22 Skin Ageprint: The Causative Factors

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INTRODUCTION

The pace of change in the demography of westernized populations has been rapid over the past century. In the corresponding affluent societies, an extraordinary shift has taken place in the age profile of the population, with older people representing a progressively growing segment. The aging "baby boomers" are now a major demographical force partly driven by the West's cultural obsession with the prevention of aging and the desire to maintain a youthful appearance. Thus, the current demographical evolution has enormous social implications. Indeed, the aging process is increasingly one of the daring topics of both the media and medical community. Any new antiaging treatment modality is avidly watched by the population. Middle-aged and even younger subjects show a craze for cosmetic dermatology when their once youthful bodies exhibit the early signs of wear and tear. In this field, breakthroughs and novel treatments fulfil some of the promises. No longer is this search a uniquely female characteristic, men also disdain an elderly demeanor. In addition to new technological advances, the future prospect of the scientific approach of skin aging relies on a better understanding of the relationships between skin biology and physiology and the ultimate clinical appearance.

The aging problem is even more complex and severe in cases where the skin has lost its protective mechanical function. The atrophy is such that the aspect of "transparent skin" is reached. Such chronic cutaneous insufficiency/fragility syndrome has been coined "dermatoporosis" (1). The clinical manifestations of dermatoporosis comprise morphological markers of fragility such as senile purpura, stellate pseudoscars, and skin atrophy. In addition, functional expression of skin fragility results from minor traumas such as frequent skin laceration, delayed wound healing, nonhealing atrophic ulcers, and subcutaneous bleeding with the formation of dissecting hematomas leading to large zones of necrosis. Dissecting hematomas bear significant morbidity that requires hospitalization and urgent surgical procedures.

FROM GLOBAL TO MOLECULAR AGING AND BACK AGAIN

All living organisms are subjected to aging. However, this event results from a multifaceted process, which is not the same in all of them. The limitation to any definition of aging lies in the diversity of life histories of the organisms. Two distinct classifications of life histories are of major importance. The first classification distinguishes between species that have a clear distinction between germ cells and somatic tissues from those that do not. The second classification makes a distinction between the semelparous species, which reproduce only once in their lifetime, and the iteroparous species, which reproduce repeatedly. The models of aging are most clearly defined in iteroparous species, which have a distinct soma separate from the germ line. Aging needs to be considerably qualified when applied to species with other kinds of life history. It is mistaken, for example, to regard the postreproductive end of life of semelparous species, which usually occurs in highly determinate fashion, as being comparable with the more protracted process of senescence in iteroparous species.

Aging of human beings is a physiological process corresponding to a progressive loss in homeostatic capacity of the body systems, ultimately increasing the vulnerability to environmental threats and to certain disease status. Nobody can escape from aging. However, it is evident that the process progresses differently among individuals of the same age. In any given subject, senescence is heterogenous among organs and also among their constitutive tissues, cells, and subcellular structures (2). Each and every organ of the human body develops and fails at its own rate, which is referred to as its age (3). This systematic aging occurs

Table 1 Core Age Markers of Each of the Body System

Aging type	Decline in	Average onset age (yr)
Electropause	Electrical activity of brain waves	45
Biopause	Neurotransmitters	Dopamine 30, acetylcholine 40, GABA 50, serotonin 60
Pineal pause	Melatonin	20
Pituitary pause	Hormone feedback loops	30
Sensory pause	Touch, hearing, vision, taste, and smell sensitivity	40
Psychopause	Personality health and mood	30
Thyropause	Calcitonin and thyroid hormone levels	50
Parathyropause	Parathyroid hormone	50
Thymopause	Glandular size and immune system	40
Cardiopause/ Vasculopause	Ejection fraction and blood flow	50
Pulmonopause	Lung elasticity and function with increase in blood pressure	50
Adrenopause	DHEA	55
Nephropause	Erythropoietin level and creatinine clearance	40
Somatopause	Growth hormone	30
Gastropause	Nutrient absorption	40
Pancropause	Blood sugar level	40
Insulopause	Glucose tolerance	40
Andropause	Testosterone in men	45
Menopause	Estrogen, progesterone, and testosterone in women	40
Osteopause	Bone density	30
Dermopause	Collagen, vitamin D synthesis	35
Onchopause	Nail growth	40
Uropause	Bladder control	45
Genopause	DNA	40

Source: From Ref. 3.

throughout the entire body from the time of about 30 to 45 years of age (Table 1). To further complicate the situation, there is regional variability of skin aging over the body. It is indeed quite evident that at any time in adult life, the face, scalp, forearms, trunk, and other body sites show different manifestations of aging. In addition, scrutinizing skin aging at the tissue level (epidermis, dermis, hypodermis, hair follicle), and further, at the cellular level (keratinocytes, melanocyte, fibroblast, dermal dendrocyte, etc.) shows a patchwork of aging severity.

Intracellular and extracellular molecules are involved differently by aging. Within each organ system, aging manifests as a progressive, approximately linear reduction in maximal function and reserve capacity at the molecular level. Some aspects of aging can be viewed as a predetermined programmed process. In addition, many of the age-associated physiological decrements are thought to result in part from environmental insults, either acute or chronic. However, in some instances, there are relatively few supportive data. To add to difficulties, physical growth and senescence are both characterized by cumulative progression of interlocking biological events. They are not always separated because at some time in the life of the organism they may proceed as if they were in tandem.

Cellular Senescence in Perspective

Granted that death is the ultimate failure of the organisms to withstand the onslaughts of an inimical environment, what is it in the aging process itself that brings about the termination of the replicative ability of cells as the individual becomes progressively older? What is it in cells and organisms that weakens their resistance to the hostile exogenous forces? How is it that some cells and organisms are programmed to die even without the assault from adverse environmental threats?

Many in vitro studies have demonstrated that the age of any tissue is strongly reflected in the behavior of cultured skin-derived cells (4). Replicative senescence of human cells is thus related to and perhaps caused by the exhaustion of their proliferative potential. According to the telomere hypothesis, somatic cells lack sufficient amounts of activity of the enzyme

telomerase to maintain the telomeric repeats in the face of the end replication problem. With each round of cell division, mortal cells lose some of their telomeric repeats (5). Since telomere length predicts the replicative capacity of cells, it may provide the best biomarker for cellular aging.

Stress-induced premature senescence (SIPS) occurs following many different sublethal stresses such as those induced by H_2O_2, other reactive oxygen species (ROS), and a variety of chemicals (6). Cells engaged in replicative senescence share common features with cells affected by SIPS, including morphology, senescence-associated β-galactosidase activity, cell cycle regulation, gene expression, and telomere shortening (6). The latter process is attributed to the accumulation of DNA single-strand breaks induced by oxidative stress. According to the thermodynamic theory of aging, the exposure of cells to sublethal stresses of various natures can trigger SIPS, with possible modulations of this process by bioenergetics. Thus, SIPS could be a mechanism of the in vivo accumulation of senescent-like cells in the skin (7).

Cellular senescence and cancer are closely related by several biological aspects, including p53 mutation (5,8,9), telomere shortening (10), vitamin A depletion (11), and defects in intercellular communications (12). The age-related mottled subclinical melanoderma, even at a subclinical stage, might be a predictive sign for a carcinoma-prone condition (13–15).

Skin Aging: 1, 2, or 7 Mechanisms?

Conceptually, human aging is one single chronological process of physiological decline progressing with age. This basic process exhibits multiple facets affecting differently the organs, tissues, and cells. This is particularly true in the skin. Over the past decades, the understanding of aging skin has considerably expanded, with a welcome emphasis on differentiating the intrinsic chronological aging changes from photoaging (Table 2) resulting from habitual chronic sun exposure (16). According to this concept, the changes observed in the skin appearance as a result of aging reflect two main processes. Firstly, the intrinsic changes in the skin are caused by the passage of time modulated by hereditary factors, along with modifications occurring inherently in the structure, physiology, and mechanobiology. Secondly, photodamage is a result of the cumulative exposure of the skin to ultraviolet (UV) exposure. Clinically, these two types of aging are manifested differently, with intrinsic aging giving rise to smooth, dry, pale, and finely wrinkled skin, and photoaging giving rise to coarse, roughened, and deeply wrinkled skin accompanied by pigmentary changes such as solar lentigines and mottled pigmentation. Differences between these two types of aging can be seen within one individual when comparing an area of skin commonly exposed to the sun, for example, the face, the neck, and the dorsal forearms, with an area commonly masked from the sun, for example, buttock skin.

This concept that is based on a duality in skin aging has been challenged because it may appear as an oversimplification in clinical practice (17). Thus, another classification of skin aging in seven distinct types was offered (Table 3). The important variables included the endocrine and overall metabolic status, the past and present life style, and several environmental threats, including cumulative UV and infrared exposures, and repeated

Table 2 Comparison of Intrinsic Aging and Photoaging

Feature	Intrinsic aging	Photoaging
Clinical appearance	Smooth texture, unblemished surface, fine wrinkles, some deepening of skin surface markings, some loss of elasticity, redundant skin	Nodular, leathery surface sallow complexion, yellowish mottled pigmentation, coarse wrinkles, severe loss of elasticity
Epidermis	Thin and viable	Marked acanthosis, cellular atypia
Elastic tissue	Increased, but almost normal	Tremendous increase, degenerates into amorphous mass
Collagen	Bundles thick, disoriented	Marked decrease of bundles and fibers
Glycosaminoglycans	Slightly decreased	Markedly increased
Reticular dermis	Thinner, fibroblasts decreased, inactive mast cells decreased, no inflammation	Thickened, elastosis, fibroblasts increased, hyperactive mast cells markedly increased, mixed inflammatory infiltrate
Papillary dermis	No grenz zone	Solar elastosis with grenz zone,
Microvasculature	Moderate loss	Great loss, abnormal and telangiectatic

Table 3 Cutaneous Aging Types

Aging type	Determinant factor
Genetic	Genetic (premature aging syndromes, phototype related, ethnic background)
Chronologic	Time
Actinic	Ultraviolet and infrared irradiations
Behavioral	Tobacco, alcoholic abuse, drug addiction, facial expressions
Endocrinological	Pregnancy, physiological, and hormonal influences (ovaries, testes, thyroid)
Catabolic	Chronic intercurrent debilitating disease (infections, cancers), nutritional deficiencies
Gravitational	Earth gravity

Source: From Ref. 17.

mechanical solicitations by muscles and external forces such as earth gravity. In this framework, the past history of the subject is emphasized. Accordingly, the global aging is considered to represent the cumulative or synergistic effects of specific features, each of them being independent from the others. Such a concept allows to individualize or integrate typical processes, including, among others, menopausal aging and smoking effects. Increased awareness of the distinct age-associated physiological changes in the skin may allow for more effective and specific skin care regimens, preventive measures, and dermatological treatment strategies in the elderly. As a consequence, the immutability of skin aging can be challenged (2,18). However, factors of skin aging share some common mechanisms (19). For instance, molecular mechanisms imply hyaluronate-CD44 pathways in the control and maintenance of epithelial growth and the viscoelastic properties of the extracellular matrix that offer new opportunities for preventive intervention (1).

Environmental Aging and Photoaging

Environmental influences produce obvious alterations to the texture and quality of the skin, the major extrinsic insults being chronic exposure to UV radiation. The action spectrum of photodamages is not fully characterized. The cumulative effects from repeated exposures to suberythemal doses of UVB and UVA in human skin are involved in these processes (20). The role of UVB in elastin promoter activation in photoaging is obvious. UVA significantly contributes to long-term actinic damage, and the spectral dependence for cumulative damages does not parallel the erythemal spectrum for acute UV injury in human beings.

Both UVA and UVB initiate a number of cellular responses, including ROS production within both dermal and epidermal cells. More specifically, cultures of human keratinocytes derived from donors of different ages and from paired sun-exposed and sun-protected sites of older donors demonstrate that both chronological aging and photoaging affect gene expression although in a quite distinct manner. Chronological aging alone, strikingly increases the baseline expression of the differentiation-associated gene small proline-rich protein (SPR2) and of the interleukin (IL)-1 receptor antagonist (RA) gene. By contrast, it has relatively little effect on the UV-inducibility of several other genes, including the proto-oncogenes c-myc and c-fos, the GADD 153 a gene inducible by growth arrest and DNA damage, and the IL-1α and 1L-β genes. Photoaging is different because it increases the UV-inducibility of c-fos, but decreases the baseline expression of the differentiation-associated genes IL-1 RA and SPR2 (21,22). The physiological impact of photodamages occurs at variable pace on the different skin structures. For instance, skin loosening and solar elastosis show clinical manifestations independently from the severity in mottled melanoderma (14).

Photoaging has profound effects on both the epidermis and dermis. The epidermis becomes atrophic compared to sun-protected areas on the same individual, often with disordered keratinocyte maturation. Histological features of photoaged skin are most apparent in the dermis where the extracellular matrix (ECM) shows marked alterations in composition (23). The collagen network of the dermal ECM is responsible for skin strength and resiliency and is intimately involved in the expression of photoaging. The major fibrillar collagen components of the dermis are of the types I and III. In photoaged human skin, precursors of both proteins are significantly reduced in the papillary dermis, and their reduction correlates with clinical severity of photoaging (24). This reduction results from a combination of reduced procollagen biosynthesis and increased enzymatic breakdown by matrix metalloproteinases

(MMPs) (25). Collagen breakdown products within photoaged dermis can negatively influence procollagen biosynthesis by fibroblasts (26). Fibrillar collagens are closely associated with the small chondroitin sulfate proteoglycan and decorin. Its distribution closely mirrors that of type I collagen in the dermis, regardless of level of extrinsic aging (27). Decorin allows interaction between the fibrillar collagens and the microfibril-forming type VI collagen that further interacts with type IV collagen, an important component of the basement membrane at the dermal–epidermis junction. Type VI collagen therefore is likely to play an important physiological role in the organization of the dermal ECM. Type VI collagen is concentrated in the papillary dermis and it seems little affected by photoaging (28). Type VII collagen was reported to be involved in the mechanism of wrinkle formation. This collagen is the major constituent of anchoring fibrils below the basement membrane providing cohesiveness between the epidermis and dermis. In photoaged skin, the number of anchoring fibrils along the basement membrane is significantly reduced, thus increasing the potential for fragility and blistering in photoaged skin (29).

The elastic fiber network supplies recoil and elasticity to the skin. The process of elastic fiber formation is under tight developmental control, with tropoelastin deposited on a preformed framework made of fibrillin-rich microfibrils. Mature elastic fibers are also encased in fibrillin and form a continuous network throughout the dermis. The elastic fiber network comprises thick elastin-rich fibers within the reticular dermis, a network of finer fibers with reduced elastin in the lower papillary dermis, and cascades of discrete fibrillin-rich microfibrillar bundles, with only discrete elastin, in the upper papillary dermis merging with the dermo-epidermal junction. Fibrillin is both a product of dermal fibroblasts and keratinocytes. The elastic fiber network shows considerable disruption in chronically photoaged skin. Firstly, photoaged skin contains abundant amounts of dystrophic elastotic material in the reticular dermis (30), which is immunopositive for tropoelastin, fibrillin, lysozyme, and immunoglobulins (31) Versican, a large chondroitin sulfate proteoglycan, appears to be regulated along with dystrophic elastin, resulting in a relative increase in photoaging. Although immunohistochemically identifiable fibrillin is present following actinic damage, the architecture and fibrillin-rich microfibrils are markedly altered. Minimally photoaged skin shows a similar marked loss of fibrillin-positive structures, implying that remodeling of the fibrillin-rich microfibrillar network is an early marker of photoaging (32).

All structural changes found in the dermis particularly affect the biomechanical properties of the skin. A cutaneous extrinsic aging score was derived from the difference between comparative photoexposed and photoprotected areas (33).

Phototype and Ethnic Aging

People of colored skin comprise the majority of the world population, and Asian subjects comprise more than half of the total earth population. The most obvious ethnic skin difference relates to skin color, which is dominated by the presence of melanin (34,35). The photoprotection derived from this polymer influences the rate of the skin-aging changes between the different racial groups. However, all racial groups are eventually subjected to the photoaging process. Generally, Caucasians have an earlier onset and greater skin wrinkling and sagging signs than other skin types, and in general, increased pigmentary problems are seen in colored skin, although East Asians, living in Europe and North America, have less pigment spots. Induction of a hyperpigmentary response is thought to be through signaling by the protease-activated receptor-2, which together with its activating protease, is increased in the epidermis of subjects with colored skin (36). Changes in skin biophysical properties with age demonstrate that the more darkly pigmented subjects retain younger skin properties compared with the more lightly pigmented groups.

Endocrine Aging

Irrespective of age, most of the skin components are under the physiological control of endocrine and neuroendocrine factors (Table 4). As such, skin is recognized as a hormone-dependent organ (37–39). Like any other system in the body, the aging process affecting the hormonal functions basically results in deteriorations expressed by hormone deficiencies, which in turn can influence the aging machinery operative in the skin (39). Quite distinct are the skin manifestations of some endocrinopathies, which may mimick or interfere with skin aging (37–39). All endocrine glands are affected by the global aging process. A few direct

Table 4 Neuroendocrine Receptors Active in the Skin

1. Adrenergic receptors
2. Androgen and estrogen receptors
3. Calcitonin gene-related peptide receptor
4. Cholinergic receptors
5. Corticotropin-releasing hormone and urocortin receptors
6. Glucocorticoid and mineralocorticoid receptors
7. Glutamate receptors
8. Growth hormone receptor
9. Histamine receptors
10. Melanocortin receptors
11. Miscellaneous neuropeptide receptors
12. Miscellaneous receptors
13. Neurokinin receptors
14. Neutrophin receptors
15. Opioid receptors
16. Parathormone and PTH-related protein receptors
17. PRL and LH-CG receptors
18. Serotonin receptors
19. Thyroid hormone receptors
20. Vasoactive intestinal peptide receptor
21. Vitamin D receptor

Abbreviations: CGRP-R, calcitonin gene-related peptide receptor; CRH-R, corticotropin-releasing hormone and urocortin receptors; GH-R, growth hormone receptor; MC-R, melanocortin receptors; NK-R, neurokinin receptors; NT-R, neutrophin receptors; PTH, parathormone; PTHrP, PTH-related protein receptors; LH/CG-R, PRL and LH-CG receptors; VIP-R, vasoactive intestinal peptide receptor; VDR, vitamin D receptor.
Source: From Ref. 39.

Table 5 Hormones and Neurotransmitters Produced by the Skin

1. Hypothalamic and pituitary hormones
2. Neuropeptides and neurotrophins
3. Neurotransmitters/neurohormones
4. Other steroid hormones
5. Parathormone-related protein
6. Sex steroid hormones
7. Thyroid hormones

Source: From Ref. 39.

consequences interfere with skin aging. They are mostly related to the declined activity of the pituitary gland, adrenal glands, ovaries, and testes.

Some hormones and neurotransmitters are synthesized by nerves, as well as by epithelial and dermal cells in the skin (Table 5). A number of environmental and intrinsic factors regulate the level of cutaneous neuroendocrine system activity. Solar radiation, temperature, humidity, as well as diverse chemicals and biological xenobiotics represent important environmental factors. Some internal mechanisms affecting the neuroendocrine system of the skin may be generated in reaction to some environmental signals or result from local biological rhythms or from local or general disease processes (37).

The paradigm of deleterious hormonal effects is presented by the influence of corticosteroids on skin atrophy. Cushing syndrome and iatrogenic effects of topical and systemic corticotherapy can equally be involved. Corticosteroids are known to regulate the expression of genes encoding collagens I,III,IV,V, decorin, elastin, MMPs 1,2,3, tenascin, and tissue inhibitors of MMPs 1 and 2 (40). However, the exact molecular mechanisms of skin atrophy induced by corticosteroids are not yet known. The corticosteroid-induced atrophy can be one of the most severe forms of skin aging corresponding to dermatoporosis (1).

The most important endocrine compound produced by the skin is vitamin D, which is a regulator of the calcium metabolism and exhibits other systemic effects as well. For example,

epidemiological evidence suggests that sunlight deprivation with associated reduction in the circulating level of vitamin D_3 may result in increased incidence of carcinomas of the breast, colon, and prostate (41). Vitamin D_3 and its analogues also modulate the biology of keratinocytes and melanocytes of the skin in vivo (42).

Growth hormone (GH) is secreted by the pituitary gland under the control of several hypothalamic and peripheral modulators that exert either positive or negative influences. The final balance among the modulating factors determines the pulsatile and circadian secretion of GH. Moreover, physiological changes occurring in particular conditions (i.e., puberty, pregnancy, aging, and severe acute illness) affect the GH secretion. The peripheral effects of GH are mainly exerted by insulin-like growth factor (IGF), produced by the liver upon GH stimulation. The circulating IGF-1 is bioavailable and functionally active depending upon its binding with the IGF-binding proteins (IGF-BPs).

Skin is a target of the GH-IGF system, which exerts a significant influence on the dermal and epidermal physiology (43). GH, IGF-1, IGF-2, and IGF-BPs are present in the skin and are involved in its physiological homeostasis, including the dermo-epidermal cross talking. Thus, not only systemic but also paracrine and/or autocrine cutaneous activity of the GH-IGF system contributes to skin homeostasis (43,44). GH supplementation induces skin changes, a part of which may correspond to some corrective effects on aging skin (45,46).

The progressive decline in dehydroepiandrosterone (DHEA) serum concentration with age, and conversely its supplementation have not demonstrated prominent effects on the skin except on sebum production.

Sex hormones manifest a variety of biological and immunological effects in the skin (47). Estrogen, alone or together with progesterone, prevents or reverses skin atrophy, dryness, and wrinkles associated with chronological aging or photoaging. Estrogen and progesterone stimulate proliferation of keratinocytes while estrogen suppresses apoptosis and thus prevents epidermal atrophy. Estrogen also enhances collagen synthesis, and estrogen and progesterone suppress collagenolysis by reducing MMP activity in fibroblasts, thereby maintaining skin thickness. Estrogen maintains skin moisture by increasing hyaluronic acid levels in the dermis; progesterone increases sebum excretion.

Both the climacteric period following menopause and the andropause decade may negatively affect the skin (47–49). Hormone replacement therapy (HRT), during the climacteric period, helps limitating these changes (50–53). However, there is a limitation because good and poor responders seem to exist (54). Smoking habit may also interfere with the treatment result (55).

Catabolic Aging

The elderly often exist on a substandard diet deficiency in many of the nutrients thought to be essential to maintain health. Protein-containing foods such as meat and fish tend to be too expensive or troublesome to prepare. Dietary faddism, confusional states, and forgetfulness are also responsible for an inadequate diet. These situations predispose skin changes that often amplify the alterations induced by age-related hormone deficiency.

Insufficient fresh fruit and/or vegetables give rise to vitamin C deficiency leading to scurvy. In this disorder, there is a defect in coagulation resulting in purpura, particularly in a punctate perifollicular pattern on the legs. In the elderly, iron deficiency is also common and may result in anemia, generalized pruritus, and some diffuse hair loss.

Essential fatty acid and vitamin A deficiencies because of dietary faddism or deprivation in the elderly cause xerosis (56). Many of the elderly are also deficient in zinc, and it has been suggested that this may be an important factor in preventing wound healing. Zinc supplementation, however, does not improve healing.

Chronic hemodialysis is another example of catabolic aging affecting the mechanical properties of skin (57,58).

Gravitational Aging

Skin of any part of the body is subjected to intrinsic and extrinsic mechanical forces. Among them, earth gravitation is important by influencing skin folding during aging. Any force generated by the skin or applied to it transduces information to cells that may in turn respond to it (59,60). The effects of mechanobiology may particularly be evidenced in the fibroblasts, dermal dendrocytes, keratinocytes, and melanocytes (61–63). Physical forces of gravity involve

mechanotransduction in the skin (64) and affect cell tensegrity and the cell mechanosensitive ion channels. As a result, the structure of the dermal extracellular matrix is affected.

The Case of Wrinkles

There is evidence that wrinkles are not related to the genuine microrelief (65,66). In addition, the microanatomical supports of wrinkles are varied (65–67). They depend on subtle changes in the structure of the superficial dermis elastotic deposits in the upper reticular dermis, loosening of the hypodermal connective tissue strands, or, universely, on hypertrophic binding of the dermis to the underlying facial muscles (66–68). The wrinkle severity rating (69) is influenced by the nature of the altered connective tissue. Similarly, the skin mechanical properties are under these influences (70,71).

Photoaged facial skin does not always present clinically with characteristic wrinkling. In some individuals, usually of light phototype, smooth unwrinkled skin and telangiectasia predominate. These people appear to be more at risk of developing basal cell carcinomas (BCC) on sun-exposed facial skin (72,73). Recent work has confirmed that there is an apparent inverse relationship between the degree of facial wrinkling and the occurrence of facial BCCs. Mechanistically, little is known regarding how these two clinical outcomes occur in response to the same environmental stimulus, namely sun exposure, but it appears that facial wrinkling may, to some extent, preclude the occurrence of BCCs in sun-exposed sites (73).

Smoking is an additional cause of wrinkling (74). Degeneration of elastic fibers by ROS and the repeated mechanical solicitations by some muscle contractions play a putative role in the formation of the smoker's wrinkles.

CONCLUSIONS

Aging is apparent at all levels of the physiology and anatomy of the body. Organs, tissues, cells, and molecules have their own aging processes that differ in their clinical relevance. The individual may perceive a global appearance of skin aging. By contrast, prevention and correction of skin aging may benefit from targeting some of the specific underlying biological processes.

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23 A Quantitative Approach to Age and Skin Structure and Function: Protein, Glycosaminoglycan, Water, and Lipid Content and Structure

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INTRODUCTION

The anatomic facets of skin are infinite, making a complete review of age-related changes in skin structure problematic. This overview, therefore, focuses on certain readily quantifiable aspects of the skin: protein, glycosaminoglycan (GAG), water, and lipid content and structure. Where possible, we address differences between intrinsic aging, physiologic aging, and extrinsic aging due to photoexposure, wind, relative humidity, and other environmental factors, although we acknowledge that this distinction is not always easily made. Ultimately, we hope to unify each topic and the general understanding of skin structure with respect to aging. This chapter reviews each category of structure and function in turn, beginning with a brief description of commonly used quantitative methods of study. Each section includes a table presenting research data, a discussion of the experimental outcomes and, wherever possible, an evidence-based summary of the effect of age on the parameter discussed.

TECHNIQUES

One quantitative method of protein study involves measurement of racemized aspartic acid in skin protein. This racemization represents a major type of nonenzymatic covalent modification of proteins initially synthesized using only L-amino acids. Aspartic acid racemization (AAR) leads to an age-dependent accumulation of D-aspartic acid in more long-lived human proteins (1). The study of AAR in total skin yields data mainly representative of collagen, which comprises the majority of dermal protein and has 10 to 20 times more aspartate residues per unit mass than elastin. However, racemization in collagen is slow because of conformational constraints of the triple helix (1). Racemization occurs more quickly in elastin. Skin samples can be purified such that, if indicated, the AAR due to elastin alone can be studied and its longevity measured (1). Elastin in various tissues including the aorta and lungs reveals high levels of AAR. This indicates a lack of turnover and accumulation of elastin damage in diverse aging tissues, possibly as part of programmed aging (1).

Raman spectroscopy is a nondestructive analytical method for determining the structure and conformation of molecular compounds. Although these results are not quantitative, per se, they are highly informative, as they do not require sample preparation or pretreatment and thus eliminate much potential interference. Recently, near-infrared Fourier transform (NIR-FT) Raman spectroscopy has emerged as being specially suited for the investigations of biologic material (2). NIR-FT Raman spectroscopy exploits an effect wherein a small amount of monochromatic light scattered by a substance has a frequency that differs from that of the incoming beam. This frequency difference represents the vibration frequency of the chemical bonds in the structure being analyzed. Frequency shifts can be analyzed and presented as spectra, with bands characteristic for chemical bonds in the examined molecules (2). This method gives highly reproducible results with only minor differences seen in spectra of different skin types (2).

PROTEINS

Collagen

Table 1 summarizes data pertaining to skin collagen. Collagen, which comprises approximately 70% to 80% of the dry weight of the dermis, is primarily responsible for the skin's tensile strength. Each collagen molecule consists of three polypeptide chains, each containing about 1000 amino acids in their primary sequence. In the collagen molecule, the α-chains are wrapped around each other to make a triple-helical conformation (3). In chronologically aged skin, the rate of collagen synthesis, activity of enzymes that act in the posttranslational modification, collagen solubility, and thickness of collagen fiber bundles in the skin all decrease (4,5). Also, the ratio of type III to type I collagen increases with increasing age (4,6). In photoaged skin, however, collagen fibers are fragmented, thickened, and more soluble (4). The increased fragmentation of collagen, especially in photoaged skin, is secondary to upregulation of collagen-degrading matrix metalloproteinases (MMPs) by UV radiation (7,8). In addition to the acute upregulation of these enzymes by UV exposure, the same MMP enzymes are also gradually increased during chronological aging (9). Aside from increased degradation and fragmentation, chronologically aged skin exhibits decreased fibroblast function and decreased mechanical stimulation, resulting in reduced collagen synthesis and replacement (10). It is plausible that both reduced collagen deposition in elderly skin and enhanced degradation of collagen in photoaged skin could explain the development of dermal atrophy and might relate to poor wound healing in the elderly.

Histological data, though not quantitative, reveal important information about orientation and arrangement of collagen fibers in skin. Lavker et al. compared skin from the upper inner arm of old (age 70–85) and young (age 19–25) individuals using light, transmission electron, and scanning electron microscopy (14). Interestingly, they suggested that the upper inner arm might be an optimal site for analyzing sun-protected skin, as it is not exposed to the pressure deformations and reformations occurring in the buttock. They found that in young adults, collagen in the papillary dermis forms a meshwork of randomly oriented thin fibers and small bundles. The reticular dermis consists of loosely interwoven, large, wavy, randomly oriented collagen bundles. However, the collagen within each bundle is packed together closely (14). In aged skin, the density of the collagen network appears to increase. This likely reflects a decrease in ground substance that would otherwise form spaces between the collagen fibers (14). Also, rather than appearing in discrete rope-like bundles of tightly packed fibers, collagen forms aggregates of loosely woven, mostly straight fibers. As fibers become straighter in aged skin, there is less room for the skin to be stretched, so tensile strength increases (14). Using immunoelectron microscopy, Vitellaro-Zuccarello et al. found similar age-related trends in skin collagen. They also noted greater intensity of collagen III staining in subjects older than 70 years (15). Hence histological and more recent methods are in agreement, revealing that increased age is associated with decreased collagen content and straightening of collagen fibers forming looser bundles, an increased type III-type I collagen ratio, and decreased ground substance.

From a biochemical standpoint, chronological aging induces increased markers of oxidation, glycoxidation, lipoxidation, and glycation in skin collagen (13). In particular, skin collagen's cross-linking lysine residues undergo significant oxidative changes with age. Lysine oxidase, a copper-dependent enzyme, converts lysine to allysine at all ages. Recently it has been shown that allysine is further oxidized to a stable end product, 2-aminoadipic acid. This oxidative change results in significant accumulation of 2-aminoadipic acid in collagen of aged skin; increased oxidative end product is also seen in diabetes, renal failure, and sepsis (13).

Elastin

Table 2 summarizes skin elastin data. The skin's intact elastic fiber network, which occupies approximately 2% to 4% of the dermis by volume, provides resilience and suppleness. This network shows definite changes associated with aging, especially between the ages of 30 and 70. In sun-exposed skin, an excessive accumulation of elastoic material occurs. Accumulation of new elastin in response to photoaging is also apparent from upregulation of the elastin promoter activity and increased abundance of elastin mRNA (1,16). Bernstein et al. compared photoaged skin with intrinsically aged skin, and found a 2.6-fold increase in elastin mRNA, a

(*text continued on page 250.*)

Table 1 Data: collagen

Source	Methods	Results	Notes
Shuster et al. 1975 (11).	Caucasian males: 74; Caucasian females: 80, aged 15–93 yr: biopsies were taken from the midpoint of extensor aspect of forearm using high-speed 5-mm punch. Some postmortem samples were included. Samples were defatted in acetone, dried to constant weight, and hydrolyzed; their hydroxyproline content was measured.	Linear decrease in absolute collagen content with age, 1% per yr. Collagen density decreases with age ($p< 0.001$). A significant relationship is noted between skin thickness and collagen content for all males ($p < 0.001$) and for females > 60 yr ($p < 0.001$).	Collagen decreases with age. This method may be subject to preparation artifacts.
Gniadecka et al. 1998. (12).	Study of Caucasians included 10 people aged 74–87 yr and 10 people aged 22–29 yr. Obtained Raman spectra from buttock skin and forearm skin, using NIR-Raman spectroscopy.	Photoaged skin: collagen fibers are fragmented, thickened, and more soluble, elastin fibers form conglomerates, and amount of GAG increases. Chronologically aged skin: changes are more subtle. Despite an overall increase in the number of collagen fibers, these are thinner and less soluble. Also there is a relative increase with age in the collagen III:collagen I ratio.	Raman spectroscopy allows a detailed analysis without preparation artifacts. The information here agrees with many of the other studies represented in this table.
Lovell et al. 1987 (6).	Strips of abdominal skin obtained at laparotomy or postmortem from 30 subjects aged 0–90 yr. Some samples were cut and acid hydrolyzed; hydroxyproline content was determined using an automated amino acid analyzer and the total collagen calculated from hydroxyproline estimations. Collagen content was calculated both per unit weight of freeze-dried skin and per unit surface area. Other skin samples were digested with CNBr, and type I:type III ratios were calculated using SDS polyacrylamide gel electrophoresis. Pepsin digestion and HPLC separation of denatured α-chains were also used to calculate type I:type III ratios. Indirect immunofluorescence enabled analysis of frozen skin samples antibody-labeled for types I, II, IV, and V collagens.	Hydroxyproline content and estimates of total collagen content did not vary significantly with age. SDS gel electrophoresis showed type III collagen content of skin samples from two young donors (age 5 yr) was 20–23%. In people aged 14–65 yr, measurements were relatively constant; showed content of type III collagen to be 18–21.5%. In people older than 65 yr, there was greater variation; levels of type III were increased and were as high as 31%. The HPLC method was less conclusive because pepsin digestion and separation of collagen component chains were incomplete, especially in skin samples from older individuals. Immunofluorescence data showed no gross changes in the distribution of various collagen types during aging.	Electron microscopy measurements represented a small study population and are therefore somewhat questionable ($n = 5$). Hydroxyproline approximations of collagen content appear more reliable, as they include 29 subjects, dispersed between age 3 mo and 82 yr; however, this still reflects a small number of subjects in any given age range. The shift in collagen type with aging prompts the question of whether this was due to increased type III synthesis or decreased type I synthesis. This study does not provide an answer.

(*Continued*)

Table 1 Data: collagen (*Continued*)

Source	Methods	Results	Notes
	Scanning electron microscopy was used to study the diameters of bundles of collagen fibers.	Scanning electron microscopy: decrease in number of collagen fiber bundles per unit area in the papillary area with increasing age. The oldest subject (82 yr) had reduced bundle width ($p < 0.0001$) compared with 4 other subjects aged 15–58 yr.	
Fisher et al. 1996 (7).	Adult buttock skin (number of subjects not clarified) was irradiated with 2 MED UVB in vivo (twice the dose required to cause barely perceptible reddening). Irradiated and adjacent nonirradiated sites were removed at various times following irradiation and snap frozen. RNA contents were then analyzed using northern blot. Band intensities were quantified using a PhosphorImager. Protein content in skin was quantified using Western blot. Nuclear extracts from irradiated and nonirradiated skin were also temporally analyzed for AP-1 and NF-κ B binding to double-stranded DNA probes by electrophoretic mobility shift assays.	Transcription factors NF-κ B and AP-1 showed 2.5–3-fold increased binding to DNA within 15 min of irradiation, lasting up to 4 hr after irradiation. Induction of interstitial collagenase, streptomelysin 1, and 92 kDa gelatinase mRNAs were maximal (6–60-fold, $p < 0.05$) at 16–24 hr, and returned to near baseline within 48–72 hr. Gelatinase mRNA of 72 kDa was detectable but was only elevated 1.6-fold at 24 hr post UVB exposure. Confirmation that mRNA rises corresponded with actual collagenase protein rises was done using Western blot.	Within minutes of UVB exposure, transcription factors AP-1 and NF-κ B showed increased binding to DNA, stimulating synthesis of various collagenase mRNAs, which resulted in increased synthesis of collagenase proteins. Authors provide direct, in vivo mechanistic evidence of UVB exposure resulting in increased collagenases in skin.
Fisher et al. 1997 (8).	Caucasian adults underwent irradiation of skin at 4 separate buttock sites, with each site exposed 1, 2, 3, or 4 times to radiation delivered at 48-hr intervals at ½ MED. Skin specimens were obtained from irradiated and adjacent nonirradiated sites at 24 hr after the last exposure.	After a single exposure to ultraviolet irradiation, collagenase and 92-kDa gelatinase activity were elevated 4.4 ± 0.2 times the value in nonirradiated skin and 2.3 ± 0.4 times, respectively. Collagenase and gelatinase activity remained maximally elevated after the 2nd, 3rd, and 4th exposures on days 3, 5, and 7, respectively	Repeated UV exposure leads to sustained induction of the MMPs.
Varani et al. 2000 (9).	Seventy-two subjects provided skin samples from sun-protected areas; age groups compared were 18–29 yr, 30–59 yr, 60–79 yr, and 80+. Levels of MMP-1 were assessed using Western blot, and levels of MMP-9 and MMP-2 were assessed using gelatin zymography with scanning laser densitometry for quantitation. Levels of type 1 and 3 pro-collagen were measured using Western blot.	In the 80+ yr group compared with the 18–29 yr group, there was a 40%, 52%, and 82% increase in MMPs 1, 9, and 2, respectively, ($p < 0.01$, 0.05, and 0.001). In the 60–79 yr group compared with the 18–29 yr group, there was a 23%, 20%, and 44% increase in MMPs 1, 9, and 2, respectively, ($p < 0.05$, NS, and $p < 0.05$, respectively). There was a 52% decrease in type 1 pro-collagen expression in 80+ yr-olds versus 18–29 yr-olds ($p = 0.022$).	Chronologic aging results in elevated expression of MMPs and decreased expression of types 1 and 3 pro-collagen.

