Barrier Function of the Skin: "La Raison d'Être" of the Epidermis

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The primary function of the epidermis is to produce the protective, semi-permeable stratum corneum that permits terrestrial life. The barrier function of the stratum corneum is provided by patterned lipid lamellae localized to the extracellular spaces between corneocytes. Anucleate corneocytes contain keratin filaments bound to a peripheral cornified envelope composed of cross-linked proteins. The many layers of these specialized cells in the stratum corneum provide a tough and resilient framework for the intercellular lipid lamellae. The lamellae are derived from disk-like lipid membranes extruded from lamellar granules into the intercellular spaces of the upper granular layer. Lysosomal and other enzymes present in the extracellular compartment are responsible for the lipid remodeling required to generate the barrier lamellae as well as for the reactions that result in desquamation. Lamellar granules likely originate from the Golgi apparatus and are currently thought to be elements of the tubulo-vesicular trans-Golgi network. The regulation of barrier lipid synthesis has been studied in a variety of models, with induction of several enzymes demonstrated during fetal development and keratinocyte differentiation, but an understanding of this process at the molecular genetic level awaits further study. Certain genetic defects in lipid metabolism or in the protein components of the stratum corneum produce scaly or ichthyotic skin with abnormal barrier lipid structure and function. The inflammatory skin diseases psoriasis and atopic dermatitis also show decreased barrier function, but the underlying mechanisms remain under investigation. Topically applied "moisturizers" work by acting as humectants or by providing an artificial barrier to trans-epidermal water loss; current work has focused on developing a more physiologic mix of lipids for topical application to skin. Recent studies in genetically engineered mice have suggested an unexpected role for tight junctions in epidermal barrier function and further developments in this area are expected. Ultimately, more sophisticated understanding of epidermal barrier function will lead to more rational therapy of a host of skin conditions in which the barrier is impaired. ***Key words: ceramides/desquamation/Golgi/keratinocyte/lipid. J Invest Dermatol 121:231–241, 2003***

Life on dry land requires the presence of a barrier to water loss to prevent desiccation (Attenborough, 1980). That the skin provided this barrier was intuitively obvious, but it was not until the 1940s that the stratum corneum (SC) clearly emerged as the specific site of this barrier (Winsor and Burch, 1944; Blank, 1953). Although the typical "basket-weave" appearance of the SC in routine histologic sections does not give the impression that it could function as an effective barrier, this is an artifact of tissue processing. In fact, as can be seen on frozen sections of the epidermis and in fortuitous electron microscopic sections (**Fig 1**), the corneocytes are tightly opposed to each other. The barrier to water permeation is not absolute and the normal movement of water through the SC into the atmosphere is known as transepidermal water loss (TEWL) and constitutes part of insensible water loss. The SC is also the principal barrier to the percutaneous penetration of exogenous substances, both accidentally encountered as well as deliberately applied. Epidermal barrier function and the related field of percutaneous absorption have been active areas of investigation in both academia and industry for many years; the information presented in this review is focused on the water barrier function of the epidermis and is intended as an overview, highlighting key points with relevance to both clinicians and basic scientists.

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Abbreviations: LG, lamellar granule; SC, stratum corneum; TEWL, transepidermal water loss; AcylGlcCer, acylglucosylceramide(s); NMF, natural moisturizing factor.

LIPIDS COMPRISE THE PERMEABILITY BARRIER

In the 1950s and 1960s, experiments were done showing that solvent extraction of epidermis dramatically increased water permeability, implicating lipids in cutaneous barrier function (Berenson and Burch, 1951; Onken and Moyer, 1963; Matoltsy *et al*, 1968; Scheuplein and Ross, 1970; Sweeny and Downing, 1970). Although some earlier studies had noted the pronounced changes in lipid composition that accompany keratinocyte differentiation, it was not until the pioneering studies of Gray, Yardley, and colleagues in the 1970s that an accurate picture of epidermal and SC lipid composition was established (reviewed in Yardley and Summerly, 1981). Thin layer chromatographic analysis of the solvent extracta-

