates inflammation via production of leukotrienes.

Macule—A macule is a flat, discolored spot of any shape less than 1cm in diameter.

Magnetic resonance imaging—Non-invasive imaging using magnets and radio waves which force hydrogen atoms in the body to line up in a certain way.

MAPK—Mitogen-activated protein (MAP) kinase pathways

Matrikines™—Messenger molecules, matrikines™ are capable of regulating cell activities. They interact with specific receptors to activate certain genes involved in the process of extracellular matrix renewal and cell proliferation. With age these mechanisms become progressively weaker

Medicalization—The tendency by society to increasingly label natural biological events as being treatable through biomedical techniques. An example is menopause, a "condition" which is increasingly treated medically.

Meissner corpuscles—Types of mechanoreceptors, responsible for sensitivity to light touch located just beneath the epidermis within the dermal papillae. They are distributed throughout the skin, but concentrated in areas especially sensitive to light touch, such as the fingertips, palms, soles, lips, tongue, face, and the skin of the male and female genitals.

Melanin—Melanin is a dark-colored polymer in the skin derived from the amino acid tyrosine. Phaeomelanin contains cysteine and is brownish-red,

while eumelanin is black. Both pigments are produced by melanocytes and are found in dark granules called melanosomes. These travel along dendritic processes and find their way to keratinocytes which therefore take on a dark color.

Melasma—Melasma appears on the face as a roughly symmetrical group of dark brown patches of pigmentation during pregnancy. It usually fades after pregnancy is completed.

Melasma (or "chloasma or "mask of pregnancy")—Brown pigmentation (often on cheeks, forehead, upper lip, and neck) which develops in women during pregnancy or with oral contraceptives or hormone replacement. The cause is the combination of estrogen hormone and UV exposure. It usually fades after pregnancy is completed.

Merkel cells—Large oval cells found in the skin, associated with the sense of touch, and are responsible for the highly malignant skin tumor known as Merkel cell carcinoma.

Mesenchymal—It refers to the mesenchyme origin, which is derived from embryonic mesoderm.

METI—Ministry of Economy, Trade & Industry, Japan.

MHLW—Ministry of Health, Labor & Welfare, Japan.

Microdermabrasion—A general term for the application of tiny rough grains to buff away the surface layer of skin

Minimal Erythema Dose (MED)—72FR49070 Proposed Rule: UVA Testing and Labeling August 27, 2007. The MED is the quantity of erythema-effective energy required to produce the first perceptible, redness reaction with clearly defined borders at 16 to 24 hours post-exposure. In 1999, the Sunscreen Drug Products For Over-The-Counter Human Use; Final Monograph FR 64. No., 98 May 21, 1999 Final Rule defined MED as: *Minimal erythema dose (MED)*. The quantity of erythema-effective energy (expressed as Joules per square meter) required to produce the first perceptible, redness reaction with clearly defined borders.

MMP—Matrix metalloprotease. One of a group of enzymes that degrade structural proteins of the skin's extracellular matrix.

MMP1—A Matrix metallopreteinase enzyme principally responsible for cleaving fibrillar collagen (Types I & III) and laminin.

MMP-1 (collagenase)—An enzyme that degrades collagen I and III and laminin.

MMP-2 and MMP-9 (gelatinases)—Enzymes that degrade gelatin and collagen types IV and VI.

MMP-3 (stromelysin)—An enzyme that degrades fibronectin, gelatin, and collagen types IV and VI.

Mottled hyperpigmentation—One of the most striking clinical aspects of actinic aging due to UV exposure is the heterogeneity of skin pigmentation. Some areas appear completely depigmented while others are brown (there are also intermediate colors such as yellow).

Mycoses fungoides—A later stage of Sezary syndrome; a chronic, progressive proliferation of abnormal mononuclear cells in the dermis. This can progress to indurated lesions and tumors of the skin.

Myrtle (Myrtus)—A genus of one or two species of flowering plants in the family of Myrtaceae, native to southern Europe and north Africa. It was sacred to the Greek goddess of love, lust and beauty, Aphrodite.

NAD—National Advertisement Division.

NADPH—The reduced form of nicotinamide adenine dinucleotide phosphate. This is the principal source or reducing equivalents in biosynthetic pathways.

NARB—National Advertising Review Board.

Nascent—Just beginning to be formed

Natural moisturization factor (NMF)—A complex mixture of low molecular weight hygroscopic compounds formed within the corneocytes by degradation of the protein filaggrin, and help maintain hydration of the stratum corneum.

NCSN—New Chemical Substance Notification.