Varani et al. 2006 (10).	Young (18–29 yr) versus old (80+ yr) subjects participated. Replicate 2- and/or 4-mm punch biopsies of sun-protected hip skin were obtained from each individual. For fluorescence microscopic and ultrastructural analysis and for the assessment of type 1 pro-collagen levels, 4-mm punches were used and 2-mm biopsies were used for routine light microscopy and for isolation of dermal fibroblasts in culture.	Fibroblasts from young skin produced greater type 1 pro-collagen than those from old skin (82 ± 16 versus 56 ± 8 ng/mL, $p < 0.05$) A reduction in mechanical stimulation in chronologically aged skin was inferred from greater percentage of cell surface attached to collagen fibers (78 ± 6 versus $56 \pm 8\%$, $p < 0.01$) and more extensive cell spreading (1.0 ± 0.3 versus 0.5 ± 0.3, $p < 0.05$) in young versus old skin.	Authors hypothesize that old fibroblasts have an age-dependent reduction in the capacity for collagen synthesis while simultaneously experiencing a loss in mechanical stimulation resulting from fewer intact collagen fibers.
Sell et al. 2007 (13).	Human skin samples from 117 people, aged 10–90 yr, were obtained at autopsy. Amounts of 2-aminoadipic acid and 6-hydroxynorleucine (a marker of allysine) in the collagen were determined in acid hydrosylates of processed samples using ion-monitoring gas chromatography. Quantitative contents of 2-aminoadipic acid and 6-hydroxynorleucine were compared in young and old subjects as well as in those with histories of diabetes, renal failure, and sepsis.	2-aminoadipic acid ($p < 0.0001$), but not 6-hydroxynorleucine ($p = 0.14$) significantly increased with age, reaching levels of 1 and 0.3 mmol/mol lysine at late age (mean 82 yr), respectively. Significant increases in 2-aminoadipic acid, but not 6-hydroxynorleucine, were also seen in patients with diabetes ($p < 0.0001$, levels of 2-aminoadipic acid up to < 3 mmol/mol), renal failure (levels of 2-aminoadipic acid up to 8 mmol/mol), and especially sepsis ($p = 0.0001$).	2-aminoadipic acid, a pan-marker for all forms of lysine oxidation, significantly increased in aging human skin. Levels of its precursor, allysine, are in steady state, suggesting ongoing oxidation of allysine to form the stable end product, 2-aminoadipic acid.

Abbreviations: CNBr, Cyanogen bromide; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; MED, Minimal erythemogenic dose; AAR, aspartic acid racemization; GAGs, glycosaminoglycans; MMPs, matrix metalloproteinases.

Table 2 Data: Elastin

Robert et al. 1988 (19).	Analyzed 6-mm punch biopsies of skin from buttock and upper inner arm of 50 individuals (40 males, 10 females). Used a specific elastic staining procedure, then automated computerized image analysis. Calculated percent of surface area covered by elastic fibers, length, and number of elastic fibers per unit surface area in superficial (papillary) and deep (reticular) dermis as a function of age.	Percentage surface area coverage by elastic fibers increased with age in superficial and deep dermis: Males: superficial dermis, $r = 0.66$; $p < 0.001$. Deep dermis, $r = 0.56$, $p < 0.01$. Similar correlation in females did not reach significance because of small sample size. Mean fiber length also increased with age in the superficial and deep dermis: $r = 0.036$, $p < 0.02$. Number of elastic fibers per unit surface area showed no significant change with age in either the superficial or deep dermis.	Continuous increase with age in the length and relative surface area of elastic fibers. This appears to contradict these authors' rheological studies on the same patients that show a continuous decrease in skin elasticity with age (19). The authors attribute this to the possibility of continuous enrichment with age in polar amino acids, carbohydrates, lipids, and calcium of the skin elastic fibers. These structural changes in elastic fibers may interfere with their proper functioning.
Ritz-Timme et al. 2003 (1).	Skin samples measuring 2 × 2 cm were taken from ventral abdomen during autopsy. Paraffin sections of all samples were examined histologically. Then, elastin was purified and AAR was quantified. Specific ages are not given.	In total skin, specimens displayed slight age-dependent increases in D-aspartyl residues; in purified elastin the rate of increase was rapid and highly correlated with age ($r = 0.98$).	This paper goes into great detail, which is beyond the scope of this overview, but does provide interesting insight into elastin degradation.
Ritz-Timme et al. 2002 (21).	Review paper.	Accumulation of new elastin in response to photoaging can be seen from upregulation of the elastin promoter activity and increased abundance of elastin mRNA. However, de novo synthesis of elastin in adult tissues is ineffective.	This paper provides a review of AAR and its role in skin aging.

Bernstein et al. 1994 (16).	Sixteen males aged 49–66 yr. Punch biopsies measuring 4 mm taken from the sun-damaged neck and photoprotected buttock. Studied samples using northern blot analyses, transient transfections with a human elastin promoter/reporter gene, and immunohistochemical staining with elastin and fibrillin antibodies. Analyzed samples in pairs to determine effects of photoaging.	Northern analysis of frozen sections: up to a 2.6-fold increase in elastin mRNA in exposed versus nonexposed skin. Analysis of mRNA from fibroblast cultures: 5.3-fold increase in elastin expression and 2.5-fold increase in fibrillin expression in photodamaged skin. Transient transfection of cultured cells revealed 5-fold increase in elastin promoter activity. Score for elastin staining in superficial dermis, protected skin: 0.62 ± 0.52; exposed skin: 5.0 ± 0.76. ($n = 8$, $p < 0.000001$). Score for fibrillin staining in superficial dermis, protected skin: 0.75 ± 0.46; photoaged skin: 3.1 ± 0.64 ($p < 0.0001$).	This study does not include a very broad age range. Also, since it used several different methods to establish mechanistic details, each method had a very small sample size (3 in Northern blot analysis of total RNA from frozen sections, 3 in Northern blot analysis of fibroblast culture, 2 for transient transfection method, and 8 for immunohistostaining method). The increase in elastin promoter activity and mRNA do not account for the degree of accumulation of elastoic material seen histologically in superficial and middermis of photoaged skin. It is suggested that most of the material staining as elastin in photoaged skin is structurally abnormal. Authors propose that elastin degradation may be slower than production, with accumulation of partially degraded elastic fibers.
Seite et al. 2006 (17).	Ninety-one skin biopsies taken from unexposed (buttock area) skin, ages 21–80 yr, 30 specimens from semiexposed (forearm skin), ages 22–64 yr, and 24 specimens from severe exposure (facial skin), ages 45–65 yr. UV exposure's (280–400 nm) influence on lysozyme deposition measured using 122 samples from buttock skin, ages 20–40 yr. Measurement of elastin and lysozmye via direct immunofluorescence with computer-aided quantitation.	Reduced elastin content with age in buttock skin (groups aged 61–80 yr had significantly less elastin than groups aged 21–50 yr). Relative amounts of elastin in the face of subjects aged 51–70 yr were abnormally high compared with buttock and forearm skin of those age groups. UVA (320–400 nm), especially long-wave UVA (340–400 nm), induces lysozyme deposition in elastin fibers to a significantly greater extent than simulated solar radiation (280–400 nm). 25% of elastin fibers in buttock skin were covered with lysozyme, compared to 66% of elastin fibers in facial skin, supporting the association of lysozyme with solar elastosis. Lysozyme was shown to inhibit degradation of elastin fibers by human leukocyte esterase.	Authors use an ample sample size and explain their methodology thoroughly to reveal mechanistic explanation for solar elastosis based on increased deposition of lysozyme in sun-exposed skin; lysozyme inhibits human leukocyte esterase to prevent proper degradation of elastin and allow accumulation of partially degraded fibers. The lower elastin content in sun-protected, older skin compared with sun-protected younger skin implies that human leukocyte esterase naturally works uninhibited to reduce elastin content with age; however, the UV-induced accumulation of lysozyme inhibits elastin's degradation with age in sun-exposed skin.

Abbreviations: AAR, aspartic acid racemization; UV, ultraviolet.

5.3-fold increase in elastin expression, and a 5-fold increase in elastin promoter activity in photodamaged skin (16). However, these apparent increases in elastin synthesis do not account for the massive accumulation of elastoic material seen histologically in photoaged skin (16). Some attribute this to elastin degradation being slower than synthesis, leading to an accumulation of partially degraded fibers. Recent work has revealed that proteins such as elafin and lysozyme, expression of which is induced by UVA radiation, prevent elastin degradation by human leukocyte (neutrophil) elastase (17,18). In purified skin elastin, the amount of racemized aspartic acid increases rapidly and is highly correlated with age ($r = 98$) (1). This indicates that skin's elastin, like elastin in the aorta and lung, is long-lived and accumulates damage over time (19,20).

In innate aging, fragmentation of elastic fibers results in decreased number and diameter. Computerized image analysis of elastin-stained skin biopsies from the buttock and upper inner arm reveals an age-related increase in mean elastin fiber length and percentage surface area coverage in the dermis, but these fibers are thought to be abnormally enriched in polar amino acids, carbohydrates, lipids, and calcium (19). Through different mechanisms, photoaging and intrinsic aging ultimately result in a deficiency of functional, structurally intact elastic fibers (5). The finer oxytalan fibers in the papillary dermis are depleted or lost altogether; eluanic and elastic fibers become progressively abnormal. These alterations largely account for the widely recognized decrease in the skin's physiological elasticity with increased age (19).

Examination of intrinsically aged skin elastin and fibrillin with immunohistochemical staining revealed that elastin was located in the papillary dermis just below the basement membrane, as small fibers mostly oriented perpendicular to the epidermis. In the deeper dermis, fibers were thicker and oriented differently. Areas surrounding adnexal structures and larger vessels in the deep dermis were also intensely stained (16). Photoaged skin demonstrated similar small-diameter fibers just below the basement membrane within a zone lacking excessive staining, which was of variable thickness (16). This may correspond to the SLEB (subepidermal low echogenic band) seen in ultrasound imaging. Beneath this area of relatively sparse staining was a region of poorly formed, clumped, thick fibers. This staining pattern occupied the superficial to middermis, below which staining again resumed its well-defined pattern as seen in sun-protected skin (16).

Elastin therefore exhibits numerous age-related changes, including slow degradation and accumulation of damage in existing elastin with intrinsic aging, increased synthesis of apparently abnormal elastin in photoexposed areas, and abnormal localization of elastin in the upper dermis of photodamaged skin. These factors lead to the histologically evident elastoic accumulation and contribute to characteristic changes in ultrasound images of aged skin.

General Protein Structure

Table 3 summarizes data pertaining to other facets of skin protein structure. Through Raman spectroscopy, little difference is seen between photoexposed and protected areas in young individuals; the majority of proteins in young skin are in helical conformation. Intrinsically aged skin shows slightly altered protein structure, and photoaged skin reveals markedly altered protein conformation, with increased folding and less exposure of aliphatic residues to water (2,12). Amino acid composition of proteins and free amino acids in aged skin also differ significantly from those of young skin, including an increase in overall hydrophobicity of amino acid fractions from the elderly (22). Since free amino acids are believed to play a key role in stratum corneum (SC) water binding, this shift in their composition, combined with the evidence of altered tertiary protein structure, provides insight into the increased incidence of xerosis in aged individuals.

Aside from protein structure, the level of expression as well as the spatial distribution of certain proteins in the skin appear to change with intrinsic aging and photoaging. For example, in normal skin, the extracellular matrix protein 1 (ECM1) is mainly expressed in the basal cell layers of the epidermis and in dermal vessels. The protein's expression is increased throughout the epidermis of photoaged skin but significantly reduced in the basal and upper epidermal layers of intrinsically aged, UV-protected skin (23). Acute exposure to UV radiation also induces increased expression of the protein throughout the epidermis in healthy young skin (23). Hence, UV-related stress may also influence distribution and expression of various proteins involved in maintaining skin structure.

Table 3 Data: Generalized Protein Structure

Source	Method	Results	Notes
Gniadecka et al. 1998 (12).	Study of Caucasians included 10 people aged 74–87 yr and 10 people aged 22–29 yr. Obtained Raman spectra from buttock skin and forearm skin, using NIR-Raman spectroscopy.	Young group: Little difference in spectra of photoexposed versus photoprotected sites. Most skin proteins were in a helical conformation. Older group: Intrinsically aged (buttock) skin: Resembled young dorsal forearm or buttock, except for a significant ($p = 0.008$) shift of amide I peak position toward lower frequencies in older skin, suggesting minor conformational changes of protein structure. Photoaged (dorsal forearm) skin: In addition to the amide I band, the amide III band was also significantly shifted to lower frequency compared with aged photoprotected and younger skin spectra. Also, decreased intensity of the amide III band indicated severe conformational changes in protein in structure, with an increase in protein folding and less exposure of aliphatic amino acids to surrounding water.	In young skin, most proteins are in helical structure and we do not see much difference between sun-exposed and sun-protected regions. Chronologically aged skin has proteins in slightly altered conformation. Photoaged skin has proteins in markedly altered conformation, with increased folding and less exposure of aliphatic residues to water. This enables the proteins in photoaged skin to bind less water.
Jacobson et al. 1990 (22).	Amino acid composition was quantified in 3 fractions isolated from scales of SC from the lower leg. The three fractions studied were free amino acids (FAA), soluble hydrolysate (SH) and whole-cell hydrolysate (WCH). “Old” subjects ($n = 20$) were 60 yr or older; “young” subjects ($n = 20$) were 30 yr or younger.	In normal subjects, each of the 3 fractions showed significant difference ($p < 0.03$) in amino acid composition as a function of age. The FAA and SH fractions revealed an increase in hydrophobic amino acids.	This is an interesting study that goes into great detail regarding specific amino acid composition, which is beyond the scope of this overview. Nonetheless, the general shift toward increased hydrophobicity is an important trend that should be noted.
Gniadecka et al. 1998 (2).	Used NIR-FT Raman spectroscopy to examine 3-mm punch biopsies from buttock, lower leg, back, and arm in 44 individuals aged 18–35 yr.	Most proteins in the whole skin and SC were in α-helix conformation. This was supported by the frequencies of amide I and III maxima and by a strong C-C stretch band at 935 cm^{-1}.	These data further support that in young skin, proteins are mostly in α-helical conformation.

(*Continued*)

Table 3 Data: Generalized Protein Structure (*Continued*)

Source	Method	Results	Notes
Sander et al. 2006 (23).	Evaluated buttock skin and photoexposed skin of 12 young (<30 yrs) and 12 older (66–73 yrs) subjects. ECM1 expression was investigated using immunohistochemistry with densitometric image analysis for semiquantitative results. Acute UV exposure was created by irradiating buttock skin over 10 days with a solar simulator.	In normal human skin, ECM1 is expressed mainly in basal cell layers of epidermal keratinocyes and dermal vessels. Intrinsically aged, UV-protected skin showed a significantly reduced expression in basal (~10% decreased staining intensity) and upper (~8% decreased staining intensity) epidermal cell layers compared with young skin ($p < 0.05$). In photoaged skin, expression is significantly increased in the lower (~15% increased staining) and upper (~18% increased staining) epidermis compared with age-matched UV-protected sites ($p < 0.01$). Acute photoexposure also results in marked increased epidermal ECM1 expression (~8–10%, $p < 0.05$).	Semiquantitative data reveal acute and chronic UV-related stress, which appears to influence expression and distribution of ECM1. Future studies may reveal similar impacts of intrinsic and photoaging on other skin proteins.

Tables 1 to 3 present age-related data on collagen, elastin, and generalized protein structure, respectively. See text for discussion.
Abbreviations: AAR, aspartic acid racemization; FAA, free amino acids; SH, soluble hydrolysate; WCH, whole-cell hydrolysate; ECM, extracellular matrix protein; ECM1, extracellular matrix protein 1; SC, stratum corneum; UV, ultraviolet.

GLYCOSAMINOGLYCANS

GAGs are composed of specific repeating disaccharide units. Those attached to a core protein are referred to as proteoglycans and are found widely distributed throughout the skin. GAGs most often present in human skin are hyaluronic acid (not attached to a protein core) and the proteoglycan family of chondroitin sulfates, including dermatan sulfate (24). GAGs are especially important in skin because they bind up to 1000 times their volume in water. Therefore, skin hydration is highly related to the content and distribution of dermal GAGs, especially hyaluronic acid (24).

Table 4 summarizes data regarding GAGs. GAGs increase in photoaged skin compared with young or intrinsically aged skin (12,24). This seems paradoxical, as photoaged skin appears leathery and dry, unlike newborn skin, which also contains high levels of GAGs. Confocal laser scanning microscopy reveals that GAGs in photodamaged skin are abnormally deposited on elastoic material, rather than diffusely scattered as in young skin (24). This aberrant localization may interfere with normal water binding by GAGs, despite their increased number.

A recent study that used laser capture microdissection and quantitative real-time PCR (polymerase chain reaction) on punch biopsy specimens from human buttock skin found that decorin, a proteoglycan, may have a significant role in collagen fiber diameter (26). Decorin mRNA is expressed in the reticular, but not the papillary dermis, and like other GAGs, levels of decorin are increased with chronological age. It is suggested that a decreased collagen-decorin ratio may contribute to the changes in ECM structure in aging skin (26).

WATER

In young skin, most of the water is bound to proteins and, appropriately, is called bound water (2). This is important for the structure and mechanical properties of many proteins and their mutual interactions. Water molecules not bound to proteins bind to each other, and are called tetrahedron or bulk water (2).

Data pertaining to water structure and aging are summarized in Table 5. Intrinsic aging does not appear to alter water structure significantly (12). However, in photoaged skin, Raman spectroscopy reveals an increase in total water content. Again, this seems paradoxical, as aged skin is often dry and weathered. However, structurally, significantly more of the water in aged skin is in tetrahedron form. Thus, as proteins are more hydrophobic and folded, and GAGs are clumped on elastoic material, they interact less with water, and water in aged skin binds to itself instead. This lack of interaction between water and surrounding molecules in photoaged skin likely contributes to its characteristically dry and wrinkled appearance.

LIPIDS

The "brick and mortar" model is often employed to describe the stratum corneum's protein-rich corneocytes embedded in a matrix of ceramides, cholesterol, and fatty acids, and smaller amounts of cholesterol sulfate, glucosylceramides, and phospholipids. These lipids form multilamellar sheets amid the intercellular spaces of the stratum corneum, and are critical to the SC's mechanical and cohesive properties, enabling it to function as an effective water barrier (28). Changes in SC lipid content have been linked to skin conditions such as xerosis and possibly atopic dermatitis (28).

Table 6 summarizes data pertaining to skin lipids and age. Many authors agree that overall lipid content of human skin decreases with age (28–30). Using high performance thin-layer chromatography (HPTLC), Rogers et al. found a 30% decrease in the face, hand, and leg of older subjects, but the older group only extended to age 50. No significant change was seen in proportional composition of lipid classes or ceramide species (28). Schreiner et al. used small-angle X-ray diffraction to compare lipid composition in subjects aged 23 to 27 years with subjects aged 63 to 69 years (31). They did not see any overall difference in lipid quantity or composition between the groups. However, the aged group consisted of only four subjects, and again, included a narrow age range. Saint-Leger et al. studied the lower legs of 50 subjects and

Table 4 Data: Glycosaminoglycans

Source	Method	Results	Notes
Bernstein et al. 1996 (24).	Included 6 males, aged 52–60 yr, with significant photodamage. Punch biopsies measuring 4 mm were taken from the sun-damaged posterior neck and sun-protected buttock. Histometrically studied GAG content of papillary dermis using immunoperoxidase stains specific for hyaluronic acid and chondroitin sulfate. Expressed measurements as percent of fields stained positively for these GAGs. Studied location of GAGs using confocal laser scanning microscopy, staining specifically for GAGs and elastin.	Significant increase in GAG staining in sun-damaged versus sun-protected skin from the same individuals: Hyaluronic acid: Sun-protected: 13.7 $\pm$ 1% Sun-exposed: 24.4 $\pm$ 0.5% ($p < 0.05$). Chondroitin sulfate: Sun-protected: 6.77 $\pm$ 0.25% Sun-damaged: 23.37 $\pm$ 0.6% ($p < 0.0001$). Superficial dermal GAGs in sun-damaged skin are clumped and deposited almost exclusively on the solar elastoic material, rather than diffusely between the fine network of collagen and elastic fibers as in normal (photoprotected) skin, wherein concentration of dermal GAGs is greatest just beneath the epidermis and decreases gradually with increasing depth.	One would expect that increased GAG content would give skin a youthful appearance, as it does in newborn skin. These authors state that the abnormal location of GAGs in photodamaged skin may explain the apparently paradoxical weathered appearance of photodamaged skin despite increased GAGs. This study does not consider possible anatomical variation between neck and buttock, separate from the factor of photodamage. Furthermore, the narrow 52–60-age range limits the study to one of photoaging and does not consider intrinsic aging.
Takahashi et al. 1996 (25).	To quantify main disaccharide units of skin GAGs, high-performance lipid chromatography was used after labeling with 1-phenyl-3-methyl-5-pyrazolone. After comparing 6 “sun-exposed people” with 6 other “sun-protected people,” the authors compared sun-exposed and sun-protected skin within 6 individuals.	Total amount of disaccharide units in sun-exposed skin was significantly greater than that in sun-protected skin ($p < 0.05$). Also saw a decrease in the ratio (δ)Di-HA (disaccharide-hyaluronic acid)/δDi-4s (disaccharide-dermatan sulfate) in photoaged skin.	This article addresses photoaging but not intrinsic aging. The increase in GAGs in photodamaged skin agrees with results of Bernstein et al. (above). The significance of the increased hyaluronic acid-dermatan sulfate ratio is unclear.

Lochner et al. 2007 (26).	Full-thickness punch biopsies isolated from human buttock skin of 5 young (21–35 yr) and 5 older (61–68 yr) subjects. Distribution and expression of collagens 1 and 3 and decorin mRNA were measured using laser capture microdissection and quantitative real-time PCR in young versus old subjects. Decorin and collagen expression were also measured before and after single exposure with two minimal erythemal doses of simulated solar irradiation after 24 hr.	Decorin mRNA is expressed in the reticular but not in the papillary dermis. Expression is 105% higher in older than in younger subjects. Simulated solar exposure resulted in downregulation of decorin mRNA in both groups [−35% in young; −35% in older). Collagens I and III expressions were downregulated with increasing age (29% and 60% lower levels in collagens I and III mRNA, respectively, in older subjects compared with young) and after single UV irradiation (21% decrease seen with collagen I and 60% seen with collagen III).	Small sample size, but otherwise convincing evidence of decreased expression of decorin and collagens I and III with age and also with UV radiation. The exact mechanistic significance is unclear, but authors concluded that decreasing collagen to decorin ratio inflected by both age and UV irradiation may affect collagen bundle diameter in aging skin.

Table 4 summarizes data on age-related changes in GAG structure and localization. See text for discussion.
Abbreviations: GAGs, glycosaminoglycans; Di-HA, disaccharide-hyaluronic acid; δDi-4s, disaccharide-dermatan sulfate; UV, ultraviolet; PCR, polymerase chain reaction.

Table 5 Data: Water Structure

Source	Method	Results	Notes
Gniadecka et al. 1998 (12).	Study of Caucasians included 10 people aged 74–87 yr and 10 people aged 22–29 yr. Obtained Raman spectra from buttock skin and dorsal forearm skin, using NIR-Raman spectroscopy.	Younger group: Most water molecules in young skin were bound to other macromolecules (the 180 cm-1 band was absent). Saw no significant difference in water content or structure in sun-exposed versus sun-protected skin. Older group: Intrinsically aged (buttock) skin: no significant difference in water content or structure compared with young skin. Photoaged (dorsal forearm) skin: increased content of nonbounded water (180 cm-1 band present). Total hydrogen-bonded water is significantly decreased in photoaged skin ($p = 0.03$). Saw an overall (30%) increase in water content of photoaged skin.	In young skin, water is primarily present in bound form. This does not appear to change with intrinsic aging. However, in photoaged skin, overall water content increases and proportionally shifts such that less of it is in bound form.
Gniadecka et al.1998 (2).	Used NIR-FT Raman spectroscopy to examine 3-mm punch biopsies from the buttock, lower leg, back, and arm in 44 people ages 18–35 yr.	Over 90% of water in whole skin is present in the bound form.	These data support that in young people, water is mainly present bound to macromolecules.
Wright et al. 1998 (27).	MRI chemical shift imaging was used to noninvasively study nine volunteers of both sexes. Obtained localized (1) H spectra of the skin, quantified free water content, normalized to skin thickness.	Relative concentration of free water in the skin, normalized to skin thickness, was slightly greater in older subjects and intanned subjects.	These data are consistent with other studies showing overall increase in the free, unbound water content of aged and sun-exposed skin.

Table 5 presents data pertaining to age and skin water structure. See text for discussion.

Table 6 Data: SC and Skin Surface Lipids

Source	Methods	Results	Notes
Schreiner et al. 2000 (31).	Included 10 normal subjects, aged 25.5 ± 2.5 yr, 10 subjects with dry skin, aged 30 ± 6 yr, and 4 subjects aged 66 ± 3 yr. Performed small-angle X-ray diffraction and lipid analysis on whole SC samples from the lower legs. Measured the percent and quantity of different barrier lipid classes.	No significant difference in the number of total ceramides, free sterols, and FFAs between younger and older groups. While not statistically significant, there was an apparent increase in percentage of FFA and compensatory decrease in percentage of ceramides.	According to this study, lipid compositions of different skin types do not differ significantly; however, the aged group only consists of four subjects.
Rogers et al. 1996 (28).	Included 28 female Caucasians, aged 21–50 yr. Studied lipid composition from 8 sequential SC tape strippings of face, hand, and leg. Corneocytes were removed from the tape strippings by sonication in methanol, and lipid extracts were treated and separated using HPTLC on 20 × 10 cm plates. HPTLC plates were developed, dried, and stained, then quantitated using a scanning densitometer at 420 nm. Samples of ceramide fractions from the leg site were also used to analyze ceramide 1 esterified fatty acids in relation to age in the following groups: 26–29 yr ($n = 9$), 41–43 yr ($n = 9$), 57–60 yr ($n = 10$).	All lipid classes decreased with increasing age [Overall, saw a 30% decrease in lipid content in aged subjects. Decrease was most marked for all ceramide species (1–6) in the face and hand, and for cholesterol in the face ($p < 0.05$)]. Percentage ratios of each of the major lipid classes and of the individual ceramide species remained constant. Esterified FA analysis: Levels of ceramide 1 linoleate decreased with increasing age. There were no significant age-related changes in other ceramide 1 esterified FAs or in FFA species.	This study included a fairly narrow age range and does not consider changes in elderly skin. Also, the authors only took 8 strippings from each site; this does not necessarily include the whole SC. This study did account for differences in the amount of SC removed by measuring protein content in strippings and normalizing the mass of each lipid fraction removed.
Saint-Leger et al. 1988 (30).	SC lipids were collected from the right and left legs of 50 subjects of varying ages.	The SC lipid profile was generally constant from age 50 yr upward. Aging was associated with a decrease in sterol esters and triglycerides. Changes in lipids did not seem to account for the increasing xerosis in aged populations.	This is again a very site-specific study. While the sample size is probably adequate, the findings in SC taken from individuals' legs may or may not be generalizable to other body surfaces. The question of how or whether lipid content affects xerosis may also require further study.
Cua et al. 1995 (32).	Included 7 females aged 24.9 ± 1.1 yr, 7 males aged 28.7 ± 0.5 yr, 7 females aged 75.3 ± 2.4 yr, and 8 males aged 73.8 ± 1.2 yr. Measured 11 anatomical regions' SSL contents.	Skin surface lipid content was not statistically different between age groups on all regions except for the ankle, where lipid content was lower in the elderly ($p < 0.05$).	This study accounts for the intraindividual variation one might expect of the skin covering different body surfaces and nicely controls for gender and age. A similarly designed study with a greater number of subjects could be very useful for further confirmation.

Table 6 presents data for age-related changes in lipid content and composition. See text for discussion.
Abbreviations: SSL, skin surface lipid; SC, stratum corneum; FFA, free fatty acids; FAs, fatty acids.

found that the lipid profile was constant from age 50 upward; overall, aging was associated with a slight decrease in sterol esters and triglycerides (30). Cua et al. noted significant regional variation within individuals as they studied 11 sites on 29 people, comparing individuals in their third decade of life with those in their eighth decade. Interestingly, they, too, found little relationship between skin surface lipid content and age, except on the ankle, where the elderly demonstrated decreased lipid content (32). From these contradictory studies, it is difficult to conclude with certainty whether lipid content decreases with age. Many confounding factors may hinder such studies, including seasonal and diurnal variation, general interindividual variation, and the use of several different methodologies by different researchers.

CONCLUSIONS

Collagen becomes less soluble, thinner, and sparser in intrinsically aged skin, but is thickened, fragmented, and more soluble with photoaging (12). UV exposure leads to activation of transcription factors that stimulate increased production of collagenases and MMPs, which lead to breakdown of skin collagen and other proteins (7–9). Also, the ratio of type III to type I

Table 7 Recommendations for Future Studies

Problem	Recommendation
Interindividual variation	**A.** Larger sample sizes to justify significance. Given the tremendous range of variation in skin bioengineering parameters, researchers should consider designing studies using power calculations, wherein before the study, they determine what would be a clinically relevant difference in measurement between young skin and old skin (δ), a significance level (α, usually 0.05) and power level (1-β, usually 8). For a test comparing means of measurements, sample size needed for significance at the specified level is calculated as follows: $n = 2^*[z_{(1-\alpha/2)} + z_{(1-\beta)}]^2/(\delta/s)^2$ Values for $z_{(1-\alpha/2)}$ and $z_{(1-\beta)}$ for 5% significance and 80% power, respectively, are 1.96 and 0.8416. The use of power calculations assumes a normal distribution of the data and equal numerical allocation of participants to the groups being compared (33).
	B. Given the likely significant effects of age-associated hormonal changes on the skin (34), studies should attempt to normalize for or at least regularly report differences in hormonal status among groups tested.
Intraindividual variation	**A**. The average of three measurements should be recorded at any test site; i.e., statistics should analyze the mean of three skin thickness measurements on a certain region of the skin to help minimize "noise" of individual variation.).
	B. Standardization of skin sites tested; e.g., we could agree to focus research on a limited number of specific areas that ideally address issues of lifelong environmental exposure; we suggest, somewhat arbitrarily: 1) the midvolar forearm, exactly halfway between the centers of the wrist and elbow joints 2) the middorsal forearm, exactly halfway between the centers of wrist and elbow joints 3) the mid upper inner arm, exactly halfway between the mid-axilla and elbow joint 3) the exact center of the forehead 4) the upper-mid buttock, 3 cm below the iliac crest and halfway between the midline and left or right border, so as to minimize effects of lifelong compression and decompression that occurs on the lower buttock 5) the midline of the back, over spinous process of T2.
Instrument and other measurement-related variation	**A.** Standardization of, or at the very least accurate and consistent reporting of settings used on bioengineering tools, including frequency settings and gain swept curves on ultrasonography machines.
	B. Since diurnal variation has been demonstrated in skin thickness and echogenicity studies (4, 35, 36), and may also be a factor in blood flow and water content, studies should be designed to minimize this variation; all individuals should be tested at the same or comparable time of day, and timing of the measurements should be reported, if not standardized.

Table 7 presents several recommendations that may help to decrease variation among future studies.

collagen is reported to increase with age (6,12,15). Histologically, young collagen is randomly organized into a meshwork of loosely interwoven bundles. Age leads to a loosening within these bundles and straightening of collagen fibers, increasing skin's tensile strength (14). Biochemically, the aging process leads to progressive oxidation of collagen's lysine residues resulting in accumulation of 2-aminoadipic acid (13).

Elastin is a long-lived protein in human skin; it appears to accumulate damage with age and sun exposure. New elastin is synthesized in greater quantities in aged skin, but it is thought that this synthesis results in abnormally structured elastin (16,21). Also, elastin degradation does not appear to keep pace with new synthesis in aged skin. This results in massive accumulation of elastoic material, especially in photoaged skin; degradation is further impaired by UV-induced expression of proteins elafin and lysozyme (17,18). The abnormal structure of this excessive elastin prevents it from functioning as it does in young skin.

Studies of primary and tertiary skin protein structure in aged skin reveal an environment unfriendly to water, with an overall increase in hydrophobic amino acids and greater folding such that aliphatic residues are more hidden from water (12,22). Also, although total amounts of GAGs appear to be increased in aged skin, these are abnormally localized on the elastoic material in the superficial dermis; thus, they are unable to bind water as well as if they were scattered appropriately throughout the whole dermis (24). Hence it is not surprising that, although aged skin contains increased amounts of water, most of this water is bound to itself in tetrahedral form, rather than being bound to proteins and GAGs as it is in young skin (12). These factors together likely contribute to increased xerosis and withered appearance of aged skin.

While it tends to be an accepted assumption that lipid content decreases with age, quantitative studies are contradictory. Some indicate a marked age-related decrease in skin lipids, at least up to age 50 (28), while others indicate little or no relationship (31,32). Future studies may be benefited by increased sample size and standardization of method, body site, and season of study. Table 7 presents suggestions for greater standardization, and hopefully greater consensus, among future studies.

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24 Glycation End Products

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INTRODUCTION: THE GLYCOXIDATIVE AGING PROCESS

Numerous studies have been carried out in an attempt to elucidate the biochemical and molecular mechanisms of the aging process. The basic biochemical process underlying the aging process was first introduced in 1956 with the free radical theory of aging (1). This theory states that oxidative damage to DNA and other cellular components is the main driving force behind aging. More recent versions of this theory predict that mitochondria are a major source of reactive oxygen species (ROS) that cause oxidative damage. The idea that genetically damaged mitochondria accumulate with time and are causally responsible for the aging phenotype via a disturbed energy production and excessive ROS production is at the core of the so-called mitochondrial theory of aging (2). In 1989, Monnier proposed the Maillard theory of aging, stating that the fundamental aging process might be mediated by the Maillard reaction (the nonenzymatic reaction between reducing sugars and proteins, also known as the glycation process) (3). The free radical-glycation/Maillard reaction theory of aging brings those two views together. It suggests that free radicals (ROS) and reactive carbonyl species (RCS) from Maillard reactions may represent interactive elements of a more complex biochemical pathway. The age-related deterioration then results from the cumulative damage induced by ROS, by RCS, and by their interactions (4). Glycation of mitochondrial proteins results in the excessive formation of intracellular superoxide (5). It was recently shown that senescent human fibroblasts are characterized by a partial uncoupling of the respiratory chain, resulting in increased proton leakage and enhanced electron transport activity (6). Stöckl et al. (7) even suggested a cause-effect relationship between impaired mitochondrial coupling and premature senescence. Others have proposed a key role for high-level ROS-generating enzymes of the NOX family NADPH oxidases in causing age-related diseases (8,9). Oxidative damage to DNA has been found to be an important determinant of life span at least in lower organisms such as *Drosophila melanogaster*. Studies in higher organisms argue for a role of oxidative stress in age-related disease, especially cancer; however, the data remain inconclusive on whether oxidative stress determines life span (10). The general consensus appears to be that the aging process is multifactorial and that it results from an accumulation of damage with an underlying glycoxidative mechanism. Our interpretation of the glycoxidative model of aging is presented schematically in Figure 1.

We will now focus on how glycation contributes to the aging process in skin.

FORMATION OF GLYCATION END PRODUCTS

The nonenzymatic protein glycation reaction was originally described by Maillard to explain the browning reactions taking place during food preparation (11). The initial phase of the Maillard reaction results in the reversible formation of early glycation products. During this process, several reactive intermediates are generated, including ROS and/or RCS like (methyl)glyoxal and glycolaldehyde. The reaction can progress beyond that stage, leading to the formation of a variety of products collectively termed "advanced glycation end products (AGEs)". Heat-generated AGEs that are formed in common foods represent "exogenous" forms of Maillard reaction products, which are ingested on a daily basis. Cigarette smoke was found to contain RCS that are able to react with proteins to form AGEs (12). The "endogenously" formed Maillard products are formed in vivo when reducing sugars or reactive intermediates react with body proteins. Carbohydrates (sugars) are part of a healthy

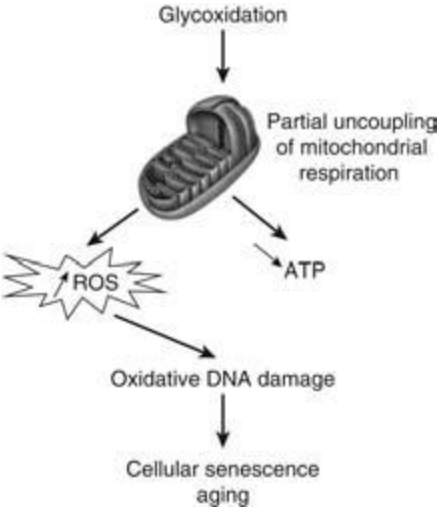

Figure 1 Glycoxidative model of aging. Glycoxidative damage, induced by ROS and RCS, causes impairment of the oxidative phosphorylation process in mitochondria. This leads to mitochondrial leakage of free radicals and less-efficient ATP production, resulting in oxidative DNA damage, cellular senescence, and aging. *Abbreviations*: ROS, reactive oxygen species; RCS, reactive carbonyl species. *Source*: From Refs. 4, 7, 10.

diet. They supply the body with energy, mainly in the form of glucose. When excess (reducing) sugars are available, they can react with other molecules such as proteins. Both exogenous and endogenous forms of AGEs are believed to play a role in aging and disease through what has been generally termed "carbonyl stress" (13).

Some AGEs are characterized by intra- and intermolecular cross-linking with typical fluorescent properties (14). Their formation can be induced in model proteins by incubation with reducing sugars (e.g., fructose) or RCS (e.g., methylglyoxal) in vitro. The progressive formation of AGEs can be followed with fluorimetric detection at wavelengths associated with AGEs (excitation/emission wavelengths of 370/440 nm). The speed of formation depends on the nature of the glycating species. As shown in Figure 2, the in vitro formation of fluorescent AGEs in a model protein (bovine serum albumin, BSA) occurs faster with glycolaldehyde than with 3-deoxyglucosone or methylglyoxal. The reaction can take up to several weeks when less-reactive sugars such as glucose or fructose are used to initiate the reaction.

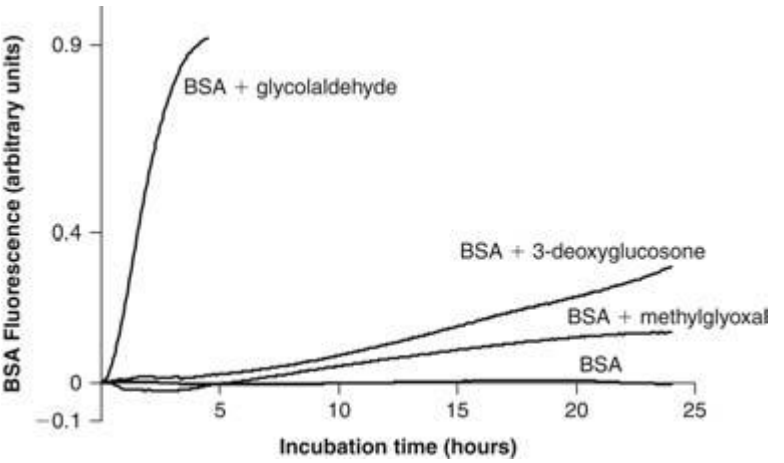

Figure 2 Kinetics of the formation of fluorescent AGEs in the model protein BSA upon incubation with glycation inducers glycolaldehyde, 3-deoxyglucosone, and methylglyoxal. Fluorimetric detection at wavelengths associated with AGEs (excitation/emission wavelengths of 370/440 nm). *Abbreviations*: AGE, advanced glycation end product; BSA, bovine serum albumin.