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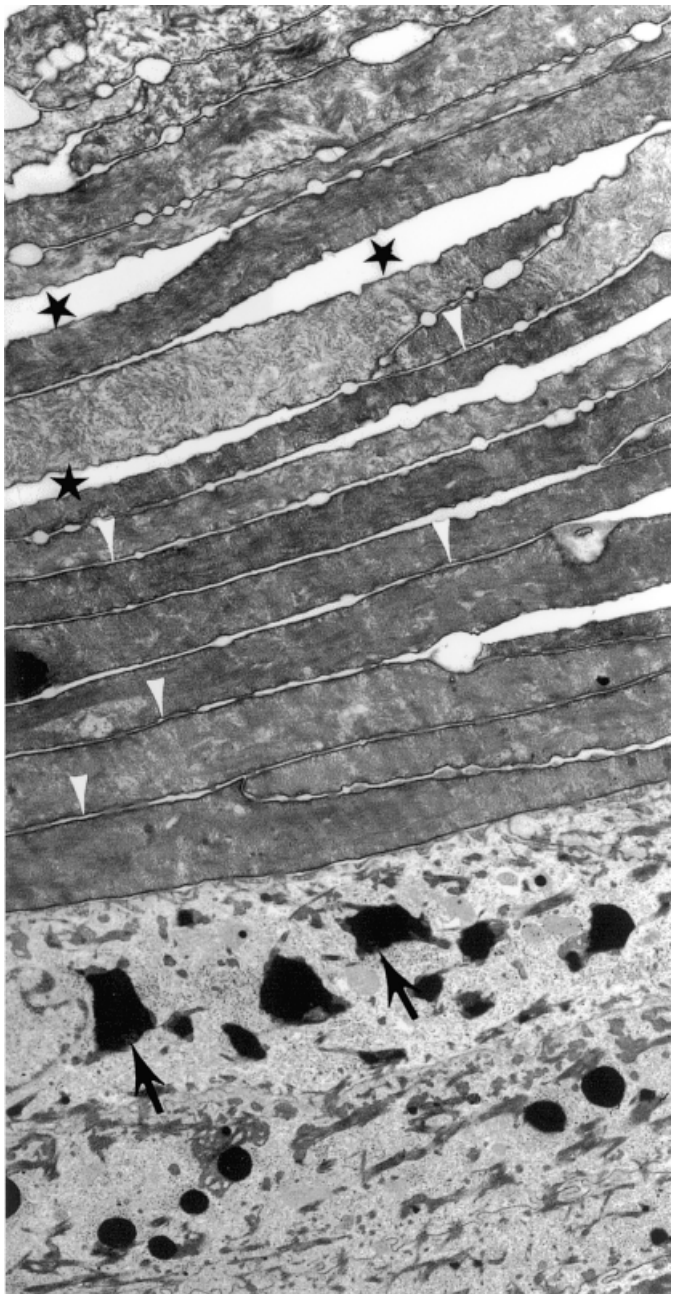

Figure 1. Electron micrograph showing the upper stratum granulosum and SC of an organotypic mouse keratinocyte culture. This figure shows a portion of a micrograph previously published in Madison *et al* (1988). *Arrows*, keratohyalin granules; *arrowheads*, intercellular spaces between closely opposed corneocytes; *stars*, artifactual separation. The intercellular spaces appear empty in this osmium tetroxide postfixed specimen. Original magnification × 6000.

ble lipids from SC reveals an unusual lipid composition consisting of a roughly equimolar mixture of ceramides (45–50% by weight), cholesterol (25%), and free fatty acids (10–15%) plus less than 5% each of several other lipids, the most important of which is cholesterol sulfate. The detailed structures of the ceramide species of pig, mouse, and human skin were determined in the 1980s (Wertz and Downing, 1983; Long *et al*, 1985; Madison *et al*, 1990) with refinements still being published (Robson *et al*, 1994; Doering *et al*, 1999a; Stewart and Downing, 1999; Hamanaka *et al*, 2002; Ponec *et al*, 2003). Human SC ceramide structures are shown in **Fig 2**.