NDA—New Drug Application.

NDSL—Non-Domestic Substance List.

Necrosis—From the Greek, Nekros, meaning dead. Cells undergo necrosis when they undergo morphological changes and eventual lysis, with release of pro-inflammatory signals to provoke their removal with consequent damages to the surrounding tissue.

Neoplastic diseases—Cancerous diseases originating in an irregular disorganized growth in a tissue or organ which usually formes a distinct mass, known as a tumor. This growth may take on a benign or malignant form.

NFkB—Nuclear factor kappa B, pro-inflammatory transcription factors.

NGOs—Nongovernmental organizations.

NHP—Natural Health Product, a product category *regulated as drugs* in Canada. Products containing ingredients of natural origin with a therapeutic function or claim are NHPs regulated by Health Canada's Natural Health Products Directorate (NHPD). Each NHP must possess a Natural Product Number (NPN). Products generally include homeopathic products, traditional herbal medicines, and other herbals.

NHPD—Natural Health Products Directorate.

NICNAS—National Industrial Chemicals Notification & Assessment Scheme.

Nitric oxide synthase (NOS)—An enzyme that mediates inflammation via production of nitric oxide.

NOHSC—National Occupational Health & Safety Commission.

NONS—Notification of new substances.

Northern blotting—The process of adsorbing RNA to a solid support (normally a membrane) and probing the RNA with a complimentary oligonucleotide corresponding to a gene of interest. The complimentary DNA probe is labeled with either a radioisotope or in a manner to facilitate its detection using chemiluminescence. This process can be used

to determine if a given gene is being expressed, and can also provide semi-quantitative data on the level of gene expression when comparing samples of RNA derived from cells or tissues undergoing different treatments.

Northern transfer analysis—A method of detecting gene expression by assessing the amount of RNA fragments by separating them electrophoretically and transferring to a special paper which binds them covalently, followed by hybridization with probes of radioactive RNA or single-stranded DNA.

Novameter—Instrument to measure skin dryness by conductance.

NSN—New substance notification.

Oligopeptide—A molecule composed of a few amino acids linked to one another. Oligo means a few.

Oncogenes—Genes that when activated or overexpressed cause cancer.

OPC (oligomeric proanthocyanidin)—A flavonoid polymer with antioxidant activity.

Optical coherent tomography—Imaging technique that produces high resolution cross sectional images or topographic maps.

OTC—Over the counter.

Ovariectomy—Surgical removal of the ovaries.

p53—Is a protein of mol. wt. 53,000 found in nearly all cells. It is a transcription factor and normally acts as a molecular policeman in monitoring genome integrity. Mutations in the p53 gene are present in about 50 percent of human cancers.

Papillae index—Density of papillae at the dermal-epidermal junction.

Paracrine signaling—A form of cell-cell biochemical crosstalk, in which the target cell is in proximity to the signal-releasing cell.

Parenchymal—Refers to parenchym origin. Parenchym refers to tissue of an organ that is distinct from connective tissue.

Peptides—Peptides are small proteins, the basic structural unit of collagen. The appearance of skin mainly depends on the Collagen structure, the most important protein family of the dermal connective tissue. Collagen is involved in a large array of biological functions including maintenance of structural integrity, cell adhesion, tissue remodeling, and skin repair. By prompting collagen synthesis, peptides contribute to decrease the visual appearance of wrinkles and increase skin firmness and thickness.

Periorbital—Around the eyes.

Permeability coefficient (K_p)—Defined as the steady state flux (J_{ss}) per concentration of the active in formulation (C_v) $K_p = J_{ss}/C_v$.

Phytosphingosine—One of the common long-chain bases found in ceramides and other sphingolipids. It is like dihydrosphingosine, except that it has an additional hydroxyl group on carbon 4.

PIF—Product information file.

Pilosebaceous units—A hair follicle with an associated sebaceous gland.

PLA—Product License Application, part of the FDA-required biologics marketing applications

PLOD—procollagen-lysine2-oxoglutarate 5-dioxygenase; the enzyme involved in post translational of pro-collagen.

PMA—Pre-market approval.

Polynomial function—A mathematical expression in which a number of variables and constants are combined using standard mathematical operations. Once values are set to the variables, a polynomial function can be evaluated.

