18 Effects of Natural Moisturizing Factor and Lactic Acid Isomers on Skin Function

Clive R. Harding and Anthony V. Rawlings

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18.1 INTRODUCTION

Dry, flaky skin remains one of the most common and vexing of human disorders. Although there is no unambiguous definition of this dermatosis, it is characterized by a rough, scaly, and flaky skin surface that often becomes fissured, particularly during the winter months of the year. The observation that low moisture content is a prime factor precipitating the condition was made by Irwin Blank over 50 years ago,[1] and in many respects these pioneering studies heralded the dawn of moisturization research. Since that time many researchers have investigated the complex process of stratum corneum (SC) maturation in both normal and dry skin and have begun to unravel the biological and physical implications of SC moisturization.

In order to maintain water effectively within the skin the epidermis undergoes a process of maturation or terminal differentiation to produce a thin, metabolically inert, barrier, the SC. This heterogeneous structure has been likened to a brick wall in which the anucleated nonviable cells, termed corneocytes are represented as bricks embedded in a continuous matrix of specialized intercellular lipids (mortar).[2] Each individual corneocyte can be viewed simplistically as a highly insoluble

protein complex, consisting primarily of a keratin macrofibrillar matrix, stabilized through inter- and intra-keratin chain disulfide bonds, and encapsulated within a protein shell called the cornified cell envelope (CE). This latter structure is composed of a number of specialized proteins[3] which are extensively cross-linked through the action of at least two members of the transglutaminase family.[4] Given that elements of the internal keratin matrix are also linked to the interior aspect of the cornified envelope (through both disulfide linkages and again by the action of transglutaminase[5]), each corneocyte can be likened to a single, intricately cross-linked "macro-protein." This extensive protein interaction imparts great strength and insolubility to the corneocyte, an essential feature for the "brick" component of this structure. The overall integrity of the SC itself is achieved primarily through specialized intercellular protein structures called corneodesmosomes[6,7] that effectively rivet the corneocytes together, but which ultimately must be degraded to facilitate desquamation.

The visual appearance of dry skin is now generally accepted to be the consequence of the altered scattering and reflection of light off the rough skin surface resulting from abnormal desquamation. This perturbation to the ultimate step of terminal differentiation emphasizes a critical and often overlooked role of water in the SC, namely, its importance for the activity of a variety of hydrolytic enzymes involved in various aspects of SC maturation and desquamation.[8–11] When the tissue becomes desiccated a loss of overall hydrolytic enzyme activity affects many biochemical processes within the SC. The most widely appreciated symptom of this enzymatic failure is the visible scaling associated with ineffective corneodesmosomal degradation.[12,13] However altered activity of several other enzymes including transglutaminase[14] and lipases[15] can contribute to the formation of dry skin.

Therefore in order to maintain its flexibility, integrity, and critical catabolic activity the SC must remain hydrated, and in healthy skin the tissue contains greater than 10% water.[1,16] In the absence of water the SC is an intrinsically fragile structure, which readily becomes cracked, brittle, and rigid. The maintenance of water balance in the SC is therefore vital to this tissue and is preserved through three major biophysical mechanisms. The first of these is the intercellular lamellar lipids that provide a very effective barrier to the passage of water through the tissue.[17,18] The second mechanism is provided by the proteinaceous corneocytes themselves that also play an important role in contributing to the water barrier.[19] Given that there is only a gradual age-related decline in lipid levels within the SC it is believed that the dramatic increase in corneocyte size plays an important role in keeping water loss (as measured by transepidermal water loss [TEWL]) at a comparable level in young and old skin.[20] The final mechanism is provided by the natural moisturizing factor (NMF), a complex mixture of low molecular weight, water-soluble compound, which is present within the corneocytes.[21] Collectively, the NMF components have the ability to bind water against the desiccating action of the environment and thereby maintain tissue hydration. Historically we have thought of the NMF as an exclusively intracellular component although clearly the consequences of corneodesmosomal lysis and the processing of glycosylated ceramides within the SC invoke the potential presence of intercellular humectants as well.

Usually these three biophysical mechanisms interact precisely to provide a highly efficient barrier against water loss and retain water within the tissue to maintain flexibility and catabolic activity. Nevertheless, this barrier is continually prone to perturbation by both external forces (UV, low RH, cold temperatures, and surfactants), and internal factors (cutaneous disease, psychological stress, and diabetic complications). With decreased performance of the water barrier the increased loss of water from the tissue ultimately leads to the formation of dry skin.

For a proper appreciation of the underlying biochemistry of dry skin we should consider this common condition as a dysfunction of one or more of the vital processes that generate and protect the water-holding capacity of the SC. With this concept in mind, in this chapter we will focus initially on the generation and critical importance of the NMF to SC function. Second, we will consider the effects of topically applied NMF components, and in particular the effects of lactic acid and its isomers, on the alleviation of dry skin symptoms. Finally, we will consider briefly the technologies that can influence NMF generation through stimulation of the synthesis of the NMF-precursor molecules.

TABLE 18.1
The Chemical Composition of NMF

	%
Free amino acids and urocanic acid	40.0
Pyrrolidone carboxylic acid	12.0
Lactate	12.0
Sugars, organic acids, peptides, unidentified materials	8.5
Urea	7.0
Chloride	6.0
Sodium	5.0
Potassium	4.0
Ammonia, uric acid, glucosamine, creatine	1.5
Calcium	1.5
Magnesium	1.5
Phosphate	0.5
Citrate, formate	0.5
Glycerol	ND
Hyaluronic acid	ND

ND. Not determined in this analysis but detected in stratum corneum.

18.2 NATURAL MOISTURIZING FACTOR

18.2.1 THE ROLE OF THE NMF IN THE STRATUM CORNEUM

The NMF consists primarily of amino acids or their derivatives such as pyrrolidone carboxylic acid (PCA) and urocanic acid (UCA) together with lactic acid, urea, citrate, and sugars[22] (Table 18.1). These compounds are collectively present at high concentrations within the cell and may represent 20 to 30% of the dry weight of the SC.[23] The importance of the NMF lies in the fact that the constituent chemicals, particularly the PCA and lactic acid salts, are intensely hygroscopic. These salts absorb atmospheric water and dissolve in their own water of hydration, thereby acting as very efficient humectants. In essence, the amount of NMF in the SC determines how much water it can hold for any given relative humidity (RH). In the absence of NMF the SC can only absorb significant amounts of water at 100% humidity, a situation that seldom occurs. It is important to remember that the highly structured intercellular lipid lamellae provide a barrier to reduce the highly water-soluble NMF from leaching out of the surface layers of the skin.[24]

Although the properties of several of the individual components of the NMF have been studied extensively, our understanding of the contribution of individual components and their synergistic behavior to the overall properties of the SC remains relatively poor. Recently, the potent water binding molecule hyaluronic acid has been shown to be naturally present in the SC[25], and the importance of glycerol, present at low concentrations, has been emphasized by the elegant studies of Verkman and coworkers.[26] This group has shown that there is a specific transporter of glycerol in the epidermis[27] and the loss of this protein is associated with major perturbations in SC water retention and mechanical properties.[26] Glycerol is also derived from sebaceous triglyceride breakdown, and again to emphasize the importance of this molecule studies by Fluhr and colleagues have indicated that topically applied glycerol can completely restore the poor quality of SC observed in asebic mice (no sebaceous secretions) to normal.[28] The identification of glycerol and hyaluronic acid in the SC is relatively recent, and in any classical consideration of NMF composition and function these two molecules have been ignored, and moisturization research has focused on four major

intrinsic components: lactate, free amino acids (FAA), PCA, and urea. Fox et al.[29] investigating the humectant capabilities of sodium lactate, demonstrated a 60% increase in water content at 60% RH, whereas, in contrast under the same conditions, glycerol only provided a 38% increase. Laden and Spitzer,[30] after studying the composition of NMF, concluded that since amino acids themselves are relatively nonhygroscopic at skin pH, PCA itself must contribute significantly to the SC water binding capacity. Although it has been demonstrated that sodium lactate is slightly more hygroscopic than sodium PCA at 50% RH,[31,32] both of these salts contribute significantly to the hygroscopicity of the SC. Biologically, this property allows the outermost layers of the SC to maintain liquid water against the desiccating action of the environment.