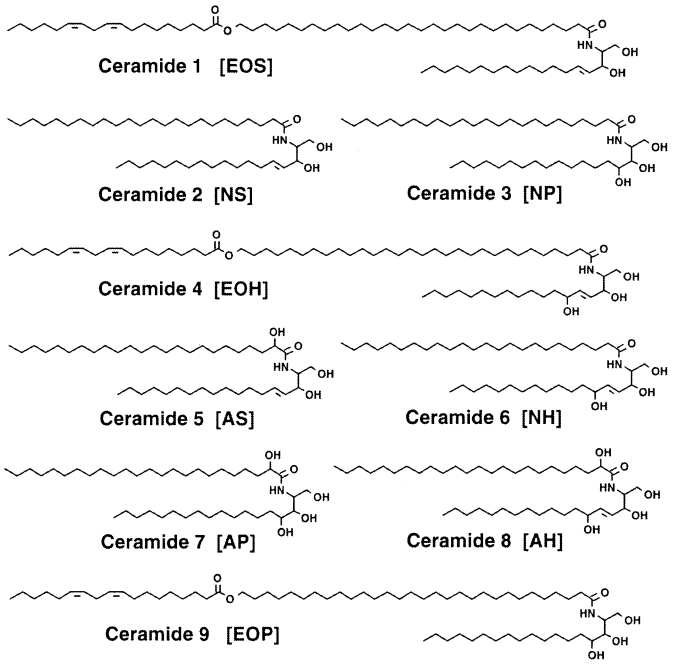

Figure 2. Structures of the free ceramides of human SC. Numbers 1 to 8 represent thin layer chromatographic mobility with ceramide 1 being the least polar and ceramide 8 the most polar. Ceramide 9 (EOP) has recently been discovered (Ponec *et al*, 2003) and has a thin layer chromatographic mobility between that of ceramide 2 and ceramide 3. The letters in parentheses give the ceramide classification as suggested in Motta *et al* (1993).

STRATUM CORNEUM LIPIDS ARE ORGANIZED AS STACKED MEMBRANE SHEETS IN THE INTERCELLULAR SPACES

Early freeze fracture electron micrographic studies of the epidermis had demonstrated the presence of broad continuous lipid sheets in the extracellular spaces of the SC (Breathnach *et al*, 1973; Elias and Friend, 1975; Elias *et al*, 1977), but these lipid membranes were not visible in conventional electron microscopy. Fixation with ruthenium tetroxide, however, which is more reactive than the usual osmium tetroxide fixative, clearly demonstrates the stacked and patterned lipid sheets in the extracellular spaces of the SC (Madison *et al*, 1987; **Fig 3**). All of the free fatty acids and the amide-linked fatty acid chains in the ceramides are nonbranched and have no double bonds. This allows for tight lateral packing and the formation of highly ordered gel phase membrane domains, which are less fluid and less permeable than typical liquid crystalline phospholipid-dominant biologic membranes. Cholesterol may provide some necessary fluidity to the membranes, which might otherwise be too rigid and possibly brittle. Numerous biophysical studies of SC structure suggest the presence of coexisting liquid crystalline and gel phase domains in the membranes of the SC. This concept was suggested by Forslind (1994) and presented as the "domain mosaic" model; recently a new model for the existence of fluid phases within the lamellae, the "sandwich model", was presented by Bouwstra *et al* (2000). Norlen (2001b), however, has very recently proposed a different "single gel phase" model that he feels is more consistent with the documented barrier properties of the SC. There are still many unanswered questions about the exact way in which the SC lipids are organized at the molecular level and this is an active area of research (reviewed in Bouwstra *et al*, 2003). Understanding the physical structure of the membranes is critical to understanding their function as a barrier, both to water and to other substances, and ultimately to understanding the mechanisms of barrier disruption in a variety of skin diseases.

STRATUM CORNEUM BARRIER LIPIDS ORIGINATE FROM LAMELLAR GRANULES

Lamellar granules (LG) are small organelles with a bounding membrane, most prominent in the granular cell layer of the epidermis and visible only by electron microscopy. They contain stacks of lipid lamellae (**Fig 4*a***) composed of phospholipids, cholesterol, and glucosylceramides (Freinkel and Traczyk, 1985) that are the precursors of the SC intercellular lipids (reviewed in Landmann, 1988). Late in epidermal differentiation, at the transition from granular cell to corneocyte, LG are thought to fuse with the plasma membrane of the granular cell and discharge their lipid membranes into the intercellular space (**Fig 4*b***). Along with the lipids, LG secrete a group of acid hydrolases (Freinkel and Traczyk, 1985; Grayson *et al*, 1985; Menon *et al*, 1986, 1992), which break down the phospholipids and convert glucosylceramides to ceramides. The enzyme responsible for the latter reaction (Holleran *et al*, 1994), β-glucocerebrosidase, is the enzyme defective in Gaucher disease. Although in most Gaucher patients residual enzyme activity is sufficient to catalyze the cutaneous reaction, there is a subset of patients with severe enzyme deficiency who present as collodion babies, have abnormal barrier function, and die in the neonatal period (Sidransky *et al*, 1992).