ACCUMULATION OF GLYCATION END PRODUCTS IN SKIN

Measuring Glycation End Products in Skin

Since the endogenous formation of AGEs is a slow process, the body's long-lived proteins such as collagen are mostly susceptible to AGE accumulation. Therefore, the formation and accumulation of AGEs have been extensively studied in the skin. The presence of glycation end products in the skin has even been proposed as a marker and predictor for the progression of certain systemic diseases (15). The initial method for quantifying AGEs was based on the measurement of collagen-linked fluorescence in tissue biopsies (16). Later, more specialized techniques were developed to analyze specific AGEs such as N^{ε}-(carboxymethyl)lysine and pentosidine (17). More recently, the in vivo measurement of skin fluorescence at wavelengths associated with AGEs (including excitation/emission wavelengths of 370/440 nm) has been introduced as a noninvasive clinical tool to study age-related phenomena in human skin (18). Skin autofluorescence has recently been shown to be correlated with the presence of specific AGEs (19). Skin autofluorescence measurement was then proposed by these authors as an independent predictor for the development of microvascular complications in type 2 diabetes mellitus (20). We have been using a similar method to measure in vivo skin autofluorescence at wavelengths associated with AGEs (including excitation/emission wavelengths of 370/440 nm) in a healthy population. The data were collected with an LS 50B fluorescence spectrometer (PerkinElmer, Waltham, MA, U.S.A.) equipped with a fiber-optic cable (Fig. 3).

Accumulation of Glycation End Products as a Function of Age and Body Weight

In humans, AGEs have been demonstrated to accumulate in the body as a function of age (21) and in age-related diseases (22). Obesity and overweight are risk factors for various disorders, including diabetes (23). Even in nondiabetic individuals, glycated hemoglobin values were shown to increase progressively with age and obesity (24). The increase in overweight and obesity prevalence is evident in the western societies over the past few decades. More recently, this trend of weight gain was observed in other countries, e.g., in Asian countries (25,26), and it is expected to develop worldwide. Although excessive weight gain is a complex, multifactorial chronic condition involving genetic, physiological, metabolic, and psychological components, a dramatic increase in energy-rich food intake accompanied by a significant reduction in physical activity are major contributors to obesity. It is therefore of interest to investigate whether weight gain and obesity have an impact on the accumulation of AGEs in skin.

Long-term consumption of the reducing sugar fructose was shown to accelerate the endogenous induction of collagen-linked fluorescence and skin collagen cross-linking in rats (27). Increasing evidence suggests that reduction of sugar intake by caloric restriction in various species leads to decreased carbonyl stress and reduced accumulation of AGE

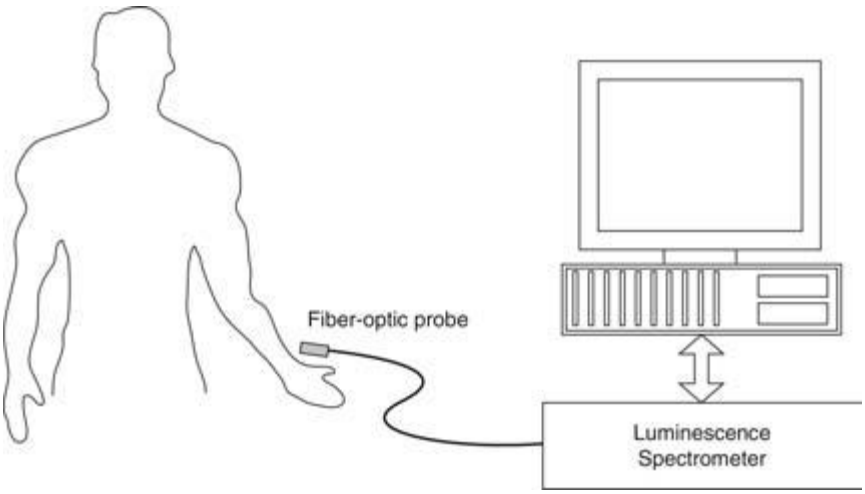

Figure 3 Experimental setup for in vivo measurement of skin autofluorescence at wavelengths associated with AGEs (excitation/emission wavelengths of 370/440 nm) using an LS 50B fluorescence spectrometer (PerkinElmer, Waltham, MA, U.S.A.) with a fiber-optic cable. *Abbreviation*: AGE, advanced glycation end product.

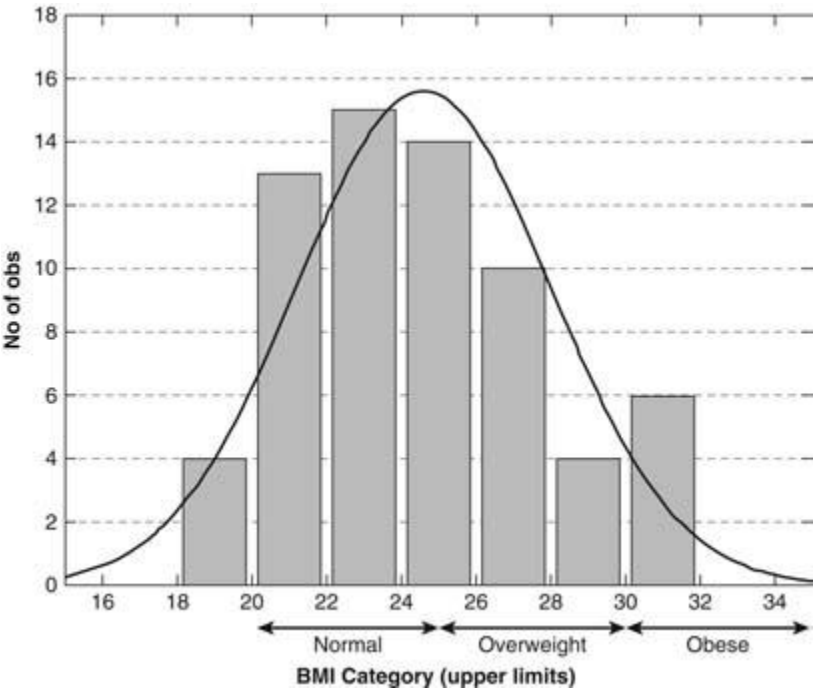

Figure 4 The distribution of BMI for test panel described in the text. The distribution was close to normal with a median of 24.2. A total of 37 volunteers had a normal BMI, 23 were overweight, and 6 were obese. *Abbreviation*: BMI, body mass index.

products in skin collagen (28,29). In calorie-restricted mice, the rate of glycated collagen and AGE accumulation in skin were also shown to be slowed down, which was inversely related to maximal life span (30).

We used in vivo skin fluorescence to evaluate the association between body fat levels (on the basis of impedance measurement) of healthy but slightly overweight individuals and their AGE-related skin fluorescence. A panel of 66 female Caucasian nonsmoking healthy volunteers living in Belgium, aged between 21 and 59 years (median 42 years), was enrolled in this study. As indicated in Figure 4, the body mass index (BMI, calculated as weight in kg divided by the square of height in m) of the volunteers was close to normal and ranged from 18.3 to 32.0 (median 24.2). Skin color was measured with a chromameter (Minolta, Osaka, Japan) and expressed as individual typology angle (ITA°). The ITA-value decreases with increasing pigmentation of the skin. Multiple linear regression techniques were used to take into account the impact of skin color on the in vivo AGE-related fluorescence (excitation/emission wavelengths of 370/440 nm). As shown in Table 1, the fluorescence intensity attributed to AGEs increased as a function of panelist age ($p = 0.008$) and with increasing percentage of body fat ($p = 0.04$) (independent of the age effect). These data suggest that within a population of healthy women with weight from normal to slightly overweight, an increasing level of fluorescent AGEs accumulates in the skin as a function of age and body weight. In a separate study, we have found that within a test panel of 448 female Caucasian volunteers

Table 1 Statistical Outcome of the Multiple Linear Regression Analysis on the Association Between In Vivo Skin Fluorescence and Percentage Body Fat, Chronological Age, and Skin Color (ITA)

	Fat (%)		Chronological age		Skin color (ITA°)	
Fluorophore	Partial regression coefficient	*p*-value	Partial regression coefficient	*p*-value	Partial regression coefficient	*p*-value
AGEs (370/440)	**0.67**	**0.04**	**0.58**	**0.008**	**2.48**	**$<10^{-6}$**

Statistically significant parameters are indicated in bold.
Abbreviation: AGE, advanced glycation end product.

Table 2 Statistical Outcome of the Multiple Linear Regression Analysis on the Association Between In Vivo Skin Fluorescence and Smoking Behavior, Chronological Age, and Skin Color (ITA)

	Smoking behavior		Chronological age		Skin color (ITA°)	
Fluorophore	Partial regression coefficient	*p*-value	Partial regression coefficient	*p*-value	Partial regression coefficient	*p*-value
AGEs (370/440)	**9.43**	**0.03**	**0.32**	**0.02**	**2.22**	**$<10^{-6}$**

Statistically significant parameters are indicated in bold.
Abbreviation: AGE, advanced glycation end product.

living in the New York area, the independent contribution from body weight and chronological age on the skin AGE-related autofluorescence was confirmed in the group of panelists younger than 40 years ($n = 147$). In the older panelists ($n = 301$), the effect of age on the AGE-related fluorescence was much stronger and became dominant over the effect of body weight (31).

Accumulation of Glycation End Products as a Function of Age and Smoking Behavior

On the basis of a systematic review of 25 prospective cohort studies, it was recently concluded that active smoking is associated with an increased risk of type 2 diabetes (32). Literature data suggest that smoking causes mitochondrial dysfunction via inhibition of complex IV activity (cytochrome c oxidase) (33). This mitochondrial dysfunction could contribute to increased endogenous production of ROS (34). Cigarette smoke is also considered to be an inducer of glycoxidative reactions (12). Nornicotine, a constituent of tobacco and metabolite of nicotine, can catalyze Maillard-like reactions under aqueous conditions. The plasma of smokers as compared with nonsmokers contains higher concentrations of nornicotine-modified proteins (35). A significant rise in the mean levels of the glycation product fructosamine and total plasma glycated proteins were found in smokers when compared with nonsmoking controls (36). An increase in dermal collagen cross-linking can be induced in rats by exposure to cigarette smoke (37). We used in vivo skin fluorescence to evaluate the association between smoking behavior and AGE-related skin fluorescence in normal healthy individuals.

A panel of 94 female Caucasian healthy volunteers living in Belgium, aged between 18 and 81 years (median 43 years), was enrolled in this study. There were 16 active smokers and 78 nonsmokers. Multiple linear regression techniques were used to take into account the impact of skin color on the in vivo AGE-related fluorescence (excitation/emission wavelengths of 370/440 nm). As shown in Table 2, the fluorescence intensity attributed to AGEs increased as a function of panelist age ($p = 0.02$) and with smoking ($p = 0.03$) (independent of the age effect). These data suggest that within a population of healthy women an increasing level of fluorescent AGEs accumulates in the skin as a function of age and smoking behavior.

CONSEQUENCES OF GLYCATION END PRODUCT FORMATION AND ACCUMULATION IN SKIN

Structural Consequences

The glycation process is characterized by the formation of AGEs with intra- and intermolecular cross-links and with typical fluorescent properties. It is well known that the accumulation of cross-linked AGEs alters the structural properties of tissue proteins. The cross-linking also reduces their susceptibility to being removed by catabolic processes, thereby contributing further to the accumulation (38). The structural changes resulting from a slow accumulation of AGEs in the skin contribute to the modification of the skin's biomechanical properties. At the clinical level, they have been associated with an increase in stiffness or brittleness of the skin (39,40). This is in agreement with data showing that in vitro glycated skin samples are characterized by a loss of biomechanical properties (41). The structural changes are not solely restricted to the dermal compartment of the skin. In a diabetic population, it was shown that glycation of plantar epidermal proteins could play an important role in the stiffening of plantar skin (42). The glycation process can therefore play an important role in explaining the changes in mechanical properties of the skin that occur over several decades of life (43,44).

Functional Consequences

The accumulation of AGEs in long-lived proteins and the resulting structural changes occur rather slowly. The glycation process can have more important short-term consequences as well. The presence of AGEs can increase cellular oxidative stress and promote inflammatory reactions (45). The in vivo formation of reactive intermediates such as methylglyoxal can cause immediate cellular damage by causing the deactivation of enzymes with an important role in cellular defence or survival (46).

Deactivation of enzymes involved in maintaining energy homeostasis such as creatine kinase is expected to result in a generalized decline in biological performance (47). Creatine kinase is responsible for transferring the energetic phosphor group between creatine/creatine phosphate and adenosine triphosphate (ATP). Clinical studies with ^{31}P nuclear magnetic resonance spectroscopy have indicated that the capacity to regenerate energy levels after a mild stress is compromised in older skin (48). As illustrated in Figure 5, creatine kinase is susceptible to in vitro deactivation upon incubation with methylglyoxal as well as with a cigarette smoke extract. Methylglyoxal decreases mitochondrial membrane potential and intracellular ATP levels, suggesting that carbonyl stress-induced loss of mitochondrial integrity could contribute to the cytotoxicity of the intermediate glycation products (49). As previously shown in Figure 1, the consequence of mitochondrial damage is a partial uncoupling of the mitochondrial respiration, mitochondrial leakage of free radicals, and less-efficient ATP production, resulting in oxidative DNA damage, cellular senescence, and aging (4,7,10).

Deactivation of protective enzymes such as catalase, superoxide dismutase, glutathione reductase, and peroxidase will result in a reduced antioxidant defense capacity of the cells. The importance of the activity of the superoxide dismutase (SOD)/catalase duo has been clearly established. SOD catalyzes the dismutation of the superoxide anion into oxygen and hydrogen peroxide. Then catalase will neutralize hydrogen peroxide to oxygen and water. Sun exposure results in a disturbed catalase to SOD ratio, at least in the upper layers of the skin (50). Low catalase activity levels have been measured in the skin of patients with vitiligo (51), polymorphic light eruption (52), and *Xeroderma Pigmentosum* (53). In vitro studies emphasize the importance of maintaining the optimal ratio between the different antioxidant enzymes and its relation to cellular senescence and sensitivity to oxidative stress (54). The observation that the life span of *Caenorhabitis elegans* could be extended after supplementation with SOD/catalase mimics supports the hypothesis that an increased efficiency at which ROS can be neutralized by antioxidant enzymes may counteract the aging process (55).

Finally, deactivation of enzymes involved in the DNA repair process can have major consequences for cellular viability and tissue functioning. Defects in cellular DNA repair

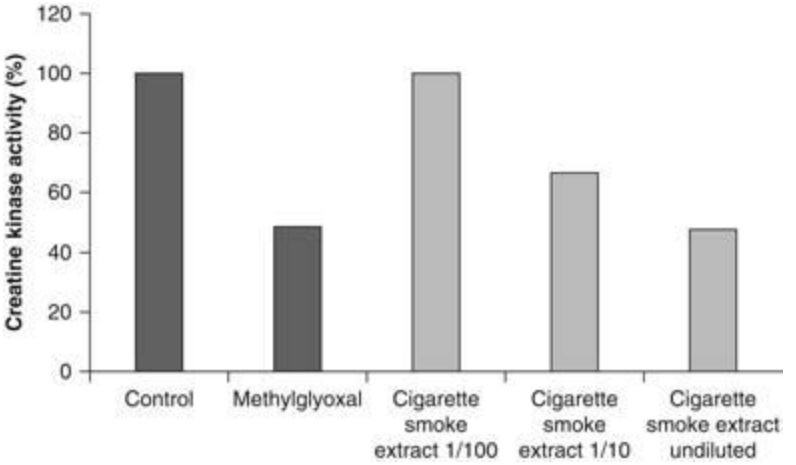

Figure 5 A cigarette smoke extract prepared by smoking six research cigarettes from Tobacco Health Research, University of Kentucky (ref.: 2R4F, 11/2001) into 7 mL of HPLC grade water, containing 7 μL of Triton X−100. In vitro incubation of creatine kinase with cigarette smoke condensate caused a dose-dependent decrease of creatine kinase enzyme activity. The undiluted cigarette smoke condensate caused 50% loss of enzyme activity, which was similar to the effect caused by the glycation intermediate methylglyoxal at 4.5 mM.

processes have been linked to genome instability. This then potentially leads to an accumulation of mutations, cellular dysfunction, and aberrant phenotypes at the tissue and organism levels. Depending on which part of the DNA repair process is affected, the clinical manifestation of those genetic defects occurs in the form of heritable cancers and/or premature-aging syndromes (56,57).

TECHNOLOGIES TO CONTROL GLYCATION IN SKIN

Protecting Against Glycation End Product Formation

The overwhelming evidence linking the consequences of the formation of glycation end products in the skin with aging has strongly stimulated the development of technologies aimed at slowing down AGE formation. Technologies for either the prevention of AGE formation in the skin or the reduction of existing AGEs have been proposed by various laboratories around the world. We intend to limit this review to the description of technologies aimed at preventing the glycation process and its consequences. In our hands, none of the ingredients that claimed to reduce the amount of preexisting AGEs (so-called AGE breakers) have shown to reduce the extent of AGE cross-linking in our in vitro model systems. This does not exclude the fact that other mechanisms may lead to their removal in a more complex in vivo system.

To characterize AGE-modified proteins used in studies of formation and structural effects of glycation, an in vitro assay was developed that surveys the content of cross-linked proteins with characteristic fluorescent properties. The assay procedure involves the high performance liquid chromatography (HPLC) separation by gel filtration of adducts resulting from the reaction between a model protein and a glycation inducer at 37°C. The quantification is based on fluorimetric detection at wavelengths associated with AGEs (excitation/emission wavelengths of 370/440 nm). An example is shown in Figure 6, with creatine kinase as the model protein before (A) and after (B) incubation with the glycation inducer glycolaldehyde. The higher molecular weight adducts elute earlier and have the characteristic fluorescent properties.

This procedure can then be used to evaluate the efficiency of glycation inhibitors in preventing the formation of AGEs in vitro. Aminoguanidine can be used as a positive control with well-known anti-glycation activity (58). As shown in Figure 6 (C), this compound significantly prevented the development of higher molecular weight AGEs in our in vitro testing model and can be proposed for topical use in products aimed at protecting the skin from the damages induced by the glycation process.

In any preventative technology, it is essential to control the initial steps of the reaction process. As described before, the glycation and oxidation reactions are intimately linked at an early stage of the process. For this reason, it is advisable to evaluate the activity of known antioxidants such as flavonoids for their ability to control the initial stage of the glycation process. Experiments conducted in our laboratories demonstrated that more than 75% of the flavonoid-like materials we tested had an antioxidant activity (based on the luminescent ABEL R antioxidant test kit for vitamin C type antioxidants) similar to their anti-glycation effect. In line with published data, the overall outcome suggested that the presence of a hydroxyl function at the C-3′ and C-4′ position of the B-ring is a requisite for both antioxidant (59) and anti-glycation activity (60) (see Fig. 7). An additional series of experiments using an ex vivo skin model confirmed the activity of a blend of eight antioxidants in preventing the damage caused by UV exposure in glycated skin (unpublished results). Finally, we demonstrated in a clinical study that the topical application of a product containing a mixture of antioxidants as well as aminoguanidine and another scavenger of RCS on a group of human volunteers for a period of two months resulted in a significant reduction of the skin fluorescence intensity at the wavelengths attributed to AGEs (unpublished results).

These data suggest that the control of the glycation process requires a multifactorial approach rather than the simple blockage of the Maillard reaction to inhibit the formation of a covalent bond between a reducing sugar and a protein. The many different steps occurring during the initial stage of the glycoxidation process, leading to the formation of the early glycation products, followed by the development of AGEs, cannot be controlled by one single molecule. On the contrary, a mixture of antioxidants with proven efficacy in protecting cellular

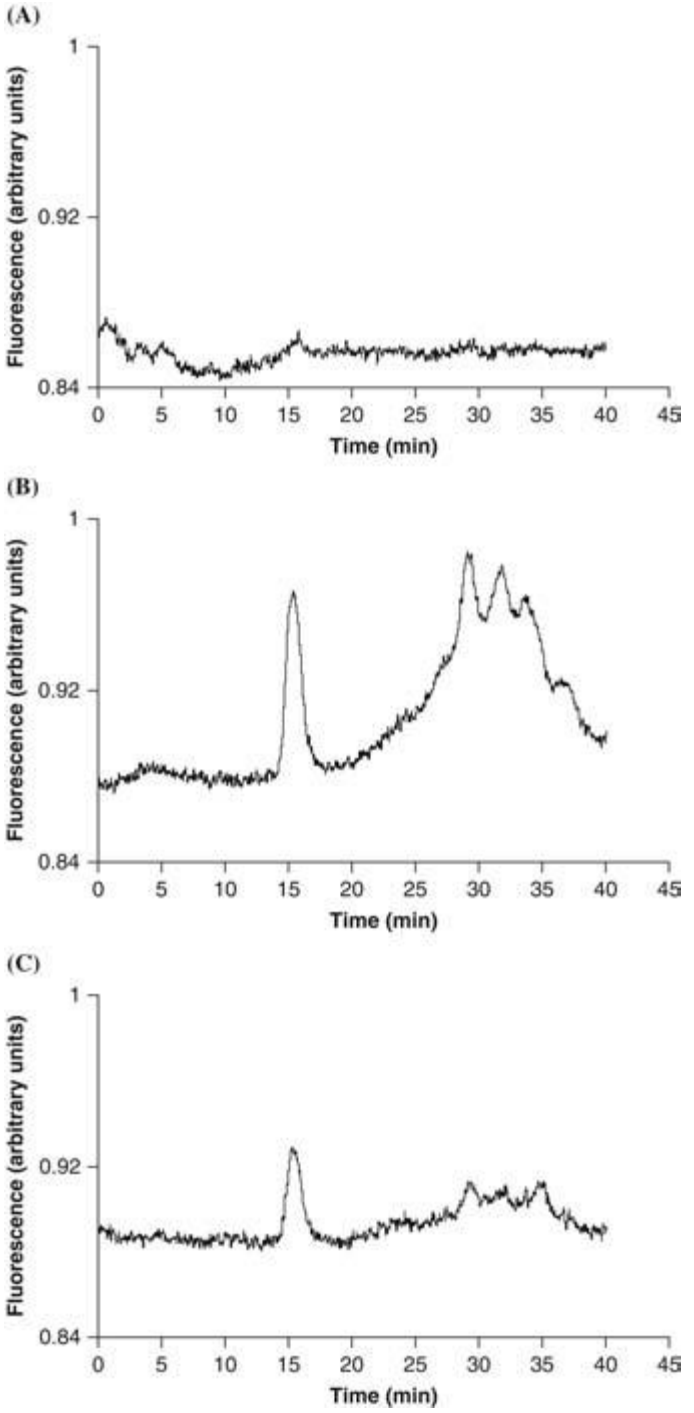

Figure 6 The formation of fluorescent AGEs in the model protein creatine kinase upon incubation with glycolaldehyde. Creatine kinase was glycated in vitro and subjected to gel filtration HPLC analysis with fluorimetric detection at wavelengths associated with AGEs (excitation/emission wavelengths of 370/440 nm). The enzyme migrates as multiple peaks in the retention window between 15 and 40 minutes. The chromatogram of untreated creatine kinase is depicted in the top panel (**A**) and shows no fluorescent signal. Incubation of creatine kinase with the glycation inducer glycolaldehyde (panel **B**) resulted in the appearance of fluorescent peaks with higher molecular weight that are attributed to the glycation of creatine kinase. Coincubation with the glycation inhibitor aminoguanidine partially prevented the formation of the fluorescent protein modifications (panel **C**). *Abbreviation*: AGE, advanced glycation end product.

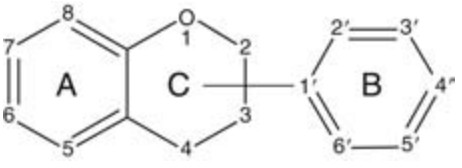

Figure 7 Basic structure of flavonoids

lipids and proteins as well as the mitochondrial and nuclear DNA together with ingredients that directly inhibit the glycation process (e.g., aminoguanidine) should be used in the same preparation to provide the skin with a more complete protection and thereby slow down the aging process.

Protecting Against Consequences of Glycation

Previously in this chapter, we reviewed the structural consequences of glycation, which result in the loss of skin resilience and elasticity because of the cross-linking of elastin and collagen in the dermis. As this cross-linking reaction is nonenzymatic and occurs slowly, it could be prevented by the regular topical application of products containing the anti-glycation ingredients we described above. The indirect consequences of the glycation process can have, in our opinion, far more impact on the skin, as they potentially undermine all the essential functions of the skin cells. As described above, the early stage of the glycation process involves the formation of RCS such as methylglyoxal, glyoxal, glycolaldehyde, and 3-deoxyglucosone. This can increase cellular oxidative stress, promote inflammatory reactions, and deactivate some of the key enzymes responsible for the protective (e.g., catalase, superoxide dismutase), reparative, as well as the metabolic function (e.g., creatine kinase) of the cells.

In an in vitro system, it is possible to prevent the loss of enzymatic activity using the technologies described above. An example is shown in Figure 8, whereby aminoguanidine prevents the loss of creatine kinase activity induced by incubation with methylglyoxal (47). In a complex in vivo system, it is advisable to use this in combination with ingredients that will compensate for the deficient metabolic activity, the lower protective enzyme capacity, and the reduced DNA repair enzyme activity.

Treatment with a blend with creatine, the reduced form of β-nicotinamide adenine dinucleotide (NADH), and *N*-acetyl-L-carnitine helps to replenish the energy reserve pool while reducing intracellular ROS generation in older skin cells (61).

The UV stable synthetic SOD/catalase mimic manganese complex EUK-134 can be used topically to compensate for a loss of antioxidant enzyme activity (62,63) and as a result help restore the protective capacity of the skin against oxidative damage. The indirect consequence is a 50% reduction of the UV-induced cellular apoptosis (sunburn cells) after treatment with nanomolar concentrations of EUK 134.

Similarly, the glycation-induced loss of DNA repair activity can be boosted with DNA repair enzymes, e.g., UV endonuclease from micrococcus luteus and 8-oxoguanine DNA glycosilase (OGG1). They are encapsulated in a liposomal preparation and marketed by AGI dermatics under the trade name of liposomal UV endonuclease and liposomal OGG1, respectively. A significant amount of research has been done (64,65) to prove that the topical application of a preparation containing a liposomal suspension of these two enzymes is

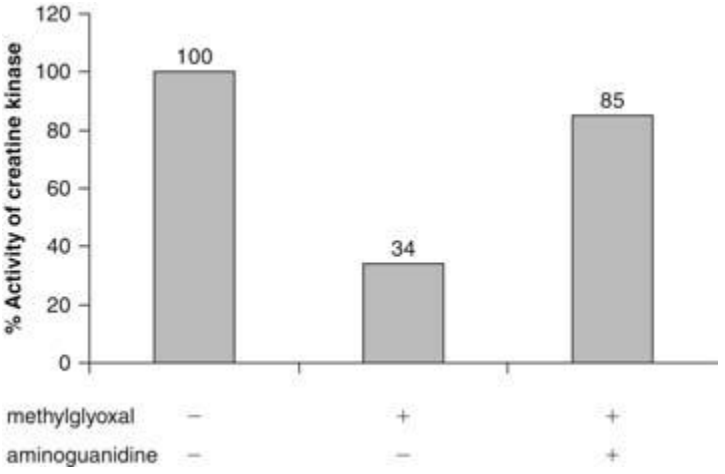

Figure 8 In vitro incubation of creatine kinase with the glycation inducer methylglyoxal decreased the creatine kinase enzyme activity by 65%. The loss of enzyme activity could partially be prevented by the glycation inhibitor aminoguanidine.

reducing the amount of DNA damage, and as a result reduces significantly the amount of cellular apoptosis.

Used together, these technologies provide a broad-spectrum protection from the direct as well as indirect damages caused by the interaction between sugars and our skin's proteins.

By interacting at every step of the process described in Figure 1, it is possible to minimize the impact of the glycation reactions on cellular viability, not only by preventing the Maillard reaction from occurring but also by restoring the enzymatic activity, which is essential for the natural protection mechanisms of the cells. Technologies that optimize metabolic activity restore the protective capacity and boost DNA repair will thereby enhance the cellular resistance to stress induced by exposure to the environment and slow down the aging process.

CONCLUSIONS

There has been an interesting evolution over the last 10 years in understanding the causes of the premature skin-aging process. From the very general free radical theory of aging, to the more specific Maillard theory of aging, the scientific research seems to seek deeper into the real causes of the premature dysfunction of cellular protection and repair mechanisms. It has been postulated that the defense mechanism expressed by mammalian cells is the result of an evolutionary process that has allowed cells to adapt to an oxidative, DNA damaging environment. Assuming this is true, it becomes difficult to understand why exposure to the environment can still result in premature skin aging.

Two important factors could explain this condition:

1. A significant boost in the level of exposure to oxidative stress due to an increase in environmental pollution and higher levels of UV exposure (more frequent travels and depletion of the ozone layer).
2. A malfunctioning of the cellular defense mechanisms caused by deactivation of the protection and repair mechanisms, leading to the accumulation of deficient lipids, proteins, and DNA.

The gradual increase in the level of oxidative stress caused by the increased pollution of the world's atmosphere is a well-accepted fact that we do not intend to discuss within the scope of this chapter. The deactivation of the cellular defense mechanism however is of far more interest, especially in the light of the gradual increase in prevalence of obesity and cigarette smoke exposure over the past few decades. Both these factors cause AGEs to accumulate faster in the skin.

In this chapter, we have reviewed how the glycoxidation process has an impact not only on the structural proteins of the skin such as keratins, collagen, and elastin but more importantly on the functional enzymes with an essential role in providing the cells with the capacity to energize, repair, and protect skin cells.

In complete agreement with Monnier (3), we believe that the glycoxidation process plays a major role in the premature-aging process, as it is involved in three major processes involved in the accumulation of damage in the cells (Fig. 1).

1. The glycoxidation process affects the cell's metabolic function by causing damage to mitochondrial proteins, resulting in a heightened release of intracellular ROS.
2. The glycoxidation process causes a deactivation of the essential enzymatic antioxidant activity, thereby further enhancing the oxidative damage to cellular components.
3. The glycoxidation process is linked to the deactivation of the enzymatic DNA repair mechanism, thereby contributing to the accumulation of DNA damage.

As a result, we see an increase in apoptosis or cellular senescence, possibly even the development of cells with erroneous genomic structure. The combination of these three factors can therefore explain why the glycation process contributes to skin aging.

The faster accumulation of AGEs in skin as a function of body weight and smoking behavior is believed to be accompanied by an enhanced level of damage induced by the glycoxidation process and a concomitant reduction of the cellular defense mechanisms.

The technologies we have described in this chapter can intervene at multiple steps in this sequence of events. The topical application of products containing a well-balanced mix of technologies to control glycoxidation and its consequences can help to restore a functional level of cellular defense. These products should therefore be of great help in maintaining cellular homeostasis and preventing premature skin aging.

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25 Spectrophotometric Intracutaneous Analysis (SIAscopy)

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WHAT IS SIASCOPY?

Spectrophotometric intracutaneous analysis (SIA)scopy is a skin-imaging technique that allows the rapid, noninvasive in vivo quantification and assessment of (eu) melanin, (oxy) hemoglobin, and dermal collagen within human skin. A powerful feature of SIAscopy is that it produces independent linear measurements of each of these endpoints, which can also be mapped over the skin, producing images called SIAscans. SIAscopy was originally developed for the assessment of malignant melanoma (1) where the accurate assessment of melanin, blood, and collagen has been shown to increase diagnostic accuracy (2) for the disease.

SIAscopy measures underlying histological parameters through the use of a model of tissue coloration, providing a cross-reference between spectral measurements and histology. The model is constructed by computing the spectral composition of light remitted from the skin, given parameters specifying its structure and optical properties, providing a unique mapping between the spectral measurements and the histological parameters (3). For each histological component, a parametric image is then created, providing the magnitude of each at all pixel locations. This approach requires two inputs: the first is a set of parameters that characterize a given tissue by specifying its components, their optical properties, their quantities, and their geometry; the second is a method for computing the remitted spectra from the given parameters.

CONSTRUCTION OF THE MATHEMATICAL OPTICAL MODEL OF HUMAN SKIN

The skin consists of a number of layers with distinct functions and optical properties as shown in Figure 1. Light incident to the skin penetrates the superficial layers, and while some of it is absorbed, much is remitted back and can be measured.

The stratum corneum is a protective layer consisting of keratinized squamous cells (corneocytes), and it varies in thickness across the body. Apart from forward scattering of incident light, it is optically neutral (4). The epidermis is composed of several layers of differentiating keratinocytes and also contains pigment-producing cells, melanocytes, and their product, the melanins. The melanins are complex heteropolymers that strongly absorb short-wavelength radiation, i.e., light in the blue part of the visible spectrum and radiation in the ultraviolet (UV) waveband (in the latter case, therefore, acting as a filter to protect the deeper layers of the skin from the well-documented harmful effects of UV radiation). Within the epidermal layer, there is very little scattering and that which does occur is forward directed. This means that all light not absorbed by melanin can be considered to pass into the dermis. The dermis is composed largely of collagen fibers, and in contrast to the epidermis, it contains sensors, receptors, blood vessels, and nerve endings. Hemoglobin, present in blood vessels within the dermis, acts as a selective absorber of light. The dermis consists of two structurally different layers, papillary and reticular, which differ principally in the size of collagen fibers. The scale of the collagen fibers in the papillary dermis (diameter of an order of magnitude less than the incident visible light) makes this layer highly scattering, i.e., any incoming light is scattered with a proportion directed back toward the skin surface. The scatter is greatest at the blue end of the spectrum, decreasing with increasing wavelength

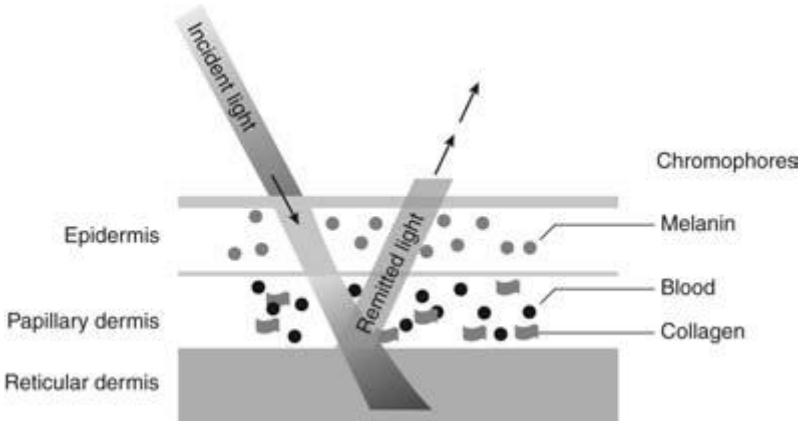

Figure 1 A schematic representation of skin layers (*labels on the left*) and chromophores (*labels on the right*).

(through green and red and into the infrared). Within the reticular dermis, in contrast, the larger scale of collagen fiber bundles causes highly forward-directed scattering (1). Thus, any light reaching this layer passes deeper into the skin and does not contribute to the remitted spectrum.

From these first principles, therefore, the mathematical optical model for normal skin has three layers corresponding to epidermis, upper papillary dermis (with prevalence of blood), and lower papillary dermis. The range of wavelengths used by the SIAscope technique, from 400 to 1000 nm, covers the entire visible spectrum and a small range of near infrared. Recently, the model has been verified by comparing its output to that generated by a stochastic Monte Carlo method using a public domain implementation (Figs. 2 and 3) (5).

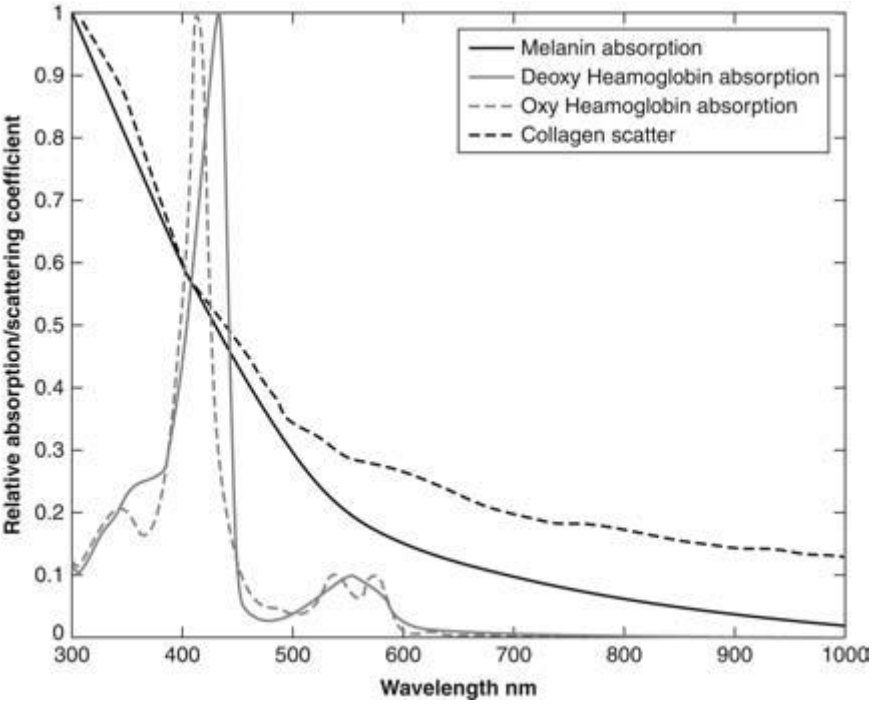

Figure 2 Absorption coefficients of principal components of human skin.

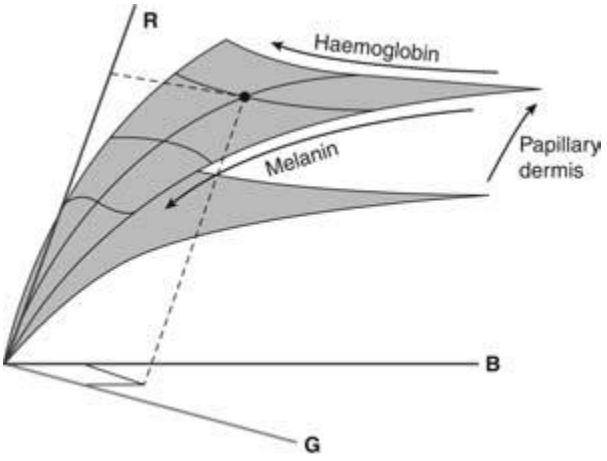

Figure 3 Schematic relationship between two reference systems: color system, with axes R, G, and B; and the histological components hemoglobin, melanin, and collagen.