Traditionally, it was believed that this liquid water plasticized the SC, keeping it resilient by preventing cracking and flaking which might occur due to mechanical stresses. However, under conditions of reduced RH, when water can only provide a transient effect, topically applied lactic acid achieves a long-term plasticization of the SC. Similarly, while developing a skin cream designed to reduce dry and flaky skin, Middleton, (through measuring changes in SC extensibility and water-holding capacity) showed that at around 80% RH sodium lactate and sodium PCA were as effective as other moisturizing agents. Although their benefits were essentially lost on rinsing the SC with water,[33] lactic acid-treated skin retained some residual plasticization benefit. Recent data indicates that lactate plays a critical role in influencing the physical properties of the SC. Lactate and potassium were found to be the only components of the NMF analyzed (although PCA was not analyzed) that correlated significantly with the state of hydration, stiffness and pH in the SC.[34]

Urea, another principle component of the NMF, has also been demonstrated to have similar effects,[35] although no direct comparison with either PCA or lactic acid has been reported.

The general mechanisms by which the NMF components influence SC functionality have been studied extensively. From a physical chemistry perspective the specific ionic interaction between keratin and NMF, accompanied by a decreased mobility of water, leads to a reduction of intermolecular forces between the keratin fibers and increased elastic behavior. Recent studies have emphasized that it is the neutral and basic FAA[36] in particular that are important for helping keratin acquire and maintain its elastic properties. Consistent with these observations Sakai et al.[37] reported that the ratio of acidic amino acids to total amino acids correlated to the resonant frequency a measure of skin stiffness.

These observations clearly emphasize how the NMF is critical for maintaining physical properties of the SC. However, as our understanding of the terminal differentiation and SC maturation process has increased, it has become clear that by maintaining free water in the SC, the NMF also facilitates critical biochemical events. As indicated earlier the coordinated activity of specific proteases and lipases is essential for optimum SC function, and these hydrolytic processes can only function in the presence of water that is effectively maintained by the water-retaining capacity of the NMF. Perhaps the most striking example of this is the regulation of a number of intracellular proteases within the corneocyte that, as we discuss in the next section, are ultimately responsible for the generation of the major elements of the NMF itself.

The generation and maintenance of an acid pH within the SC, the so-called "acid mantle" is critical to the correct functioning of this tissue and there is evidence of a pH gradient within the tissue.[38] Studies from Elias and coworkers point to an essential role of free fatty acids generated through phospholipase activity as being vital for SC acidification,[39,40] whilst Krein and Kermici[41] have recently proposed that UCA plays a vital role in the regulation of SC pH. However, studies on the histidase-deficient mouse (which cannot generate UCA from free histidine), indicate that SC pH in these animals is within the normal range, and this observation rather argues against the importance of UCA.[42] Nevertheless it is likely that other NMF components contribute significantly to the overall maintenance of pH. Collectively the NMF and free fatty acids (derived from phospholipid, ceramide, and sebum breakdown) contribute toward a physiologically important and gradual acidification of the SC toward the skin surface. Although a detailed consideration of the influence of pH on many enzymatic activities within the SC is beyond the scope of this chapter, there is a growing realization

that pH directly regulates barrier formation and homeostasis. Alterations of pH away from its acidic norm of 4.5 to 6.5 is associated with loss of SC integrity and cohesion. This perturbation is due in part to the inappropriate activation and activity of serine proteases involved with desquamation.[43]

18.2.2 THE ORIGIN OF THE SKIN'S NMF

The precise origin of the lactic acid and urea components of the NMF remains ill defined. They may be derived from the general breakdown of proteins and amino acids (e.g. following arginase activity on arginine). It has also been proposed that urea, like lactate may also be derived in part from sweat.[44] The presence of sugars in the SC represents primarily the activity of the enzyme ∃-D-glucocerebrosidase as it catalyzes the removal of glucose from glucosylceramides to initiate lipid lamellae organization in the deep stratum corneum.[15] In addition the degradation of corneodesmosomes will also release sugars from these glycosylated proteins.[45] Hyaluronic acid, is known to be synthesized in the epidermis by the hyaluron synthase family of enzymes, at least one of which is synthesized by keratinocytes.[46] This glycosaminoglycan may indeed be responsible for the Alcian blue staining reported in the SC by the team led by Voorhees.[47] Finally, staining of isolated corneocytes and CE with a range of fluorescently labeled lectins has revealed the presence of N-acetylglucosamine.[48] The persistence of lectin staining following the harsh isolation procedures required for CE evaluation suggests that these sugars are covalently attached, but they may subsequently be released by ∃-D-glucosaminidase known to be present in the tissue.[48]

Historically a major focus of interest has been the origin of the FAA and their derivatives within the SC, which together represent over 50% of the NMF. Studies conducted by Scott and Harding during the early 1980s[49–52] lead to the conclusion that all of the amino acid components of the NMF were derived specifically from a single, high molecular weight, histidine-rich protein, which represented the major component of the F type keratohyalin granules (KHG).[53] Based upon the ability to these histidine-rich proteins to aggregate keratin fibers *in vitro* into macro-structures reminiscent of the keratin pattern seen in the SC *in vivo*, Dale and coworkers named this class of basic proteins filaggrins,[54] and the phosphorylated precursor protein subsequently became known as profilaggrin.

Although studies by other groups have confirmed that filaggrin is a major source of intercellular FAA,[55] it is probably incorrect to accord it as the status of being the *only* source of these components in the SC. Studies by Jacobsen and team concluded that the FAA composition of human SC could not be accounted for simply by the known amino acid composition of filaggrin.[56] Based on our understanding of the spectrum of catabolic activities intrinsic to the corneocyte we can now consider at least two sources for the FAA present in the stratum corneum: filaggrin and corneodesmosomes. In addition, SC keratins also undergo a small decrease in molecular weight during SC formation and may make a minor contribution (unpublished observations).

Corneodesmosome hydrolysis initiated deep within the SC may lead to the production of an *intercellular* pool of osmotically active solutes. Warner, in studying the disruptive nature of hydration on human stratum corneum ultrastructure observed the presence of water-filled cisternae in the intercellular space and suggested the site of corneodesmosomal degradation as a focal point for production of NMF.[57] The precise contribution of intercellular humectancy to SC function remains to be established and caution must be taken with extrapolation of data from these hyperhydration studies. The dramatic size of some of the cisternae observed in these studies lead Warner to suggest that leaching of NMF from within the corneocyte could contribute to the phenomenon. Nevertheless, the presence of water around corneodesmosome has also been reported in studies where the tissue was not subjected to such extremes of hydration.[58] It is of interest to note that Nguyen[59] has also proposed the presence of intercellular (although in this case filaggrin-derived) humectants following observations of keratinocyte behavior *in vitro*.

Recently, a protein named hornerin has been identified in mouse skin.[60,61] Based on its amino acid sequence and distribution in skin it has been proposed to fulfill a similar role to profilaggrin/filaggrin in murine skin. The conditions under which it is expressed remain to be determined but it is tempting to

speculate that this protein may compensate for the absence of profilaggrin synthesis in the flaky mouse mutant, recently described by Presland.[62] In this mouse model although profilaggrin is synthesized, it is a truncated form that is not proteolytically converted into filaggrin (and hence to FAA) in the SC. Despite this defect the mouse SC recovers from a marked barrier impairment and dry flaky skin after birth to produce a functionally normal SC within three weeks. Based on the importance of profilaggrin synthesis, and the consequences of its failure to be synthesized, it would be anticipated that this gene-deletion would lead to persistent abnormal scaling and dryness. Clearly the mouse can compensate for this deficiency, and although the NMF composition of this mouse model has not been evaluated it is assumed that there is a compensatory degradation of a protein with a profilaggrin-like amino acid composition to derive the appropriate NMF profile of FAA. Alternatively, compensation via increased lipid synthesis and altered cornified envelope composition may occur. There may of course be a far simpler explanation. Normal adult mouse skin has a dramatically reduced filaggrin level compared with its neonatal counterpart, and the need for filaggrin and its derivatives may decline naturally as other mechanisms mature within the skin, and most noticeably the animal grows a coat of fur protecting against moisture loss and UV irradiation.