Other enzymes involved in the lipid metabolic changes that occur after extrusion of LG contents include acid sphingomyelinase and secretory phospholipase A_2, both of which have been shown to be required for permeability barrier function (Jensen *et al*, 1999; Elias *et al*, 2000; Schmuth *et al*, 2000). Lysosomal acid lipase activity is also present in LG (Madison *et al*, 1998), but its exact function has not been determined. Some of the proteases that appear to regulate desmosome breakdown and contribute to desquamation (see below) may also be delivered via LG (Sondell *et al*, 1995).

Concurrent with the complex changes in lipid composition that occur following extrusion of LG contents, the short stacks of membranes reorganize structurally to form patterned lamellar sheets as shown in **Fig 3**. This transformation has been suggested to occur via edge-to-edge fusion of the lipid stacks (Landmann, 1986) and calcium may promote this process (Abraham *et al*, 1987). Based on knowledge of the lipid composition of the lamellae and their electron microscopic appearance in ruthenium tetroxide-fixed sections, a model for their biochemical structure has been proposed (Swartzendruber *et al*, 1989).

LIPIDS UNIQUE TO KERATINIZING EPITHELIA MAY PLAY A SPECIFIC ROLE IN MEMBRANE FORMATION

LG are particularly enriched in a lipid unique to keratinizing epithelia, acylglucosylceramide (AcylGlcCer). This unusual lipid has a very long chain ω-hydroxy fatty acid moiety (C28–36) with linoleic acid (an essential fatty acid) ester-linked to the ω-hydroxyl group (Abraham *et al*, 1985; **Fig 5**, top). The interior lipid lamellae of LG have been suggested to arise from the flattening and stacking of lipid vesicles (Landmann, 1986) and AcylGlcCer has been proposed to function as a molecular rivet to accomplish this process (Wertz and Downing, 1982; **Fig 5**, bottom). The fatty acid chain is long enough to span completely a lipid bilayer and allow the linoleate tail to insert into a leaflet of a neighboring bilayer as shown in **Fig 5** (bottom). **Figure 4**(***a***, right) schematically shows the interior structure of a LG as flattened and stacked lipid vesicles. Evidence to support this model includes the ability of AcylGlcCer to cause the flattening and aggregation of lipid liposomes *in vitro* (Landmann *et al*, 1984). Menon *et al* (1992) have suggested an alternative model of accordion-like pleating of lipid membranes to explain the appearance of LG contents followed by "unfurling" after extrusion. AcylGlcCer could function as a rivet in this model as well. As the biophysics of membrane dynamics and the function of the Golgi apparatus (see below) are better understood, new models of LG assembly/extrusion may well emerge. After extrusion of LG contents, AcylGlcCer is deglycosylated, along with the rest of the glucosylceramides, to produce acylceramide (**Fig 6**, middle structure). Acylceramide is thought to perform the same molecular rivet role in the SC lamellae as AcylGlcCer does in the LG, and there are X-ray diffraction data to support this concept (Schreiner *et al*, 2000).