CONTACT SIASCOPY

Contact SIAscopy uses a small handheld scanner (Fig. 4), which is placed in contact with the skin. This contact ensures that the distance of the skin from the lens is known and fixed, which allows exact calibration of the spectral imaging used. This control of imaging geometry allows the synthesis of accurate gray scale concentration maps each of (oxy) hemoglobin, (eu) melanin, collagen, and the position of melanin relative to the dermo-epidermal junction (Fig. 5). A small

Figure 4 SIAscope.

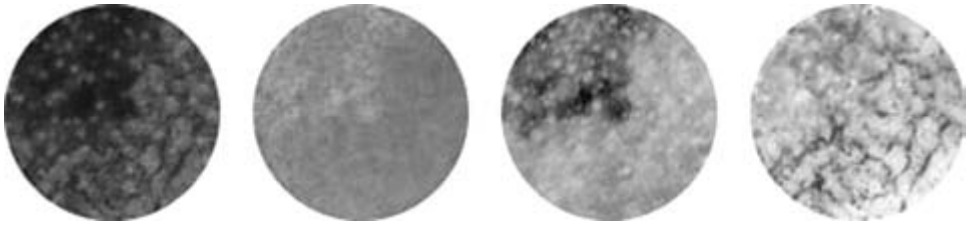

Figure 5 SIAscans showing, from left: color, collagen, melanin, and hemoglobin.

amount of matching fluid is used to ensure that optical aberration due to the refractive index of air is removed effectively.

The contact SIAscope provides a rapid and convenient method for assessing and characterizing intrinsic and extrinsic skin aging and also assessing the effects of cosmetic products, for example, the reduction of solar lentigines (6,7).

CHROMOPHORE MAPPING

We have given the term "chromophore mapping" to the synthesis and subsequent analysis of the gray scale molecular concentration maps produced by SIAscopy. These maps are readily accessible by a wide range of image analysis techniques, allowing sophisticated analysis of the arrangements of chromophores within them. We used these techniques in a study performed on 400 female Caucasian subjects aged 10 to 70 years, recruited in equal five-year cohorts (7), and demonstrated remarkable relationships for the melanin, hemoglobin, and collagen endpoints (obtained using a contact SIAscope) with age, consistent with ingoing hypotheses relating to the extent and timetabling of expression for each of these optical skin components. Moreover, sufficient dynamic range was present within the data to allow the use of this technique to track changes in these chromophores because of treatment.

SURFACE ANALYSIS

Further development of the contact SIAscope method has yielded an analysis of fine surface topograhical features ("microtexture" and fine lines and wrinkles) (8). This technique uses the fact that light returning from the skin is a blend of deeply scattered light and direct surface reflection resulting from the stacked nature of the stratum corneum. If the deeply scattered light is isolated and removed, the remaining directly reflected light carries information pertaining to the skin surface. Other workers have used this phenomenon by acquiring separate images taken in different polarization states (9). This is a useful technique, but requires specialist hardware and can suffer from registration problems between image sets.

Highly detailed information regarding the surface of the skin can be obtained using contact SIAscopy because the instrument polarizers operate only within the visible region of the spectrum and do not interact with infrared light (Fig. 6). This results in suppression of surface reflection in the visible spectrum only (which is why a matching fluid is required to obtain accurate collagen measurements). By using a technique described in (8), a prediction of

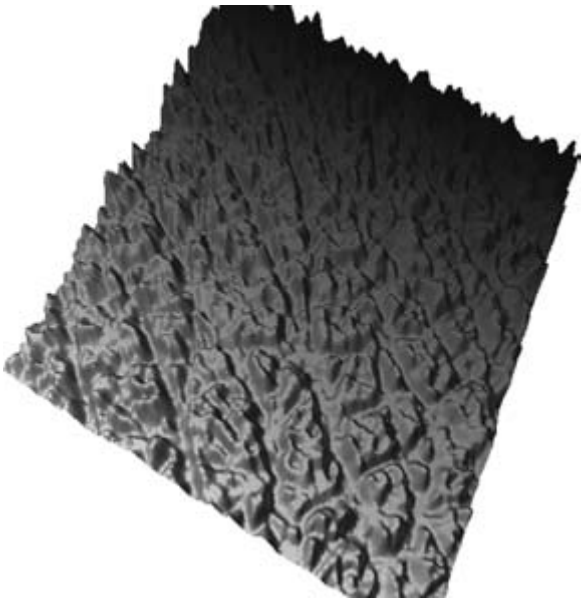

Figure 6 Skin surface micro architecture measured with a SIAscope.

what the polarized infrared signal should be is made from the polarized-visible spectra and then compared with the actual unpolarized infrared measurement. The difference is then converted into a measurement of direct surface scatter. The result is a detailed map of skin topology, which is both rapid and simple, lending itself naturally to large scale cosmetic testing and development.

HYDRATION

An adaptation of the surface analysis technique can also be used to assess skin hydration levels in vivo, allowing investigation into the effects of skin moisturizers and also diseased skin conditions such as eczema. This technique operates on the principle that direct surface reflection measured by the surface analysis technique is lower in hydrated stratum corneum. An example of skin moisturization changing over time following the application of a topical moisturizer is shown in Figure 7, with the false color images showing the spatial changes in hydration over a 10-minute period.

NONCONTACT SIASCOPY

Noncontact SIAscopy (NCS) uses a digital camera as a broad-band spectrometer to recover chromophore information over a wide area. The same mathematical model underpinning contact SIAscopy is used to create a mathematical model of the camera response to varying amounts of hemoglobin and melanin (10). The mathematical model is based on the Bayer filter response curves, the light-sensitive array that sits at the focal point of a digital camera, and the spectral power distribution of the light source (usually a flash) used with the camera. To measure the response curves of the Bayer filter, a double-monochromator is used to illuminate it with specific and highly calibrated narrow ($<$10 nm) wavebands of light.

A problem still exists, however, because the geometry of the scene being imaged is not known and, therefore, calibration of the measured information is difficult. To overcome this problem, a ratio of different Bayer filter wavebands forms the input to the mathematical model. The use of ratios removes the artifacts of geometry, as they are present equally in all wavebands. From first principles, this approach reduces the number of chromophores that can be measured, such that NCS is able to measure only hemoglobin and melanin. NCS is, however, extremely flexible allowing imaging of full faces, or, indeed, any body part (if the

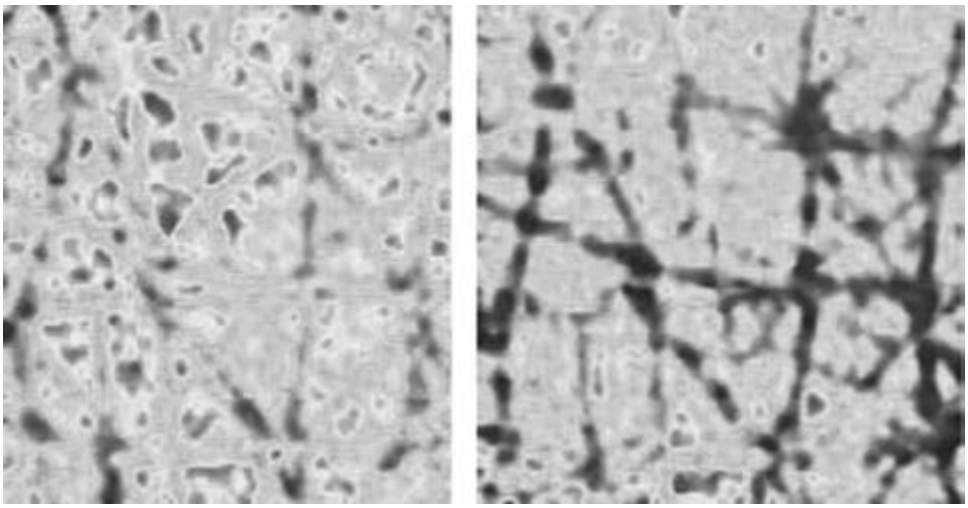

Figure 7 Changes in hydration levels in human skin (same area) following application of a topical moisturizer (before and 10 minutes after application). "Dry" skin (*left*) and "moisturized" skin (*right*) with pseudocolor scale indicating hydration state.

Figure 8 Noncontact SIAscopy in use.

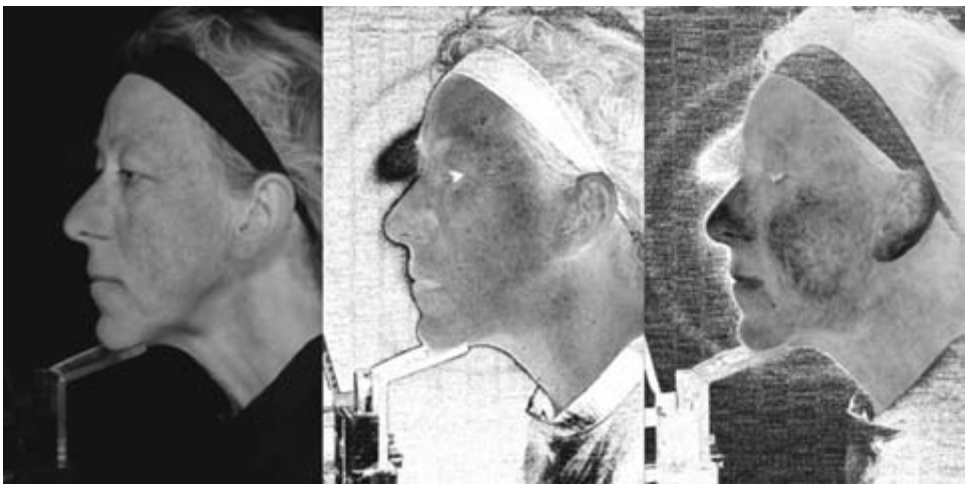

Figure 9 NCS images with (*left to right*) cross-polarized color, melanin, and hemoglobin maps.

camera is used apart from the stand). Apart from the ability to measure large fields, NCS also has the advantage of eliminating the potential artifacts of pressure "blanching" that can potentially occur with any skin contact measure (Fig. 8).

Figure 9 shows NCS melanin and hemoglobin SIAscans. Localized subsurface hyperpigmentation can clearly be seen in the melanin SIAscan and telangiectasia in the hemoglobin SIAscan.

Because the NCS technique now allows routine acquisition of full-face melanin and hemoglobin chromophore maps, the method has proven an ideal clinical partner in assessing the effects of cosmetic treatments. In a recent double-blinded study, NCS was used to provide a quantitative means of measuring the effect of a vehicle containing 2% *N*-acetyl glucosamine (NAG) and 4% niacinamide (N) versus a vehicle control, applied topically, full-face, twice-daily for eight weeks, to two groups of 100 females aged 40 to 60 years, respectively, on melanized hyperpigmented spots (11). Analysis of the NCS melanin maps demonstrated clear treatment effects for the NAG + N combination versus vehicle control, resulting in a significant ($p < 0.05$) reduction in melanin spot area fraction and a significant ($p < 0.05$) increase in melanin evenness.

Finally, it should be noted that a separate study has shown an excellent correlation between NCS-derived melanin concentrations and eumelanin concentrations in human skin biopsies, spanning Fitzpatrick skin types I-VI (12). It must be concluded, therefore, that large-field chromophore mapping by NCS brings a new level of sensitivity and specificity to measurement of human skin color.

CONCLUSION

Whereas even modern high-resolution imaging still only describes skin appearance, SIAscopy explains it by separating the molecular components responsible for that appearance in the first place. In this way, SIAscopy provides the clinician and researcher alike with a powerful new tool to both measure and characterize human skin.

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26 The Visioscan-Driven ULEV and SELS Methods

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INTRODUCTION

Melanocytes and melanins are largely responsible for the phototype-related color of the skin. The varied colors of skin depend on the nature, amount, size, and distribution of melanin pigment produced by melanocytes and transferred to keratinocytes. The system is governed by the epidermal melanin unit corresponding to a functional entity composed of one melanocyte and its neighboring keratinocytes into which the melanosomes are transferred. Chronic ultraviolet (UV) radiation provides a positive signal to the exposed epidermal melanin unit. In this instance, the number of active melanocytes increases, and individual melanocytes are stimulated to produce more melanins. In addition, melanosome transfer from melanocytes to the surrounding keratinocytes is enhanced through the intervention of the protease-activated receptor 2 (1).

According to the individual melanin phenotype, age, and cumulative UV exposure, the skin commonly develops a discrete-to-severe mottled appearance. Freckles in youths and solar lentigines in older individuals are the clinical representatives of these influences. Such aspects are due to an increase in the keratinocyte melanin content (melanotic hypermelanosis), which may or may not be associated with melanocytic hyperplasia. The resulting mottled pigmentation is an early key feature of photoaging.

Photography under UVA light, which is highly absorbed by melanin, is a convenient way to highlight any discrete variation in skin pigmentation. Provided that the lighting is kept constant and the camera calibrated beforehand, this technique highlights any change in pigmentation. By using a UVA source, any skin blemish can thus be conveniently assessed by a regular photography system (2–7). As such, UV photography is used as a diagnostic tool, but it has rarely been employed for measuring pigmentation. Indeed, the latter application is not satisfactory because casual equipments generate shadows superposed to the skin pigmentation. To respond to this drawback, a charge couple device (CCD) camera equipped with an internal UV-emitting unit (VisioScan® VC98, C+K electronic, Cologne, Germany) was developed (Fig. 1). The video sensor chip must be closely applied to the skin surface to avoid shadows. The uniform illumination of the skin brings out a sharp picture of a 6 mm × 8 mm area of the skin surface. The high resolution of the system allows close assessments of the skin. The connection of the VisioScan VC98 to the computer is made through an image digitalization unit, which configures the image in pixels of 256 gray levels, where 0 is black and 256 is white. This leads to the so-called UV-light enhanced visualization (ULEV) method.

SUBCLINICAL MELANODERMA

The mosaic subclinical melanoderma (MSM) is a mottled pattern enhanced under ULEV examination (8,9). This feature is particularly prominent in photosensitive individuals with a phaeomelanin-enriched phenotype. Using this method, the increased contrast between the faint or almost invisible hyperpigmentation and the surrounding skin is the combined result of a greater reflection of UVA than visible light by collagen, and its greater absorption by melanin inside the epidermis. The boundary between the subclinical melanoderma and the surrounding skin is sharply marked. Since UV rays penetrate less deeply into the skin than visible light, it is considered that UV photography and the ULEV method catch mainly the pigmentation inside the epidermis. Thus, the pigments localized in the dermis are not detectable using these methods. Therefore, a clear distinction can be made using ULEV between an enhanced melanotic hypermelanosis and a dermal melanoderma. This distinction is useful because the former type is accessible to treatment and the latter type is unresponsive and remains as a melanin tattoo. These two conditions coexist, for instance, in melasma.

Figure 1 VisioScan camera.

Several MSM patterns have been identified (8–11). They are listed in Table 1. Spotty perifollicular dots (Fig. 2) are commonly seen on the scalp and face, occasionally extending to other seborrheic regions (8,10–12). This physiological pattern is recognized as early as during adolescence, and it does not seem altered by aging. By contrast, the interfollicular region exhibits a combination of pinpoint lesions, small macules, and globular macules (Fig. 3). This pattern appears later in life and is subject to variations with cumulative photoexposure and aging. These hypermelanotic blemishes may become confluent, and they appear more prominent on chronically sun-exposed skin. This aspect is regarded as an early sign of

Table 1 Patterns of Subclinical Skin Mottling

- Pinpoint: minute irregularly distributed darker spots
- Follicular dots: speckled perifollicular darker rings
- Small macules: small interfollicular darker areas
- Globular macules: accretive and circinate confluence of smaller macules
- Streaky macules: elongated darker areas along wrinkles
- Confluent macules: massive darker areas

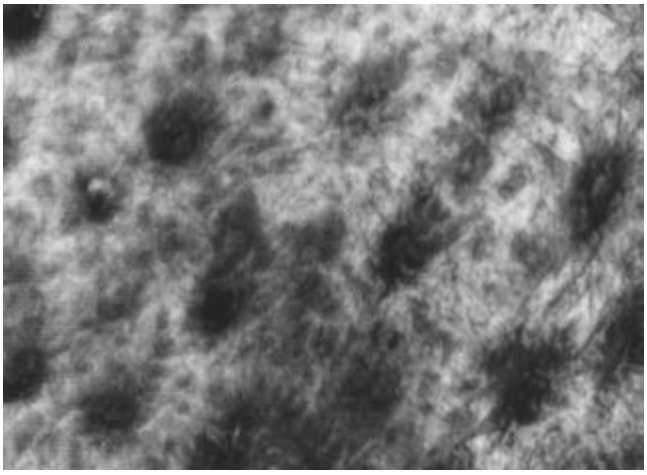

Figure 2 Perifollicular dots of subclinical melanoderma.

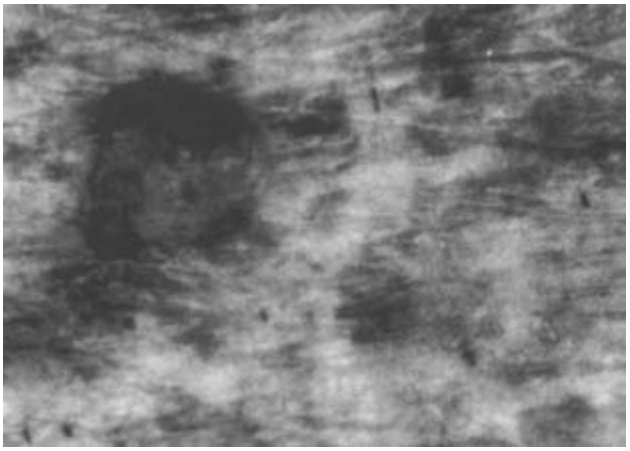

Figure 3 Macular interfollicular subclinical melanoderma.

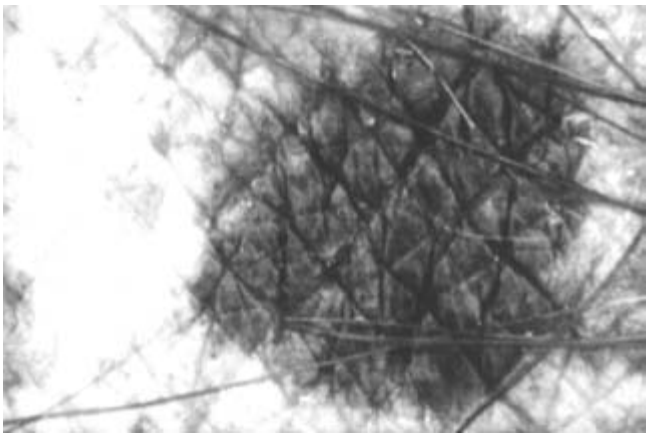

Figure 4 "Brown spot" on photoaged skin.

photoaging (9,13,14). Still another aspect is given by the streaky pattern elongated along wrinkles. This aspect is typically present on the sunny side slope of facial frown lines (8).

ASSESSMENT OF WHITENING AGENTS

The ULEV method can be employed for assessing the efficacy of cosmetic whitening products (8,15–18). Image analysis of the pictures offers objective quantification of the depigmenting effect on the epidermal melanin unit. The method has been applied to the assessment of "brown spots" (Fig. 4) corresponding to solar lentigines or incipient pigmented seborrheic keratoses (15,16,18). Such evaluations are more easily handled than the bleaching effect on melasma (17). Using the ULEV method, the observed effects of whitening agents cannot be distinguished according to the putative biological effects on tyrosinase, protease-activated receptor 2 or any other step of melanization.

Beyond conventional cosmetic whitening agents, some drugs can alter the activity of the epidermal melanin unit. Both topical corticosteroids and vitamin D analogues decrease the MSM severity as assessed by the ULEV method (14).

SKIN SURFACE MICRORELIEF AND SCALINESS

The quantitative evaluation of the skin surface microrelief is of interest in assessing some of the therapeutic and cosmetic interventions as well as for the determination of the severity in irritation damages to the skin. A measurement where the skin can be directly monitored

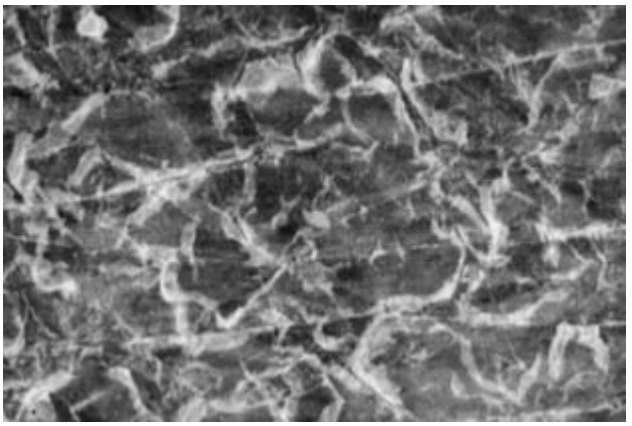

Figure 5 Scaly aspect of the skin.

optically using an image digitalization process without any sampling or replica collection should represent a great advance in technology (19–21).

The regular observation of ULEV pictures reveals any hyperkeratotic and scaly aspect of the skin surface. The lesions look whitish in contrast with the gray aspect of the normal-looking skin (19–21). The SELS (surface evaluation of living skin) method is an additional way, allowing direct and noninvasive measurement of the skin topography using the VisioScan camera (22). The actual picture of the skin can be presented with false colors and viewed either two dimensionally or tridimensionally. Four different parameters have been selected to be displayed and analyzed in the evaluation of the skin surface. Skin smoothness (SE_{sm}) is calculated from the average width and depth of the wrinkles. Skin roughness (SE_r) represents the opposite parameter to the first. Scaliness parameter (SE_{sc}) aims at representing the level of dryness of the stratum corneum (SC). The wrinkle parameter (SE_w) is calculated from the proportion of horizontal and vertical wrinkles.

In the SELS assessment, swelling the stratum corneum with water or a moisturizer does not appear to affect the roughness of the skin surface, but the value of the scaliness parameter and the number of wrinkles decrease, while smoothness increases.

The UVA of the VisioScan VC98 highlights desquamation (Fig. 5). A semiquantitative assessment is possible, but the interpretation of data is not always straightforward. For improving this assessment using the VisioScan, the transparent, sticky Corneofix® F20 tape (C+K electronic, Cologne, Germany) is slightly pressed onto the skin surface. The superficial corneocytes stick to the tape and are harvested for the assessment of their numbers and sizes. As the light is absorbed differently because of the different thickness of corneocyte clumps, they appear as dark pixels in the image. Looking at the gray level distribution in the histogram, a quantitative assessment can be performed. The regular VisioScan software distinguishes five scaliness levels. The drawback of this automatic cutting is that the shutter does not properly recognize the background in some images. In these instances, the observer can set the shutter manually by comparing the original and the segmented image with an accuracy of $\pm 0.1\%$.

COMEDOGENESIS

Microcomedones and keratin-filled funnel-like acroinfundibula can be easily identified using ULEV examination (Figs. 6 and 7). The method can thus be useful in the assessment of comedogenic and comedolytic compounds. Acne physiopathology is also highlighted by this method.

SEBUM EXCRETION

Sebum flow dynamics can be assessed using lipid-sensitive films (23). The assessment benefits from image analysis of the sebum-enriched spots. The VisioScan VC98 camera can be used for that purpose (24). The head of the camera is covered by the opaque microporous lipid sensitive Sebufix® F16 tape (C+K electronic, Cologne, Germany) before being applied to

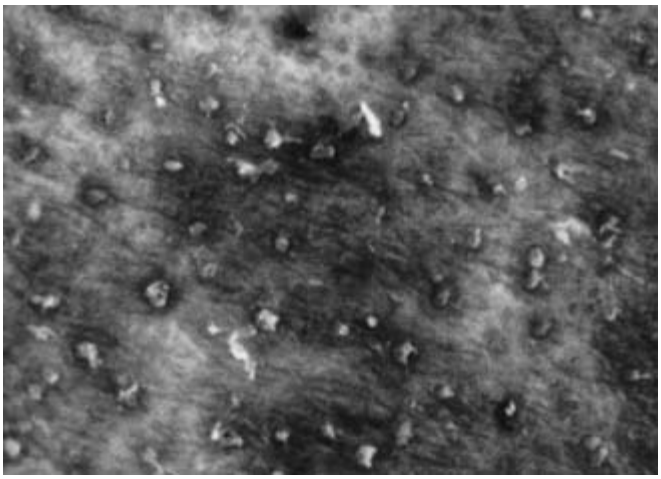

Figure 6 Microcomedones.

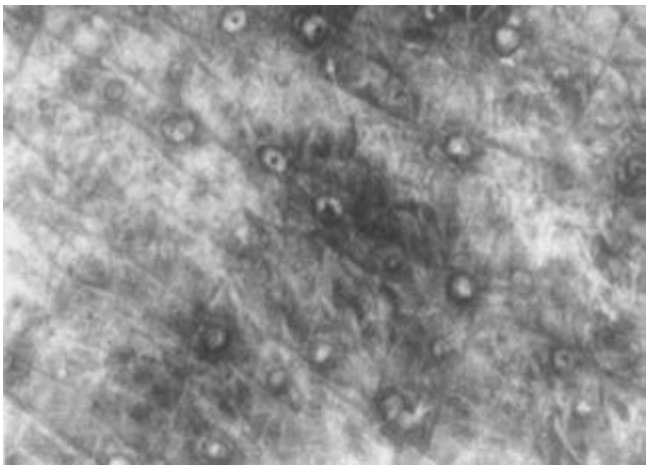

Figure 7 Open comedones.

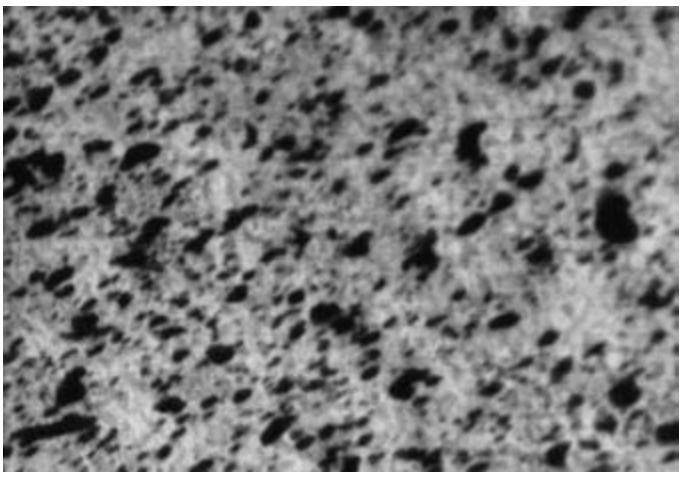

Figure 8 Sebum droplets in a Sebufix foil.

the skin. After about 30 seconds, the mean instant sebum follicular output (SFO) corresponding to the area of the transparent spots of lipid droplets is assessed using computerized image analysis (Fig. 8). The face of the Sebufix F16 in contact with the skin is glue free. Thus, the sebum can enter the micropores of the sebum-sensitive foil without any restriction. This leads to a short

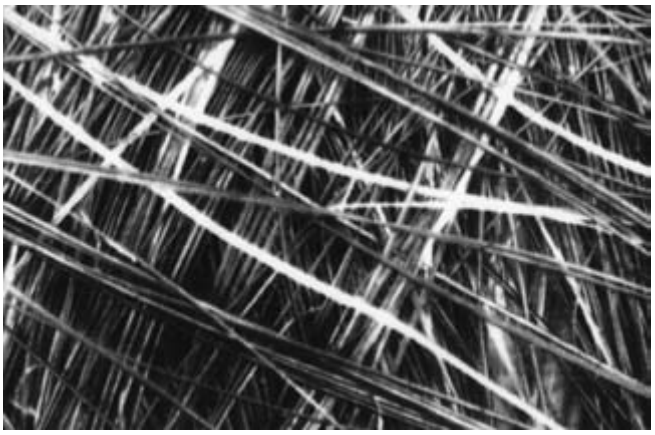

Figure 9 Gray hairs dispersed in normally pigmented hairs.

measuring time after which the foil is ready to be evaluated. This requirement is important to avoid any occlusion effect. Indeed, occlusion leads to stratum corneum swelling and to increasing skin temperature, thus influencing the sebum measurements.

The more oily the skin, the shorter the collecting time is necessary. Studies performed any time after cleansing the skin is thus possible. The supplied holder presses the lipid-sensitive film lightly but constantly on the skin and leads to reproducible measurements. The special foil specifically measures the sebum output from the follicular reservoir. With this procedure, the sebum output at the skin surface can be monitored live in real time.

HAIR GRAYING

The loss of melanin content in the hair shaft is a natural manifestation of aging leading to apparent hair whitening. Hair graying can be observed as early as 20 years of age in Caucasians and 30 years in Africans; it has been reported that on average, in a cohort of Caucasians, 50% of people had at least 50% gray hair by age 50 (25). The biological processes underlying hair graying remain unclear (26). In addition, the process of hair graying is difficult to assess and quantify in vivo. The ULEV method highlights this physiological phenomenon by enhancing the contrast between graying hair, which appear bright white, and the other hair shafts (Fig. 9).

TRICHOBACTERIOSIS

Some bacterial species tend to clump along hair shafts. They are embedded in a biofilm. This condition corresponds to trichobacteriosis formerly called trichomycosis. The ULEV method is a convenient way to observe these structures, which appear as bright white sheaths encasing the base of the hair shafts (Fig. 10). This aspect is probably related to the fluorescence exhibited

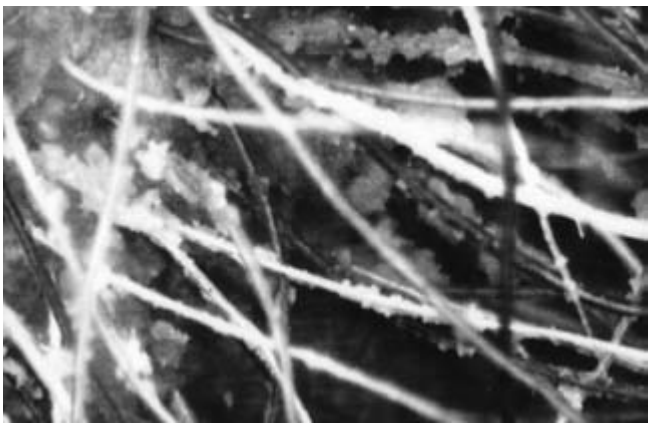

Figure 10 Trichobacteriosis characterized by bacterial sheaths encasing hairs.

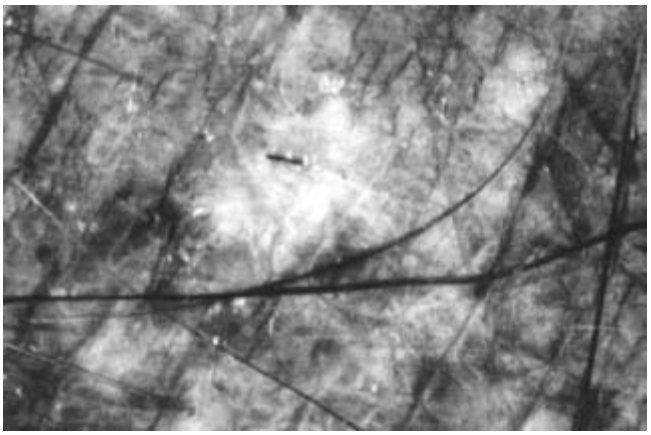

Figure 11 Area of depigmented skin in the background of mosaic subclinical melanoderma.

by trichobacteriosis under Wood's light. A similar phenomenon can occur at the site of other fluorescent lesions such as erythrasma and pityriasis (tinea) versicolor.

DANDRUFF ASSESSMENTS

Under ULEV examination, dandruffs appear as small white objects dispersed along hair shafts (27,28). Because of the high contrast with hair, a quantitative assessment is made possible without any specific sampling procedure.

RISK ASSESSMENT OF SKIN CANCERS

A correlation was found between MSM severity and the risk for developing actinic keratoses and basal cell carcinomas on facial skin and the scalp (29–34). A peculiar aspect corresponds to focal depigmentation suggesting the destruction of some epidermal melanin units (Fig. 11). Such finding could help in identifying early adult life subjects at risk of skin cancers.

CONCLUSION

UVA photography and its more recent development, using a CCD camera equipped with an internal UVA-emitting unit, are useful by different aspects. The MSM revealed by the ULEV method is one of the earliest clinical signs of photoaging. The same method also highlights scaliness and desquamation as well as a series of other potential specific conditions mentioned in this chapter.

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27 New Trends in Antiaging Cosmetic Ingredients and Treatments: An Overview

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INTRODUCTION

Aging is a natural process in which both intrinsic aging, also described as biological aging, and photoaging lead progressively to a loss of structural integrity and physiological function of the skin (1–4).

With intrinsic aging, structural changes occur in the skin as a natural consequence of the biological changes over time and produce a certain number of histological, physiological, and biochemical modifications. Intrinsic aging is determined genetically (influence of gender and ethnic group), variable in function of skin site, and also influenced by hormonal changes. Visually it is characterized by fine wrinkles. By comparison, "photoaging" is the term used to describe the changes occurring in the skin, resulting from repetitive exposure to sunlight. The histological, physiological, and biochemical changes in the different layers of the skin are much more drastic. As a result, the photodamaged skin may present various symptoms such as deep wrinkles, actinic keratoses, solar elastosis, yellowing, pigmentation disorders and premalignant lesions, skin atrophy, senile purpura, telangiectasia, laxity, and roughness.

The demographic changes in the industrialized populations (United States, Europe, and Japan) are the consequence of the rise in the number of elderly persons: the life expectancy for men (about 80 years of age) and women (far above 80 years). Women with a longer average life expectancy will spend a long lifetime in the menopausal status. They will enjoy their leisure time and are very much aware of the concept of preservation of the youthful appearance (face and whole body) and good health (4). These categories of seniors (3 or 4×20 years) have more financial means to spend on cosmetic products than do the very young population. Consumers are focused on their health and appearance, creating a great demand for antiaging cosmetic and oral treatments. We observe the following trends in these demands: an appeal for "Green and Natural," more natural and less invasive cosmetic substitutes instead of cosmetic surgery and skin injections without the side effects. Modern topical antiaging cosmetic products can be characterized as cosmeceutical preparations: formulated with "natural," functional active ingredients and with innovative delivery systems (penetration enhancers, nanoparticles, and porous delivery systems) (5). The entrance of a large number of men older than 60 years, who like women want to preserve their appearance and good health, has provoked the development of a specific range of skin care and antiaging products for men. The aims of this chapter are (*i*) to describe the histological, physiological, biophysical, and biochemical characteristics of biological aging and photoaging, respectively, (*ii*) to describe the clinical visual and tactile examinations and bioengineering methods to qualify and/or quantify these symptoms, and (*iii*) to give a critical overview of the different topical antiaging cosmetic ingredients and treatments. Also, oral antiaging food supplement will be mentioned. Excellent review articles on skin aging have been published (1–4,6).

INTRINSIC SKIN AGING

Intrinsic aging or chronologic or biological aging is, by definition, unavoidable, since it represents the biological effect of time on the skin, which is not influenced by repetitive sun exposure. The skin that normally ages is smooth with some deepening of the surface (small wrinkles) and loss of isotropy in the microrelief. Histologically, there is an epidermal and dermal atrophy, gradual reduction in the amount of collagen, hyperkeratosis, and some atrophy—a reduced number of melanocytes, Langerhans cells, and fibroblasts. A shortening in telomere DNA and metabolic oxidative damage are believed to play a major role in the

intrinsic aging process. Also, the glycation reaction on skin proteins occurs with time and contributes to the intrinsic aging (see the chapter "Glycation End Products" by Declercq et al.) (7). The synergy of intrinsic aging and photoaging during the life span produces a deterioration of the cutaneous barrier. Aged skin is more susceptible to extreme dryness, itching, cutaneous infections, autoimmune disorders, vascular complications, lack of elasticity, and an increased risk for premalignant and malignant lesions. The major intrinsic skin aging factors are anatomical variations, gender, ethnicity, and hormonal changes (4).

Ethnicity

It is obvious that differences in pigmentation have the greatest effect on aging. High levels of melanin pigmentation are protective with regard to aging. Black skin shows smaller differences between exposed and unexposed sites. Skin cancer incidence rates between white Caucasian and Africans indicate that pigmentation provides a significant level of protection from UV radiation. It appears that wrinkling in Asian skin occurs later and with less severity than in Caucasian skin, probably due to ethnicity but also due to differences in lifestyle and diet. For a complete recent review of the properties of skin related to race, sex, and site, the reader is referred to the chapter "Biophysical Characteristics of the Skin: Relation to Race, Sex, Age, and Site" by Couturaud (8).

Anatomical Skin Sites

Large variations in some skin properties (hydration, transepidermal water loss, epidermal lipids, sebum secretion, and mechanical properties) have been observed with respect to the studied body site. There are also large differences in skin thickness in function of the body site, ranging from very thin on the eyelids to more than 5 mm on the sole of the feet. A regional variation is clearly observed when considering the quantity and composition of lipids in the stratum corneum. Because of thickness and sebum secretion, the viscoelastic properties of the skin are very different at the forehead, nose, and cheeks compared with the forearm.

Gender and Hormonal Changes in Cutaneous Tissues

The influence of gender on the physicochemical properties of the skin is often reported in the literature (8); however, no clear-cut conclusive information on the influence of sex on intrinsic aging is reported. Nevertheless, there are significant morphological differences according to sex: total skin thickness is greater for men on most skin sites.

The changes of estrogen levels in the female skin when comparing pre- with postmenopausal women have well been described in the literature. Intrinsic aging in men is a progressive phenomenon: one observes a smooth decline in skin properties in function of time. The same curve shows clearly a sharp acceleration of decline around 50 to 60 years, which corresponds to the passage from pre- to postmenopause.

EXTRINSIC SKIN AGING

Chronic repetitive exposure of human skin to solar UV rays causes marked morphological, histological, biochemical, and biophysical changes that are described as photoaging. As already mentioned here above, the clinical signs of photoaging are fine and coarse wrinkles, actinic keratoses, solar elastosis, yellowing, pigmentation disorders and premalignant lesions, skin atrophy, senile purpura, freckles, solar comedones, telangiectasia, laxity, roughness, and extreme dryness. Most of the alterations are apparent in the dermis of photoaged skin and include massive accumulation of abnormal elastic fibers, loss of collagen, and increase of GlycoAminoGlycans (GAGs). The fibrous network degenerates into amorphous masses modifying the structure of the matrix. UV-mediated damage to elastic fibers and fibroblasts provokes the accumulation of this elastotic material. Alteration in the dermal extracellular matrix in photoaged skin could involve the production of abnormal matrix molecules by the fibroblasts. Also is noted the increase of activity of metal-binding matrix-degrading enzymes (9).