Interestingly, the human genome project has indicated the presence of a hornerin-like gene close to profilaggrin on chromosome 1q21, but its expression has not been reported or studied in man to date. It remains to be established whether, in otherwise healthy skin, an age-related decline in the ability to synthesize profilaggrin can be compensated for by synthesis of another proteolytically labile protein.

Despite the continued inconsistencies in our understanding of the contribution of nonfilaggrin-derived proteins to intracellular NMF, the synthesis and controlled proteolysis of filaggrin, remains pivotal to our understanding of how the barrier responds to changes in the external environment,[63,64] and how abrupt changes in RH can induce abnormalities in barrier homeostasis.[65]

18.2.3 SYNTHESIS AND DEGRADATION OF PROFILAGGRIN

Studies conducted in our laboratory indicated that profilaggrin was rapidly dephosphorylated during the transition of the mature granular cell into the corneocyte and then underwent selective proteolysis to form lower molecular weight, highly basic species within the SC.[50]

However, regardless of the putative structural function proposed for this family of proteins within the SC, by Dale in a landmark paper[52] it was clear that keratin-aggregation, was at best, a transient role restricted to the early formation of the SC. Radiolabel pulse chase,[49] immunohistochemical,[66] and biochemical studies[50] confirmed that filaggrin, with the exception of a minor incorporation into the cornified CE,[4,67] and occasional persistence due to altered processing[68] does not persist beyond the deepest two or three layers of the SC (Figure 18.1). First, it becomes extensively deiminated through the activity of the enzyme peptidyl deiminase (PAD),[52] which serves to reduce the affinity of the filaggrin/keratin complex. Second, it is rapidly and completely degraded through small peptides to FAA. Finally, specific constituent amino acids are catabolized further to form specialized components of the NMF.

Foremost among these catabolites are PCA itself (derived primarily by the nonenzymatic cyclization of glutamine[49]), and UCA, a natural UV-absorber[69] formed by the action of the enzyme histidase on histidine[70] (filaggrin catabolism summarized in Figure 18.2).

18.2.4 CONTROL OF FILAGGRIN HYDROLYSIS

Although the precise nature of the protease system (filaggrinases) catalyzing filaggrin breakdown remains to be identified, they are primarily serine proteases. The actual "trigger" that initializes the proteolysis at a discreet but variable location within the SC is the water activity gradient present across the tissue. The discovery of this mechanism was elucidated following careful observation of changes in filaggrin distribution during SC maturation of fetal and newborn skin.[63] In normal adult
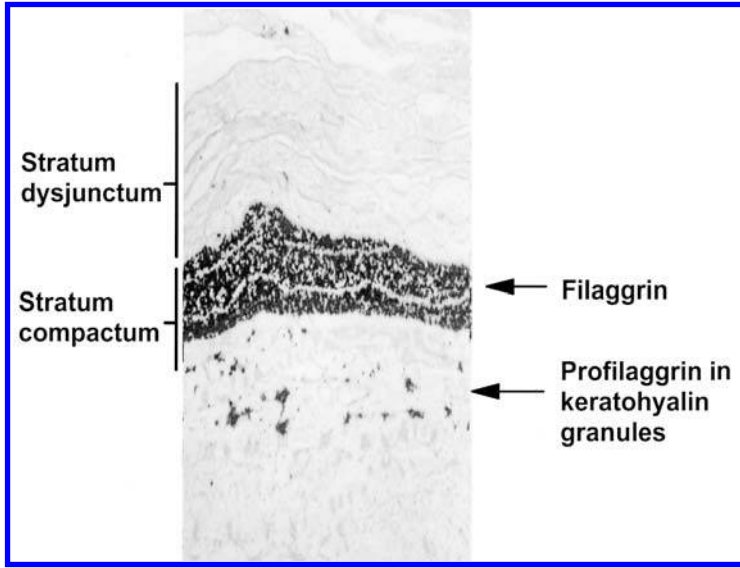
FIGURE 18.1 Distribution of filaggrin in human stratum corneum. Immunoelectron micrograph of human facial skin (9-year-old male). Ultrathin sections were incubated with rabbit-antihuman filaggrin followed by incubation with goat-antirabbit/colloidal gold (5 nm diameter).
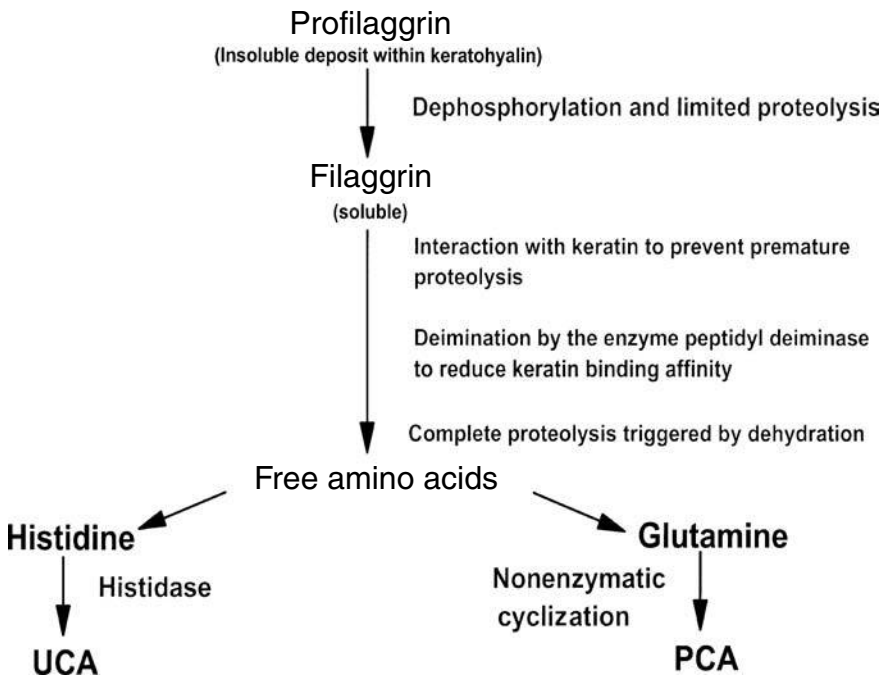

FIGURE 18.2 Schematic representation of profilaggrin catabolism during terminal differentiation.

skin filaggrin is only detected in the innermost layers of the SC (Figure 18.1), whereas in newborn and fetal tissue there is no indication of any proteolytic breakdown of filaggrin in the outer regions. However, within a few hours of birth, the breakdown of filaggrin is initiated in these regions. This triggering could be prevented in a very humid environment, which indicates the possibility that

the water content of the SC is a critical factor. Subsequent studies on filaggrin breakdown in isolated SC revealed that hydrolysis only occurred if the SC was maintained within a certain RH range (70 to 95%). Similarly, if the skin was occluded for a long period[68] filaggrin hydrolysis was blocked, the corneocytes remained filled with the protein, and the NMF level of the SC fell close to zero.

It is now appreciated that the water activity gradient within the SC and the water flux through this tissue at rest and following damage, are intimately involved in several aspects of tissue homeostasis, notably in relation to water barrier repair.[71] However, the observations on the control of filaggrin catabolism, originally made over 25 years ago, represent some of the earliest studies to demonstrate and emphasize the dynamic nature of SC maturation.

At first sight the process by which the skin generates the NMF within the SC seems absurdly complex. However, the logic of Nature's complexity becomes apparent once it is appreciated that the epidermis cannot afford to generate NMF, either within the viable layers or within the newly formed immature corneocyte itself, due to the risk of osmotic damage. It is imperative that the activation of the filaggrin protease systems is delayed until the corneocyte has flattened, strengthened and moved far enough out into the dryer areas of the SC to be able to withstand the osmotic effects of the concentrated NMF pool. The epidermis circumvents the potentially harmful effects of osmotic pressure resulting from the inappropriate premature hydrolysis of filaggrin through two strategies. First, profilaggrin, once synthesized, is precipitated within KHG where it acts as an insoluble and, most importantly, an osmotically inactive repository of the NMF. Second, the interaction between keratin and filaggrin forms a proteolytically resistant complex, which prevents premature proteolysis of the filaggrin (an intrinsically labile protein containing 10 to 15 mol% arginine residues[49]) during the intensely hydrolytic processes, which accompany SC formation.