Acylceramide and its precursor are the two principal carriers of linoleic acid in the SC and living epidermis, respectively. It has been known for years that essential fatty acid deficiency results in poor cutaneous barrier function and increased TEWL (reviewed in Wertz *et al*, 1987). These effects correlate with replacement of linoleic acid by oleic acid in AcylGlcCer and acylceramide (Melton *et al*, 1987), a substitution that results in al-

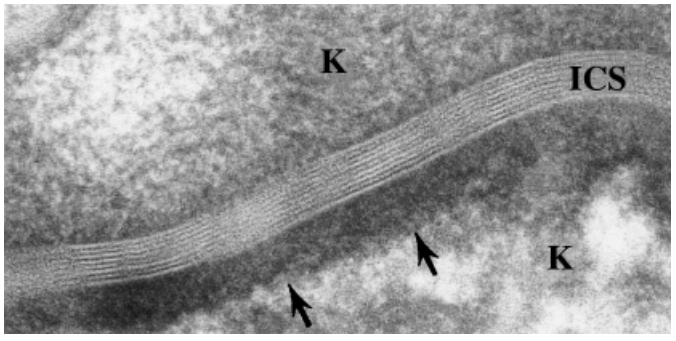

Figure 3. Electron micrograph showing the stacked and patterned lamellar membrane sheets in a single intercellular space in mouse SC postfixed with ruthenium tetroxide. The cornified envelope of the lower corneocyte is clearly visible (*arrows*); ICS, intercellular space; K, keratin contents of the corneocytes bordering the intercellular space. Original magnification × 200,000.

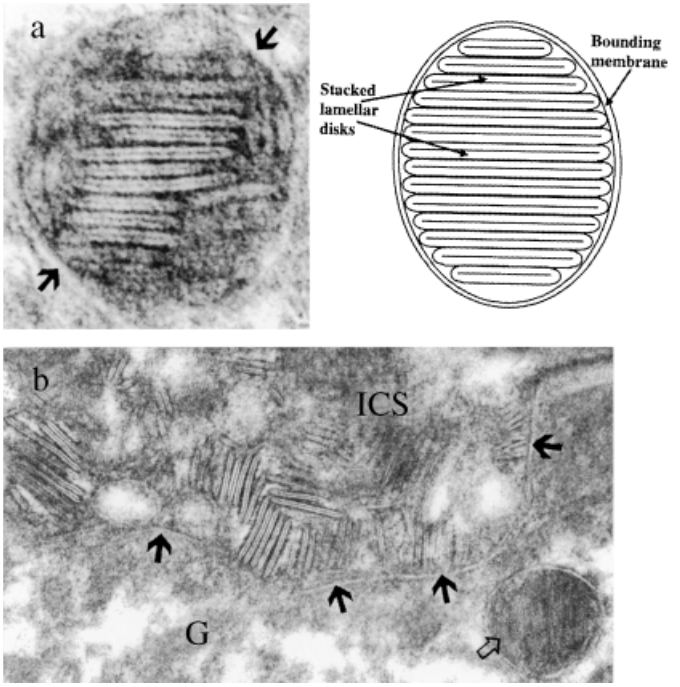

Figure 4. (a) Left: electron micrograph of a single LG in mouse epidermis. A lower magnification view of the same LG was used in a previously published figure (Madison *et al*, 1987). *Arrows*, bounding membrane. Original magnification × 300,000. Right: a schematic diagram of a LG as suggested by Landmann (1986). (*b*) Electron micrograph of extruded LG contents in the intercellular space at the junction of the granular layer and the SC. G, granular cell; ICS, intercellular space; *Arrows*, granular cell plasma membrane; *open arrow*, LG. Original magnification × 125,000.

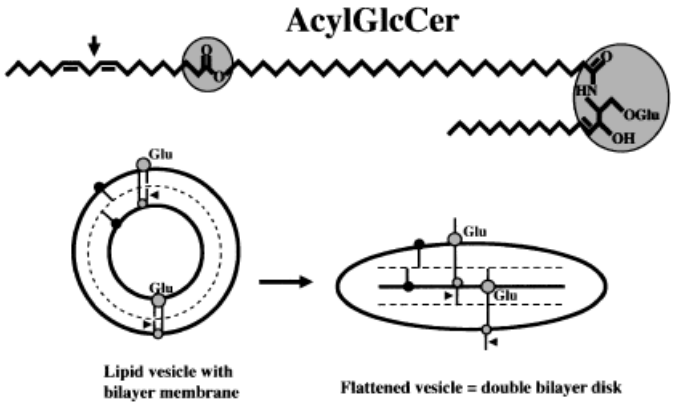

Figure 5. Top: structure of acylglucosylceramide. Bottom: proposed model of acylglucosylceramide function in the flattening and stacking of lipid vesicles to generate the double bilayer structure of the internal LG lamellae. The orientation of acylglucosylceramide is not known and both possibilities are shown. The vesicle and double bilayer disk are shown in cross-section. *Arrowheads*, linoleate moiety; Glu, glucose.

tered biophysical properties of the SC lamellae (Bouwstra *et al*, 2002) and increased water permeability.