Exposure to UV Light

Research in the field of photaging has resulted in a better understanding of the molecular mechanisms of the aging process. UV light penetrates skin, and depending on its wavelength, it interacts with different skin layers located at different depths. Shorter wavelengths (UVB,

280–320 nm) are mostly absorbed in the epidermis, affecting predominantly keratinocytes. Longer-wavelength UV light (UVA, 320–340 nm) penetrates deeper and can interact with keratinocytes and dermal fibroblasts. The melanin-pigmented cells absorb UV light and thus protect skin cells from exposure to UV radiation. UVA light mostly acts indirectly through generation of reactive oxygen species (ROS). "ROS" is a general term for oxygen-derived species (superoxide anion, hydroxyl radical, and certain peroxides). ROS exert a multitude of effects such as lipid peroxidation, activation of matrix metalloproteinases and generation of DNA, and mitochondrial DNA damage (10,11). UVB light can also generate ROS, its main mechanism of action is the interaction with DNA, provoking DNA damage. The skin presents an antioxidant defense mechanism, including an enzymatic and nonenzymatic system. As an enzymatic system, we have superoxide dismutases, catalase and a selenium glutathione peroxidase, converting superoxide anion in hydrogen peroxide and hydrogen peroxide in water, respectively. As endogenous antioxidants we find glutathione, α-lipoic acid, coenzyme Q, etc. Other antioxidants, such as vitamins and polyphenolic compounds, can be obtained from diet. An increase of ROS (depletion of the antioxidant system) will cause oxidative stress, leading to potential tissue damage.

Sun damage also creates a state of chronic inflammation, with the release of proteolytic enzymes by the inflammatory system, disrupting the dermal matrix (1). Sunburn is a well-known acute effect of sun exposure and is clinically visible as an erythema triggered by inflammation. After a certain threshold of UV exposure is reached, a delayed and prolonged vasodilatation allows the passage of lymphocytes and macrophages in the tissues, which induces inflammation, leading to a histological appearance of chronic inflammation (9). The intake of antioxidants or anti-inflammatory compounds was suggested to reduce erythema provoked by UV irradiation (11).

Effect of Smoking

It is now well established that smoking has an aggravating effect on skin aging. Cigarette smoke represents one of the greatest exogenous sources of free radicals. The skin is diectly and intensively exposed to cigarette smoke and to smoke from exhalation. Even external exposure to cigarette smoke (secondhand cigarette smoke) prematurely ages the skin. Smoking provokes elastosis, telangiectasia, skin roughness, and premature wrinkles on facial skin due to the vascular constriction of nicotine (12). A clear dose-response relationship has been observed between smoking and wrinkling (4). Smoking also increases ROS formation and is an important risk for cutaneous squamous cell carcinoma.

DESCRIPTION OF THE DIFFERENT OBJECTIVE EVALUATIONS OF PHOTOAGING

Evaluation of Microrelief and Wrinkles

Wrinkles in the Facial Skin

Facial wrinkles are the most characteristic morphological changes observed in photoaged skin. Therefore, many quantificative methods to analyze wrinkles have been developed (13). First, direct visual evaluation of the face (front and lateral views) and rating of different well-selected aspects of photoaging (fine and coarse wrinkle, roughness, yellowing solar lentigines) by experienced examinators using a numerical scale are very popular in use and rather simple to carry out. The subjects themselves can judge their degree of photoaging and the efficacy of cosmetic products on the severity of wrinkles. Different clinical studies have shown that there is more or less a good correlation between the rating of the subjects and the evaluation of experienced investigators. Although visual evaluation is rather subjective and evaluator dependent, both methods are widely used in clinical testing of wrinkle-smoothing products. The same visual evaluation of the face can be carried out on macro digital photographic pictures obtained using a standard setup for positioning the face of the subject and lateral illumination. Evaluation of the wrinkles using silicone skin replicas has been employed complementary to visual examinations. Skin microrelief and coarse wrinkles (crow's feet) can be taken using these replicas. Many techniques using different principles are now available to analyze the replicas (mechanical and laser profilometry, two-dimensional image analysis, confocal micoscopy, etc.).

In vivo instruments have been developed to study directly the morphological structure of the skin. The PRIMOS® system is based on the image analysis with image triangulation using a micro-mirror device projection on the skin (14). It is possible to measure the skin topography by using a UVA-light video camera Visioscan VC98® with a SELS® software (Surface Evaluation of the Living Skin) technique providing the standard roughness parameters (Rt, Rm, Rz, and Rp) (15). The microrelief of the skin surface and particularly the isotropy/anisotropy of this microrelief can be evaluated from the images obtained using a charge coupled device (CCD) visible video camera (Intuiskin IntuiPro Pack®, Crolles, France). Image analysis gives the classical roughness parameters and the degree of anisotropy of the skin surface (16).

Reviscometer® (17)
Aged skin is characterized, among other symptoms, by profound changes in the orientation of the collagen fibers in the dermis. In young skin, these fibers are very small and oriented almost in all directions (isotropy). When the skin gets older, thickening of the fibers occurs with a preferential orientation of the thick collagen fibers (anisotropy). The degree of isotropy/anisotropy in the dermis can be evaluated by measuring the propagation speed of an acoustic shock wave applied on the skin surfaces following different orientations with a Reviscometer® (18). The propagation speed of a shock wave applied on the skin surface at different angles gives a degree of isotropy/anisotropy clearly related to skin aging.

Viscoelastic Properties of the Skin Using the Cutometer®
Photoaging will provoke profound changes in the structure of the dermis and consequently changes in the mechanical properties of this layer. The Cutometer® (19) is a suction device that measures the vertical displacement of the skin when a small vacuum (suction) is applied on the surface. The vertical displacement of the skin can be measured in function of vacuum (stree-strain curve) or in function of time when suddenly vacuum is applied and released (strain-time curve). From the strain versus time curve, the pure elastic, pure elastic recovery, and viscoelastic properties can be quantified and are clearly related to the degree of aging and skin laxity.

Ultrasound Measurements at 20 MHz
Querleux et al. (20) described on echographic pictures of photoaged skin, between the epidermis and reticular dermis, a dark echogenic band, which has been called subepidermal low echogenic band (SLEB). Several studies have proposed that the evaluation of the SLEB (thickness, number of low echogenic pixels, and the ratio of the number of pixels in the SLEB to the number of pixels in the deeper dermis) to be markers of photoaging (21–23). Also, the mean total thickness of the dermis and its density can be computed.

Hydration Measurements
Typical photoaged skin shows symptoms of roughness and dryness, although extreme dryness (ichtyosis) can be present on nonexposed skin areas (lower part of the legs in very old persons). Typical hydrating and smoothing products are used in the treatment of dryness. In addition, most antiaging cosmetic preparations contain hydrating ingredients and skin-smoothing ingredients. Quantitative hydration evaluation of the upper layers of the skin, e.g., horny layer and upper epidermis, are numerous: electrical impedance and capacitance measurements (24), Fourier transformed infrared spectroscopy with an attenuated total reflection unit, FTATR (25), and confocal Raman microscopy (26). The last two instruments, although they give quantitative data directly related to the amount of water present in the horny layer, are less used in routine clinical research because of the high price of purchase. Most routine hydration measurements are carried out using the electrical impedance/conductance properties [Dermalab®, Hadsund, Denmark (27), Skicon®, Hamamatsu, Japan (28), DPM Nova®, Portsmouth, New Hampshire (29)] or capacitance properties [Corneometer®, Cologne: Germany (30) and MoistureMeter®, Kuopio, Finland (31)] of an alternating electric current applied on the skin surface. It must be pointed out that the data of these instruments (electrical units or arbitrary units) are related to hydration but not linearly proportional to the percentage of water present in the horny layer.

CRITICAL OVERVIEW OF THE TOPICAL ANTIAGING INGREDIENTS AND TREATMENTS

Before starting to give an overview of the different categories of antiaging ingredients, it is important to make the following remarks.

As mentioned above, the clinical symptoms of photoaging are multiple: fine and coarse wrinkles, actinic keratoses, solar elastosis, yellowing, pigmentation disorders, premalignant lesions, skin atrophy, senile purpura, freckles, solar comedones, telangiectasia, laxity, roughness, and extreme dryness. Obviously, it is impossible with one antiaging cream/gel to treat all these symptoms simultaneously. Some symptoms such as actinic keratoses, purpura, pigmentation disorder, or telangiectasia can be reduced only with focused treatments containing specific ingredients. For example, a skin-whitening product, which reduces skin pigmentation problems on the face, hands, and arms.

As a consequence, we will discuss in this chapter the most cited clinical symptoms of photoaging, such as fine and coarse wrinkles, skin laxity, roughness, and dryness. With the exception of dryness (horny layer), these clinical symptoms are related to major alteration of the dermis.

Cosmetic or Cosmeceutical Antiaging Products?

The antiaging "active" ingredients, which will be described hereafter, are cosmetic products with properties very similar to a pharmaceutical product (drug-like benefits)—cosmeceuticals (32,33). However, the EEC legislation and particularly the FDA do not recognize such category as "cosmeceuticals" and consider these formulations as cosmetic products. The term cosmeceutical or its synonym terminology is often misused in cosmetic advertising and may be misleading to the consumer. He interprets a cosmeceutical to be similar to a pharmaceutical product and that these formulations have passed with success the tests for efficacy, safety, and quality control as required for a medical preparation (33). Indeed, product testing may also be warranted by the companies to document claimed efficacy and to support marketing (34). Generally speaking, the quality control testing on ingredients and safety testing are of good quality, and the used ingredients are mostly safe. However, these ingredients may not be as efficient as claimed, and the concentrations used in these formulations will not necessarily correspond to an "effective" concentration. This can be the case with many plant extracts with antioxidant properties. There are no proven effective topical antiaging ingredients/or treatment that completely eliminates the symptoms of skin photoaging, but there are products and treatments that can visibly reduce or slow down these symptoms: it is more correct to consider reduction of the appearance of aged skin. Many cosmetic products claim to reduce the clinical signs of photoaged skin; however, there are very few scientific, randomized, double-blind, placebo-controlled, clinical studies to support these claims (35). Finally, many antiaging claims are based on in vivo testing on cells or simple skin models but not in vivo on a sufficient number of human subjects.

Moisturizers

The classical moisturizers are used for treating dryness in the photoaged skin: polyols (glycerin, propylene glycol, butylene glycol and sorbitol), urea, lactic acid and salts, hyaluronic and salts, pyrrolodone-5-carboxylic acid and salts (PCA), panthenol, amino acids and proteins (collagen and proteins from wheat, rice, silk, soybean, and oat). See the chapter "Hydrating Substances" by Lodén (36). More sophisticated peptides and proteins are presently used as moisturizers. It concerns generally more lipophylic quaternary *N*-alkyl derivatives of proteins or small polypeptides with long side chains (ester binding) to increase the lipophilic character: binding to the horny layer and a better percutaneous absorption. Recently, the use of small peptides, which mimic the amino acid sequence of collagen or enzymes (biomimetic peptides), has been proposed as mosturizers (37). For a better percutaneous penetration, small fragments of hyaluronic acid were also suggested (38).

Surface-Smoothing Agents

Surface-smoothing silicone derivatives (39) or filmogen proteins such as quaternized proteins or silk, rice and oat (36), and skin feel agents (40) are used in antiaging products. The high adsorption to the skin surface provokes a smoothing of the skin surface and is at the same time humectant.

Retinoids

The use of retinoic acid as a topical antiaging ingredient has been extensively investigated. The efficacy of this topical drug has been scientifically proved: reversing fine wrinkles, non-homogenous skin pigmentation and rough skin surface (41). However, the typical side effects of topical application of retinoic acid, known as retinoid dermatitis, occur in most patients: erythema, scaling, and pruritis limit the use of this topical drug. Cosmetic research on similar molecules with the same antiaging properties but without the irritant side effects are going on. Retinol, retinal, and different retinol derivatives (retinyl palmitate = lipophilic derivate and retinyl propionate = hydrophilic derivative) are frequently proposed in topical antiaging treatments (42–44). Retinol and retinal must be metabolized in the skin to the active trans-retinoic acid. The incoporation of retinol and probably also retinal in cosmetic preparations poses the problem of stability (slow oxidation of retinol in function of time). New delivery systems and derivatives of retinol may solve the problem of stability of these preparations.

α- and β-Hydroxy Acids

Hydroxy acids such as glycolic acid and lactic acid at lower concentrations than those used in dermatological peelings are designed to remove the superficial epidermal layers of the skin and give a younger appearance (exfoliation and rejuvenation) (45,46). There is an effect on the synthesis of ceramides by the keratinocytes and on the lipidic structure of the stratum corneum (modulation of the barrier function). Stimulation of the epidermis and dermis cells. Sort of soft chemical peeling without the side effects of higher concentrations (redness, burning, and swelling). The use of more lipophilic derivatives of β-hydroxy acid (C8-lipohydroxy acid) has recently been proposed as a treatment of photoaged skin. The slower rate of penetration of this soft lipophilic exfoliating agent (reservoir effect in the horny layer) assures an effect at lower concentration with less side effects (47).

Antioxidants

In the field of cosmetic treatments, antioxidants are widely innovative ingredients in topical antiaging applications. The chapter "Antioxidants" by Weber et al. gives an overview of the current state of research on the use of antioxidants in topical cosmeceutical applications (48). The chapter on "Oral Cosmetics" by Demeester et al. (11) explains the benefits of antioxidants in antiaging oral cosmetics. The target of these active ingredients is to counter the ROS molecules produced in the skin. The most important antioxidants are vitamin C (49,50), vitamin E (51,52), coenzyme Q (53), and α-lipoic acid (54). The combined topical use of retinoids, niacinamide, *N*-acetyl glucosamine, and moisturizing peptides has recently been reported (55).

Some double-blind, vehicle-controlled antiaging treatments over long periods have been reported in the literature.

The topical application of a cream containing a mixture of folic acid and creatine showed improvements in the mechanical properties of the skin (56). Also, an improvement of the photoaged dermal matrix by topical application of a cosmetic "antiaging" product containing a lipoentapeptide, white lupin, and retinyl palmitate was reported by Watson et al. (44).

Plant antioxidants have recently been investigated with promising results: polyphenols in green tea, soya isoflavones genistein and daidzein, tannins in pomegranate, and resveratrol in seeds of grape.

The topical use of resveratol, a polyphenol from red grapes with great antioxidant activity in skin care formulation, has been reported by Baxter (57). The efficacy of soybean extract against photoaging was investigated in a double-blind, vehicle-controlled clinical study by Wallo et al. (58), indicating the promise of the soya isoflavones. Finally, the effects of green tea extracts in the clinical and histological appearance of photoaging skin were examined by Chiu et al. (59).

UV Filters

Protection of the skin against photoaging is ensured by the use of broad-spectrum UV filters either by radiation absorption or by light reflection. As a consequence, one reduces the total lifetime UV dose. Most cosmetic antiaging creams and lotions contain a mixture of UVA and UVB filters with SPF around 15 to 20.

Table 1 List of the Plant Extracts Mostly Used in Commercial Antiaging Cosmetics

Sesamum indicum, Prunus Amygdalis dulcis, Phyllanthus umblica, Siegesbeckia orientalis, Theobroma cacao, Bytospermum parkii, Mangifera indica, Mentha piperada, Aleurits moluccana, Glycurrhiza glabra, Arcostaphylos uva, Imperata cylindrica, Centella asiatica, Echinacea purpurea, Camelia sinensis, Thea sinensis, Hordeum vulgare, Crithium maritimum, Plantago lanceolata, Phellodendron amurense, Spirea ulmaria, Artemisia vulgaris, Santalum album, Rosmarinus officinalis, Centella asiatica, Curcuma longa, Aloe vera, Arnica calendula, Ginkgo biloba, various algae such as Fucus vesiculosus, Laminaria flexicaulis, Ascophyllum nodosum.

Small Lipopolypeptides

Is palmitoyl pentapeptide a new skin rejuvenation compound? It is a relatively small molecule: an ester between palmitic acid and a pentapeptide chain to enhance oil solubility for the sake of better skin penetration. The typical sequence of one of these pentapeptides is Lys-Thr-Thr-Lys-Ser, a sequence, which mimics the specific sequence of a domain in procollagen type I (Pal-KTTKS) or Matrixyl® (60–62). In vitro studies have shown that the addition of Pal-KTTKS stimulates the synthesis of key constituents of the skin matrix: collagen, elastin, and GAGs. In vivo studies with the topical use of the lipo-mimetic pentapeptides are currently under way and show an improvement of skin appearance (63,64).

Miscellanous Plant Extracts

When looking at the composition of most commercial antiaging formulations, one always notices the use of plant ingredients. Table 1 gives an overview of the plant extracts mostly used. The rationale of antiaging efficacy of these plant extracts is mainly based on folk medicine, general anecdotic or subjective information, and much less on sound clinical published studies concerning double-blind, vehicle-controlled antiaging treatments over long periods.

A Stretch-Mark Cream Which Was Used as an Antiwrinkle Cream

The last marketing furor in Europe and United States was regarding an antiaging product, a commercial product named Strivectin-SD® (65), a rather surprising story. As claimed by the company in their adverisement, originally the product was developed as a cream for treating stretch marks on abdomen and legs. This body cream contains many ingredients, including a patented Striadil Complex® (plant extracts such as Phyllantus emblica, Siegesbeckia orientalis, and others) and the patented antiaging lipo-pentapeptide. Preliminary clinical trials with Strivectin-SD showed that the product was able to reduce the appearance of existing stretch marks. Then it appeared that the product could also be used on the face for treating photoaging. So far, the scant clinical data are encouraging (61). The major question remains: Is there a sound scientific basis under the HYPE? How does this new antiaging cream exactly work? Besides the lipo-pentapeptide derivative, which are the other possible "active plant ingredients"? The formulation contains 24 different plant ingredients. Further clinical antiaging studies with these peptide-containing formulations and plant extracts are necessary in the future before these efficacy claims can be confirmed. At this stage, only preliminary antiaging testing results of this new cream were presented (6).

ORAL ANTIAGING COSMETICS

The term "oral cosmetics" is used with respect to dietary supplements, which claim to have beneficial physiological effect on skin, hair, or nails; these are preparations for oral use only such as capsules, tablets, liquids, or granulates. Oral cosmetics are mainly focused on antiaging and slimming/anticellulite preparations. Clearly, the oral route of admnistration requires special product characterizations such as toxicology, bioavailability, and the metabolization of its components. Furthermore, these clinical trials should be randomized, placebo controlled and double blind to minimize the bias of the results of the studies. In addition, any change in dietary habits or lifestyle should be avoided, and the intake of other food supplements should be kept under control during the complete study. An overview of the most recent antiaging oral cosmetics available and their results is given in the chapter "Oral Cosmetics" by Demeester et al. (11).

CONCLUSIONS

The importance of antiaging cosmetics is actually well established. Many molecules are promising "active" ingredients to slow down the different symptoms of photoaging. Even though a growing amount of scientific literature deals with clinical antiaging studies, there is still a need of further investigation to demonstrate the efficacy of these various ingredients in topical formulations.

However, a sound approach of antiaging strategy is a lifestyle, which does not have any marketing appeal but is probably effective. It consists of a healthy balanced diet, regular physical exercise, avoidance of smoking and excessive alcohol, a good basic day and night skin care treatment, use of sunscreens with high SPF during the day, maximal sun avoidance, particularly for those with a light complexion (phototypes I and II) and those with an outdoor lifestyle (35). The synergy of combining the topical use of antiaging creams with oral food supplements appears to be an interesting approach in the future.

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28 Antioxidants

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INTRODUCTION

In the field of dermatology, antioxidants are a widely used and innovative ingredient in topical applications. This chapter is intended to provide an overview of the current state of research on the use of antioxidants in cosmeceutical applications as well as safety aspects. The most important antioxidants, vitamin E, vitamin C, thiols, and flavonoids, will be introduced and their intriguing cooperation, as well as their role in signal transduction events, will be discussed.

The body is continuously exposed to oxidants. Endogenous sources arise as a consequence of normal metabolic pathways. For example, mitochondrial respiration produces superoxide and hydrogen peroxide, while enzymes such as lipoxygenase, xanthine oxidase, and NADPH oxidase produce hydroperoxides and superoxide. Exogenous oxidants arise from environmental pollutants such as smoke, smog, UV radiation, and diet. In response to these oxidants, a number of systemic antioxidants are available whose function is to scavenge reactive oxygen species, preventing damage to macromolecules such as lipids, DNA, and proteins. Antioxidant protection arises from molecules synthesized as part of metabolism, e.g., glutathione (GSH) and uric acid; essential vitamins, which must be taken in from the diet, e.g., vitamin E and C; and enzymes, which decompose reactive oxygen species, e.g., superoxide dismutase, catalase, and GSH peroxidases. These systems provide protection in various intra- and intercellular compartments. Usually there is a tight balance between oxidants produced and antioxidant scavenging; however, under certain conditions, the balance can be tipped in favor of the oxidants, a condition called "oxidative stress." Potentially oxidative stress can be caused by an increase in the number of oxidants, for example, as a result of cigarette smoking or UV irradiation, or by a deficiency of any one important antioxidant. This is of major concern since oxidative stress has been implicated in a number of conditions including atherosclerosis, skin cancer, and photoaging.

VITAMIN E

Vitamin E is the major lipophilic antioxidant in skin, and it is the most commonly used natural antioxidant in topical formulations. It is found in all parts of the skin, the dermis and epidermis as well as in the stratum corneum (SC), and is believed to play an essential role in the protection of biomolecules from oxidative stress.

Vitamin E is a family of eight naturally occurring isoforms: four tocopherols (α-,β-,γ-,δ-form) and four tocotrienols (α-,β-,γ-,δ-form) (1) (Fig. 1). All forms consist of a chromanol nucleus that carries the redox-active phenolic hydroxyl group and a lipophilic tail. While tocopherols contain a phytyl side chain, the isoprenoid tail of the tocotrienols is polyunsaturated, making the chain more rigid. The side chain is anchored in lipid membranes while the nucleus is located at the lipid/aqueous interface. Even though the radical scavenging activity

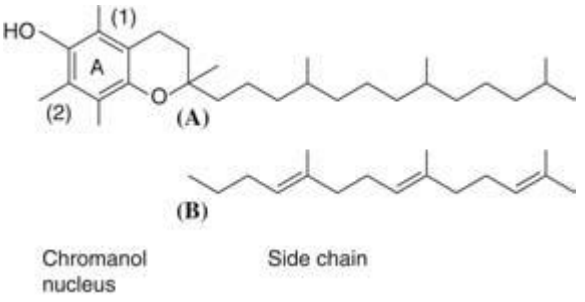

Figure 1 Naturally occurring forms of vitamin E. (**A**) Tocopherols contain a saturated side chain, (**B**) while the isoprenoid side chain of tocotrienols is polyunsaturated. The α-forms contain both methyl groups on the chromanol nucleus (1,2), while the β-forms contain only methyl group (1), the γ-forms only (2), and the δ-forms none.

of the different isoforms is essentially identical, their biological activity after oral administration differs dramatically (1). This phenomenon can be explained by the existence of an α-tocopherol transfer protein in the liver that positively selects RRR-α-tocopherol and incorporates it into VLDL, which leads to recirculation of the α-tocopherol pool, while this transfer protein does not recognize the other forms, which are therefore excreted more rapidly (2).

In skin, as in the other human organs, α-tocopherol is the predominant form of vitamin E with 5 to 10 higher concentrations than γ-tocopherol. Delivery of vitamin E to the SC occurs in two different modes. On the one hand, it is stored into differentiating keratinocytes and moves up into the newly formed SC, which leads to a gradient-type distribution of α-tocopherol with decreasing concentrations toward the skin surface (3). On the other hand, vitamin E is secreted by sebaceous glands and reaches the SC from the outside. In sebaceous gland-rich regions such as the face, this delivery mechanism is responsible for the enrichment of the outer SC with vitamin E (4). Sebaceous gland activity is low in children and starts to increase during puberty to reach a plateau at the age of 19 and remains relatively constant until it starts to decline in women starting in the cohort of 50 to 59 years of age and in men starting from 70 years of age. (5). Thus, children as well as women older than 50 years and men older than 70 years may have a compromised mechanism of vitamin E delivery to the skin surface. These collectives may potentially benefit from topical vitamin E supplementation.

After oral supplementation, vitamin E reaches the skin via sebaceous glands after a period of at least two to three weeks (6). These findings demonstrate that the sebaceous gland route is a significant delivery mechanism for vitamin E.

Various oxidative stressors have been shown to deplete vitamin E, among other antioxidants. In the epidermis, at least four minimal erythemal doses (MEDs) of solar-simulated ultraviolet (ssUV) radiation are needed to deplete vitamin E (7), while doses as low as 0.75 MED are capable of destroying vitamin E in the human SC (3). Mouse experiments have shown that a dose of 1 ppm × 2 hr of ozone (O_3) depletes SC vitamin E (8). Since this concentration of O_3 is higher than the naturally occurring levels of tropospheric O_3, the biological relevance of these findings for human skin is not yet clear. Benzoyl peroxide is used for the treatment of acne. A single application of a 10% w/vol formulation of benzoyl peroxide almost completely depletes SC vitamin E in vivo (9).

α-Tocopherol is widely used as an active ingredient in topical formulations. After topical application, it penetrates readily into the skin (10). Since the free form of vitamin E is quite unstable and light sensitive (it absorbs in the UVB range), the active hydroxyl group is usually protected by esterification with acetate. This increases the stability but renders the compound redox inactive. When administered orally, vitamin E acetate is hydrolyzed quantitatively in the intestines. There is some controversy, however, as to whether α-tocopherol acetate can be hydrolyzed in human skin. Chronic application of α-tocopherol acetate leads to an increase in free vitamin E in both the rat (11) and the mouse (12), where it was recently shown that UVB increases the hydrolysis of α-tocopherol acetate by induction of nonspecific esterases up to 10- to 30-fold (13). While one study suggested that bioconversion of α-tocopherol acetate does not occur in human skin (14), significant hydrolysis was demonstrated in recent studies using a human epidermis-tissue culture model and in vivo (15). A 0.15% formulation of vitamin E acetate increased the SC far more than oral supplementation with 400-IU α-tocopherol (16). A rinse-off application of vitamin E was also able to increase the vitamin E.

The availability of the free form of vitamin E needs to be considered when analyzing possible health benefits. The majority of studies have been carried out in animal models, while

only limited data exist for human studies. Lipid peroxidation is inhibited after topical application of α-tocopherol (17). Several studies indicate that topically applied α-tocopherol inhibits UVB-induced photodamage of DNA in a mouse model (18) and keratinocyte cultures (Trolox®, water-soluble derivative of vitamin E) (19). Protection against Langerhans cell depletion by UV light was observed after topical application of α-tocopherol in a mouse model (20). α-Tocopherol and its sorbate ester were studied in a mouse model of skin aging. Both antioxidants were found to be effective, sorbate even more so than α-tocopherol (21). Systemic administration of vitamin E in humans (only in combination with vitamin C) increased the MED and reduced changes in skin blood flow after UV irradiation (22,23). A rinse-off application of vitamin E was able to increase the vitamin E content in the barrier lipids. It was also able to decrease the formation of squalene monohydroperoxide from squalene by low-dose UVA (8 J/cm^2 (24).

Yet, several studies indicate that α-tocopherol acetate is not as effective as free vitamin E when applied topically. Inhibition of DNA mutation in mice was 5 to 10 times less effective (19). Also, in a mouse model, unlike free vitamin E, the acetate form seemed to be ineffective (25). In summary, even though some health benefits of vitamin E supplementation have been shown, there is still a need for controlled studies in humans under physiological conditions. So far vitamin E was found to have anticarcinogenic, photoprotective, skin-stabilizing properties. This topic is reviewed in detail by Thiele et al. (26).

The safety of vitamin E supplementation is a topic of current discourse. For years, oral application of vitamin E has been regarded harmless. Recent meta-analyses discuss an increase in overall mortality after oral vitamin E supplementation. However, another large meta-analysis found no overall increase in cancer mortality by vitamin E (27). In the framework of the SUVIMAX study, a recent publication suggests increased risk of skin cancer in women taking a mixture of vitamin E, vitamin C, β-carotene, selenium, and zinc, but not in men (28). A panel of experts concluded from clinical trial evidence that vitamin E supplements appear safe for most adults in amounts $\leq$ 1600 IU (1073 mg RRR-α-tocopherol or the molar equivalent of its esters (29). Safety data were obtained for oral supplementation. No large controlled studies exist for topical supplementation of vitamin E.

Recently, the tocotrienol forms of vitamin E have become a focus of interest, since they have been found to be more efficient antioxidants in some model systems than tocopherols (30). Even if they are not bioavailable after oral supplementation, topical application circumvents the exclusion by α-TTP in the liver. In fact, free tocotrienols readily penetrate into mouse skin (10), and tocotrienol acetate is hydrolyzed in skin homogenates and in murine skin in vivo (24). Topical application of a tocotrienol-rich fraction has been demonstrated to protect mouse skin from UV- and O_3-induced oxidative stress (31,32).

Benzoyl peroxide (BPO) is used for the treatment of acne. During seven daily applications of a 10% w/vol formulation of benzoyl peroxide, endogenous SC vitamin E was progressively depleted. Vitamin E in the SC was significantly retained after α-tocotrienol (5% w/vol) supplementation for seven days. α-Tocotrienol supplementation significantly mitigated the BPO-induced lipid peroxidation. The transepidermal water loss was increased 1.9-fold by seven BPO applications, while there was no difference between α-tocotrienol treatment and controls (9). In conclusion, tocotrienols bear a potential that yet remains to be explored.

VITAMIN C

Ascorbic acid or vitamin C is one of the most important water-soluble antioxidants, which is present in high amounts in the skin. While most species are able to produce ascorbic acid, humans lack the enzymes necessary for its synthesis. Deficiency in ascorbic acid causes scurvy, a disease already described in the ancient writings of the Greeks (33). Apart from the pure antioxidant function ascorbic acid is an essential cofactor for different enzymes. The antioxidant capacity of vitamin C is related to its unique structure (Fig. 2). Due to its pKa1 of 4.25, it is

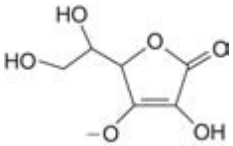

Figure 2 Structural formula of vitamin C as the mono-anion ascorbate.

present as a monoanion at physiological pH, which can undergo a one-electron donation to form the ascorbyl radical with a delocalized electron and can be further oxidized to result in dehydroascorbic acid. Dehydroascorbic acid is relatively unstable and breaks down if it is not regenerated (see antioxidant network). In vitro ascorbic acid can scavenge many types of radicals, including the hydroxyl ($OH^{\bullet}$), the superoxide ($O_2^{\bullet -}$), and water-soluble peroxyl ($ROO^{\bullet}$) radicals as well as other reactive oxygen species such as O_3, and quenches singlet O_2. Because of its relative reduction potentials, ascorbate can reduce Fe(III) to Fe(II), which in turn can decompose hydrogen peroxide (H_2O_2) to the dangerous hydroxyl radical. Therefore, vitamin C can exert pro-oxidant effects in the presence of unbound iron (Fenton's reagent).

In the skin, vitamin C is found in all layers. In SC, it forms a similar gradient as vitamin E with decreasing concentrations toward the outside. Vitamin C is depleted by O_3, UV radiation, and BPO. One of the earliest discoveries of vitamin C benefits in the skin was the observation that it stimulates collagen synthesis in dermal fibroblasts (34). Recently, a pretranscriptional role of vitamin C has been described (35). Also, vitamin C is essential in the formation of competent barrier lipids in reconstructed human epidermis (36).

Several studies have investigated protective effects of vitamin C against oxidative stress. UVB-induced immunotolerance as a marker of damage to the immune system could be abrogated by topical application of vitamin C to murine skin (37). UVB-induced sunburn cell formation was mitigated by vitamin C in porcine skin (38). While one study reported a postadministrative protective effect of vitamin C-phosphate against UV-induced damage in mice (39), another study found no such effect in humans (40). Systemic application of vitamin C in combination with vitamin E protected against UV-induced erythema in humans (22). Another study described protection against erythema, sunburn cell formation, and thymidine dimer formation in pigs by a combination of vitamin E (1%) and vitamin C (15%) (41). In a keratinocyte cell culture system, vitamin C reduced UVB-induced DNA damage (19). In mice, an anticarcinogenic effect of vitamin C was described (42). However, no data regarding such benefits exist in humans.

Since vitamin C is not very stable, it is difficult to incorporate it into topical formulations. Esterification with phosphate is used to circumvent this limitation. In vitro experiments demonstrated that Mg-ascorbyl-2-phosphate penetrates the murine skin barrier and is bioconverted into free ascorbate (43).

THIOL ANTIOXIDANTS

Thiols share an oxidizable sulfydryl (SH) group. Glutathione (GSH) is a tripeptide (Fig. 3) whose SH group at the cysteine can be oxidized, forming a disulfide (GSSG) with another GSH. Physiologically, more than 90% of the GSH is in the reduced form. GSH peroxidases use GSH oxidation to reduce H_2O_2 and other water-soluble peroxides. The synthesis of GSH by the human cell is stimulated by *N*-acetyl-cysteine (NAC), which is hydrolyzed to cysteine intracellularly. Moreover, NAC acts as an antioxidant itself. Lipoic acid (1,2-dithiolane-3-pentanoic acid or thioctic acid, LA) is a cofactor of multienzyme complexes in the decarboxylation of α-keto acids. Applied as the oxidized dithiol dihydrolipoic acid (DHLA) it is taken up by cells and reduced by mitochondrial and cytosolic enzymes [NAD(P)H dependent]. It thereby forms an efficient cycle, since it can in turn regenerate GSSG to GSH and stimulate the GSH synthesis by improving cysteine utilization (44).

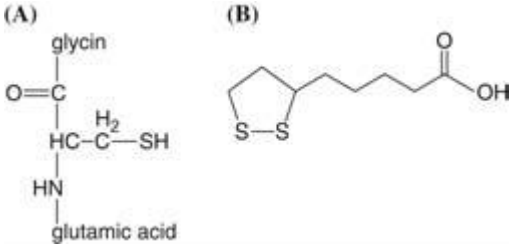

Figure 3 Chemical structures of thiols: (**A**) GSH consisting of glycine, cysteine, and glutamic acid. (**B**) Lipoic acid in its oxidized form as a disulfide.

General provisos in the use of thiols in skin applications are the typical smell and the poor solubility of LA in aqueous solutions below pH 7. Yet, several thiol agents have been tested for protective effects in the skin. For oral as well as topical application in mouse models, GSH-ethyl esters and GSH-isopropyl esters proved to be more efficient than free GSH. Oral supplementation decreased the formation of UV-induced tumors (43) and the formation of sunburn cells (45). Topical treatment partially inhibited UV-induced immunosuppression (46). NAC was able to reduce UVA-induced DNA damage in fibroblasts (47) and protected mice against UVB-induced immunosuppression after topical application (46) in a mode that did not involve de novo GSH synthesis (48). Lipoic acid was demonstrated to penetrate into mouse skin (49), while oral supplementation of lipoic acid has actually been shown to have an anti-inflammatory effect in mice (50) and to prevent symptoms of vitamin E deficiency in vitamin E-deficient mice (51).

POLYPHENOLS

Flavonoids are widely distributed plant pigments and tannins occurring in barks, roots, leaves, flowers, and fruits. Their roles in plants include photoprotection and contributing to the plant color. Consequently, our diet contains flavonoids, which can be found in a variety of foods from green vegetables to red wine.

In spite of the fact flavonoids have been used in traditional medicine for several centuries, it was not until 1936 that their first biological activity, the vitamin C-sparing action, was described by Rusznyak and Szent–Györgyi. As a result, they received the name of "Vitamin P." Flavonoids, also referred to as "plant polyphenols," have been recognized as potent antioxidants. Their free radical-scavenging and metal-chelating activities have been extensively studied. Nonetheless, given their polyphenolic structure (Fig. 4), the electron- and hydrogen-donating abilities constitute the major feature of their antioxidant properties (52). By opposition to the antioxidants previously described, flavonoids are not part of the endogenous antioxidant system but still interact with it through the antioxidant network (see below).

Among the applications found in traditional medicine, flavonoids account for anti-inflammatory, antiphlogistic, and wound-healing functions. Their effect on skin inflammation has been thought for a long time to be limited to the inhibition of the activity of 5-lipoxygenase and cyclooxygenase. However, recent studies suggest a more subtle mode of regulation of the inflammatory reaction by flavonoids. In fact, flavonoids such as silymarin, quercetin, genistein, and apigenin are effective inhibitors of NF-κB, a proinflammatory transcription factor, thereby reducing the transcription of proinflammatory genes and preventing inflammation (53–55).

Oral supplementation and topical application of green and black tea polyphenols show beneficial effects against UV radiation (UVR)-induced skin carcinogenesis in mice (56–58). In addition, these flavonoids as well as silymarin were found to prevent UVR-induced inflammation, ornithine decarboxylase expression, and activity (59), all of these events being potential contributors to carcinogenesis (60).

Procyanidins, also named "condensed tannins" are flavonoids found in pine bark (Pycnogenol®), grape seeds, and fruits, for instance. By direct protein interaction, they were shown to protect collagen and elastin, two dermal matrix proteins, against their degradation (61). Furthermore, some of these procyanidins exhibit a remarkable effect on follicle hair proliferation (62), thus extending the therapeutic applications of flavonoids to alopecia. Although the flavonoids are not part of our endogenous antioxidant defenses, they display a broad spectrum of properties particularly helpful in preventing UVR-caused deleterious effects in human skin.

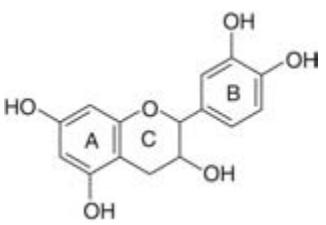

Figure 4 Chemical structure of catechin, a flavane, as an example of a flavonoid. Flavanes share a common base structure (rings A, B, C, one), which is hydroxylated in different patterns.

THE ANTIOXIDANT NETWORK

When antioxidants react with an oxidant, they are converted to a form that no longer functions as an antioxidant and is said to be consumed. In order for the oxidized product to function again, it needs to be recycled to its native form. The antioxidant network describes the ability of the antioxidants to recycle and regenerate oxidized forms of each other, thereby providing extra levels of protection (Fig. 5). Thus, the process is synergistic, and the net antioxidant protection is always greater than the sum of the individual effects.