In summary, these mechanisms are part of an elegant, self-adjusting moisturization process within the SC that allows it to respond to different climatic conditions. This mechanism ensures that it is only as filaggrin containing corneocytes migrate upward from the deepest layers and begin to dry out (and the water activity within the cell decreases) that certain proteases, by a poorly understood mechanism, are activated and the NMF is produced. The point at which this hydrolysis is initiated is independent of the age of the corneocyte[66] and is dictated ultimately by the environmental humidity. When the weather is humid the proteolysis occurs almost at the outer surface. In conditions of extreme low humidity the proteolysis is initiated deep within the tissue so that all but the innermost layers contain the NMF required to prevent desiccation. An appreciation of filaggrin form, function and fate helps to understand the water distribution, altered morphology and swelling properties of isolated SC maintained at differing hydration levels,[72] and offers an explanation for the transient reduced water-holding capacity of the SC of newborn infants.[73]

Immunocytochemical and stereological studies indicate that corneodesmosome hydrolysis is also initiated in approximately the same layer of the SC as where filaggrin is rapidly hydrolyzed. These dramatic changes in protein distribution account for the differential staining properties of the stratum dysjunctum/compactum (unpublished observations) and suggest that the enzymatic activation of the two classes of proteases (one *intra*cellularly based for filaggrin hydrolysis and one *inter*cellularly based for corneodesmosome digestion) are carefully coordinated.

18.2.5 NMF LEVELS AND DRY SKIN CONDITIONS

The failure to either make or process (pro)filaggrin is a major problem for the skin and is associated with various dermatological disorders. The symptoms of ichthyosis vulgaris[74] are closely associated with an inability or failure to make profilaggrin. The absence of KHG histologically has been known for many years, and the NMF content of corneum in ichthyosis vulgaris patients is close to zero. Likewise, in psoriatics there is again a paucity of KHG and the associated SC is essentially NMF deficient.[75] Recently, it has been proposed that the very presence of KHG, revered for over a century as the defining characteristic of the granular layer, are in fact a histological artifact.[76] If this is indeed the case then it is likely that the unusual dual acidic and basic characteristics of

the highly phosphorylated profilaggrin are responsible for the putative aggregation artifact leading to the formation of an insoluble precipitation (which we call KHG) during histological processing. The unusual properties of isolated profilaggrin (then known as the histidine-rich protein) were first noted by Ugel in 1970.[77]

It is noteworthy that research continues to hint at additional roles for profilaggrin within the granular cell to corneocyte transition. An inability to dephosphorylate profilaggrin, following the deletion of the serine-protease Matriptase is associated with dramatic impairment of many stratum corneum events, and has lead to the suggestion that profilaggrin dephosphorylation is *the* pivotal event initiating terminal differentiation.[78] Such a critical role is supported by the unusual metabolism of profilaggrin observed in oral epithelium, particularly in the case of the hard palate, where an inability to process profilaggrin leads to an altered keratin pattern and a highly keratinised tissue.[79] Just as the physical properties intrinsic to this protein may represent the driving force for KHG formation (by artifact or by design), then the dramatic changes to these properties once profilaggrin is desphosphorylated (and cleaved) may initiate a cascade of events in the rapidly changing late granular cell. Although it is clear that in all these conditions several aspects of keratinization are impaired, the inability to produce or retain NMF within the SC appears to be a significant factor contributing to the overall manifestation of the skin problem.

Reduced NMF levels are also implicated in the more common dry skin conditions. Subjects with atopic dermatitis have decreased levels of NMF,[80] and FAA levels have been reported to decrease significantly in dry, scaly skin induced experimentally by repetitive tape stripping.[81] Additionally, a significant correlation exists between SC hydration state and the FAA content of elderly individuals with skin xerosis.[82]

Traditionally, components of the NMF are measured following extraction of corneocytes recovered from superficial tape-strippings, or from direct extraction of the skin surface by attaching open-ended chambers to the skin and eluting with small volumes of aqueous buffers or dilute surfactant solutions. By analysing sequential tape strips recovered from the same site profiles of how NMF levels change with depth can be constructed. These profiles indicate that the levels of NMF decline markedly toward the surface of the skin. This is typical of normal skin exposed to routine soap washing where much of the readily soluble NMF is washed out from the superficial SC.[83]

Individual NMF species can be measured by High Performance Liquid Chromatography (PCA and UCA), colorimetric assays (FAA) or by enzymatic assays (lactate and glycerol).

Most recently Puppels and co-workers to determine the concentration of defined NMF component non-invasively *in vivo* in the SC have pioneered the use of confocal Raman microscopy.[84] Figure 18.3 shows depth profiles for the major filaggrin derived components, urea and lactate obtained using this technique. Evidence of leaching from the skin surface is characteristically seen in most profiles and the precipitous drop off in levels of filaggrin derived components deeper in the SC indicates the boundary at which filaggrin hydrolysis is rapidly initiated.

Our own studies have suggested that there is a significant age-related decline in the level of certain NMF components, most noticeably PCA (unpublished studies). The decline in PCA production probably reflects the cumulative effects of actinic damage as it was observed in SC recovered from the back of the hand (photodamaged) of elderly individuals, but not from the inner aspect of the biceps (photoprotected) in the same population. Taken together with electron microscopy studies that report decreased numbers of KHG in senile xerosis,[85] these results suggest that the intrinsically lower NMF levels present in aged skin, compared with young skin, reflect a general reduced synthesis of profilaggrin. In addition, it is likely that in aged skin the loss of NMF becomes more pronounced as elderly individuals also show an age-related decline in water barrier repair.[86]

However, a recent publication from Takashashi and Tezuka has suggested that the content of FAA in the SC is actually *increased* in both senile xerosis and in "normal" aged skin compared to young.[87] Indeed these observations are consistent with earlier observations on the age-related increase in the levels of certain FAA primarily found in filaggrin (serine, glutamic acid, glycine) made by Jacobsen.[56]
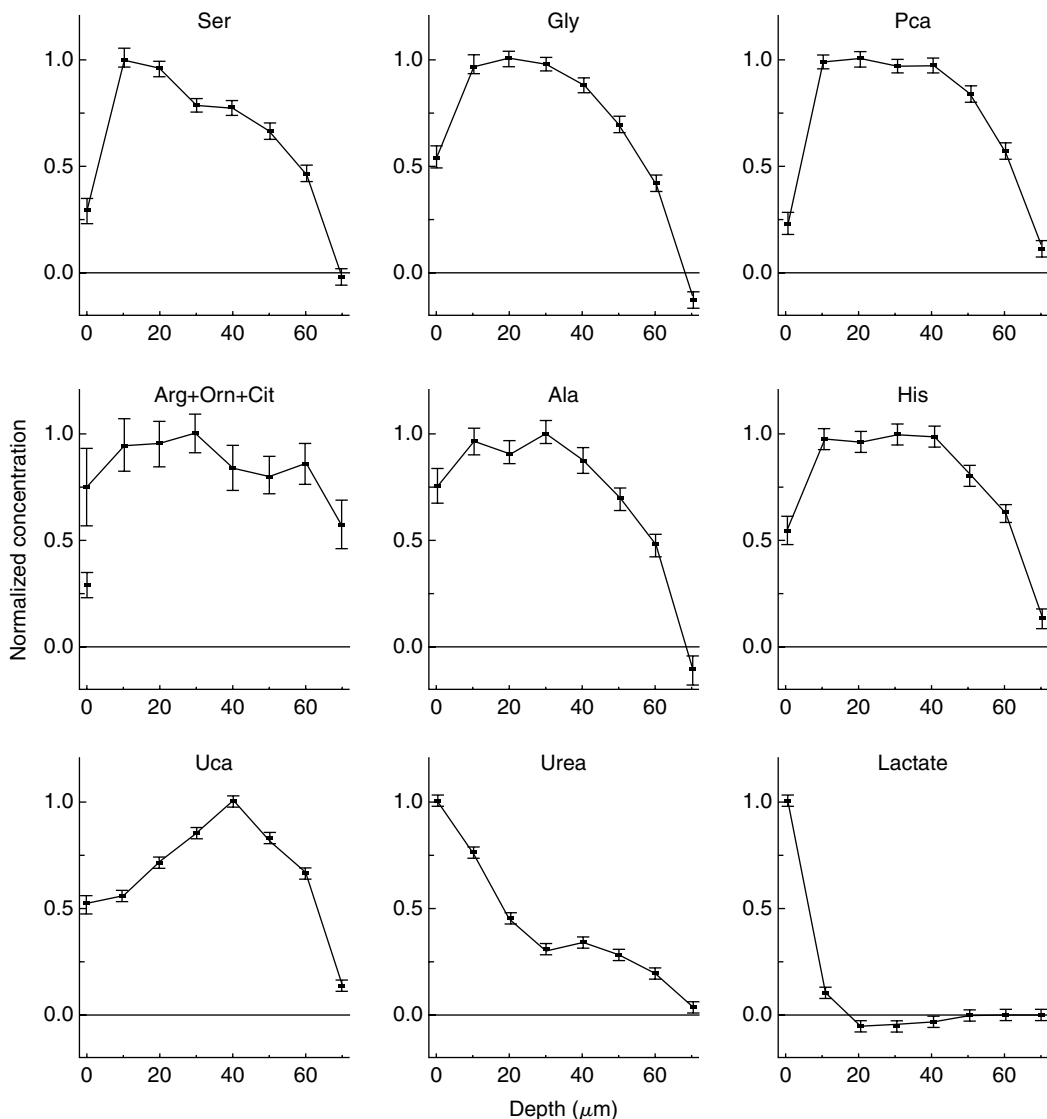
FIGURE 18.3 Semiquantitative *in vivo* concentration profiles of NMF and sweat constituents in the stratum corneum of the thenar as determined by Raman spectroscopy (From Caspers, P.J., Lucassen, G.W., Carter, E.A., Bruining, H.A., and Puppels, G.J. l. *J. Invest. Dermatol.*, 116, 434–442, 2001).