THE LIPID ENVELOPE

Each corneocyte has an approximately 10 nm thick tough peripheral protein envelope, called the cornified envelope, that is composed of several structural proteins, notably involucrin and loricrin, cross-linked by sulfhydryl oxidases and transglutaminases (reviewed in Kalinin *et al*, 2002). The interior surface of the cornified envelope is linked to the bundles of keratin filaments that fill the intracellular compartment of corneocytes. The multiple layers of corneocytes in the SC contribute a tough and resilient framework for the intercellular lipid lamellae. On the exterior (extracellular) surface of the cornified envelope is a covalently bound layer of very long chain ω-hydroxyceramides called the lipid envelope (Swartzendruber *et al*, 1987; Wertz and Downing, 1987; **Fig 6**, bottom). This structure can be seen on electron microscopy of SC that has been solvent extracted to remove all of the free lipid, as shown in **Fig 7**. Evidence suggests that the ω-hydroxyceramides are ester-linked to involucrin amino acid residues (Downing, 1992; Marekov and Steinert, 1998) and transglutaminase has been shown to be capable of catalyzing this reaction (Nemes *et al*, 1999). Specific three-dimensional conformations for involucrin that would allow for attachment of lipids on the exterior surface and other envelope proteins on the interior surface of the cornified envelope have been proposed (Lazo and Downing, 1999; Kajava, 2000). Surprisingly, however, both involucrin

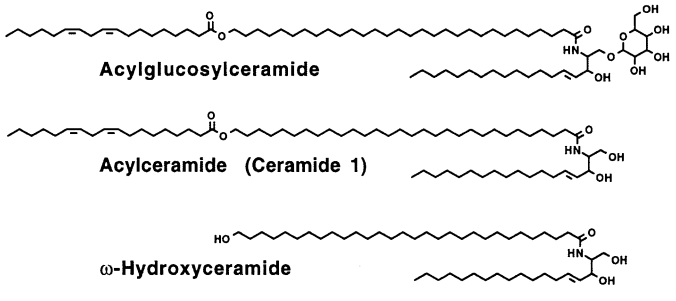

Figure 6. The very long chain ω-hydroxy fatty acid-containing ceramides of mammalian epidermis. LG acylglucosylceramides are the precursors of the acylceramides (ceramide 1 (EOS) is shown) in the SC intercellular lamellae and the ω-hydroxyceramides of the lipid envelope. See text for details.

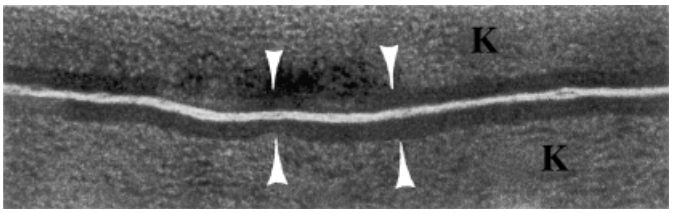

Figure 7. Electron micrograph of mouse SC that has been solvent extracted to remove all of the free lipid. A lucent band (the lipid envelope) remains on the exterior surface of the cornified envelopes of adjacent corneocytes. In solvent-extracted SC the lipid envelopes are tightly opposed and a narrow dark line can be seen where they join (see Wertz *et al*, 1989 for a more detailed discussion). The dominant component of the lucent band is very long chain ω-hydroxyceramides derived from LG acylglucosylceramides (likely from the LG bounding membrane; see text and Fig 6) and covalently bound to cornified envelope proteins. K, keratin contents of two adjacent corneocytes; *arrowheads*, cornified envelopes. Ruthenium tetroxide postfixation, original magnification × 125,000.

(Djian *et al*, 2000) and loricrin (Koch *et al*, 2000; Jarnik *et al*, 2002) knockout mice have relatively normal-appearing cornified envelopes and no epidermal phenotype, suggesting great redundancy in the components of the epidermal barrier (Steinert, 2000). This also implies that other envelope proteins are able to bond with ω-hydroxyceramides and, indeed, new envelope-associated proteins continue to be discovered (Cabral *et al*, 2001; Marshall *et al*, 2001).