The major systemic antioxidants vitamin E, vitamin C, and GSH are present in different cellular compartments, and all have the ability to interact with one another. Typically the radicals formed on the antioxidants are more stable and longer lived than the damaging radicals produced in vivo, which is mostly due to a delocalization of the unpaired electron. Thus, they have more chance to interact with each other and be reduced than to react with macromolecules. Vitamin E is the major chain-breaking antioxidant, protecting biological membranes from lipid peroxidation, which is a difficult task considering the ratio of phospholipids molecules to vitamin E is ~1500:1. However, vitamin E is never depleted because it is constantly being recycled. When vitamin E becomes oxidized, a radical on vitamin E is formed (chromanoxyl radical). In the absence of networking antioxidants, this radical can either become pro-oxidant by abstracting hydrogen from lipids or react to form nonradical products (consumed). However, a number of antioxidants are known to be able to reduce the chromanoxyl radical and regenerate vitamin E (63). These include vitamin C (64), ubiquinol, and GSH (65). Vitamin C, the most abundant plasma antioxidant and first line of defense, can reduce the tocopheroxyl radical, forming the ascorbyl radical. Interactions between vitamins E and C have been demonstrated in various systems both in vivo [reviewed in (66)] and in vitro [reviewed in (67)]. The ascorbyl radical is practically inert and oxidizes further to form dehydroascorbic acid. This can be reduced back to native vitamin C by GSH. This process is known to occur both chemically (68) and enzymatically (69) in both erythrocytes (70) and neutrophils induced by bacteria (71); the latter may relate to a host of defense mechanisms. Glutathione is the major intracellular antioxidant. Oxidized GSSG is constantly recycled to GSH enzymatically by GSH reductase, thus providing a constant pool of GSH. Glutathione recycling relies on NAD(P)H as the electron donor. Thus, metabolic pathways involved in energy production provide the ultimate electron donors for the antioxidant network. It is also known that GSH can directly recycle vitamin E (65,72), as well as ubiquinol (73), another lipophilic antioxidant which itself is recycled in mitochondria as part of the electron transport chain.

Certain supplements are also known to contribute to the network by recycling antioxidants. Lipoic acid is a prime example since this potent antioxidant can recycle ascorbate, GSH, and ubiquinol in vitro [reviewed in (74)]. Recently, it has been demonstrated that flavonoids may also play a networking role since they are also able to recycle the ascorbyl radical (75). Thus, there exists a very organized defense system against free radical attack, which ultimately serves to protect and recycle antioxidants in various cellular compartments.

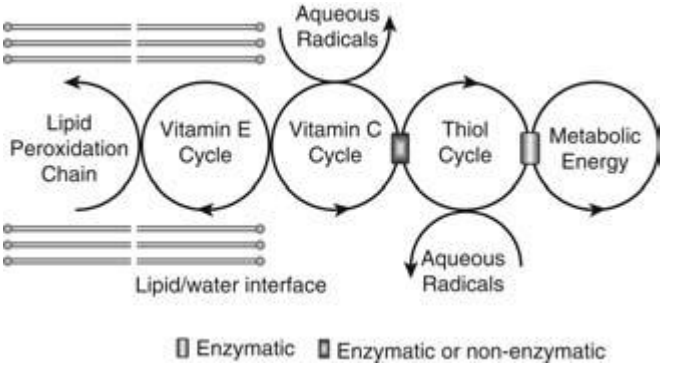

Figure 5 Schematics of the intertwined action of the antioxidant network. An ascorbate molecule can either recycle the vitamin E radical arising from breaking the lipid peroxidation chain or scavenge an aqueous radical. Glutathione can either regenerate ascorbate or scavenge a radical enzymatically. Glutathione itself then can be regenerated by the cellular metabolism.

REGULATION OF GENE TRANSCRIPTION

The skin is the largest human organ permanently exposed to a variety of stresses, among which oxidative insults such as UVR and ozone exposure account for the etiology of many skin disorders. However, oxidative damage is not responsible for all biological effects engendered by these stressors in the skin. Indeed, UVR causes changes in the expression of genes encoding proinflammatory cytokines, growth factors, stress response proteins, oncoproteins, matrix metalloproteinases (MMPs), etc. (76). Although the immediate target(s) of UVR is (are) still unknown, certain kinases and transcription factors can be activated by UVR, thereby increasing gene transcription (77). One transcription factor, NF-κB, appears of particular interest for the skin, since the lack of its inhibitory protein, IκBα, is associated with the development of a widespread dermatitis in knockout mice (78,79). Furthermore, reactive oxygen species, such as the ones produced after UVR, are suspected to play an important role in the activation of NF-κB (80). Consequently, antioxidants have been found to be among the most potent NF-κB inhibitors.

Vitamin E has specific effects on signaling events: Tocotrienols downregulate the 3-hydroxyl-3-methylglutaryl-coenzyme A (HMG CoA) reductase, a key enzyme of the mevalonate pathway that produces cholesterol (81). Inhibition of side-pathways of HMG-CoA-reductase is known to have shown anticarcinogenic effects. α-Tocopherol is able to inhibit protein kinase C, an important factor of atherosclerosis (82). Protein kinase C also regulates the activity of collagenase (MMP-1), an enzyme that degrades skin collagen. In cell culture models, α-tocopherol inhibits MMP-1 via inhibition of protein kinase C(83). Recently, it was demonstrated that vitamin E inhibited the UV-induced expression of metalloelastase and thus may inhibit the development of solar elastosis, the hallmark of sun-induced damage.

However, clinical studies are required to assess the effectiveness of these antioxidants, including the flavonoid silymarin, α-lipoic acid, and the GSH precursor *N*-acetyl-*L*-cysteine, on skin inflammatory disorders. Using high-throughput procedures such as the cDNA arrays for instance (84), the evaluation of the antioxidants on the whole genome is henceforth possible. These studies will only confirm the hypothesis that antioxidants are responsible for a much broader action spectrum than their antioxidant functions per se and extend their role on more subtle regulatory mechanisms of the gene expression.

PERSPECTIVES

The general role of antioxidants in the protection against oxidative stress is well established. In skin applications, antioxidants are a promising tool to mitigate oxidative injury. Even though a growing amount of literature deals with skin protection by antioxidants, there is still a need for investigation. Especially, clinical human studies need to be carried out to demonstrate the efficacy of antioxidants in topical formulations.

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29 UV Filters

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INTRODUCTION

The presence of UV filters in skin care and cosmetic products represents a key benefit that cosmetics can provide consumers. The hazards of ultraviolet (UV) light exposure are well known. It is estimated that the incidence of non-melanoma skin cancer in the United States exceeds one million cases per year (1). UV-induced or photoaging accounts for most of the age-associated changes in skin appearance (2). UV radiation (UVR) damages the skin by both direct effects on DNA and indirectly on the skin's immune system (3).

In animal models, sunscreens prevent the formation of squamous cell carcinomas of the skin (4). The regular use of sunscreens has been shown to reduce the number of actinic or precancerous keratoses (5) and solar elastosis (6). Daily sunscreen use on the hands and face reduced the total incidence of squamous cell carcinoma in an Australian study (7). Sunscreens also prevent immunosuppression (8). Double-blind photoaging studies show consistent improvement in the "untreated" control groups partly because of the use of sunscreens by all study subjects (9).

The effect of sunscreen use on melanoma is less clear. A meta-analysis of population-based studies of population-based case control studies found no effect of sunscreen use on risk for melanoma (10). Nevertheless, observational studies suggest that intermittent or intense sun exposure is associated with increased risk for melanoma (11), supporting the hypothesis that preventing sunburn especially in childhood may reduce the lifetime risk of melanoma.

The cosmetic formulator has an expanding menu of active sunscreen ingredients for incorporation into a variety of cosmetic formulations. Selection is restricted by regulatory agencies in the country in which the final product is to be marketed. This chapter will concentrate on reviewing available UV filters.

DEFINITIONS

UVR reaching the Earth's surface can be divided into UVB (290–320 nm) and UVA (320–400 nm). UVA can be further subdivided into UVA I (340–400 nm) or far UVA and UVA II (320–340 nm) or near UVA.

The sun protection factor (SPF) is defined as the dose of UVR required to produce 1 minimal erythema dose (MED) on protected skin after application of 2 mg/cm^2 of product divided by the UVR to produce 1 MED on unprotected skin. A "water-resistant" product maintains the SPF level after 40 minutes of water immersion. A "very water-resistant" or "waterproof" product is tested after 80 minutes of water immersion. If the SPF level is diminished by immersion, a separate SPF level may be listed. A "broad-spectrum" or "full-spectrum" sunscreen provides both UVB and UVA protection. Ideally, this includes both UVA I and UVA II coverages.

HISTORY

Acidified quinine sulfate was proposed for use as a chemical sunscreen in the 1890s (12). At the beginning of the 20th century, Unna found aesculin, a chestnut extract used in folk medicine for many years, to be more effective. Two UV filters, benzyl salicylate and benzyl cinnamate, were first incorporated into a commercially available sunscreen emulsion in the United States in 1928 (13). In the early 1930s, phenyl salicylate (salol) was used in an Australian product (14).

Para-aminobenzoic acid (PABA) was patented in 1943, leading to the development of PABA derivative UV filters. During World War II, red veterinary petrolatum (RVP) was used by the U.S. military, encouraging the development of further UV filters in the post-war period.

In the 1970s, increased interest in commercial sunscreen products led to refinements and consumer acceptance of these products over the next two decades. Facilitated by growing awareness as to the hazards of UVR, higher SPF products became the norm. Daily use consumer products containing UV filters, including moisturizers, color cosmetics, and even hair care products, have become more prevalent in the past decade. Concerns related to the adequacy of sunscreen protection for the prevention of melanoma and photoaging in the last few years have led to greater interest in broad-spectrum sunscreen UV protection throughout the entire UVA range.

REGULATORY

United States

Sunscreen products in the United States are regulated by the FDA as over-the-counter (OTC) drugs. The final monograph for sunscreen drug products for OTC human use (Federal Register 1999: 64: 27666–27693) established the conditions for safety, efficacy, and labeling of these products. A recently proposed amendment (Federal Register 2007: 72: 49070–49122) further elaborates on UVB (SPF) and UVA testing and labeling. As active ingredients in drug products, they are listed by their United States Adopted Names (USAN). There are 16 approved sunscreen ingredients (Table 1). All permitted UV filters can be used with any other permitted filters except avobenzone. The latter cannot be used with PABA, octyl dimethyl PABA, meridamate, and titanium dioxide (TiO_2). Maximum allowable concentrations are provided. Minimum concentration requirements were dropped, providing that the concentration of each active ingredient is sufficient to contribute a minimum SPF of not less than 2 to a finished product. A sunscreen product must have a minimum SPF of not less than the number of active sunscreen ingredients used in combination multiplied by 2.

The proposed recent amendment allows products with SPF values above 50, but the SPF declaration for sunscreens with SPF values above 50 are limited to SPF 50+. The term "sunblock" is prohibited. The term "UVB" is to be included before the term "SPF" on the principal product display panel. Newer labeling requires the listing of UV filters not only as active ingredients but also their concentration in the product. To address the inadequacies of any single UVA rating system, the FDA proposes a combination of spectrophotometric (in vitro) and clinical (in vivo) UVA testing procedure to allow for a nonnumerical UVA protection four-star rating system, with one star being low protection and four stars being the

Table 1 FDA Sunscreen Final Monograph Ingredients

Drug name	Concentration (%)	Absorbance
Aminobenzoic acid	Up to 15	UVB
Avobenzone	2–3	UVAI
Cinoxate	Up to 3	UVB
Dioxybenzone	Up to 3	UVB, UVAII
Ensulizole	Up to 4	UVB
Homosalate	Up to 15	UVB
Meradimate	Up to 5	UVAII
Octocrylene	Up to 10	UVB
Octinoxate	Up to 7.5	UVB
Octisalate	Up to 5	UVB
Oxybenzone	Up to 6	UVB, UVAII
Padimate O	Up to 8	UVB
Sulisobenzone	Up to 10	UVB, UVAII
Titanium dioxide	2 to 25	Physical
Trolamine salicylate	Up to 12	UVB
Zinc oxide	2 to 20	Physical

Abbreviations: FDA, Food and Drug Administration; UV, ultraviolet.

highest. The in vivo study to be used is the persistent pigment-darkening (PPD) method (15). The Boots adaptation of the Diffey/Robson method was proposed for in vitro testing in the recent amendment.

Europe

In Europe, sunscreen products are considered to be cosmetics, their function being to protect the skin from sunburn. The Third Amendment of the European Economic Community (EEC) Directive provides a definition and lists the UV filters that cosmetic products may contain. Table 2 lists UV filters that are fully permitted as amended most recently through commission directives 2003/83/EC and 2005/9/EC. As cosmetic products, cosmetic or *International Cosmetic Ingredient Dictionary* (INCI) nomenclature is used as listed in the *CTFA International Cosmetic Ingredient Dictionary*. The European Union (EU) allows, at the date of covering this chapter, several ingredients not available in the United States (see discussion below). The EU has added TiO_2 to the approved list. Zinc oxide (ZnO) is not included in this list but may be used as a cosmetic ingredient.

A more recent commission directive 2006/647/EC provides further guidance on UVA/UVB efficacy claims. The PPD method is clinically recommended. For in vitro testing, the critical wavelength method (16) is to be used, in contrast to the Boots' adaptation recommended by the FDA.

Australia

Sunscreens in Australia are regulated as therapeutic goods. The latest edition of Australian Standard 2604 was published in 1993 as a joint publication of Australia and New Zealand. Sunscreen products are classified as either primary or secondary, depending on whether the primary function of the designated product is to protect from UVR as opposed to a product with a primary cosmetic purpose. SPF designations greater than 30 are not permitted (SPF 30+ represents the maximum designation). In general, Australian Approved Names (AAN) for allowed active sunscreen ingredients are the same as FDA drug nomenclature with a few differences.

Other Countries

Most non–EEC countries follow the EEC directive. Many other countries follow U.S. trends with their own provisions. In Japan, sunscreens are classified as cosmetics. Regulations for each individual country need to be consulted for selection of the various UV filters for incorporation into a sunscreen product to be marketed in a given jurisdiction (17).

MECHANISM OF ACTION

UV filters have been traditionally divided into chemical absorber and physical blockers on the basis of their mechanism of action. Chemical sunscreens are generally aromatic compounds conjugated with a carbonyl group (13). These chemicals absorb high-intensity UV rays with excitation to a higher-energy state. The energy lost results in conversion of the remaining energy into longer lower-energy wavelengths with return to ground state. The evolution of modern sunscreen chemicals represents a prototype study in the use of structure-activity relationships to design new active ingredients and has been well reviewed elsewhere (18).

Physical blockers reflect or scatter UVR. Newer microsized forms of physical blockers may also function in part by absorption (19). Sometimes referred to as "non-chemical" sunscreens, they may be more appropriately designated as inorganic particulate sunscreen ingredients.

NOMENCLATURE

Sunscreen nomenclature can be quite confusing. They may be referred to by their chemical or trade name. In the United States, individual sunscreen ingredients are also assigned a drug name by the OTC monograph. Annex VII of the EU may use either a drug or chemical name. Australia has its own approved list of names (AAN). Table 3 lists the most commonly used names, including their primary listing in the INCI designation (20).

Table 2 List of UV Filters That Cosmetic Products May Contain (EEC Directive Annex VII – Part 1)

Reference number	Substance	INCI name	Maximum authorized concentration (%)
1	4-Aminobenzoic acid	PABA	5
2	*N,N,N*-trimethyl-4-(2-oxoborn-3-ylidenemethyl) anilinium methyl sulfate	Camphor benzylkonium methosulfate	6
3	Homosalate (INN)	Homosalate	10
4	Oxybenzone (INN)	Benzophenone -3	10
6	2-Phenylbenzimidazole-5-sulfonic acid and its potassium, sodium, and triethanolamine salts	Phenylbenzyimidazole sulfonic acid	8 (expressed as acid)
7	3,3'-(1,4-Phenylenedimethylene)bis[7,7-dimethyl-2-oxo-bicyclo-(2,2,1)hept-1-ylmethanesulfonic acid] and its salts	Terephthalidene dicamphor sulfonic acid	10 (expressed as acid)
8	1-(4-Tert-butylphenyl)-3-(4-methoxyphenyl) propane-1,3-dione	Butyl methoxy dibenzoyl-methane	5
9	α-(2-Oxobron-3-ylidene)toluene-4-sulfonic acid and its salts	Benzylidene camphor sulfonic acid	10 (expressed as acid)
10	2-Cyano-3,3-diphenyl acrylic acid, 2-ethylnexyl ester	Octocrylene	10 (expressed as acid)
11	Polymer of *N*-(2 and 4)-[(2-oxoborn-3-ylidene)methyl] benzyl acrylamide	Polyacrylamidomethyl benzylidene camphor	6
12	Octyl methoxycinnamate	Ethyl hexyl methoxy-cinnamate	10
13	Ethoxylated ethyl-4-aminobenzoate	PEG-35 PABA	10
14	Isopentyl-4-methoxycinnamate	Isoamyl *p*-methoxy cinnamate	10
15	2,4,6-Trianilino-(*p*-carbo-2'-ethylhexyl-1'-oxy)-1,3,5-triazine	Octyl triazone	5
16	Phenol,2-(2H-benzotriazol-2-yl)-4-methyl-6-(2-methyl-3-(1,3,3,3-tetramethyl-1-(trimethylsilyl)oxy)-disiloxanyl)propyl) (drometrizone trisiloxane)	Drometrizone Trisiloxane	15
17	Benzoic acid, 4,4-((6-(((1,1-dimethylethyl)amino)carbonyl)phenyl) amino)-1,3,5,triazine-2,4-diyl)diimino) bis-cbis(2-ethylhexyl)ester)	Diethylhexyl butamido triazone	10
18	3-(4'-Methylbenzylidene)-d-t camphor	4-Methylbenzylidene camphor	2
19	3-Benzylidene camphor	3-Benzylidene camphor	2
20	2-Ethylhexyl salicylate	Octyl salicylate	5
21	4-Dimethyl-amino-benzoate of ethyl-2-hexyl	Octyl dimethyl PABA	8
22	2-Hydroxy-4-methoxybenzophenone-5-sulfonic acid and its sodium salt	Benzophenone-5	5 (of acid)
23	2,2'-Methylene-bis-6-(2H-benzotriazol-2yl)-4-(tetramethylbutyl-1,1,3,3,-phenol)	Bisoctyltriazol	10
24	Monosodium salt of 2-2'-bis-(1,4-phenylene) 1H-benzimidazole-4,6-disulphonic acid	Bisamidazylate	10 (of acid)
25	(1,3,5)-Triazine-2,4-bis ((4-(2-ethyl-hexyloxyl)-2-hydroxy)-phenyl)-6-(4-methoxyphenyl)	Anisotriazine	10
26	Dimethicodiethylbenzalmalonate (CAS No 207574-74-1)		10
27	Titanium dioxide		25
28	Benzoic acid, 2-(4-(diethylamino)-2 – hydrobenzoyl)-, hexylester	Dithylamino hydroxybenzoyl hexyl benzoate	10

Abbreviations: EEC, European Economic Community; UV, ultraviolet; PABA, para-aminobenzoic acid; INCI, International Cosmetic Ingredient Dictionary.

Table 3 Sunscreen Nomenclature

CAS #	Drug name (FDA)	INCI name	Colipa #	EU reference #	Trade names	Solubility	Spectrum
150-13-0	Aminobenzoic acid	PABA	S 1	1	4-Aminobenzoic acid	Hydrophilic	UVB
70356-09-1	Avobenzone	Butyl methoxydibenzyl methane	S 66	8	Parsol 1789	Lipophilic	UVA I
104-28-9	Cinoxate	Cinoxate				Lipophilic	UVB
92761-26-7	Ecamzule	Terephthalydene dicamphor sulfonic acid	S 71	7	Mexoryl SX	Hydrophilic	
27503-81-7	Ensulizole	Phenylbenzimidazole sulfonic acid	S 45	6	Eusolex 232, Neo Heliopan Hydro	Hydrophilic	UVB
118-56-9	Homosalate	Homosalate	S 12	3	Eusolex HMS	Lipophilic	UVB
134-09-8	Meradimate	Menthyl anthranilate			Dermoblock MA, Neo Heliopan, Type MA	Lipophilic	UVA II
6197-30-4	Octocrylene	Octocrylene	S 32	10	Escalol 597, Eusolex OCR, Uvinul N-539-50	Lipophilic	UVB
5466-77-3	Octyl methoxycinnamate	Octyl methoxycinnamate	S 28	12	Neo Heliopan AV, Parsol MCX, Eusolex 2292	Lipophilic	UVB
88122-99-0	Octyl triazone	Octyl triazone	S 69	15	Uvinul T-150	Lipophilic	UVB
118-60-5	Octisalate	Octyl salicylate	S 20	8	Escalol 587, Eusolex BS, Uvinul O-18	Lipophilic	UVB
131-57-7	Oxybenzone	Benzophenone-3	S 38	4	Eusolex 4360, Neo Heliopan, Uvinul M40	Lipophilic	UVB, UVA II
21245-02-03	Padimate O	Octyl dimethyl PABA	S 78	17	Escalol 507, Eusolex 6007	Lipophilic	UVB
4065-45-6	Sulisobenzone	Benzophenone-4	S 78	17	Escalol 577, Uvinul MS 40	Lipophilic	UVB, UVA II

Abbreviations: FDA, Food and Drug Administration; INCI, International Cosmetic Ingredient Dictionary; PABA, para-aminobenzoic acid.

INDIVIDUAL UV FILTERS

Sunscreen ingredients may be considered by dividing them into larger overall classes by chemical structure. They may also be classified by their absorption spectrum. Although the lists of UV filters approved by the various regulatory agencies may seem quite extensive, fewer are used with any degree of frequency. The discussion, which follows, will concentrate on those listed in Table 3.

UVB

PABA and Its Derivatives

PABA was one of the first chemical sunscreen chemicals to be widely available. Several problems limited its use. It is very water soluble, was frequently used in alcoholic vehicles, stained clothing, and was associated with photodermatitis. Ester derivatives of PABA, mainly octyl dimetyl PABA or padimate O, became more popular with greater compatibility in a variety of more substantive vehicles and a lower potential for staining or adverse reactions. Amyl dimethyl PABA and glyceryl PABA (glyceryl aminobenzoate) are no longer used.

Padimate O or octyl dimethyl PABA is a most potent UV absorber in the mid-UVB range. Because of problems with PABA formulations, marketers have emphasized the "PABA-free" claim. Although still used (21), it is confused with PABA, limiting its use. The decline in the use of this PABA derivative along with the demand for higher SPF products has led to the incorporation of multiple active ingredients in a single product to achieve the desired SPF.

Cinnamates

The next most potent UVB absorbers allowed by the FDA monograph, the cinnamates, have largely replaced PABA derivatives. Octinoxate or octyl methoxycinnamate is the most frequently used sunscreen ingredient (21). Octyl or ethylhexyl methoxycinnamate is in order of magnitude less potent than padimate O and requires additional UVB absorbers to achieve higher SPF levels in a final product. Cinoxate (ethoxy-ethyl-*p*-methoxycinnamate) is less-widely used. Isoamyl *p*-methoxycinnamate (EU no. 14) is available in Europe.

Salicylates

Salicylates are weaker UVB absorbers. They have a long history of use but were supplanted by the more efficient PABA and cinnamate derivatives. They are generally used to augment other UVB absorbers. With the trend to higher SPFs, more octisalate or octyl salicylate (ethylhexyl salicylate) is being used followed by homosalate or homomenthyl salicylate. Both materials have the ability to solubilize oxybenzone and avobenzone. Trolamine or triethanolamine salicylate has good water solubility.

Camphor Derivatives

Not approved by the FDA for use in the United States, there are six camphor derivatives approved in Europe, and 4-methylbenzylidene camphor (EU no. 18) is the most widely used one (21).

Octocrylene

2-Ethylhexyl-2-cyano-3,3 diphenylacrylate or octocrylene is chemically related to cinnamates. It can be used to boost SPF and improve water resistance in a given formulation. Octocrylene is photostabile and can improve the photostability of other sunscreens. It is expensive and can present difficulties in formulation.

Phenylbenzimadazole Sulfonic Acid

Phenylbenzimadazole sulfonic acid or ensulizole is a water-soluble UVB absorber that can be used in the water phase of emulsion systems, in contrast to most oil-soluble sunscreen ingredients, allowing for a less-greasy, more aesthetically pleasing formulation such as a daily use moisturizer containing sunscreen. Phenybenzimidazole sulfonic acid boosts the SPF of organic and inorganic sunscreens. It can also be used in clear gels owing to its water solubility.

Triazones

Octyl or ethylhexyl triazone (EHT) is a UVB filter available in Europe (EU no. 15). It is eligible to enter the FDA sunscreen monograph through the FDA time and extent application (TEA) process (22), but not yet available. Diethylhexyl butamido triazone (DBT) (methylene bis-benzotriazol tetramethylbutylphenol—EU no. 23) or Tinasorb M is a newer, more efficient UVB filter with improved solubility over EHT. (23). An anisotriazine or bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT) or Tinasorb S (EU no. 25) is a new broadband filter, which also provides UVA protection (22), as does Tinasorb M, methylene-bis-benzotriazolyl tetramethyl-butyl phenol (MBBT) or bisoctyltriazol (EU no. 23). Both Tinasorb ingredients have also been submitted for the FDA approval through the TEA process.

UVA

Benzophenones

Although oxybenzone or benzophenone-3 absorbs most efficiently in the UVB range, absorption extends well into the UVA II range. It is used primarily as a UVA absorber, but boosts SPF values in combination with other UVB absorbers. Oxybenzone is supplied as a solid material and has poor solubility and a relatively low extinction coefficient. Sulisobenzone or benzophenone-4 is water soluble, somewhat unstable, and used with less frequency.

Menthyl Anthanilate

Meradimate or menthyl anthralate is a weak UVB filter, with absorption mainly in the near UVA portion of the spectrum. It is less effective than benzophenones in this range and is less widely used.

Butylmethoxydibenzoylmethane

Avobenzone or Parsol 1789 is the only current pure UVA filter approved by the FDA for use in OTC sunscreens in the United States. It has been used in Europe for a considerably longer period. It provides strong absorption in the UVA I range with peak absorption at 360 nm. Since standards for measuring UVA protection in the United States have only been proposed, a minimum use concentration has been set at 2% with a maximum of 3%.

Photostability refers to the ability of a molecule to remain intact with irradiation. Photostability is potentially a problem with all UV filters. This issue been raised specifically with avobenzone (24). This effect may degrade other sunscreens in a formulation, including octyl methoxycinnamate. Octocrylene and some of the newer suncreens, including BEMT, stabilized avobenzone (25). Non-UV filters such as diethylhexyl 2,6 napthalate may also be used (22). Overall formulation with avobenzone is therefore critical in this regard.

Tetraphthalydine Dicamphor Sulfonic Acid

3,3'-(1,4-phenylenedimethylene)bis[7,7-dimethyl-2-oxo-bicyclo-(2,2,1)hept-1-yl methanesulfonic acid (EU no. 7) or Mexoryl SX is a UVA blocker more recently available in Europe with comparable (26) or superior efficacy to avobenzone (27). Only specific formulations by the patent holder with this ingredient have been approved in the United States.

Physical Blockers

Some of the original sunblocks were opaque formulations reflecting or scattering UVR. Color cosmetics containing a variety of inorganic pigments function in this fashion. TiO_2 and ZnO are chemically inert and protect through the full spectrum of UVR. They offer significant advantages. Poor cosmetic acceptance limited the widespread use of these two ingredients until microsized forms became available. By decreasing particle size of these materials to a microsize or ultrafine grade, it is less visible on the skin surface.

Micropigmentary sunblocks function differently than opaque sunblocks of pigmented color cosmetics by absorbing and not simply reflecting or scattering UVR (19). By varying and mixing particle sizes, differing levels of photoprotection are achieved throughout the UV spectrum. In addition to avobenzone, micropigmentary TiO_2 and ZnO offer the best available protection in the UVA II range.

Photoreactivity has been raised as an issue with these materials. Both TiO_2 and ZnO are semiconductors potentially absorbing light and generating reactive species (28). These effects have been demonstrated in vitro (29). Coating these materials reduces their photochemical reactivity. The in vivo relevance of these effects has not been demonstrated, and both materials have a long history of safe use. Physical blockers also have the significant advantage of lowered skin irritancy potential.

Titanium Dioxide

TiO_2 was the first micropigment extensively used. Advantages include a broad spectrum of protection and inability to cause contact dermatitis. The use of rutile as opposed to anastase crystal forms of TiO_2 lessens photoactivity. Newer materials are amphiphilic designed to be dispersed in both water and oil emulsion phases. Particle size and uniformity of dispersion is key to achieving SPF. Primary particle size may be 10 to 15 nm with secondary particle assembly to 100 nm. Particle size needs to be less than 200 nm to achieve transparency.

Despite advances in the technology and understanding of these materials, whitening remains a problem secondary to pigment residue. Adding other pigment-simulating fleshtones may partially camouflage this effect. The net effect may be that the user is inclined to make a less-heavy application of product effectively lowering SPF (30). "Hybrid" formulations employing a combination of chemical absorbers with inorganic particulates may represent a practical compromise.

Zinc Oxide

ZnO was added as an active sunscreen agent for the FDA OTC sunscreen monograph with avobenzone. Reduced to a particle size of less than 200 nm, light scattering is minimized and the particles appear transparent in thin films (31). ZnO has a refractive index of 1.9, as opposed to 2.6 for TiO_2, and therefore causes less whitening than TiO_2. ZnO attenuates UVR more effectively in the UVA I range (32) with a peak at 360 nm. Microfine TiO_2 at an equal concentration offers somewhat more protection in the UVB range. Fine particle ZnO is not approved as a sunscreen ingredient in the EU. It is approved as a cosmetic colorant and as a general cosmetic ingredient.

FORMULATION

A detailed discussion of incorporating UV filters into various vehicles to achieve defined goals for efficacy and aesthetics is beyond the scope of this chapter and has been well reviewed elsewhere (33). Briefly, the first step is to determine the type of product, SPF and UVA efficacy levels, aesthetics, and nonsunscreen claims desired. Sunscreen actives are chosen realizing that most products use multiple actives comprising up to 35% of the final formulation. Sunscreen ingredients are among the most expensive used in cosmetic formulation. Vehicle type determines which actives can be used on the basis of polarity and solubility characteristics of individual filters. The most commonly used sunscreen actives are oils significantly affecting greasiness. Inorganic particulates such as ZnO and TiO_2 tend to make products feel dry and drag on application. Emulsions are the most popular vehicles to ideally incorporate active ingredients into both water and oil phases for greater efficiency. The final products, rheological profile and polymer levels, determine that a uniform film coats the surface of insure UV efficacy. Water resistance needs to be considered (34). Lastly, a photostable system needs to be designed.

ADVERSE REACTIONS—TOXICITY

In a longitudinal prospective study of 603 subjects applying daily either an SPF 15+ broad-spectrum sunscreen containing octyl methoxycinnamate and avobenzone or a vehicle cream, 19% developed an adverse reaction (35). Interestingly, the rates of reaction to both the active and vehicle creams were similar, emphasizing the importance of excipient ingredients in the

vehicle. The majority of reactions were irritant in nature. Not surprisingly, a disproportionate 50% of the reacting subjects were atopic. Less than 10% of the reactions were allergic, with none of the subjects patch tested actually found to be allergic to an individual sunscreen ingredient.

Subjective irritation associated with burning or stinging without objective erythema from some organic UV filters (36) is the most frequent sensitivity complaint associated with sunscreen use. This is most frequently experienced in the eye area. Longer-lasting objective irritant contact dermatitis may be difficult to distinguish from true allergic contact dermatitis. In a post-market evaluation of sunscreen sensitivity complaints in 57 patients, 20 patients had short-lasting symptoms, 26 long-lasting symptoms, and 11 mixed or borderline symptoms (37). Half of the patients were patch and photopatch tested, and only three showed positive reactions to sunscreen ingredients.

Contact and photocontact sensitivity to individual sunscreen ingredients has been extensively reviewed (38). Considering their widespread use, the number of documented allergic reactions is not high (36). PABA and PABA esters accounted for many of the early-reported reactions, but with a decrease in their use, an increase in reactions to benzophenones was reported (39). Skin reaction is probably higher with benzophenones than other UV filters (40). Fragrances, preservatives, and other excipients account for a large number of the allergic reactions seen (38).

Virtually all sunscreen ingredients reported to cause contact allergy may be photoallergens. Although still relatively uncommon, sunscreen actives seem to have become the leading cause of photocontact allergic reactions (41,42). Individuals with preexisting eczematous conditions have a significant predisposition to sensitization associated with their impaired cutaneous barrier. The majority of individuals who develop photocontact dermatitis to sunscreens are patients with photodermatides (38).

Contact sensitivity is not an issue with the use of physical blockers. However, concerns with dermal penetration with the use of nanoparticles have more recently been raised. Several studies examining the dermal penetration of pigmentary particles greater than 100 mm and microfine TiO_2 and ZnO would indicate that they do not penetrate the skin and remain in the stratum corneum (40).

Some organic sunscreens show estrogenic activity in screening toxicological assays (43). In vivo studies would question the relevance of this in humans (44). Clearly, the benefits of sun protection outweigh these risks.

CONCLUSION

A limited menu of UV filters for incorporation into sunscreen products is available to the formulating chemist, depending on regulatory requirements in an individual country or jurisdiction. With the demand for higher SPFs, the trend has been to use more individual and a wider variety of agents in newer products. Recent research in sunscreen efficacy has emphasized the need for products protecting against the full UV spectrum with a limited number of available agents. Regulatory agencies are often slow to approve new ingredients. Rules governing the approval of new ingredients by the EEC are more flexible.

Sunscreen efficacy remains very dependent on vehicle formulation. Solvents and emollients can have a profound effect on the strength of UV absorbance by the active ingredients and at which wavelengths they absorb (45). Film formers and emulsifiers determine the uniformity and thickness of the film formed on the skin surface, which in turn determines SPF level, durability, and water resistance (46). Lastly, product aesthetics play a large role in product acceptance, particularly with sunscreens being incorporated into daily use cosmetics. These constraints provide the sunscreen formulator with significant challenges in developing new and improved formulations.

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30 Sun Protection and Sunscreens

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INTRODUCTION

Visible sunlight is only a tiny part of the whole radiation spectrum emitted by the sun (400–700 nm). By increasing wavelength greater than 700 nm, one enters the invisible radiation range called infrared radiation (IRR); by decreasing wavelength lesser than 400 nm, one enters the ultraviolet radiation (UVR) domain. Overexposure to the sun's invisible rays can be harmful to human skin. The damage can be immediate with visible effects, such as erythema and sunburn, leading to cell and tissue degradation. The damage can also be long term, and the cumulative effects of prolonged exposure are now increasingly recognized to be the possible cause of degenerative changes in the skin such as premature wrinkling and skin cancer. Indeed, many skin changes that often are identified with aging actually result from damage by too much sun exposure (1,2).

DEFINITIONS

The shorthand notations for wavelength ranges in the UVR and IRR defined by the Commission Internationale de l'Éclairage (CIE) are closely related to the absorption depth of radiation in tissue. UVR from both sunlight and artificial sources is subdivided into three sections termed "UVA," "UVB," and "UVC" from the longer to shorter wavelengths: UVA from 400 to 320 nm, UVB from 320 to 290 nm, and UVC less than 290 nm. The UVA section is further divided in two subsections: UVA I (longer wavelengths 400–340 nm) and UVA II (shorter wavelengths 340–320 nm). The longer the wavelength and the higher the number, the deeper the UV penetrates the skin. The shorter the wavelength and the lower the number, the greater the energy level of the light and the more damage it can do. UVC, e.g., is highly efficient in causing sunburn and could destroy the skin but, fortunately, it is completely absorbed by ozone in the highest part of the earth's atmosphere. The sunlight's UVR at the surface of the earth is therefore constituted by variable proportions of UVB and UVA passing right through the atmosphere, even on a cloudy day. This variability is due to different factors such as latitude, height of the sun above horizon (time of the day), altitude, atmospheric conditions, etc. As a rule, the amount of UVA reaching the earth's surface may be considered to be 10 to 30 times greater than that of UVB (3,4).

IRR is also subdivided into three sections termed "IRA," "IRB," and "IRC," but from the shorter to the longer wavelengths: IRA (or near-IR) from 780 to 1400 nm, IRB from 1400 nm to 3000 nm, and IRC from 3000 nm to 1 mm. Contrary to UVR, the longer the wavelength and the higher the number, the less deep the IRR penetrates the tissue. Deep-penetrating IRA does not cause any strong sensation of heat. But this is the case with the longer wavelengths IRB and particularly IRC, which, at sufficiently high intensities, may damage or destroy the skin (2).

BASICS ON PROTECTION

Ultraviolet Radiation

Skin Effects of UVR

It has long been known that UVB is the principal cause of acute sunburn and tanning (4–6). Therefore, sunscreen efficacy is predominantly directed against UVB radiations (the relative contribution of UVA radiation to sunburn is considered to be only 15–24%). In addition, exposure to UVB radiation is immunosuppressive, mutagenic, and carcinogenic.

Meanwhile, the importance of the biological effects of UVA has been recognized (5,6). UVA induces significant photobiological reactions, mostly of indirect nature and requiring the presence of oxygen, such as immediate and delayed tanning reactions and new melanin formation. Importantly, there is now considerable evidence that UVA definitively contributes to long-term degenerative changes of the skin such as significant connective tissue damage (premature skin aging) and cancer formation, and may also contribute to UVB-induced carcinogenesis (6,7).

UV Filters, Sunscreens, Photostability

Protection against the effects of UVR in the skin is achieved by specially designed molecules (i.e., UV filters) incorporated in suitable formulations (sunscreens) such as creams or lotions, oils, gels, sticks, etc. (8). However, in view of the growing photobiological knowledge about the mechanisms of UVR-induced effects in the skin, this definition must now be revised to include new concepts. Besides providing a minimum sunburn protection, modern sunscreens are now required to provide a significant protection against UVA-induced effects (5,6). Further, they may not only contain chemicals that absorb, reflect, or scatter UVR but also chemicals that interfere with secondary reactions such as generation of free radicals and reactive oxygen species (ROS) in the skin, generation of inflammatory mediators, photoexcitation of different molecules, etc., and ultimately exert long-term protective effects on degenerative skin damage (8). Consequently, non-UVR-absorbing, UVR-reflecting, or UVR-scattering molecules with a pharmacological action in the skin may also be incorporated in sunscreens, endowing the corresponding products with expanded properties (8–12).

UV filters used in cosmetic sunscreen formulations are roughly considered belonging to two groups (13): organic molecules deliberately selected for their UVR-absorbing capacities (i.e., organic UV filters) and particles that absorb, reflect, or scatter UVR. Particles may be inorganic (i.e., metal oxides) or organic (microfine polymeric molecules) (8,13,14).

Both classes aim at preventing UVR from striking the skin. By absorbing UVR, UV filters are transferred to an excited electronic state from where the energy may dissipate into molecular vibrations (organic molecules) and into heat via collisions with surrounding molecules. However, when an efficient dissipation of the absorbed energy is not possible via, e.g., fluorescence, phosphorescence, heat, or internal conversion (isomerization) of an organic molecule, the UV-filter may break and irreversibly change its chemical structure. The molecule is not photostable. Consequently, the performance of the sunscreen may be altered (8,15–17).

Free Radicals, Free Radical Scavengers, and Antioxidants

Photochemical reactions due to UVR are inextricably coupled with the chemistry of free radicals. The photostability of UV filters is only one aspect of the reaction that may occur in cosmetic products applied on sunlight-exposed skin. The role of ROS and, among these, of free radicals has now been recognized as a possible cause of skin damage (4,7,18–20). Exposure of the skin to the UVR leads to the generation of a multitude of ROS. These cause injury by reacting with molecules such as lipids, proteins, nucleic acids; and by depleting the skin of its natural endogenous antioxidant defenses, a condition now arises termed "oxidative stress" (4,18,21). Thus, incorporation of molecules being able to control ROS in sunscreens should be beneficial in terms of the so-called photodamage or actinic damage of the UV-exposed skin.