Given that profilaggrin synthesis has been shown clearly to undergo a significant age-related decline,[85,87] on several body-sites (although reportedly not the face[88]) then as discussed earlier, the conclusion must be that other sources of protein are contributing to the overall FAA pool.

Further speculation however is unwarranted at this time as it is likely that choice of body site, the nature of induction of xerosis (natural versus surfactant/solvent induced), differing methods of RT–PCR and filaggrin extraction and quantification protocols may all contribute to the current lack of clarity in our understanding.

Studies in UV and hexadecane damaged skin indicate that the endo- and exo-proteases (filaggrinases) responsible for filaggrin degradation are extremely robust enzymes, effectively degrading all filaggrin present in the SC during and immediately after an acute insult.[66] Nevertheless,
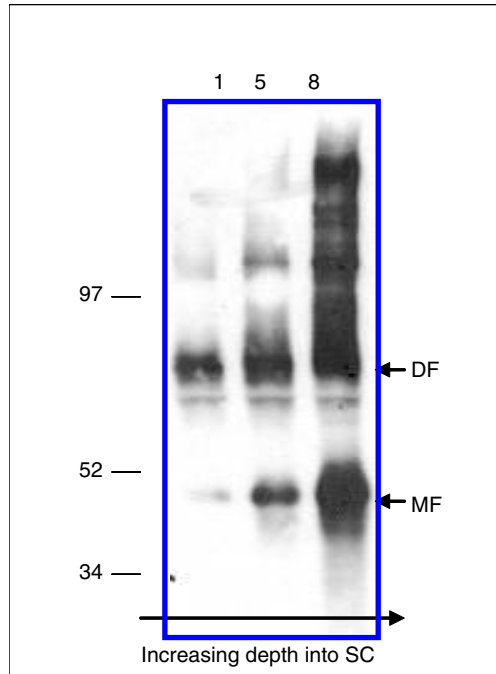
FIGURE 18.4 Persistence of filaggrin-related material in superficial SC. Samples of SC collected by consecutive cyanoacrylate stripping of human forearm skin, extracted, analyzed by PAG electrophoresis, and probed with antifilaggrin antibody following Western blotting (strippings 1, 5, and 8 shown). Mature filaggrin (MF) decreases toward skin surface and is absent from most superficial tape-stripped sample. Higher molecular-weight, deiminated, and protease resistant filaggrin variant (DF) persists into most superficial layers. Molecular weight calibration in kDa indicated on left.

we have recently observed that in aged photodamaged skin there is a minor perturbation in filaggrin processing leading to the persistence of a high molecular weight filaggrin-related material in superficial SC (see Figure 18.4). It appears that in some individuals an imbalance in the activity between the enzyme PAD and general filaggrinase activity may lead to the formation of a form of filaggrin in which complete deimination (through continued PAD activity) renders the protein refractive to filaggrinase activity. Essentially the complete conversion of arginine to citrulline residues on the filaggrin proteins removes trypsin-sensitive protease sites on the normally protease-labile protein. As we have previously shown that it is filaggrin degradation rather than filaggrin deimination that is sensitive to changes in external RH,[68] it is likely that frequent changes in environmental humidity may exacerbate dry skin conditions in part by favoring the formation of this "protease-resistant" filaggrin.

Finally, we recently reported that the allelic polymorphism recognized in the profilaggrin gene may be linked to a predisposition toward dry skin.[89] The profilaggrin gene codes for either a 10, 11, or 12 filaggrin-repeat, and therefore an individual can be 10:10, 10:11, 11:11, 10:12, 11:11, or 12:12. Using a PCR-based approach we have determined individual profilaggrin allelotypes and identified an inverse association between the 12 repeat allele and the frequency of self-perceived dry skin ($n = 89$, $p = 0.0237$). This novel observation could not be explained by a simple reduction in NMF production, and provides further circumstantial evidence for profilaggrin itself (rather than filaggrin or NMF) playing a critical role in epidermal differentiation.

Clearly in dry flaky skin conditions where corneodesmosome degradation is frequently and characteristically perturbed then generation of amino acid-derived intercellular humectancy will also be decreased potentially leading to a further reduction in protease activity.

In summary, the various processes leading from profilaggrin synthesis to conversion to filaggrin and then to NMF are under tight control. However, these controls are perturbed in different ways by a range of factors including UV-light, exposure to surfactants, and, of course, rapid changes in environmental humidity. It is generally accepted that these very different causes can all lead to reduced NMF and contribute to the complex phenomenon known as dry skin.

18.3   THE EFFECT OF TOPICALLY APPLIED NMF

Moisturizing ingredients have been used widely in skin care products for the treatment of dry skin for many years. In fact the use of oils for smoothing skin is reported as early as 2300 B.C., although it was not until the work of Blank in the 1950s[1] that research focused on water-imbibing substances to retain moisture in the SC. This section will discuss briefly the effects of PCA, urea, glycerol, and lactic acid on human SC function *in vivo*.

18.3.1   PYRROLIDONE CARBOXYLIC ACID

A considerable amount of work has been performed evaluating the effects of PCA and its salts *in vitro*. However, surprisingly only a limited amount of work has been reported on the influence of PCA topically applied on human skin. In one such study Middleton and Roberts[90] demonstrated that lotions containing PCA were more effective at treating dry skin compared to a placebo lotion.

18.3.2   UREA

Urea is a major component of the NMF, and it has been used in hand creams since the 1940s. This unique physiological substance has proven to be a potent skin humidifier and descaling agent[91] and in high concentrations it has been shown to be an effective treatment for dry skin, being more efficacious than salicylic acid and petroleum jelly.[92] Urea containing moisturizers are also reported to influence barrier properties of the skin, reducing TEWL,[93–96] increasing skin capacitance, and reducing irritant reactions. Corresponding lotions containing glycerol as the humectant had no comparable effect on reducing TEWL. Although the precise mode of action of urea is unknown, the improved barrier function may be related to increased corneocyte size resulting from reduced keratinocyte proliferation. High concentrations of urea have also been reported recently to enhance lipid biosynthesis.[97] Finally, in combination with lactic acid, urea has also been shown to be an effective treatment of ichthyosis[98] and in combination with polidocanol urea is reported to improve juvenile atopic dermatitis.[99]

18.3.3   LACTIC ACID

Lactic acid, as well as being a component of the NMF, is also a member of the class of molecules called alpha hydroxy acids (AHAs), which exert specific and unique benefits on skin structure and function. Although originally described for the treatment of dry skin-related disorders, their pleiotropic properties include influencing skin cell renewal and other antiaging benefits, which have become the focus of considerable interest in recent years.