AcylGlcCer, the precursor of acylceramide (see above), is also the precursor of the ω-hydroxyceramides of the lipid envelope. Wertz (1996) has found that two-thirds of LG AcylGlcCer is in the bounding membrane; this suggests that lipid envelope ω-hydroxyceramides are delivered to the cell surface when LG bounding membranes fuse with the granular cell plasma membrane (Wertz, 1996; Kalinin *et al*, 2002). Recent evidence suggests that most or all or the ω-hydroxyceramides are bound by their ω-hydroxyl ends (Nemes *et al*, 1999; Doering *et al*, 1999b; Stewart and Downing, 2001). This implies that the linoleic acid tail must be removed from the ω-hydroxyl end of AcylGlcCer; the enzyme responsible for this deacylation is not known, but candidates include transglutaminase (Nemes *et al*, 1999; Kalinin *et al*, 2002) and acid lipase.[1] The uniquely long fatty acid chains of the lipid envelope ceramides span the distance of a typical plasma membrane bilayer leaving the sphingosine chains free to interdigitate with the nonbound intercellular lipid lamellae. This chain interdigitation may contribute to the patterned organization of the lamellae seen on electron microscopy. The structures of the very long chain ω-hydroxy fatty acid-containing ceramides are shown in **Fig 6**. Note that human ceramide 4 and the newly discovered ceramide 9 (**Fig 2**) also contain a very long chain ω-hydroxy fatty acid and contribute ω-hydroxyceramides to the lipid envelope. Differentiation-related changes in lipid structures are illustrated schematically in **Fig 8**.

DESQUAMATION

It has long been known from the clinical example of X-linked ichthyosis, caused by cholesterol sulfatase deficiency (Shapiro *et al*, 1978), that the hydrolysis of cholesterol sulfate in the SC is important for corneocyte desquamation. The mechanism by which excess cholesterol sulfate inhibits desquamation and its hydrolysis promotes desquamation, however, is still under investigation. Excess cholesterol sulfate has been suggested to alter the structure and function of the lipid bilayers (Zettersten *et al*, 1998;

[1]Sando GN, Howard EJ, Madison KC: Acid lipase expression in cultured human keratinocytes: Potential role in epidermal ceramide metabolism. *J Invest Dermatol* 108:554a, 1997 (Abstr.)

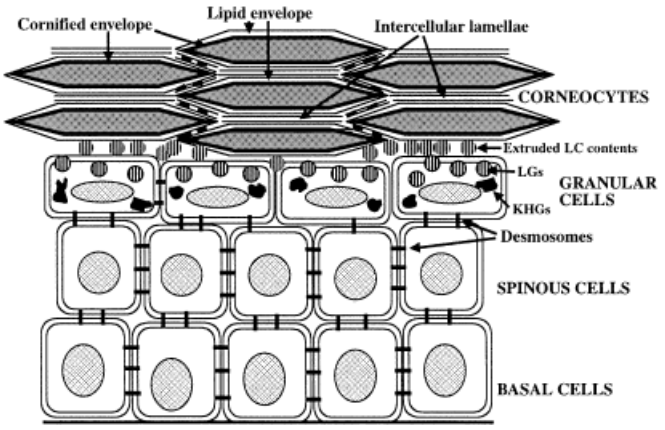

Figure 8. Schematic diagram of the epidermis, including the transformations of lipid structures that accompany epidermal differentiation. Keratin filaments are omitted. Not to scale.

Bouwstra *et al*, 1999). Proteases, especially SC tryptic enzyme and SC chymotryptic enzyme, have been implicated in desmosome breakdown and corneocyte desquamation (Hansson *et al*, 1994; Horikoshi *et al*, 1998; Brattsand and Egelrud, 1999; Simon *et al*, 2001), and cholesterol sulfate has been shown to inhibit some of their activities (Sato *et al*, 1998). A recent study showed that cholesterol sulfate inhibits transglutaminase-mediated involucrin cross-linking as well as involucrin esterification to the ω-hydroxyceramides of the lipid envelope (Nemes *et al*, 2000). Any of these mechanisms could contribute to the so-called "retention hyperkeratosis" of X-linked ichthyosis.