Infrared Radiation

Skin Effects of IRR

Interest in investigating the effects of IRR on the skin is growing, and the current understanding of the biological effects of IRR is evolving rapidly notwithstanding controversies (22,23). IRR is inseparably linked to sunlight and perceived as heat. In the skin, heat is implicated in erythema "ab igne" and elastotic degeneration, and may even be the consequence of modern way of living (24).

Chronic exposure to IRR is growing because of the increased popularity of outdoor activities combined with the lack of protection of sunscreens in the IRR range and increased

use of IRR for wellness or therapeutic purposes, including promotion of healing of acute and chronic wounds (22,23). Recent publications, however, have reported controversial results concerning the biological effects of IRR in the skin. IRR, and particularly IRA, seems to be involved in premature photoaging and also in photocarcinogenesis (2,22). On a molecular basis, upregulation of endopeptidases in dermal fibroblasts [matrix metalloproteinases (MMPs)], which are responsible for the degradation of the extracellular matrix (ECM), seems to be at the origin of these deleterious effects. Induction of ROS is also involved. On the other side, protective effects of IRR against UVB-induced changes have been reported, together with a lack of upregulation of MMPs after IRA irradiation of dermal fibroblasts. The mechanisms of this protection are not completely elucidated and seem to implicate ferritin and/or heat shock proteins (5,22,23,25–29), although protection has also been observed without the involvement of heat shock proteins (27,30,31). A general view of these results points to temperature-induced protection against oxidative stress due to UV, a mechanism that could represent a natural process of cell protection acquired and preserved through evolution (23,27,28).

Protection Against IRR

In the past, some sunscreens claimed to protect against IRR (32). However, this has been rapidly abandoned because of the lack of a proper investigation method and the obvious lack of spectral absorption of UV filters in the IRR wavelength range. However, given the possible protective effect of the IRR exposure against UVR damage, the question, nature, and mode of IRR protection should be thoroughly investigated.

Nutritional protection using targeted micronutrients with ROS scavenging activities is one such possibility. Several studies have already demonstrated that it is possible to modify some properties of the skin (33,34) or reactions to sunlight by the administration of suitable nutritional complements (35–37). Presently, nutritional protection against skin damage from sunlight is increasingly advocated to the general public, but its effectiveness is controversial (35–37). Investigation of an interaction (whatever direction) with IRA effects in the skin is presently only possible by analyzing gene expression of key enzymes in skin biopsies (7,23,30,31). Clearly, much more studies are presently needed about this controversial issue, which is not explicitly addressed in the most recent regulatory monographs on sunscreens (5,6).

SPECIFIC LEGISLATION CONCERNING SUNSCREENS

Sunscreens are subject to specific regulations in almost every country of the world. In particular, UV filters allowed to be used in sunscreens and their maximal concentrations are listed in specific regulatory documents issued by the authorities. However, the spirit of the regulations varies between countries. For example, in the United States, UV filters are considered as over-the-counter drugs, and the monograph of the final over-the-counter sunscreen drug products (6,38) issued by the Food and Drug Administration (FDA) lies down conditions for the safety, efficacy, and labeling of sunscreens. It went into effect in 2001. UVA labeling and testing was deferred until 2007, and a proposal has now been recently released (6). In Europe, UV filters are cosmetic ingredients and are regulated by the Cosmetic Directive last amended for the seventh time in 2003 (39).

However, in every country, and notwithstanding a cosmetic status, sunscreens and the basis on which their efficacy is being claimed are viewed as important public health issues. For example, the European Commission has recently issued a recommendation on the efficacy of sunscreen products and the claims made relating thereto, which sets out claims that should not be made in relation to sunscreen products, precautions to be observed including application instructions, and the minimum efficacy standard for sunscreen products to ensure a high level of protection of public health (5). The same concerns are addressed in the proposed rules for sunscreens, which were recently issued by the FDA (6).

Safety requirements for the registration of chemical UV filters are important and are based on a stringent risk/benefit assessment (8,40). The safety dossier of a UV filter is evaluated and approved by national and international health authorities such as the Scientific Committee on Consumer Products (SCCP) in Europe and the FDA in the United States.

EVALUATION OF THE EFFICACY CLAIMS

Protection Against UVB: the Sun Protection Factor

The protective efficacy of a sunscreen against UVB is expressed as the sun protection factor (SPF). This is universally accepted and acknowledged in the most recent regulatory issues (5,6,38,41). The SPF is a number representing the ratio of the time required for a given irradiation to produce minimal perceptible erythema (MED: minimum erythemal dose, the UVR dose necessary to produce the minimal sunburn or minimal perceptible erythema 16–24 hours after exposure) in sunscreen-protected skin to the time required in unprotected skin. It should be remembered that it is not a ratio of doses, as at each lecture the skin has received the same dose (the MED). Only the irradiation times are different (38,41).

The SPF measurement method is now well established. Detailed guidelines concerning the experimental procedure have been published in many different countries (6,38,41,42). The SPF measurement methods may now be considered worldwide as close to harmonization, although minor differences in the experimental design may subsist (43). The SPF value of a given sunscreen is now considered to be the same within acceptable limits regardless of the country where the product is sold.

Protection Against UVA

The situation is different concerning the evaluation of the protection afforded by a sunscreen in the UVA range, because contrary to the measurement of the SPF, no universally accepted method exists to evaluate UVA protection. However, most recent regulatory recommendations or requirements consider the following methods for the evaluation of UVA protection of a sunscreen (5,6,44):

- in vivo measurement of afforded protection with the persistent pigment darkening method
- in vitro absorbance measurements
- in vitro determination of the critical wavelength
- in vitro determination of a UVA protection factor (UVAPF)

The requirements differ slightly between different regulatory authorities. The FDA has proposed a two-step testing [in vivo persistent pigment darkening testing and in vitro the ratio of long-wavelength UVA absorbance (UVA I) to the total UVR absorbance (UVB + UVA)] (6). In addition, if the products claim water resistance (WR), the UVA protection should be tested after the appropriate period of water immersion. The European Union (EU) has recommended the use of in vivo persistent pigment darkening test and the in vitro determination of the critical wavelength (5). In addition, the EU clearly stated that in vitro testing methods delivering equivalent results should be preferred, as in vivo methods raise ethical concerns. At the time of writing, the testing methods referred to in the European recommendation are subject to standardization by the European Committee for Standardization.

The determination methods are published and are freely available on the Internet. The Colipa (the European cosmetic, toiletry and perfumery association) has recently issued a detailed guideline on the in vitro determination of the UVAPF of sunscreen products, which now may be used instead of the in vivo persistent pigmentation darkening method, thus complying with the recommendations of the EU Commission (44).

Both the FDA proposed rules and the Colipa guidelines addressed on the photostability issue and incorporation of a pre-irradiation step to the in vitro test method. Concerning the pre-irradiation dose, the FDA proposes a dose of UVR corresponding to the SPF of the product, but reduced by a factor of one-third to represent a reasonable exposure (6,43). The Colipa method requires the determination of a UVAPF on a non-irradiated sample and then to irradiate the product with a UVA dose of 1.2 J/cm^2 multiplied by the UVAPF. The value of 1.2 J/cm^2 results from a Colipa round robin test (44). The UVAPF determined after irradiation is the one used to calculate the ratio of SPF to UVAPF, which should be less than 3 (5).

The Australian method was the first official method for evaluating the UVA protection of a sunscreen (42). It is an in vitro method involving the determination of transmission values between 320 and 360 nm (UVA II). This method was frequently used by sunscreen manufacturers to claim UVA protection for their products at a time when no real alternative

existed. However, because the Australian method takes no account of the long-wave UVA I, it has not been considered by the EU for UVA protection claim of sunscreen products.

Protection Against IRR

There is presently no standard method to evaluate efficacy claims concerning IRR. This results on one side from the current debate concerning the question if IRR effects on the skin are deleterious, or not, and on the other side from the absence of defined criteria for measurements of IRR effects in the skin, particularly concerning IRA.

Water and Sweat Resistance

WR characterizes the property of a sunscreen to maintain its degree of protection under adverse conditions, such as repeated water immersion or sweating. Because of the outdoor use of sunscreens in conditions where water immersion is usual and abundant sweating may be encountered, water and sweat resistance is very important (45). Human testing is considered to be the most acceptable and definitive method for claiming WR, and several guidelines have been published for estimating the WR of a sunscreen (38,42,45).

FDA Guideline

A product is claimed to be "WR" if it retains the same category description after 40 minutes of water immersion (2 × 20 time periods separated by a 20-minute rest period without toweling). It may be claimed "very WR" if this is the case after four immersion periods of 20 minutes each (80 minutes). The claim "waterproof" is no longer allowed. Products carrying the claims WR or very WR may also claim to be "sweat resistant" because the FDA concluded that the immersion test is a more severe test than a sweating test (45,46). It is now proposed that the time spent in water (40 or 80 minutes) would be added on the product label to ensure frequent reapplication (6).

Australian/New Zealand Guideline

The SPF of the sunscreen is determined after water immersion for not less than 40 minutes (2 × 20 minutes' time periods separated by a 5-minute rest period without toweling) (42). If WR of greater than 40 minutes is to be tested, the schedule of alternating 20 minutes immersion per 5 min rest is continued. The SPF measured after immersion determines the category classification of the sunscreen. Any claim of WR is to be qualified by a statement of the time for which the WR has been tested, up to the maximum claimed time. No statement is made about sweat-resistance.

The Colipa Guideline

The Colipa guideline for testing WR was published recently (45). Contrary to the FDA and to the Australian method, the SPF is determined on dry skin as usual before immersion. The watering conditions are ensured by 2 × 20 minutes' (40 minutes) immersion separated by a 15-minute drying period, which is very similar to the FDA requirements. The SPF is then measured again after the immersion period and the so-called mean percentage water resistance retention (%WRR) is calculated. The %WRR is the mean of the individual ratios:

$$\frac{[\text{SPF (wet skin)} - 1]}{[\text{SPF (dry skin)} - 1]} \times 100$$

A product may claim WR if the value for the 90% lower unilateral confidence limit of the %WRR is greater than or equal to 50% and the 95% confidence interval on SPF (dry skin) was within 17% of the mean SPF.

Finally, one must be aware that an important difference remains concerning the labeling of the products: A water-resistant sunscreen in the United States or Australia is labeled with the SPF measured after water exposition, whereas in Europe the SPF indicated is still the SPF measured on dry skin.

SUN PROTECTION WITH NUTRITIONAL COMPLEMENTS

As already quoted, nutritional protection against skin damage from sunlight is increasingly advocated to the general public. Several reasons are at the origin of these marketing campaigns, besides the fact that the nutritional complement market is a fast-growing area with a big development potential.

Recent results have been published showing that the mean yearly UVR exposition of the skin amounts to 200 SED (standard erythemal dose) in Europe and to 250 SED in the United States, and occurs during all-day life. Holiday exposition is shown to be only 30% and the weekend exposure 40% (summer) and 20% (winter). The remaining 10% are the daily amount, whereas children may be exposed to higher than 10% of the yearly SED (47–49). Therefore, up to 70% of the yearly exposure is all-day exposure.

In addition, recent analyses have shown that 25% to 30% of the lifelong UVR exposition occurs before 18 years of age (19,47,48). In this context, a systemic lifelong protection of the whole body (and not only the treated areas) through oral administration of targeted micronutrients makes sense and is considered by some experts as contributing to the general public health (4,19,49).

However, the protection offered by the nutritional complements is difficult to assess. In the majority of the published studies, it was measured using a standard MED determination as practiced for the measurement of the SPF. All measured protection factors did not exceed 2.

The investigated micronutrients are more or less potent antioxidants and/or free radical scavengers: vitamins or provitamins with antioxidative properties (carotenoids such as β-carotene, vitamin A, vitamin C, and vitamin E), plant extracts enriched or containing polyphenols as the most potent antioxidants known (polycopodium leucotomos, pycnogenol, green tea extract), ω-3 fatty acids, and trace elements such as selenium and zinc (5,19,33–37).

In view of these developments, the following points must be borne in mind:

- Protection measurements with the standard MED determination always showed results around 2. This raises the question if this experimental approach is the right one. But in any case, a thorough risk-benefit assessment must be conducted, as risks of long-term administration of such micronutrients in high doses are not really appreciated.
- Recent investigations have shown that a mixture of different micronutrients may show a far better activity than each micronutrient administered alone (50). Therefore, some people may face a huge amount of supplemented substances, depending on their way of living. For β-carotene, it has been demonstrated that a category of persons is definitively at risk if the amount administered is above a daily threshold (51).
- The current legislation concerning nutritional supplements is rapidly evolving, at least in Europe.

CONCLUSION

The increasing awareness about the damaging effects of sunlight has led to a significant demand for more protection from sunscreens and to an enlargement of the concept of sun protection toward global photoprotection. A controversial issue is the enlargement of this concept to the IRR wavelengths, as it is not entirely clear if IRA is damaging the skin or exerts a protective effect against UVB damage.

The demand for more protection is clearly reflected by the steady increase in the SPF. The SPF values of the majority of the sunscreens found in the market are now between 10 and 30, compared with 2 and 8 at the end of the 1980s (52). Sunscreens with SPF greater than 50 are now available. However, failure to use the sunscreen appropriately is a major concern and a great educational effort is needed concerning the amount of product applied and the frequency of use as consumers are still getting sunburned despite high-SPF sunscreens (51). This, and recent results concerning the lifelong exposition to sunlight, has led some experts to advocate systemic sun protection through administration of micronutrient supplements. This is still another controversial issue, notwithstanding the missing risk-benefit analysis for this kind of sun protection.

Finally, the traditional "sunscreen" concept has been further enlarged to "photoprotection," including UVA radiation and its consequences as a general public health issue (5,6).

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31 After-Sun Products

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THE HISTORY BEHIND AFTER-SUN PRODUCTS

It was at the beginning of the 20th century when vigilance against significant sun exposure (a relic of the 19th century) started to erode, and the roots of later attitudes toward sunbathing were already manifested. The first clinical observations associating long-term sun exposure with beneficial effects (phototherapy) as well as with skin cancer were also reported during this time (1).

The role of sunlight as a major cause of skin cancer was increasingly mentioned in popular magazines in the 1940s and 1950s. However, rapid growth of the sunscreen and later of the after-sun industry has taken place, allowing sun tanning to remain popular (2). Sunburn treatment and after-sun products were already used at the end of the 19th century. Examples of such products are lactic acid with glycerine and rose water, hydrogen peroxide, iodine, lemon or cucumber juice, and buttermilk baths (1).

AFTER-SUN-RELEVANT EFFECTS OF SUNLIGHT ON THE SKIN

Sunlight is highly energetic. Upon interaction with the skin, sunlight can be reflected, scattered, or absorbed. To initiate a physical or chemical process, light needs to be absorbed by an atom or molecule. Human skin is an abundant source of numerous chromophores with strong absorption, particularly in the UVB, UVA, and blue visible region, e.g., porphyrin, bilirubin 2, and pheomelanin (3).

Irradiation of the skin with ultraviolet radiation (UVR) is today known as a major cause of skin cancer and of local and systemic immunosuppression and as a contributor of cutaneous photoaging (4). Major changes concern all parts of the dermis and the dermal-epidermal junction (5).

UVR is proved to produce DNA damage directly and indirectly through oxidative stress. It provokes increased production of reactive oxygen species (ROS), leading to local inflammation and connective tissue degradation. Increased production of endopeptidases such as matrix metalloproteinases (MMPs) results in destruction of extracellular matrix (ECM). Alterations of the ECM result in wrinkle formation with loss of moisture and elasticity, increased skin fragility, and impaired wound healing (5). Furthermore, UVR induces synthesis of various neuropeptides (substance P, calcitonin gene-related peptide, propiomelanocortin, etc.) leading to immunosuppression and photoaging (6). UVR supports additionally epidermal thickening, reduced skin barrier function, and breakdown of tissue homeostasis (3).

Sunlight affects human skin not only via UVR but also via infrared radiation (IRR) and the combination of them. IRR is perceived as heat. It has been observed that the temperature of human skin measured inside the dermis increases to 40°C to 43°C within 15 to 20 minutes after exposition to direct summer midday sunlight (7). Heat is one of the environmental factors that amplify the effects of UVR mentioned above, reinforcing at the same time angiogenesis and dehydration (7). The skin lipids may as well get thermally destroyed, leading to further damage of the epidermal barrier. Additionally, thermal degradation of carbohydrates leads to modified osmolar environment and further dehydration. Even if the skin does not seem to have been affected by sun irradiation, increased transepidermal water loss (TEWL) is a fact and irritation of nerve endings as well (8). Other climatic conditions can reinforce dehydration. On the beach, wind is always blowing accelerating water evaporation from the skin. Because of the cooling effect of the wind, heat perception is diminished or absent, and beachgoers may consequently be staying longer in the sun without any discomfort. With increased evaporation,

the skin looses both moisture and the important water-soluble natural moisturizing substances too. In this respect, particular attention should be paid to elderly sunbathers. It is known that elderly skin is dry, exhibiting an overall dermal atrophy and reduced amounts of fibrillar collagens and elastic fibers. Thus, prolonged sunbathing may worsen the already present skin condition, leading to accelerated photoaging (9).

Under the influence of the sun, the human body increases sweat production. The horny layers of the skin swell and loosen up, facilitating penetration of the radiation into the epidermis. This may furthermore favor the effects described above (9).

Most of the effects described above are not immediately perceived by sunbathers. Rather acute reactions to sunlight irradiation include erythema, edema followed by exfoliation, tanning, and epidermal thickening, depending on exposure dose. Erythema appears 2 to 4 hours after irradiation, reaches its maximum by 24 to 48 hours, and then gradually disappears (9). Pain, in the form of tenderness to touch, is usually delayed for several hours after sun exposure (10).

The skin can manage the sunlight radiation stress only up to a limited grade of irradiation. Sunscreen protection, although greatly limiting the effects of UVR in the skin, does not completely protect against DNA damage. Small damage may be immediately restored, but repair takes time. Therefore, a sufficient resting period, of at least 12 hours, should be taken before going back to the sun (9).

SKIN CARE AFTER SUN EXPOSITION

Following intensive sun exposition, the skin needs appropriate care or treatment. Even after a sunbath without any signs of erythema, an appropriate skin care is recommended. In case of sunburn, treatment is necessary (8,9).

As a first step after every sun exposure, the skin should be washed with lukewarm water to remove sweat and superficial mud particles. Thereafter, the skin should be treated in accordance with the general schema of skin care: cleanse, vitalize, and maintain the status of the skin.

For cleansing, surfactants showing little interaction with both skin lipids and skin proteins are suitable. Products of pH, which are neutral for the skin, mildness enhancers, and moisturizing agents such as lipids, occlusives, and humectants minimize aggressive interactions with the epidermal barrier, thereby reducing skin damage (11). Recent clinical testing on leading facial cleansers shows that non-foaming, emollient-based cleansers are extremely mild to the skin while still being efficacious (12).

To vitalize and maintain the healthy status of the skin, appropriate skin care is most important after sun exposition. Restoring the hydrolipidic skin barrier is mandatory (8). Formulations containing moisturizers such as sodium lactate, urea, glycerine, panthenol, etc., are most suitable. Slightly occlusive water-in-oil formulations may further enhance the moisturizing effect by impairing TEWL (13,14). However, they should be applied after first cooling and calming the sun-irritated skin with an indicated oil-in-water formula (9,15). More recently, use of skin-like physiologic lipids or naturally occurring lipid complexes of precursor barrier lipids in the appropriate emulsion and molecular ratio has been shown to favorably influence skin repair, barrier function, and moisturization (16,17).

AFTER-SUN PREPARATIONS AND THEIR ACTIVE INGREDIENTS

Most of the after-sun formulations are emulsions (lotions, creams, and sprays) or gels containing moisturizers and actives known for their anti-inflammatory and antioxidative effects. Only cosmetic, nondrug actives will be considered in this section. Thus, active ingredients are usually of plant origin such as azulene or bisabolol (from chamomile), glycyrrhizin (from the root of liquorice), hamamelis distillate (witch hazel), or extracts from aloe vera or chamomile. Further compounds such as allantoin, panthenol, menthol, jojoba, collagen, silk amino acids, unsaturated fatty acids, and fat-soluble vitamins such as retinol (vitamin A) and vitamin E are also found (9). Formulations containing vasoconstrictive and/or tanning or astringent agents (tannins) are used to alleviate heat and tension sensations due to erythema. However, owing to the potent astringent

effect (protein precipitation), use of such preparations on irregular pigmented skin or skin of elderly people is not recommended. New developments have led to preparations facilitating or supporting repair mechanisms (9). Recent studies demonstrated the effect of specific molecular photoprotective agents. Prototype agents that antagonize, modulate, or reverse the chemistry of skin photodamage hold promise in delivering therapeutic benefits (18–20).

In general, the active components used in after-sun preparations can be divided into moisturizers, anti-inflammatory substances, antioxidants, and other ingredients.

Moisturizers

The name "moisturizer" is poorly defined and may be used to define the formula indented to be applied to the skin or the ingredient(s) incorporated in the formulation (14,21). Moisturizing substances are classical, well-known cosmetic ingredients used "to reduce the signs and symptoms of dry, scaly skin" (21). Certainly, effective moisturizers are important components of after-sun preparations for the reasons delineated previously. There is a huge amount of literature available on different moisturizers and their mechanisms of action, thus the reader is referred to very recent pertinent monographs (22–24) and to chapter 10 of this book.

Anti-Inflammatory Substances

Ingredients such as dexpanthenol, azulene, glycyrrhetinic acid, bisabolol, allantoin are incorporated into after-sun formulations to relieve erythema and its symptoms of pain, redness, and burning. The effects of these substances are generally weak. For example, dexpanthenol was shown to alleviate dry, inflamed skin in an experimental model of skin irritation due to repetitive washing. It improved stratum corneum hydration, stabilized the epidermal barrier function, and showed an anti-inflammatory effect (25–27). The combination of skin hydrating and anti-inflammatory properties in the same molecule is of course very interesting for after-sun preparations.

However, more powerful are the tanning or astringent agents. They alleviate itch and possess a local anesthetic effect (9).

Hamamelis (Witch Hazel)

For a long time hamamelis (witch hazel) has been used in the natural medicine. In United States, hamamelis is approved as an over-the-counter astringent in the external analgesic (pain relieving) and skin-protecting categories. In Germany, hamamelis extract–containing preparations are approved among others for the treatment of minor skin injuries and local skin inflammation. Extracts from the leaves or from the bark, but also flower distillate, are mainly used. Main components of the distillate are tanning agents as well as flavonoids and essential oils. Gallotannine and proanthocyanidine act constrictive at the outer membranes of the skin (28,29). Flavonoids are known for their anti-inflammatory effects on the skin (30). In several studies, the anti-inflammatory and erythema-suppressing effect of hamamelis has been demonstrated (27,31–33), even if lower than the one of hydrocortisone-containing formulations (31). However, the efficacy of the hamamelis distillate lotions has been shown to be similar to an antihistamine gel containing 0.1% dimethindene maleate. The low toxicity of hamamelis and the absence of known undesirable effects underline its favorable risk-benefit ratio supporting thus its usage in after-sun formulations.

Polyhydroxy Acids

Polyhydroxy acids (PHAs) are a special type of AHAs (α-hydroxyl acids) known to modulate keratinization, normalize stratum corneum exfoliation and thickness, and condition the barrier integrity due to the effects mediated by the α-hydroxy group. In addition, PHAs have been found to be less irritating to the skin than traditional AHAs mainly due to their larger size (34). For this reason, PHAs offer a distinct advantage over conventional AHAs (glycolic acid and lactic acid) when used on sensitive or irritated skin. In addition to the fact that many PHAs are strong humectants, several PHAs including gluconolactone, lactobionic acid, and glucoheptonolactone have also been shown to function as antioxidants. Through chelating of oxidation-promoting metals, PHAs may prove beneficial in protecting the skin from UVR-induced damage (35).

Cooling Compounds

Menthol and related "cooling agents" such as camphor, alcohol, and other compounds are widely used in cosmetics and particularly in after-sun products. The cooling and carminative actions of menthol have been repeatedly shown (36–39). Green (37) demonstrated that menthol intensified paradoxical cutaneous sensations. In the study of Yosipovitch et al. (39), menthol had a subjective cooling effect lasting up to 70 minutes in most of the volunteers. However, it did not affect cold and heat pain threshold. Recently, Green and Schoen (40) found that dynamic contact can suppress steady-state cold sensations from menthol, proving the bimodal action of menthol.

Alcohol induces an immediate short cold sensation and lowers the threshold of cold sensation. This is mainly due to its rapid evaporation.

Thus, incorporation of cooling agents such as menthol or alcohol in after-sun preparations is meaningful, as they soothe and alleviate the sensations of warmth and tenseness of sun-irritated skin, at least for a short time. This clearly improves their cosmetic acceptance.

Antioxidants

The cutaneous antioxidant defense system is complex, multilayered, and far from being completely understood. The human skin contains various antioxidants such as lipophilic ones [vitamin E with its active components tocopherol and tocotrienol, ubiquinones (coenzyme Q10), carotenoids, and vitamin A] as well as hydrophilic ones (vitamin C, uric acid, and glutathione) and enzymatic antioxidants (catalase, etc.) (41,42). Being the most environmentally exposed skin layer, the stratum corneum may particularly benefit from an increased antioxidant capacity due to topical supplementation as shown in the example of vitamin E (43,44).

While efficacy of topical antioxidant application before UVR is demonstrated in several studies (35), the effect of these agents after irritation is less obvious (44,45). Hence, use of antioxidants in after-sun preparations aims at replenishing the depleted antioxidant pool and/or at boosting or reinforcing the antioxidant defenses of the horny layer and not necessarily at a direct effect on sun-exposed skin. For this purpose, substances such as vitamins of the A, C, and E series, ubiquinones (coenzyme Q10), and/or plant polyphenols from green tea (catechins), vine (resveratrol), pomegranate (anthocyanidins and hydrolizable tannins) or soybeans (genistein) are now incorporated in many after-sun preparations (46).

Green tea extract is particularly interesting in this respect because several studies are available regarding photoprotection and anti-inflammatory activities after topical application. A protection against UVA has also been demonstrated (47–49). The principal chemical constituents of green tea are polyphenols containing (−) epigalocatechin (EGC), (−) epicatechin (EC), (−) epicatechin-3-gallate (ECG), and (−) epigalocatechin gallate (EGCG). Topical EGCG has been shown to reduce UV-carcinogenesis in mice (50–52). Furthermore, recent studies suggest that green tea polyphenols mediate almost probably their UV-protective effects via induction of DNA repair (53).

Other Ingredients

Shea Butter

Shea butter may be incorporated in the lipid fraction of after-sun formulations. Shea butter contains a highly unsaponifiable fraction consisting of terpenic alcohols and sterols found almost exclusively as cinnamic acid esters. They give shea butter its known healing properties, e.g., the elimination of superficial irritation and erythema. The unsaponifiables also contain 5% to 10% phytosterols, which are known to activate cellular growth stimulation. Moreover, shea butter contains the potent antioxidant butylated hydroxytoluene as well as various catechin compounds (54). Shea butter is a nontoxic and nonirritating material. It has a highly unsaturated glyceridic fraction, which makes it useful as UV screen. It also contains linoleic acid (55).

Clinical studies were performed with shea butter showing protecting, regenerating, wound-healing, and wrinkle-reducing effects (56). It has moisturizing, soothing, antiageing, and anti-inflammatory properties and contributes to an efficient release of active ingredients (56).

Olive Oil

Extra virgin olive oil (EVOO), which is obtained from whole fruit, is rich in phenolic compounds having potent anti-ROS activity. Recently, it has been found that EVOO painted on mouse skin immediately after UVB radiation, significantly retarded the onset and reduced the number of skin cancer and ROS-induced DNA damage. Interestingly, pretreatment with EVOO and pre- or posttreatment with regular olive oil neither retarded nor reduced skin cancer in UV-irradiated mice. Of the olive oil components tested, oleuropein has been found to reduce ROS-induced skin damage (57). These results strongly suggest that topical use of olive oil after sun bathing in humans may prevent skin cancer formation by reducing ROS-induced DNA damage. However, further human studies are needed to support recommendations in the public.

Molecular Photoprotective Agents

On the basis of the causative involvement of skin chromophores in skin photodamage, it is to be expected that molecular antagonism of photoexcited states offers a potential therapeutic opportunity for skin photoprotection. Reactivity-based approaches are widely followed for the design of photoprotective agents, including FDA-approved drugs, cosmeceuticals, and experimental therapeutics in preclinical development.

Two classes of reactivity-based agents for skin photoprotection can be distinguished on the basis of their mechanism of action: *direct antagonists of photooxidative stress* (sunscreens, quenchers of photoexcited states, antioxidants, redox modulators, and glycation inhibitors) and *skin photo-adaptation inducers* [nuclear factor erythroid 2-related factor 2 (Nrf2) activators, heat-shock response inducers, and metallothionein inducers] (20). Of the first class, quenchers are worth to be mentioned more explicitly as many of them are allowed to be used in cosmetics.

Quenchers are compounds capable of inactivating photoexcited states by direct chemical and/or physical interactions. Physical quenchers that can undergo repetitive cycles of excited state quenching without chemical depletion or the need of metabolic regeneration represent a very attractive class of compounds. Examples of such quenchers are vitamin E, ascorbate, L-proline, L-proline methylester, carotenoids (lycopene, lutein, and zeaxanthin), the microbial osmoprotectant ectoine, etc. (20). Ectoine, which can be used, as well as a strong moisturizer is already included in various kinds of marketed products (58,59).

Two further molecular photoprotective concepts are worth to be mentioned.

Few studies demonstrated that when exogenously applied, the bacterial DNA incision repair enzyme T4 endonuclease V (T4N5) is able to protect human skin in vivo from UVR-induced DNA damage. Although neither erythemal response nor microscopic sunburn cell formation were influenced by T4N5 treatment, UVR-induced upregulation of interleukin 10 and tumor necrosis factor-α were prevented (19). Interestingly, the liposomal lotion was applied after irradiation, thus as an after-sun product would be. A further experiment in xeroderma pigmentosum patients showed that the T4N5 enzyme clearly lowered the annualized rate of new lesions in these patients with defects in their DNA repair mechanisms. An interesting and perhaps fundamental finding was that the rate of new actinic keratoses and basal cell carcinomas did not increase during the six months of follow-up after discontinuation of treatment (60). This may imply that T4N5 treatment reverses a fundamental and common source of these neoplasms and is not just a cosmetic treatment (61).

The second approach is similar to the first one but uses a different DNA repair enzyme. Photolyases are repair enzymes found in fish or prokaryotic organism and normally not present in human skin. They bind to cyclobutan-pyrimidine dimers after their formation due to UVR exposure. The complex enzyme plus dimer is activated by light in the range of 300 to 500 nm, and the repair process gets induced. Here too, the photolyase from the blue algae Anacystis nidulans prepared from genetically engineered *Escherichia coli* was packed in liposomes and applied topically after irradiation. After a new irradiation with activating light of 365 nm, a reduction of 40% to 45% of the cyclobutan-pyrimidine dimers was noted. Similarly, a clear effect on immunosuppression was seen and on the expression of the pro-inflammatory ICAM-1, which was clearly decreased by UVR, but the decrease was antagonized by topical enzyme treatment (18).

Topical application of molecular photoprotective agents is a very promising approach to skin cancer prevention and totally compatible with the principle of after-sun products. Thus, one

may think of a pre-sun preparation combining antioxidants to replete the horny layer with the T4N5 enzyme to prevent formation of cyclobutan-pyrimidine dimers. Then, an after-sun preparation could provide moisturization and skin care, including photolyase to repair the last DNA changes. A liposomal lotion containing photolyase is already on the market in Germany (62).

CONCLUSIONS

Appropriate after-sun skin care is necessary to support the natural regeneration process of sun-irritated skin. Mild cleansing, cooling, moisturizing, and caring of the skin belong to the basis of a good after-sun treatment. In the last years, a lot of research has been successfully conducted to find ways to inhibit or repair sun-induced skin damage and to prevent skin cancer. Of the investigated compounds, antioxidants and molecular photoprotective agents seem to be the most promising for future application in after-sun products. However, ingredients with dual action showing UV filter properties as well as repair properties are certainly a valuable alternative. Recent studies have shown that topical caffeine as well as caffeine benzoate application induces apoptosis, thus decreasing the probability of neoplasmic transformation–irradiated cells, whereas having at the same time a sunscreen effect (63).

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32 Skin Care Products: Artificial Tanning

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INTRODUCTION

The desire for a tanned appearance along with increasing awareness of the hazards of ultraviolet (UV) light exposure has generated a renewed interest in artificial tanning products. Better formulations of sunless or self-tanners with improved aesthetics are more widely available. As consumer experience with the newer products has grown, this category has become more popular, and there has been an increasing proportion of overall sun care sales. Over 20% of young adults in both United States and Australia reported using these products in the preceding year (1,2). Individual users were also more likely to have sunburned consistent with higher use of these products in fairer Caucasians. In other studies, exclusive users of sunless tanners were more likely to practice overall sun protection (3,4) and decrease their use of tanning beds (5).

Dihydroxyacetone (DHA) is the active ingredient in sunless or self-tanners, and is responsible for darkening the skin by staining. DHA is classified in the *International Cosmetic Ingredient Dictionary and Handbook* (6) as a colorant or a colorless dye. Other agents with the potential to enhance skin pigmentation, including tan accelerators containing tyrosine and other ingredients. Tanning promoters containing psoralens, which require UV exposure, will not be discussed here (7).

HISTORY

The first mention of DHA as an active ingredient in medicine appeared in the 1920s, when it was proposed as a substitute for glucose in diabetics. In the 1950s, the oral administration of DHA was restudied as a diagnostic procedure for glycogen storage disease when it was given in large doses orally (8). When the children spit up this sweet concentrated material, the skin became pigmented in areas splattered on the skin, without staining clothing. Aqueous solutions were then applied to the skin directly, and the pigmentation reproduced (9). In the late 1950s, cosmetic tanning preparations first appeared in the marketplace. Cosmetic acceptance of these initial products was limited because of the uneven orange-brown color they imparted to the skin. With the availability of improved formulations in the 1990s, sales of sunless tanners grew exponentially as a total proportion of sun care product sales. In the last several years, operator-assisted spray tans using DHA have become popular in spas and salons.

CHEMISTRY

DHA ($C_3H_6O_3$) is a white, crystalline hygroscopic powder. This 3-carbon sugar forms a dimer in freshly prepared aqueous solution (Fig. 1). With heating to effect a solution in alcohol, ether, or acetone, it reverts to the monomer. The monomeric form is less stable, but more important in the browning reaction, which leads to the skin color change (10). DHA is stable between pH 4 and 6, but above pH 7, efficacy is lost with the formation of brown-colored compounds. A buffered mixture at pH 5 is most stable. Heating above 38°C for long periods of time will also affect stability. DHA needs to be stored in a cool, dry place, ideally 4°C and low atmospheric humidity (11). Glyceraldehyde, the isomer of DHA, is also present in the solution. Glyceraldehyde may degrade into formaldehyde and formic acid. In acidic solution (pH 4), this isomerization and therefore these latter undesirable ingredients are minimized.

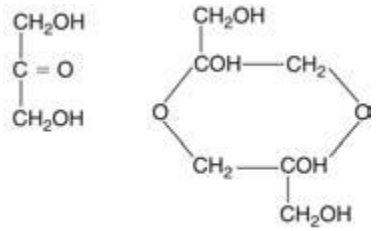

Figure 1 Chemical structure of DHA. *Abbreviation*: DHA, dihydroxyacetone.

The Maillard or browning reaction has been defined as the reaction of an amino group of amino acids, peptides, or proteins with the glycosidic hydroxyl group of sugars. DHA in the context of this reaction may be considered a 3-carbon sugar, reacting with free amino groups available as amino acids, peptides, and proteins supplied by the keratin to form products or chromophores referred to as melanoidins (12). Melanoidins have some physicochemical properties similar to naturally occurring melanin (13). Electron spin resonance has recently shown that free radicals are produced in vivo by the Maillard reaction (14).

FORMULATION

The concentration range of DHA in self-tanning products can range from 2.5% to 10%, with the usual concentration being 5% (10). Lower concentration products allow the consumer greater latitude with application since they tend to be more "forgiving" of uneven application or rough surfaces. Labeling products as light, medium, or dark can be particularly helpful with the depth of shade a function of the DHA concentration.

DHA is predominantly formulated in oil-in-water emulsions. Formulating with silicones allows the formulator to obtain the spreadability of oils, which potentially reduces streakiness with application to the skin. Minimizing particle size of the micelles in the emulsion chosen also improves uniformity of spreading the formulation on the skin surface. On the basis of the chemistry of DHA, formulations should be buffered to an acidic pH (4 to 5) and not heated in manufacturing to temperatures higher than 40°C.

After incorporation of DHA into a formulation, the pH may drop during storage, suggesting that stability may actually be increased when the pH is kept between 3 and 4 (15). The use of nonionic emulsifiers as opposed to ionic emulsifiers may also improve stability. Some thickeners such as carbomers, sodium carboxymethylcellulose, and magnesium aluminum silicate can cause rapid degradation of DHA. Hydroxyethylcellulose, methylcellulose, and silica as well as xanthan gum and polyquaternium-10 for thickening DHA-containing emulsions are better choices.

DHA can react with oxygen and nitrogen-containing compounds, collagen, urea derivatives, amino acids, and proteins. They should be avoided in the formulation of the DHA-containing vehicle. Non-nitrogen-containing sunscreen should be used if sun protection is desired. Attempts have been made to take advantage of this effect by adding amino acids to speed up the skin-darkening process, but with less substantive color results. Methionine sulfoxide, a sulfur-containing amino acid, has been used as an excipient applied before the application of the DHA-containing cream (16). Two compartment systems have been patented on the basis of this reaction.

As with moisturizing products in general, lotions are more readily accepted by consumers than are creams with ease of spreadability and aesthetics. Creams can produce a more intense tan owing to greater applied film thickness. Products may be formulated for dry-skin types by the addition of emollients and humectants. Products formulated in gel or alcoholic vehicles may be more suitable for oily skin. Newer vehicles include sprays, foams, mousses, and wipes.

MECHANISM OF ACTION

The site of action of DHA is the stratum corneum (17). Tape stripping of the skin quickly removes the color (18), as does mechanical rubbing. Deeper staining in areas with thicker stratum corneum and no staining of mucous membranes without a stratum corneum are also

consistent with this being the site of action. DHA may be used as a substitute for dansylchloride as a measure of stratum corneum turnover time (19,20). Microscopic studies of stripped stratum corneum and hair reveal irregular pigment masses in the keratin layers (21) consistent with melanoidins. These melanoidins are formed via the Maillard reaction with DHA as a sugar reacting with the amino groups supplied by the keratin.