Population doubling—A measurement used as an index of cell division occurring within a set of cultured cells. An initial count of the cells in culture is made and when the number of cells is twice the initial count, the culture has gone through a population doubling. Population doublings are typically numbered sequentially, starting with the primary culture of the cells (the first round of culturing after the cells are isolated from their donor tissue). The first time the cells double in number it is the first population

doubling, when the cell numbers double again it is the second population doubling. If the population doubling number is plotted against time, a growth curve can be generated. As normal cells enter into a high number of population doublings, the time required for a subsequent doubling increases. Once the cells reach a point where the number of cells no longer increases the cells have entered into senescence.

Postmodernism—A philosophy or view of society with several characteristics. First, postmodernism is characterized by a blending of traditional types of signs and symbols (for example, a building with both classical and modern elements). Second, is the idea that there are no "master narratives" that uniformly explain or give context to a generic, seemingly-universal story about society. Third, postmodernism is characterized by a decentering of views of people, acknowledging that the stories or perspectives of anyone are equally valid.

Principal component analysis (PCA)—A classical statistical technique commonly used for exploring correlation of variables in multivariate distributions.

Procollagen—The precursor of collagen.

Profilometry—The recording of a series of measurement to obtain a profile.

Progeria—A childhood genetic disorder that strongly resembles normal aging.

Prooxidant—A chemical with a tendency to induce oxidation.

Protein—Composed of amino acids, proteins form most of a cell's structure and cell products, which include keratin, collagen, elastin, melanin, enzymes, hormones, and antibodies. It can be of animal or vegetable source.

Proteosome—An intracellular structure that degrades proteins.

Pruritus—Itching of the skin, associated with a variety of causes such as dry skin, infection, and cancer.

PSL—Priority substances list

Psoralens—Plant furocoumarin compounds that react with DNA in the presence of UV light.

Pyrimidine (thymine dimmers)—DNA damage characterized by covalent bonding of two adjacent thymine residues within a DNA molecule, often catalyzed by UV radiation or chemical mutagens

Quality of life survey—Defining the well-being of a population.

Raman spectroscopy—Measurement of the wavelength and intensity of inelastically scattered light from molecules.

REACH—Registration, Evaluation & Authorization of Chemicals

Reactive carbonyl species—Potent mediators of cellular carbonyl stress originating from endogenous chemical processes such as lipid peroxidation and glycation.

Reactive oxygen species (ROS)—Oxygen-containing chemical species that are unstable due to the presence of unpaired electrons. In excess amounts, ROS cause cell damage.

Red tea extract—It provides increased protection against free radicals after sunbathing, and are claimed to alleviate mild sunburn (slight reddening of the skin) rapidly. Also, studies indicate that red tea may help prevent cirrhosis of the liver.

Redox potential—A measure of the affinity of a chemical for electrons; affects the reduced or oxidized state of that chemical.

Replicative senescence—A form of cellular senescence which occurs as cells go through an extended number of population doublings. With each doubling, the cells lose a certain number of telomere repeats from the terminal ends of their chromosomes. Once the telomeres are shortened to a certain critical length the cells become senescent.

Restylene—Restylane® is the first and only dermal filler in the US, composed of non-animal stabilized hyaluronic acid (NASHA™). It is proven to provide long-lasting correction of moderate-to-severe facial wrinkles and folds.

Rete Ridges—The junction between the dermis and the epidermis in most parts of the body is not flat but wavy. The epidermis penetrates into the dermis in the form of rete ridges or rete pegs.

Rhytides—Wrinkles

SCCNFP—Scientific Committee on Cosmetic Products and Non-Food Products. One of the scientific committees to provide the European Commission with the sound scientific advice needed to establish policy and guidelines pertinent to consumer safety, public health and the environment. The mandate for the SCCNFP is to address scientific and technical questions concerning consumer health relating to cosmetic products and non-food products intended for the consumer especially substances used in the preparation of these products, their composition, use as well as their types of packaging.

Sebaceous follicle—A sebaceous follicle that is not associated with a hair follicle, but has a duct running straight to the skin surface.

Seborrheic dermatitis—Inflammation manifested by flaking of the skin or reddish patches, usually in areas of the head and trunk where sebaceous glands are abundant. Caused by environmental and genetic factors.

Seborrheic keratoses—These are flesh-colored, brown or black, waxy growths that can appear anywhere on the skin (also called seborrheic warts). They tend to appear in middle age or in older people: cause unknown.

Sebum—The lipid mixture synthesized in sebaceous glands.

Secondary metabolites—Chemicals produced by a plant that are not directly related to growth, energy production or metabolism of the plant. Examples are some antioxidants or antimicrobial compounds that the plant may make to adapt to its environment.

Senescence—A term applied to cells that will no longer undergo cell division. Senescent cells are still viable; however they will no longer make the transition from the G1 phase of the cell cycle to the S phase.