The first recorded use of lactic acid was in 1943 by Stern who used it for the treatment of ichthyosis,[100] and in the early 1970s and 1980s Middleton[101] and Van Scott and Yu[102,103] demonstrated the efficacy of these short chain AHAs in ameliorating dry skin in moisturization efficacy studies.

Other researchers[104–106] have also shown that racemic mixtures of lactic acid ameliorate the common problem of winter xerosis. Typical effects of lactic acid in moisturization efficacy studies
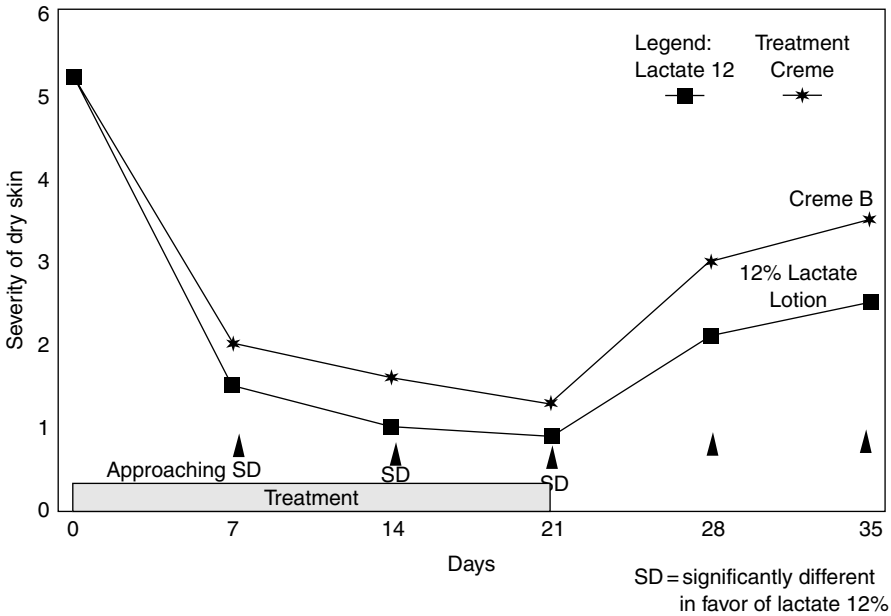
FIGURE 18.5 Improvement in dry skin condition following twice daily applications of a 12% lactic acid formulation (Reprinted by permission of publisher from Wehr, R., Krochmal, L., Bagatell, F., and Ragsdale, W.A. *Cutis*, 23, 205, 1986. Copyright 1999 by Quadrant Healthcom Inc.)

are shown in Figure 18.5. However, as is the case with other humectants, application of lactic acid alone fails to ameliorate the symptoms of dry skin, and coformulation with occlusive agents is required to help retain the humectant bound water within the surface layers of the SC. Typically, we have found that lotions containing barrier lipids (ceramides) and lactic acid provide synergistic relief of dry skin.[107] These results are similar to those found with lotions containing barrier lipids and glycerol[108] and we believe that these lotions then act by increasing enzymatic activity within the SC leading to corneodesmolysis. More recently, the relative efficacy of the different isomers of lactic acid has been studied to help decipher its mode of action in improving SC resilience. *In vitro* lactic acid increased the production of ceramides by keratinocytes, and the l-isomer was found to be more effective than the d-isomer.[109] Similar effects were observed *in vivo* where in a four-week study topically applied lactic acid increased SC ceramide levels and l-lactic acid was seen to be the most active isomer. These changes were associated with improvements in SC barrier performance measured by changes in TEWL following a challenge to skin with sodium lauryl sulfate (Figure 18.6) and by a decrease in the expression of dry skin in the regression phase of a moisturization efficacy study. Significant improvements in these parameters were observed following application of lotions containing l-lactic acid and d,l-lactic acid but not d-lactic acid. In the studies outlined previously a significant increase in the ratio of ceramide 1 linoleate to ceramide 1 oleate may also have contributed to the improvements in SC performance. Ceramide 1 linoleate is of critical importance to the SC, where it functions as an important modulator of lipid phase behavior.[110]

Recently, Berardesca et al.[111] have also reported the ability of a number of AHAs to improve SC barrier and prevent skin irritation (Figure 18.7).

In a pivotal clinical study evaluating the effects of lactic acid on photodamaged skin,[112] an 8% l-lactic acid formula was found to be statistically significantly superior to the vehicle cream in reducing the overall severity of photodamage, mottled hyperpigmentation, sallowness, and skin roughness. Furthermore, the benefit of lactic acid on skin roughness was confirmed instrumentally following laser profilometry of silicone replicas taken from the cheek area. The results indicated that
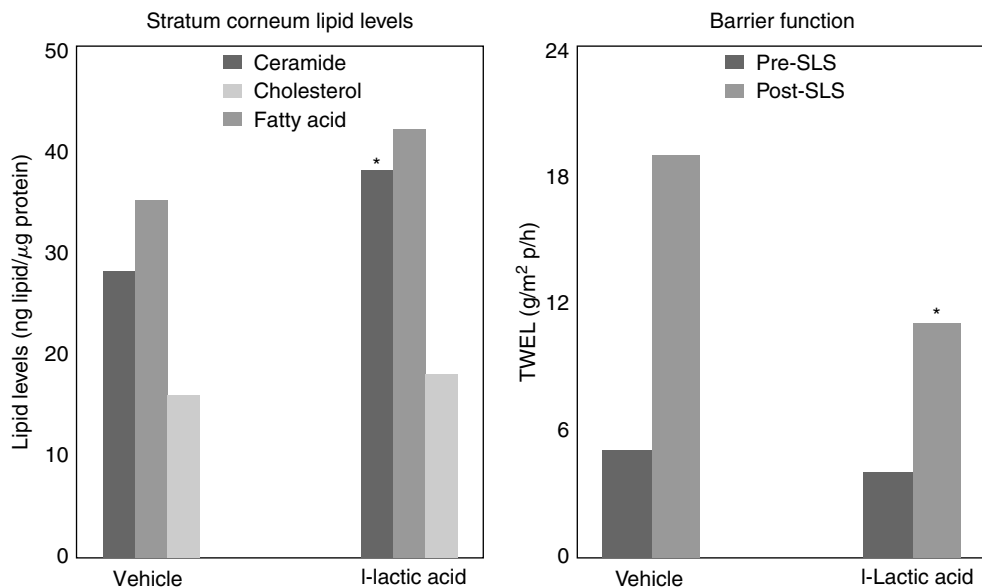
FIGURE 18.6 Effect of lactic acid on SC lipid levels and barrier function following a 1-month topical application of 4% lactic acid in an aqueous vehicle. TEWL evaluated before application of SLS patch and 24 h after removal (*$p < 0.05$). (Reprinted with permission by publisher from Rawlings, A.V., Davies, A., Carlomusto, M., Pillai, S., Zhang, K., Kosturko, R., Verdejo, P., Feinberg, C., Nguyen, L., and Chandar, P. *Arch. Dermatol. Res.*, 288, 383, 1996. Copyright 1999 by Springer-Verlag, New York.)

the l-lactic acid formula substantially reduced the roughness of the skin compared to the vehicle cream regardless of the roughness parameter calculated. Generally, the improvement in skin roughness was of the order of 25 and 10% compared to baseline values for the lactic and vehicle creams, respectively. Although the exact mechanisms that explain these observations are not known, we have shown that lactic acid imparts changes to SC lipids and increases epidermal turnover rates that should lead to the formation of smaller corneocytes. Further studies are in progress to understand more clearly the mode of action of lactic acid in the effective treatment of photodamaged skin.