APPLICATION

Following application of a typical DHA-containing self-tanning lotion, color change may be observed within an hour (22). This color change may be seen under Wood's light (black light) within 20 minutes. Maximal darkening may take 8 to 24 hours to develop. Individuals can make several successive applications every few hours to achieve their desired color. Color may last as long as five to seven days with a single application. Depending on anatomical application, the same color can be maintained with repeat applications every one to four days. The face requires fewer applications but more frequent reapplication to maintain the color than the extremities. Depth of color varies with the thickness and compactness of the stratum corneum. Palms and soles stain deepest, necessitating washing of hands after application to avoid staining. Hair and nails will color but not mucous membranes lacking a stratum corneum or keratin layer. Rougher hyperkeratotic skin over the knees, elbows, and ankles will color more unevenly as will older skin with keratoses and mottled pigmentation. Color will also be maintained longer in these areas.

As in the formulation, the pH of the skin before application may have an effect on the tonality of the skin color (10). Alkaline residues from soaps or detergents may interfere with the reaction between DHA and the amino acids on the skin surface, resulting in a less natural-appearing (more yellow) color. Wiping the skin surface with a hydroalcoholic, acidic toner just prior to DHA application may improve results. Ex vitro epidermal studies suggest that skin hydration (23) and relative humidity (24) influence the development of coloration.

Careful directions provided with these products are therefore quite important in determining consumer satisfaction. The skin may be prepared with a mild form of exfoliation. Even application is required with lighter application around elbows, knees, and ankles to avoid excessive darkening in these areas. Care also needs to be taken around the hairline where lighter hair may darken. Hands need to be washed immediately after use to avoid darkening of the palms, fingers, and nails. Skill and experience are necessary with using these products resulting in greater user satisfaction.

ADDITIVES

As commonly occurs, growth in this category has compelled both formulators and marketers to seek points of differentiation between their product and that of their competitors. Besides formulating for specific skin types, active treatment ingredients may be incorporated into DHA-containing formulations. Vitamins, botanical extracts, antioxidants, anti-irritants, and even α-hydroxy acids may be added to broaden the claims made with a given product. Addition of antioxidants can shift tonality to a more natural coloration (25). The addition of sunscreen ingredients to self-tanners warrants a more detailed discussion in the section that follows.

Some newer formulations have included colorants as used in bronzers, including dyes and caramel, to achieve an immediate makeup effect. Similarly, tinting with titanium oxide or iron oxides can provide immediate color and allow the user to more easily visualize the evenness of application. Metal oxides may however induce degradation of DHA (15). To compensate for less red absorption by the products of the Maillard reaction, erythrulose may be added as a colorant to add red to the tone.

SUNSCREEN ACTIVITY

In the United States, the FDA Tentative Final Over-the-Counter Monograph on Sunscreens (Fed. Reg. 1993) listed DHA as an approved sunscreen ingredient when used sequentially with lawsone (2-hydroxy-1,4-napthoquinone). The final monograph (Fed. Reg. 1999) removed this

combination from the approved list. The European Economic Community Directive does not list DHA as a permitted UV filter.

DHA itself has at most a modest effect on SPF (26), providing perhaps SPF 3 or 4. SPF increases with DHA concentration and number of applications (27). Low-level SPF persists for several days decreasing with loss of color (28). The brown color obtained on the skin does absorb in the low end of the visible spectrum with overlap into long UVA and may provide some UVA I protection (29). Melanoidins can act as free-radical scavengers as they demonstrate an electron spin resonance signal (14). Superficial skin coloration induced by frequent topical application of DHA in high concentrations may delay skin cancer development in hairless mice irradiated with moderate UV doses (30).

Individuals using DHA-containing tanning products need to be cautioned that despite visible darkening of their skin, these products provide minimal sun protection. Confusion may be compounded by the addition of UV filters to the formulation providing significant sun protection. The stated SPF for the product is applicable for a few hours after application, but not for the days during which the skin color change may remain perceptible.

INDICATIONS

Even with recent improvement in DHA formulations, the color achieved remains dependent on skin type. Individuals of medium complexion with skin phototypes II or III (31), as opposed to those who are lighter or darker, will obtain a more pleasing color. Individuals with underlying golden skin tones will achieve better results than individuals with rosy, sallow, or olive complexion. Older consumers with roughened, hyperkeratotic skin, or mottled pigmentation with freckling may be less pleased with their use.

Dermatologists regularly recommend these products for tanning as a safe alternative to UV exposure. They may be used to camouflage some skin irregularities such as leg spider veins. Light to medium complected patients with vitiligo who show increased contrast with the vitiliginous areas with natural or unavoidable tanning in their normal skin may also benefit (32,33). They may even provide some protection for individuals with certain photosensitivity disorders (34). Protection of uninvolved skin by DHA during psoralen-UVA treatment (PUVA) allows higher UVA exposures to be tolerated, with fewer treatments resulting in faster clearing know as turbo-PUVA (35).

SAFETY

The visible color change associated with the use of artificial tanning products might suggest to some users that these products are hazardous. On the basis of the chemistry of DHA and its toxicological profile, it can be considered nontoxic. It reacts quickly in the stratum corneum, minimizing systemic absorption. The acute toxicity of DHA was investigated for diabetics in the 1920s with oral intake well tolerated (15). The phosphate of DHA is found naturally as one of the intermediates in the Krebs cycle.

Contact dermatitis to DHA has only rarely been reported (36). As with other topical products with active ingredients, such as sunscreens, much of the reported sensitivity is secondary to other ingredients in the vehicle (37). Adverse reactions are more likely to occur on the basis of irritation and not true allergy. Ultimately, all claims related to product safety are based on testing the final formulation.

ALTERNATIVE TANNING AGENTS

Lawsone found that henna plant and juglone (5-hydroxy-1,4,-napthoquinone) derived from walnuts also stain hair, skin, and nails. They have been used for centuries for hair coloring. Both substances lack skin substantivity and readily discolor clothing (38). The skin color they produce does not resemble a natural tan.

On the basis of the underlying principle of the Maillard reaction, other molecules with a ketone function have been investigated (39). An α-hydroxy group attached with electron-withdrawing groups can also increase reactivity. Substances such as glyceraldehyde and

glyoxal (40) have been described but found ineffective. Mucondialdehyde as described by Eichler (41) is an effective agent, but associated toxicity mitigated against its use (39). Although several other aldehydes have been shown to have better color properties, stability issues limit their use (39).

CONCLUSION

Increasing consumer awareness to the hazards of UV light should fuel ongoing interest in self-tanning products. The benign toxicologic profile of DHA reinforces the notion that these products represent a safe alternative to a UV-induced tan. The results obtained with these products are dependent on the final formulation, individual application technique, and the consumers' complexion type. Greater experience in formulation combined with increasing sophistication on the part of the consumer should lead to continuing growth and satisfaction with the use of these products.

Consumers need to be clearly informed that these products do not offer significant protection against UVB. If formulated with standard sunscreens, consumers should be cautioned that the duration of UV protection is more short lived than the color change.

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33 Reconstructed Human Skin and Skin Organ Culture Models Used in Cosmetic Efficacy Testing

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INTRODUCTION

The use of animals for research purposes and especially for cosmetic efficacy testing has been a sensitive matter for several decades. Regulatory agencies and political as well as scientific communities have put constant pressure to ban the use of animals for such purposes. In Europe, this has forced the Council of the European Union to approve legislation for banning the use of animals for testing cosmetic products, starting March 2009. This implies that alternative methods require validation by the European Center for the Validation of Alternative Methods (ECVAM). To date, only a few alternative testing methods have been validated for toxicological testing using reconstructed human skin models. Nevertheless, human skin equivalents, such as reconstructed human epidermis (RHE), full-thickness (FT) skin, or skin organ culture (SOC) models have already been adopted by cosmetic laboratories as alternatives to animal experimentation for quite some time. These models not only allow to comply with the demands of regulatory authorities, animal welfare organizations, and consumers but also provide a means to improve and extend our knowledge on skin biology. Moreover, they have proven to be reliable, efficient, informative, and predictive tools for screening, bioavailability, and efficacy testing of active ingredients.

One of the main reasons for the development of reconstituted human skin (RHS) models is the fact that they mimic more closely human skin in vivo, which includes the presence of a stratum corneum, allowing topical application of both aqueous and oily solutions as well as final formulations. Three-dimensional (3D) skin models are composed of either the epidermal compartment or both the epidermal and dermal compartments. Various cells types can be incorporated within each compartment, including melanocytes and Langerhans cells in the epidermis and fibroblasts and endothelial cells in the dermis. Because the use of RHS models requires a substantial amount of expertise, including internal benchmark validation, which often requires considerable financial resources, and given the fact that these models have some inherent limitations, especially an impaired barrier function, skin organ culture models are also being used as alternatives for cosmetic efficacy testing.

In this chapter, we first present a review about the development of reconstructed skin models, followed by an overview of the commercially available ones. We review a selection of published literature using commercially available models as well as homemade models developed in research laboratories used for in-house testing of cosmetic formulation. This overview will mostly focus on studies in oxidative stress photoprotection evaluation and pigmentation assessment. A brief review of more complex and integrated skin models for clinical use is also discussed. Then, we present the results of two sets of experiments carried out using both RHE and SOC models. The first experiment deals with vitamin E prodrug, its diffusion and metabolism in RHE and human SOC models, and also its efficacy in the prevention of UV-induced lipid peroxidation in RHE. The second experiment studies the response of RHE and a pig SOC model to solar radiation–induced cytotoxicity and apoptosis and tests the efficacy of a sunscreen formulation. In conclusion, these studies on cosmetic efficacy testing confirm that both RHS and SOC models represent complementary models with a genuine added value for the cosmetic testing.

SKIN EQUIVALENTS USED IN COSMETIC EFFICACY TESTING

Basic Research on Reconstructed Human Skin

New and stricter regulations have led researchers and companies to develop in vitro tissue models to be used in the study of cutaneous biochemistry and physiology. An excellent guideline enumerates the processes used in developing these cell culture models (1). Briefly, Bell's model (2), the first in vitro reconstructed model, developed in 1979, was a dermal equivalent composed of fibroblasts in a collagen lattice. Rheinwald and Green (3) fortuitment found that under certain culture conditions a teratogenic cell lines undergoes a maturation that mimics epidermal keratinization (3). Later, the major breakthrough was the culture of keratinocytes (KCs) at the air-liquid interface (4), leading to the formation of a multilayered and differentiated epidermis, a characterisitc of all types existing in 3-D skin models. This differentiation program provided the crucial stratum corneum (SC) layer, which constitutes a barrier function for reconstructed models. As cell culture undergoes constant evolution, various models were subsequently designed by differentiating KC cultures on different supports: inert filters (5–7), dermal substrate such as collagen matrices (8,9), lyophilized collagen-GAG membranes (10), de-epidermized dermis (DED) (11), and fibroblast-populated dermal substrates (12,13). Some of the above mentioned commercially available skin models are listed in Table 1.

Morphological studies (14,15) have shown that RHS models form a multilayered epithelium, displaying characteristic epidermal structure and expressing markers of epidermal differentiation. Ultrastructurally, keratohyalin granules, lamellar bodies, and lamellar structures filled with epidermal lipids are present in the stratum granulosum and SC. The SC layer, a corneified barrier for RHS models (Fig. 1), is composed of multiple lipid lamellae located in the intercellular spaces between keratinized cells, a corneocyte lipid envelope, and desmosomal structures. This barrier is associated with an epidermis calcium gradient, similar to that found in native human skin (16). However, it has been established that there is a difference in the lipid organization, with a hexagonal SC lipids packing in RHS, whereas in native skin it is orthorhombic (17). This difference may account for the 5- to 50-fold higher penetration rate observed in RHS models for most of the substances tested (18). In addition, the recent scientific committee on consumer products (SCCP) guidelines in dermal absorption

Table 1 Commercially Available RHS and SOC Models

Company/laboratory	Skin models	Web site
Bioprédic, Rennes, France	Skin organotypic culture Natskin® (human skin), 1.0 and 1.6 cm^2	www.biopredic.com
CellSystems, St Katarinen, Germany	Reconstructed human epidermis (EST 1000) Full-thickness skin (AST 2000)	www.cellsystems.de
MatTek Corp. Ashland, MA, U.S.	Reconstructed human epidermis (EpiDerm®), 0.9 cm^2 Full-thickness skin (EpiDermFT®), 1.2 cm^2 Reconstructed pigmented epidermis, (Melanoderm®), 0.9 cm^2 Melanoma skin model, 0.6 cm^2	www.mattek.com
Phenion GmbH & Co. KG (Henkel), Düsseldorf, Germany	Reconstructed human epidermis (OS-REP) 0.63 cm^2 Full-thickness skin (Phenion FT), 1.3 cm^2	www.phenion.com
SkinEthic, (L'Oréal), Nice, France	Reconstructed human epidermis, 0.5/4 and 0.11/0.33 cm^2 in 24/96 well plate Reconstructed pigmented epidermis, 0.5 cm^2 Full-thickness skin (Episkin®), 0.38/1.07 cm^2	www.skinethic.com
StratiCell, Gembloux, Belgium	Reconstructed human epidermis Genetically modified reconstituted human epidermis Reconstructed human 3-D dermis Reconstructed full-skin equivalent	www.straticell.com

Technical specifications and ordering informations for these skin models can be found in the respective Web site. Some of these companies also supply reconstructed epithelia models (buccal, vaginal, pulmonary etc.).

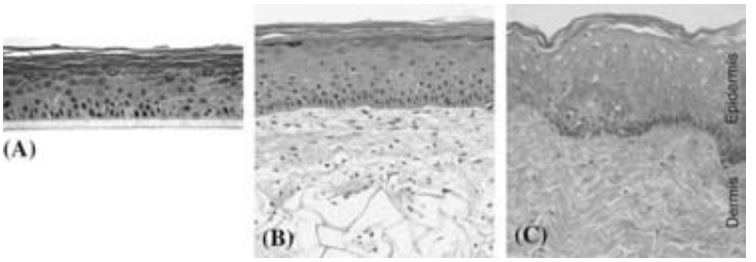

Figure 1 Histological section of different skin models. (**A**) A reconstructed human epidermis (SkinEthic Laboratories, Nice, France[a]); a polycarbonate membrane supports a stratified epidermis. (**B**) A full-thickness skin model (Phenion®[a]) with a stratified epidermis on a dermal equivalent. (**C**) Human SOC model from personal data.

of cosmetic ingredient (19) consider that RHS models as alternative membranes are inadequate for use in dermal absorption study because of this impaired barrier function. In spite of these differences (18,20), the presence of the SC on skin equivalents makes it possible to apply a great variety of active ingredients topically. In addition, through metabolism studies performed on 3-D human reconstructs and reviewed in details (21), most of 3-D skin equivalents (RHE, FT skin, or SOC) are metabolically representative of human skin and therefore can be used as a good surrogate model for human skin. For instance, similar enzymatic activities, such as phase I and phase II enzymes were found in RHE (22), making them efficient models for toxicological assessment. Other enzymatic activities, such as esterase, β-glucocerebrosidase, or 5α-reductase are also found in skin equivalents. Accordingly, reconstructed skin models were used successfully in the study of the controlled release of pro-vitamin E into free tocopherol (as referred to in details in section "Skin Absorption, Metabolism, and Antioxidant Efficiency of Vitamin E Prodrugs) (23,24), and of testosterone metabolism with androgen modulators (25).

Table 1 lists the commercially available skin models used for pharmaco-toxicological trials. Reconstructed epidermis models are first used for irritation testing, and some have already been validated or are in the process of being validated for this use. Providers now offer pigmented epidermis as well as FT skin models. However, the fact that only a handful companies are capable of producing skin models in sufficiently large quantities demands for some careful consideration. For instance, in 2006, Episkin® (L'Oréal group) acquired SkinEthic Laboratories. The resulting situation created a kind of monopoly situation in Europe (15). In addition, all models currently validated are either protected by patents or being commercialized using proprietary tissue culture procedures by only a few private companies; hence, their continued availability is dependent only and entirely on the corporate strategies of these companies and therefore cannot be guaranteed for the long-term future. In addition, although it is generally accepted that the reconstructed epidermal models currently available are all "comparable," intrinsic differences inherent to their proprietary manufacturing procedures have been causing reasonable concern in the scientific community; for instance, the test protocol transferability from one model to another is often limited and considered a bottleneck for method acceptance both at industry and regulatory levels.

Not only for regulatory toxicology testing but also in cosmetic efficacy testing, the use of these models requires great care and must include the appropriate controls and an analysis of reliable and reproducible endpoints. Accordingly, multiple endpoint analysis (MEA) including tissue viability, morphology, and the release of proinflammatory mediators should be used to determine the efficacy of a cosmetic product (26). One of these endpoints is the MTT [3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide] toxicity test (27), which measures either cellular viability and therefore the cytotoxic effects of external stress or a cosmetic formulation. We recently determined, for instance, that the release of lactate dehydrogenase (LDH) into the culture medium is a more relevant endpoint than MTT assay to study irritation and solar-induced cellular damages in SOC models (28).

Applications of Skin Equivalents in Cosmetic Efficacy Testing

Below we describe some experiments carried out to determine the effects of UVB-UVA irradiation as inducer of oxidative stress and photoaging. Studies performed to measure photoprotection and monitor the pigmentation process in a pigmented skin model are also

discussed. Finally, new and "clinical use" models of skin equivalents are presented as potentially useful tools for the testing of future cosmetic formulations.

Oxidative Stress and Photoprotection

The skin is directly exposed to external oxidative stress, and in vitro RHE models have been used in the evaluation of ozone- (29) or UVA- (30) induced lipid hydroperoxides (LPOs). Ex vivo pig and human SOC models have been also developed as tool for use in investigating short-term UV-induced damage (31) and time course and spatial distribution of UV effects (32). Such experiments have demonstrated that skin models are sensitive to UV and that topical application of various antioxidants could successfully be evaluated against UV-induced oxidative stress by reducing apoptotic response and DNA damages: combination of vitamins on a pig SOC model (33), topical application of genisteine on a human SOC model (34), or epigallocathecinegallate (35) and salicylic acid (36) in RHS. In addition, UVA dermal alterations in an human SOC model and topical application of retinoids (37) induced the formation of newly synthesized collagen, suggesting dermal repair on this "photoaged" skin model.

Moreover, although in vivo assays are essential for sunscreen testing [determination of SPF (sun protection factor) and PFA (protection factor in UVA)], in vitro techniques based on skin equivalents were developed and have shown to be very useful for these types of tests. Using an RHS model, Augustin (38) showed the deleterious effects of both UVA and UVB irradiation by measuring viability and Il-1α release assay, which were abolished following the application of sunscreens. Also the apoptotic response was also assessed following UV irradiation with and without photoprotection in RHE (39), RHS (40), and pig SOC (41) (see experimental details and results in section "Deleterious Effects of Solar Radiation and Sunscreen Efficacy Testing on Both RHE and Pig SOC"). In general, all these skin models demonstrate their usefulness and ability to model UV-induced damages, which can be reversed in presence of photoprotectant formulations.

Pigmentation

Pigmented RHS are cocultures of both KCs and melanocytes. The integration of melanocytes from different ethnic regions results in a pigmented epidermis (42) or skin equivalent (43) reflecting Caucasian, Asian, and African-American skin phenotypes. These models provide an interesting alternative to animal testing for evaluating the regulation of mammalian pigmentation by melanogenic factors and for elucidating the mechanisms of action of these factors. Using these models, it has been demonstrated that the protease-activated receptor-2 (PAR-2) pathway (44) regulates pigmentation via melanosome transfer, but only when KCs and melanocytes are in contact. Gibbs has shown (45) that a complete program of melanogenesis occurs following UVB irradiation and supplementation with 3-isobutyl-1-methyl-xanthine: melanosome synthesis, melanosome transport to KCs, supranuclear capping of KC nuclei, and tanning of the epidermis. Dysfunctionnal pigmentation can also be assessed through the use of in vitro recontructed models. A xeroderma pigmentosa skin model was reconstrcuted to study genetic hyperphotosensitivity (46). Recently, an RHS model was engineered with normal nonsegmental nonlesional vitiligo cells (47), and tested under stressed conditions (H_2O_2). First results seem to support the melanocytorrhagic hypothesis of vitiligo.

Additionally, pigmented skin tissue models provide a useful tool in the comparison of the inhibitors (kojic acid, arbutin, and hydroquinone) and activators (α-MSH and dihydroxyphenylalanine) of melanogenesis (43) after repeated topical application or systemic delivery (48). Pigmented skin tissue is also useful in the assessment of the antipigmentation effects of sunblocks.

New and "Clinical Use" Skin Models

Tissue engineering attempts to reconstruct complete skin tissue, integrating different cellular types. Most of the previously mentioned skin models are comprised of KCs, fibroblast, melanocytes, and endothelial cells. However, the skin is an immune organ, displaying an elaborated enervated system. Immunoreactive reconstructed skin containing Langerhans cells has been reconstructed. The immune response of this RHS was demonstrated by a reduction in the number of Langerhans cells and by a modification in their dendritic morphology (49) after

exposure to sensitizers or UV irradiation. Another immunocompetent reconstructed model was developed, comprising epidermal Langerhans cells, dermal dendritic cell, and endothelial cell activated to acquire HLA-DR expression (50). This model provides a complex environment-integrating vascular components to study the differentiation of interstitial dendritic cells in the dermis. Also a "neuronal" epidermal model was developed to evaluate the regeneration of sensory neurons on injured skin (51), which was mainly influenced by the extracellular matrix molecules, matrix-binding growth factors, and trophic factors.

In addition to these "dermo-cosmetic" models, tissue engineering has led to the developement of complex reconstructed models for clinical indications such as grafting ulcers, treatment of burn patients, and wound repair. Some of these models are commercially available: Apligraf™, Alloderm™, Integra™, Dermagraft™, and Orcelf™ (52). Last, but not the least, a model comprising of endothelial cells was created for testing potentially angiogenic molecules (53) as well as for treating recalcitrant leg ulcers through split-thickness skin autografting (54). As perspective, additional development in the two integrative aspects of neovascularization and reinnervation is necessary for such skin reconstructs to reach their full therapeutic potential. Clearly, such "complete" skin models would be of great interest for integrated pharmaco-toxicological trials of the future cosmetic formulations.

EXAMPLES OF COSMETIC EFFICACY TESTING USING SKIN MODELS

After this brief literature review, we report our results obtained on two sets of experimentations using both RHE and SOC models. The first one deals with a skin diffusion and metabolism study of two vitamin E prodrugs on RHE and human SOC models (24), followed by determination of the antioxidant protection on RHE. The second example (41,55) shows the biological response to UV irradiation through cytotoxicity, DNA damage, and apoptosis in both RHE and pig SOC models treated with and without photoprotection.

Skin Absorption, Metabolism, and Antioxidant Efficiency of Vitamin E Prodrugs (24)

The development of prodrug technology is intended to avoid certain undesirable properties of topically applied drugs, such as instability and pro-oxidant effects. Accordingly, cutaneous metabolism could be considered as a critical determinant of the efficacy of these topically applied molecules, especially for the delivery of a pharmacologically active molecule through the skin. A gluco-vitamin E conjugate, δ-tocopherol glucoside (δ-TG) has been synthesized by making use of β-glucocerebrosidase activity, which hydrolyzes amphiphilic β-glucocerebrosides into ceramides. The resulting glycosidic bond cleavage allows the release of active vitamin E into the SC. The skin penetration and metabolism of δ-TG were evaluated in 18-hour studies in both RHE (SkinEthic Laboratories, Nice, France) and human SOC (Biopredic, Rennes, France) models. In addition, the efficacy of delivery of free tocopherol in a 0.5% δ-TG solution was tested by means of an lipid hydroperoxide (LPO) assay prior to solar irradiation (105 mJ/cm^2) on the extracted lipids. All these experiments were assessed in comparison with α-tocopherol acetate (α-TAc), the most common vitamin E prodrug known to be converted in the skin (23,56).

Following an infinite dose prodrug solution applied on RHE, a better diffusion was observed for α-TAc. However, no metabolism was detected with α-TAc. In contrast, 20% and 50% of δ-TG was bioconverted into free tocopherol in both SC and epidermis, respectively. A kinetic study with a 0.05% finite dose of δ-TG solution applied on RHE showed that about 90% of the prodrug was converted at 18 hours. A similar experiment on viable human skin confirmed that no free tocopherol was detected from α-TAc, in spite of the fact that the amount diffused was four times higher than the amount of δ-TG (Table 2). The compartmental distribution study confirmed a delivery of free tocopherol from δ-TG. The highest amount was found in the SC, then in the epidermis and also in the dermis (Table 2). Antioxidant efficacy is obtained only if the prodrug is metabolized into free tocopherol, and this was confirmed by a 70% decrease in LPO concentration after treatment with δ-TG, in contrast to treatment with α-TAc (Fig. 2) or the positive control (irradiated lipids). After topical application, δ-TG had a reservoir effect, associated with gradual delivery of free tocopherol. This experiment enabled us to demonstrate that RHE and human SOC models are complementary. Indeed, RHE can be used to confirm the metabolism and the efficacy of an active ingredient, as a result of its low barrier function, offering better bioavailability of the molecule being tested. Finally, human

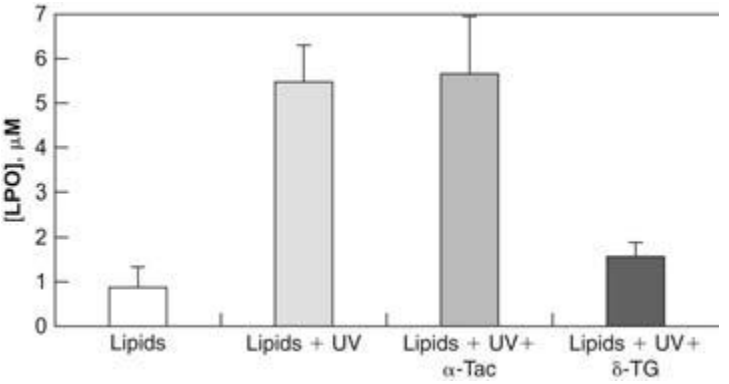

Figure 2 Concentration of lipid hydroperoxide (LPO) in lipids extracted from RHE, after 18-hour topical application of a 0.5% solution of the prodrugs with or without UV irradiation ($n = 4$) showing an antioxidant activity only when the skin model was treated with δ-TG (*last bar*).

Table 2 Compartmental Analysis [Surface, Stratum Corneum (10 Tape Strippings), Epidermis, and Dermis] of Two Prodrugs (δ-Tocopherol-Glucoside and α-Tocopherol Acetate), Their Conversion into Free Tocopherol, and Percentage of Metabolism After 18 Hours' Incubation in Viable Human Skin [Mean (S.E.)], ($n = 6$)

Analyte	Surface in μg/cm^2	Stratum corneum in μg/cm^2	Epidermis in μg/cm^2	Dermis in μg/cm^2
α-TAc	0.17 (0.04)	2.98 (0.18)	0.80 (0.27)	0.79 (0.45)
α-Toc	Not detected	Not detected	Not detected	Not detected
% of metabolite	/	/	/	/
δ-TG	3.44 (0.45)	0.78 (0.27)	0.35 (0.24)	0.16 (0.09)
δ-Toc	0.04 (0.04)	0.12 (0.07)	0.10 (0.04)	0.02 (0.02)
% of metabolite	1.1	15.3	29.2	13.1

SOC on the other hand, having a more efficient barrier function, can be used either as preclinical model or in the optimization of an active ingredient concentration to be included in the formulation.

Deleterious Effects of Solar Radiation and Sunscreen Efficacy Testing on Both RHE and Pig SOC (41,55)

Solar radiations are a major concern for human health since they have been recognized as an environmental human carcinogen, and they also contribute to the photoaging process (57–59). Today, sunfilters provide a good protection against the harmful effects of UVA and UVB radiations. However, the ban of animal testing in cosmetic industry urges to develop new alternative models to discover innovative strategies of skin photoprotection and to assess the photoprotective capacities of a sunblock formulation. In this context, we recently developed a short-term ex vivo skin organ culture model from domestic pig ears (Fig. 3) and also investigated solar-induced cytotoxicity and photoprotection. Furthermore, the results were compared to data obtained with in vitro RHE (SkinEthic Laboratories, Nice, France).

Skin samples were irradiated by using a suntest apparatus and received a single solar-simulated radiation (SSR) dose up to 275 kJ m^{-2}. This acute UV dose corresponds to about fivefold minimal erythemal doses (MEDs). The sunburn cells (SBCs) corresponding to apoptotic KCs following UVB exposure were easily detected by hematoxylin staining 24 hours postirradiation in pig skin and RHE models. These dying cells were mainly located in the basal layers of the epidermis. Furthermore, the deleterious effects of SSR were associated with the formation of thymine dimers (Fig. 4) and DNA fragmentation (Fig. 5) in both the epidermal and dermal compartments in both tissue models, as respectively assayed by immunohistochemistry and TUNEL technique (TdT-mediated dUTP nick-end labeling). None of these

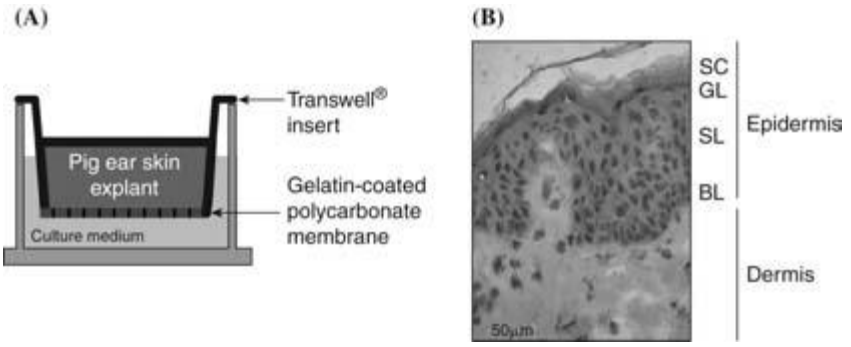

Figure 3 Pig ear SOC. (**A**) Schematic representation of the organ culture system. Skin was excised from domestic pig ears, sectioned at 500-μm thickness, and punched into 12-mm diameter discs. The explants were then seeded in gelatin-coated polycarbonate Transwell® inserts in 12-well plate prefilled with culture medium. (**B**) The multilayered structure of the porcine epidermis. Hematoxylin staining. *Abbreviations*: SC, stratum corneum; GL, granular layer; SL, spinous layer; BL, basal layer.

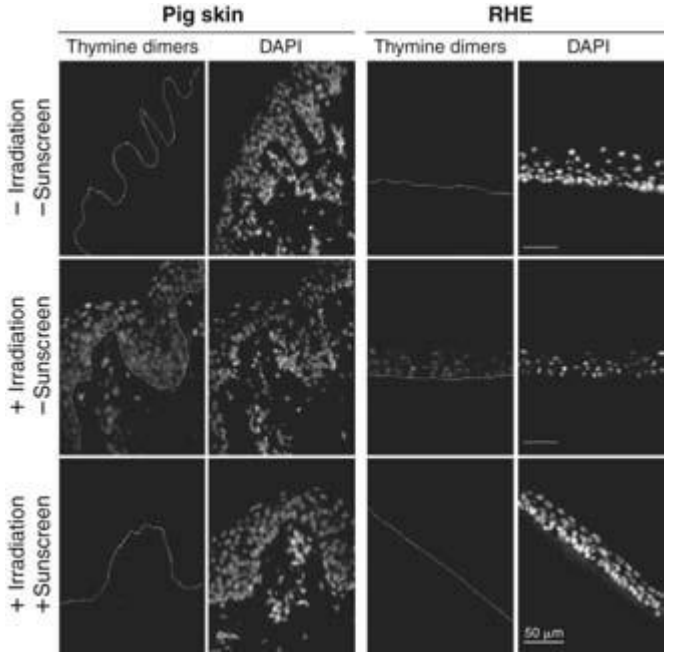

Figure 4 Thymine dimer formation after solar irradiation in pig skin and RHE. Skin was pretreated with or without sunscreen, and then exposed to solar-simulated radiation. Thymine dimers were identified by immunofluorescence 24 hours postirradiation. Note that topical application of sunscreen prevented the formation of DNA lesions in keratinocyte nuclei stained with a DNA fluorescent staining (DAPI). Dashed lines correspond to the dermal-epidermal junction and filter surface in pig skin and RHE, respectively. *Abbreviation*: RHE, reconstructed human epidermis.

cellular responses was observed in nonirradiated skin. The DNA damage was clearly correlated to SSR-induced cytotoxicity since a significant level of LDH activity and extracellular signal-related kinase 2 (ERK2) protein were recovered in the culture supernatant from the irradiated skin models (Fig. 6). The SSR-induced apoptosis involved the upregulation of the p53 tumor suppressor and the activation of the caspase-3 protease. Interestingly, caspase-3 activation was detected mainly in the basal epidermis after irradiation in pig skin organ culture. Thus, basal KCs might be more sensitive to UV exposure than suprabasal KCs and dermal fibroblasts.

To explore whether pig SOC and RHE models are suitable for investigating photoprotection, a broad-spectrum UVB+A sunscreen formulation, was topically applied on skin samples at a dose of 2 mg cm^{-2}. The experiments showed that the sunfilter provides good photoprotection without affecting skin viability in both tissue models. Indeed, sunscreen application completely abrogated not only SBCs and DNA damage formation (Figs. 4 and 5) but also the LDH/ERK2 leakage and caspase-3 activation in irradiated models. These data are

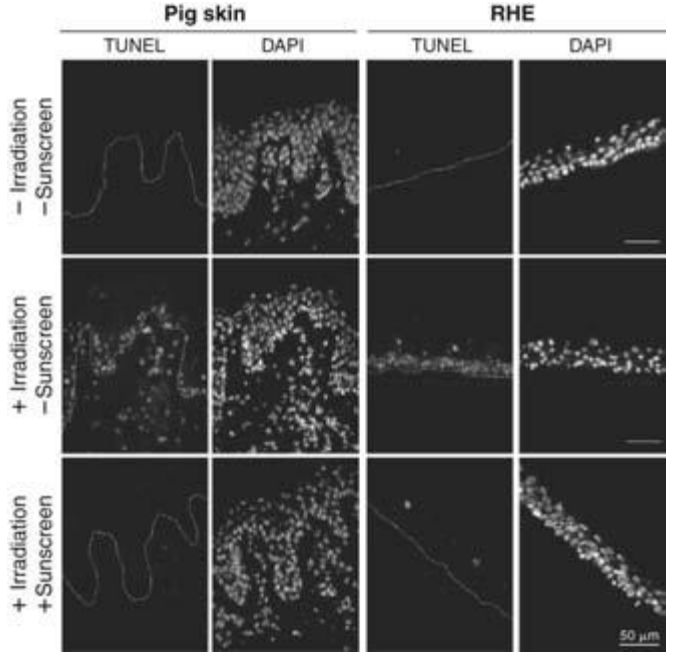

Figure 5 DNA strand breaks after solar irradiation in pig skin and RHE. Skin was pretreated with or without sunscreen, and then exposed to solar-simulated radiation. DNA strand breaks were identified 24 hours postirradiation by TUNEL reaction using fluorescein-dUTP. Note that topical application of sunscreen prevented the formation of DNA lesions in both dermal fibroblast and keratinocyte nuclei stained with DAPI. Dashed lines correspond to the dermal-epidermal junction and filter surface in pig skin and RHE, respectively. *Abbreviations*: TUNEL, dT-mediated dUTP nick-end labeling; RHE, reconstructed human epidermis.

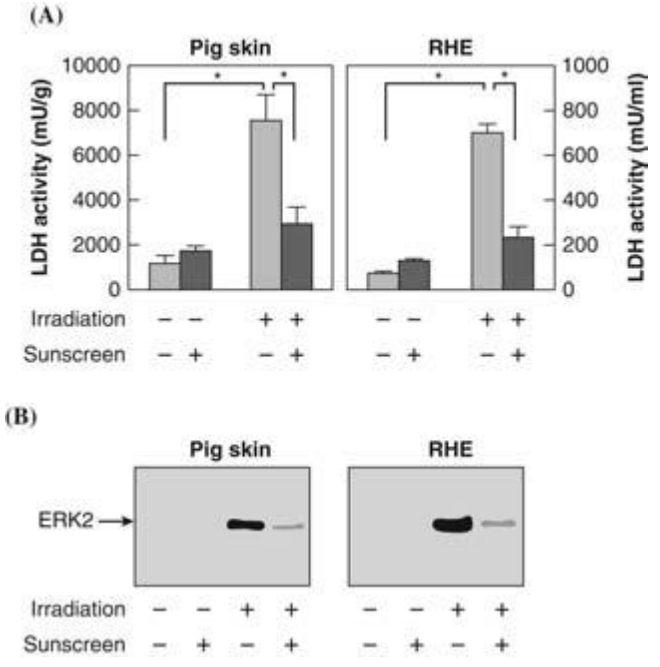

Figure 6 Cytotoxicity after solar irradiation in pig skin and RHE. Skin was pretreated with or without sunscreen, and then exposed to solar-simulated radiation. Culture media were harvested 24 hours later and tested for the presence of cytosolic proteins released from the explants. (**A**) LDH activity was measured by colorimetry using tetrazolium dye (*mean* $\pm$ *SD*, $n = 3$, $^*p < 0.01$). (**B**) ERK2 expression was analyzed by immunoblotting. Note that topical application of sunscreen reduced the leakage of both LDH and ERK2 from UV-irradiated pig skin and RHE. *Abbreviations*: RHE, reconstructed human epidermis; LDH, lactate dehydrogenase.

in agreement with previous results obtained in various skin models (31,32,39,40) and in clinical studies with sunscreen-treated volunteers (60–62).

Altogether, our results indicate that both pig SOC and RHE models are good surrogates to human tissue and that these 3-D alternative models are relevant tools to better understand SSR-induced phototoxicity and to evaluate sunscreen efficacy against UVB and UVA damage.

GENERAL CONCLUSION

A substantial amount of effort is being put into the development and validation of skin models, including reconstructed skin equivalents and skin organ culture models. Even if most of these skin equivalents are not fully validated, they are already frequently being used to prove cosmetic product effectiveness, and hence they represent a genuine added value for the cosmetics industry. The increasing availability of complex reconstructed models offer a wide range of possibilities for use in the efficacy evaluation of cosmetic ingredients, in spite of some limitations such as low barrier function. In addition, these should be provided preferentially by multiple and independent skin model suppliers as a guarantee of their availability to the cosmetic industry. The skin organ culture models are less expensive than the in vitro reconstructed models, are easy to obtain, and possess good barrier function. Accordingly, the combination of RHE and SOC models currently provides a very useful and complementary test system and represents an essential step in the pharmaco-toxicological trials needed in the development of cosmetic products, from screening on cellular models to final validation in clinical trials.

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