Serial analysis of gene expression (SAGE)—Strategy that indicates the relative level of expression of each tagged gene.

Sezary Syndrome—Flaky, exfoliative dermatitis with redness and intense itching, associated with skin infiltration of particular atypical white blood cells (mononuclear cells) also found in the peripheral blood.

Silicone Replicas—Thin layers of silicone polymer applied to areas of skin in order to quantify wrinkles and depth of wrinkles. When dry, these are peeled off, giving a uniquely individual, permanent record of the length and depth of each wrinkle. This method is most frequently used to measure periorbital wrinkles before and after treatment in order to quantify improvement.

SIRT 1—Stands for Sirtuin (Silent mating type information regulation 2 homolog), an enzyme which deacetylates Histones, and recently found to be crucial in longevity of organisms from yeast to primates.

Slot Blot Hybridization—A method of detecting gene expression by assessing the amount of RNA in an unfractionated preparation by immobilizing a sample in a manifold slot and hybridizing with labeled DNA probes that hybridizes to the immobilized RNA. Quantitation can be performed visually or by scanning with a densitometer.

SNA—Significant new activity

Solar Elastosis—Damage of elastic fibers from UV exposure with clumping, making the fibers lose their function of elastic stretching and retraction.

Solar Lentigines—are circumscribed, small, darkly pigmented macules that appears following exposure to natural or artificial UV radiation, especially in the elderly. They tend to be located on the face, upper back and the dorsum of the hands. They are considered to be markers of cumulative or intermittent intense sun exposure. They consist of hypermelanosis and a hyperproliferation of functionally active melanocytes. They do not exhibit increased pigmentation following sun exposure however, and tend to persist indefinitely.

Southern blotting—The process of adsorbing DNA to a solid support (normally a membrane) and probing the adsorbed DNA with complimentary DNA corresponding to a DNA sequence of interest. The complimentary DNA probe is labeled with either a radioisotope or in a manner to facilitate its detection using chemiluminescence. This process is used to determine if a DNA sequence of interest is present in a DNA sample, and

can also be used to provide a semiquantitative comparison of the relative abundance of the DNA sequence between different samples.

SPF (sun protection factor)—A measurement of protection against UVB damage. SPF 15 theoretically indicates protection by a factor of 15:5 hours of exposure with SPF 15 is comparable to 20 minutes with no sunscreen (20 min x 15 = 5 hours).

Sphingosine—One of the common long-chain bases found in ceramides and other sphingolipids. It is like dihydrosphingosine, except that it has a double bond between carbons 4 and 5.

Squamous cell carcinoma—A malignant form of cancer, manifested in squamous cells of the epithelium, and occurring in a variety of organs including the skin. Often induced by long-term exposure to the sun.

Stasis dermatitis—Inflammation of the lower legs due to chronic insufficiency of the veins, often accompanied by hypertension, swelling, and redness.

Steady state flux—Represents the slope of the plot of culmulative amount of drug permeated per unit area against time.

Stem cell factor—A cytokine that promotes the differentiation and growth of hematopoietic stem cells into many types of cells, among which are mast cells.

Stem cells—Stem cells are pivotal cells found in higher organisms, which are capable of self-renew and differentiate into specialized cell types such as bone cells, blood cells, neurons etc.

Stratum corneum (SC)—The outmost layer of the epidermis consisting of dead, keratin-filled cells embedded in a lipid matrix.

Stress Induced Premature Senescence (SIPS)—A form of cellular senescence which has been brought on by repeated exposure of the cells to a sublethal level of oxidative stress. The mechanism behind this type of senescence is thought to be DNA damage.

Subpapillary plexus—Arteries supplying the skin located below the dermis.

Sunburn cells—UV-induced apoptotic cells.

Super oxide dismutase (SOD)—Enzyme which scavenges free radicals by using superoxide to form its molecular body. SOD is found throughout the body and is believed by some longevity researchers to be a primary element for long life. In the skin, it is destroyed by sunlight.

Telomere—End of the chromosome, which is made of highly repetitive DNA.

Temporal—Pertaining to the temple region of the head.

TEWL—Transepidermal Water Loss.

The third age—A term used to describe that time of life after retirement and after children have grown and have left the household. With better health, this time has increasingly become extended, giving possibility to new careers or activities that may last decades until health problems intercede.

Theaflavins—Polyphenols that are formed from catechins in tea leaves during the enzymatic oxidation (fermentation) of tea leaves.