Most recently, Nakagawya et al.[34] demonstrated that topical application of potassium lactate restored stratum corneum hydration after NMF extraction and exhibited a significantly higher restorative effect than sodium lactate. The authors speculate that this is due to the structure-destructive properties of the potassium ion and may influence hydrogen bonding in the keratin matrix.

18.3.4 SACCHARIDE ISOMERATES

Mixtures of sugars, saccharide isomerates, have been shown to be effective humectants. These isomerates mimic those found naturally in skin as a result of the hydrolysis of glucosylceramides. In clinical studies Smith[113] has shown that these isomerates reduce the visual appearance of dry skin, increase skin hydration, and reduce stinging to lactic acid.

18.3.5 GLYCEROL

As glycerol has now been identified in the SC it can be considered as a component of NMF. It is the archetypal moisturizer. It enhances desquamation by acting as a corneodesmolytics, that is, it aids the proteolytic degradation of corneodesmosomes[114] (Figure 18.8.) Equally, however, it also enhances the transglutaminase mediated corneocyte envelope cross linking and ceramide esterifying
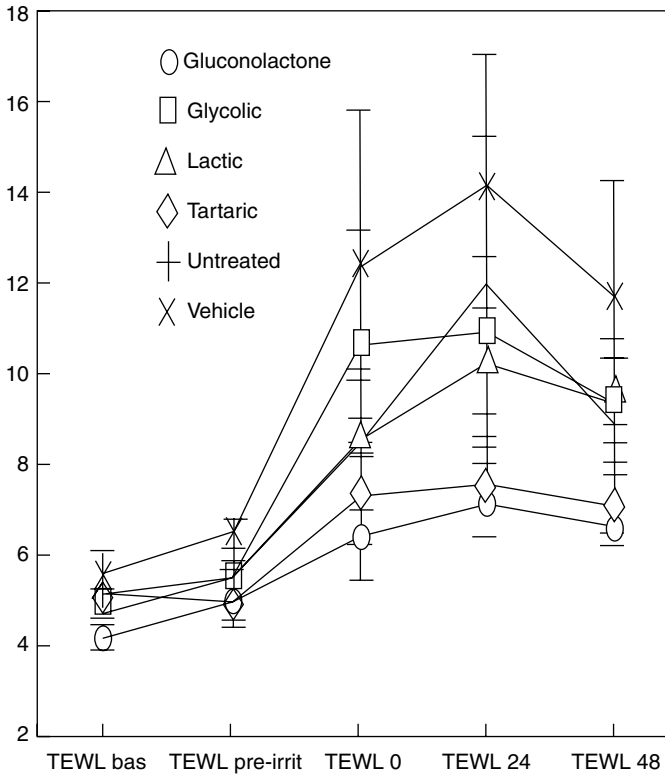
FIGURE 18.7 TEWL after sodium lauryl sulfate SLS challenge (g/m^2/h). Lower barrier damage was detected in alpha hydroxy acid treated sites compared with vehicle and untreated sites (Reprinted with permission of publisher from: Berardesca, E., Distante, F., Vignoli, G.P., Oresajo, C., and Green, B. *Br. J. Dermatol.*, 137, 934–938, 1997. Copyright 2004 by Blackwell Publishing, Oxford, UK.)

events essential for the normal functioning of the stratum corneum.[14] Nevertheless, at conventional levels of use even glycerol's effects of supplementing the NMF moisturizing system needs to be enhanced by combination with other occlusive materials. Petroleum jelly or lipid-based systems are clinically more effective when combined with glycerol,[108] and in fact a synergistic alleviation of dry skin is apparent (Figure 18.9).

18.4 ENHANCING PROFILAGGRIN SYNTHESIS

Given the importance of the profilaggrin/filaggrin family of proteins to skin condition, and the fact that synthesis declines with age and is readily perturbed by UV-irradiation, many researchers have sought to enhance synthesis.[115] A promising approach is through modulation of gene expression particularly through specific members of the nuclear hormone receptor family. Gene expression is regulated by the interplay of specific transcription factors and the nuclear hormone receptors are transcription factors that regulate many important cellular functions. This superfamily of receptors has been divided into five major subgroups depending upon their dimerization and DNA binding properties. The class II subfamily consists of nuclear receptors that form heterodimers with the retinoid X receptor (RXR).[116] Some of the transcription factors that form heterodimers with the RXR's include the retinoic acid receptor (RAR), the peroxisome proliferator receptor (PPAR), the liver X receptor (LXR) and the farnesol X receptor (FXR). Stimulation of these receptors, in particular, regulates keratinocyte proliferation and differentiation.
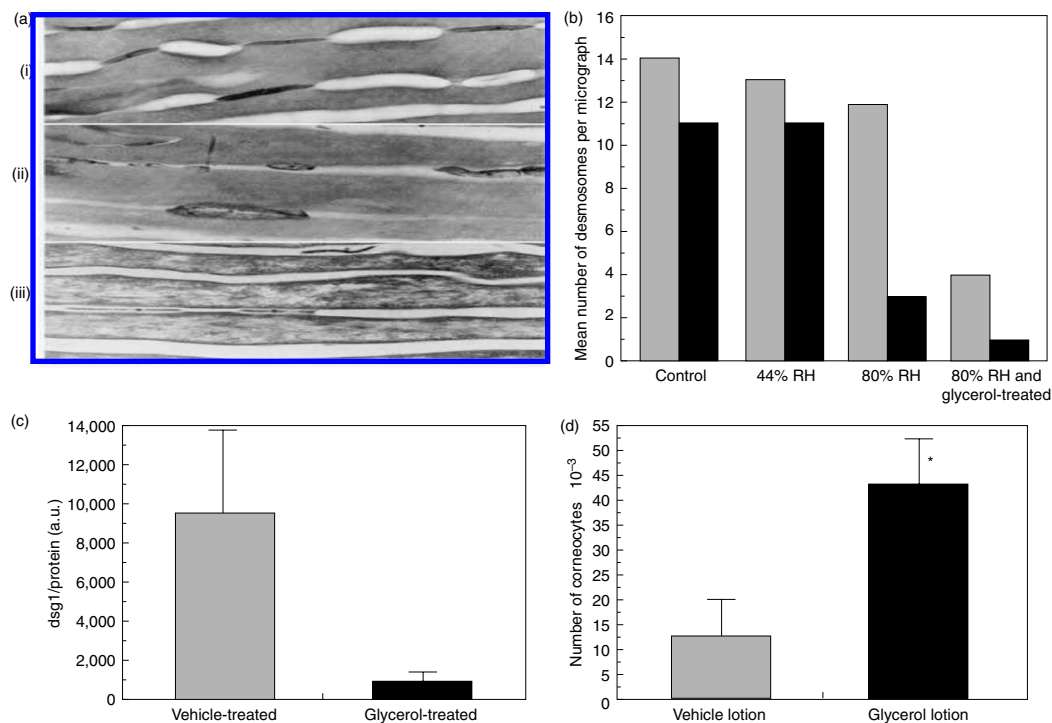
FIGURE 18.8 (a) Osmium tetroxide-fixed stratum corneum. (i) Control tissue no treatment and incubated at 44% RH. Note electron dense corneodesmosomes are fully intact. (ii) Tissue incubated at 80% RH for 7 days. Note the partial degradation of corneodesmosomes. (iii) Tissue incubated at 80% RH following 5% glycerol treatment. Note the paucity of corneodesmosomes and virtually complete degradation of their structures. (b) Comparison of the number of corneodesmosomes in control stratum corneum and stratum corneum incubated at 44% RH, 80% RH, and 80% RH following 5% glycerol treatment. Note the decrease in intact corneodesmosomes in 80% RH-treated samples and the significantly reduced number of intact (black boxes) and total (gray boxes) corneodesmosomes in glycerol-treated tissue incubated at 80% RH. (c) Comparison of the effect of 5% glycerol on desmoglein 1 digestion at 80% RH. Note the dramatic decrease in desmoglein 1 levels in glycerol-treated samples. (d) Comparison of the effect of lotions with and without the addition of 5% glycerol on corneocyte release. (Reprinted with permission of publisher from Rawlings, A.V., Harding, C.R., Watkinson, A., Banks, J., Ackerman, C., and Sabin, R. *Arch. Dermatol. Res.*, 287, 457–464, 1995. Copyright 2004 by Springer, Heidelberg.)
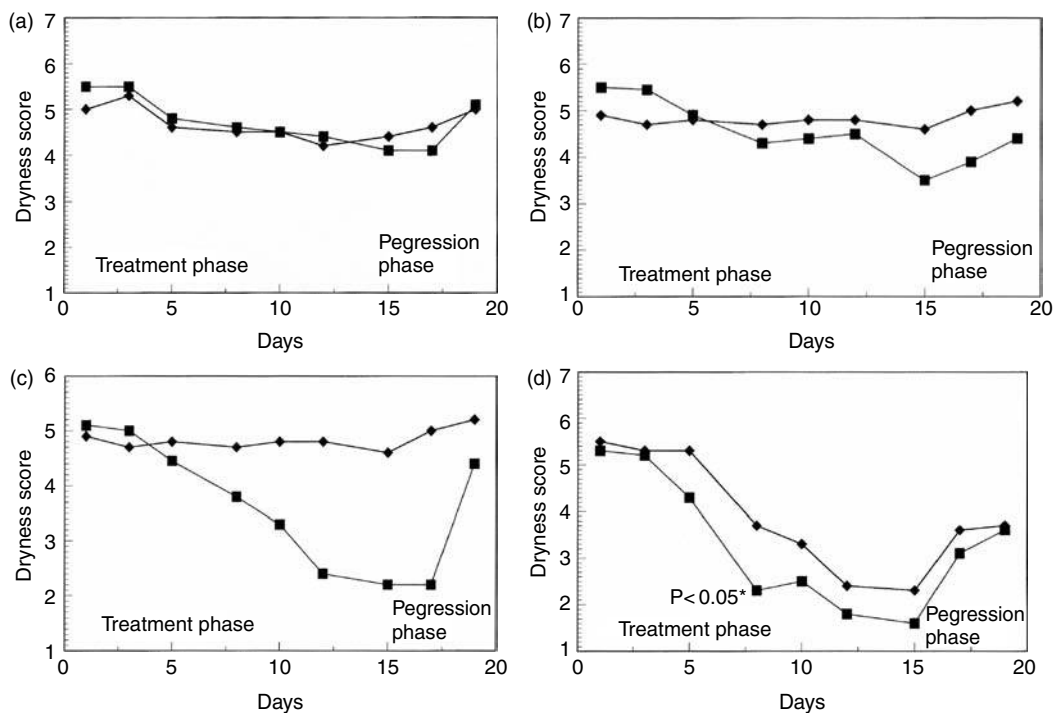
FIGURE 18.9 Moisturization efficacy tests: (a) Comparing the effect of 1% glycerol (square) to a no-treatment control (triangle). (b) Comparing the effect of a lotion containing 1% phospholipids, 2% cholesterol, and 1% stearic acid (square) to a no-treatment control (triangle). (c) Comparing the effect of a lotion containing 1% phospholipids, 2% cholesterol, and 1% stearic acid plus 1% glycerol (square) to a no-treatment control (triangle). (d) Comparing the effect of a lotion containing 1% phospholipid, 2% cholesterol, 1% stearic acid plus 5% glycerol (square) to a lotion containing 1% petrolatum, 2% cholesterol, 1% stearic acid plus 5% glycerol (triangle). (Modified from Summers, R.S., Summers, B., Chandar, P., Feinberg, C., Gursky, R., and Rawlings, A.V. *J. Soc. Cosmet. Chem.* 47, 27–39, 1996.)