Tinea Versicolor—A common superficial fungus infection of the skin (by Malassezia furfur) which causes flat, brownish or reddish, and/or white irregular patching of the skin of the trunk and upper arms.

TPD—Therapeutic Products Directorate

Transglutaminase 1—The enzyme that introduces isopeptide linkages in creation of the cornified envelope.

Transmission electron microscopy—A method for examining subcellular structure in thin secrions of tissue that uses a beam of electrons instead of light.

TSCA—Toxic Substances Control Act

Tumor suppressors—Proteins that prevent cancer development or progression. Genes encoding tumor suppressors are often deleted or mutated in cancer.

Type-I (immediate) Hypersensitivity Reaction—Immunological reaction occuring within minutes of exposure of the IgE antibody to the allergen in allergy-predisposed (sensitized) individuals. Primarily involves mast cell degranulation to release histamine, leukotrienes, cytokines, and proteases.

Ultraviolet wavelengths—Electromagnetic radiation exerted through short wavelengths, and naturally emitted by the sun. Categorized as three types (A, B, C) which are of hazardous potential to the human skin.

Upregulation—Increase in expression at the protein or messenger RNA (mRNA) level.

USDA—US Department of Agriculture.

UV—Ultraviolet radiation or solar radiation. High energy UVC is totally filtered by ozone in our atmosphere and does not reach the earth's surface. UVB is only partially filtered, so is stronger in the summer than in winter. UVB causes photoaging as well as skin precancers and cancer. Low energy UVA is not filtered by ozone and is the same in winter and summer. UVA causes photoaging and immunosuppression (which enhances skin precancers and cancers) as well as phototoxicity and photoallergy.

UVA, UVB—The Ultraviolet spectrum ranges from 200 to 400 nm. Only UVB (290–320 nm) and UVA (320–400 nm) reach the earth's surface. UVB probably causes the majority of photodamage to the skin but does not penetrate very deeply into the dermis. UVA is about 1000 times weaker than UVB but reaches the earth in a quantity about 100-fold greater than UVB and can penetrate more deeply into the skin.

VCRP—Voluntary cosmetic registration program.

Vernix Caseosa—A mixture of sebaceous lipids and exfoliated stratum corneum material (lipids + cells) that coats the skin surface late in gestation.

VISIA™ CR—Clinical Research high-definition serial photography system (Canfield Scientific, Fairfield NJ USA).

Visual Analog Scale—Common research tool to measure a response score, usually based on a ten-point system with one end of the scale being zero and the other end a ten.

VOCs—Volatile organic compounds.

Volar—Pertaining to the palm and sole.

Werner Syndrome—A type of premature aging disease caused by a defect in the WRN gene, which encodes an isoform of DNA Helicase.

Western Blotting—The process of adsorbing proteins to a solid support and probing them with antibodies which recognize proteins of interest. The antibody used to detect the protein is in turn detected with a second antibody that is coupled to an enzyme based detection system. This process can be used to determine if a given protein is present in a protein sample, and can also provide semiquantitative data on the relative amount of protein present when comparing cells or tissues undergoing different treatments.

Wild Yam Extract—It is generally derived from the wild yam root. It was shown to exhibit anti-inflammatory and it has healing properties.

Xenografts—The cell, tissues or organs that are transplanted into a different species.

Xeroderma Pigmentosum—A genetic disorder in which the cell's ability to repair DNA damage induced by UV irradiation. The accumulation of DNA damage can lead to premature aging.

Xerosis—Dryness of skin.

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Skin Aging Handbook

Anti-aging products are undergoing exceptional growth in the cosmetics industry far beyond that of general cosmetics. This book is the only available text that assembles the key pieces developers need to produce new breakthroughs for a growing market that demands quicker and more effective results. It also focuses much needed attention on the biochemical and clinical differences between Caucasian and other skin types.

Beginning with detailed descriptions of the forces driving the anti-aging market, this unique book provides readers with all the tools necessary to further research, develop, market, and sell novel products. Recent discoveries on the molecular level and novel methods of skin aging assessment are detailed, as well as the state of the rapidly changing global regulatory environment. The formulation approaches of major cosmetics companies are presented, as are their techniques for measuring skin aging *in vitro* and *in vivo*, both on the molecular and clinical levels.

Key features:

- Provides philosophical perspective on the growth of the anti-aging market.
- Covers skin types beyond Caucasian.
- Provides key pieces for developing and selling new breakthrough products.
- Includes technology from major cosmetic companies such as Aveda, Estée Lauder, Coty, Avon, and NuSkin.

ABOUT THE EDITOR

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