18.4.1 Peroxisome Proliferator Activated Receptor

Peroxisome proliferator activated receptors are a recently discovered family of nuclear transcription factors.[117–119] Three PPAR receptor types, PPAR alpha, PPAR beta and gamma/delta, PPAR gamma have been characterized. Like other nuclear receptors PPARs bind to response elements within the promoter region of the DNA of the target gene in the form of homo or heterodimers together with the ubiquitous RXR. On binding ligands, corepressors dissociate from the transcriptional machinery complex and coactivators bind to initiate gene transcription.

PPARs are activated by a wide range of molecules including the fibrate hypolipidemic drugs and a range of saturated and unsaturated dietary fatty acids, eicosanoids and prostanoids.[120,121] Recently, triterpenoids such as ursolic and oleanolic have also been reported to stimulate the alpha receptor[122] and increase filaggrin biosynthesis.

The epidermis has been shown to express the three PPAR variants with PPAR delta being the predominant subtype.[123–125] All PPAR receptors improve epidermal differentiation and increased filaggrin levels *in vitro* and in animal studies.[123,125,126] Watkinson et al.[127] recently extended these observations in a clinical study and reported that topical application of petroselinic acid, a known PPAR alpha agonist, increased epidermal filaggrin levels significantly compared with the vehicle control in a repeat patch study for 21 days (Figure 18.10). Niacinamide or its free acid is also a PPAR agonist and Oblong[128] has reported that niacinamide increases filaggrin biosynthesis by keratinocytes.
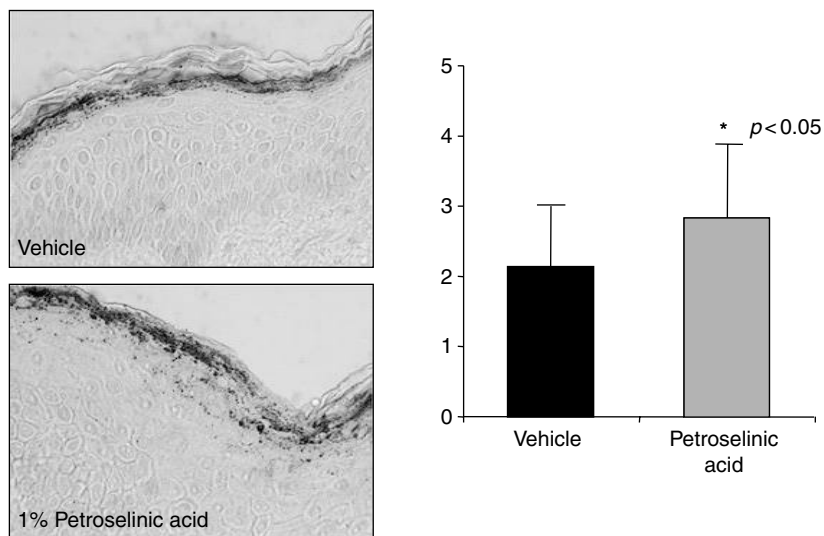
FIGURE 18.10   Increased synthesis of profilaggrin/filaggrin in human axilla skin detected by immunohisto-chemistry following a 3 week application of a 1% petroselinic acid formulation.

18.4.2   LIVER X-RECEPTOR AND FARNESOL X-RECEPTOR

Two other new nuclear receptors have been shown to increase epidermal differentiation: the LXR and the FXR. Farnesol and juvenile hormone activate the FXR leading to improved epidermal differentiation. Two genes encode for the LXR proteins, LXR alpha and LXR beta, and both are activated by various oxysterols the most potent being 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S) 25-epoxycholesterol and 7-hydroxycholesterol. Cholestenoic acid also acts on this receptor. *In vitro* these agents also increased epidermal filaggrin levels.[129,130]

18.5   FINAL COMMENTS

The NMF is essential for normal functioning of the SC. Working together with the SC lipids this pool of low molecular weight compounds assists in the retention of water within the corneocytes, a capability that is vital for the integrity of this barrier, and its mechanical properties. Hydration of the SC is also essential for the normal functioning of numerous enzymatic processes that are pivotal, not only for desquamation, but also for the generation of the NMF itself. Perturbations to either of these two biophysical mechanisms can lead to xerotic problems. Applications of lotions containing a variety of the constitutive NMF components have been shown to improve SC extensibility properties, desquamation performance, water barrier quality and to alleviate the symptoms of dry and aging skin. However, our understanding remains incomplete, and the location and activity of water within the SC and its effects on the physical and biochemical properties of this unique tissue will continue to be a quest for stratum corneum biologists for many years.

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