REGULATION OF EPIDERMAL LIPID SYNTHESIS

Epidermal barrier lipid synthesis has been studied *in vivo* in adult pigs (Hedberg *et al*, 1988; Wertz and Downing, 1990) and hairless mice (reviewed in Feingold and Elias, 2000), in fetal rat development models *in vivo* and *in vitro* (reviewed in Williams *et al*, 1998), during fetal mouse development (Doering *et al*, 2002), and in a variety of keratinocyte culture models (Madison *et al*, 1989, 1990; Jetten *et al*, 1992; Sando *et al*, 1996; Ponec *et al*, 1997; Watanabe *et al*, 1998). We know from *in vivo* metabolic labeling studies that barrier lipids are largely synthesized *de novo* from acetate (Hedberg *et al*, 1988; Wertz and Downing, 1990). The loss of phospholipids and the conversion of glucosylceramides to ceramides and AcylGlcCer to acylceramide and ω-hydroxyceramides during epidermal differentiation have been demonstrated *in vivo* as well as *in vitro* (Hedberg *et al*, 1988; Madison *et al*, 1990). As an essential fatty acid, linoleate must be derived from the circulation, but may also be recycled within the epidermis (Madison *et al*, 1989; Wertz and Downing, 1990).

Several of the enzymes known to be required for either barrier lipid synthesis or postextrusion processing are induced during keratinocyte differentiation *in vitro* (Jetten *et al*, 1992; Sando *et al*, 1996; Watanabe *et al*, 1998) or during fetal rat epidermal development (Williams *et al*, 1998). Submerged explant cultures of fetal rat skin recapitulate *in utero* barrier development and the development of the barrier can be inhibited by testosterone and stimulated by exogenous application of glucocorticoids, thyroid hormone, and estrogen and by lifting the cultures to the air–liquid interface (Williams *et al*, 1998). More recently, peroxisome proliferator activated receptor (PPARα) ligands (clofibrate, linoleic acid, oleic acid) and farnesol, a metabolite in the cholesterol biosynthetic pathway, have been shown to stimulate epidermal differentiation and barrier development in fetal rats both *in utero* and in skin explant cultures (Hanley *et al*, 1999). PPAR are nuclear hormone receptors that heterodimerize with retinoid X receptors to regulate the transcription of several genes involved in lipid metabolism and have been most studied in adipocytes. Recently, mice transgenic for a dominant-negative mutant retinoic acid receptor α were shown to have markedly decreased barrier function associated with abnormal lipid processing, implicating retinoid receptor-mediated signaling pathways in barrier formation (Attar *et al*, 1997). Which genes involved in epidermal barrier lipid synthesis or processing might be regulated by these hormones and receptors is not known, although increased β-glucocerebrosidase and cholesterol sulfatase activity following treatment with PPAR ligands has been shown (Hanley *et al*, 1999). PPAR-α knockout mice show a delay in fetal SC formation as well as decreased β-glucocerebrosidase activity, but are normal by the time of birth (Schmuth *et al*, 2002). Another study in transgenic mice has demonstrated that a member of the kruppel family of transcription factors, Klf4, is essential for normal barrier development (Segre *et al*, 1999). These and other mouse models should be of great help in dissecting the mechanisms of barrier formation at the molecular genetic level.

Artificial barrier disruption of hairless mouse skin by acetone wiping or tape stripping (as measured by increased TEWL) has been used as a model to study the events involved in barrier repair. The most commonly used approach to determine if an effect is mediated by loss of barrier rather than a nonspecific injury effect is to immediately cover one site with a vapor impermeable membrane and compare the response to an uncovered site. Studies using this model have shown acute barrier disruption to stimulate epidermal proliferation and increase mRNA levels and activities of several (but not all) of the enzymes involved in barrier lipid synthesis, in particular those associated with fatty acid, cholesterol, and ceramide synthesis (Feingold and Elias, 2000). In general, vapor-impermeable and semipermeable membranes inhibit the stimulatory response and delay barrier repair, the degree of inhibition correlating with the degree of impermeability. The molecular mechanisms by which barrier disruption produces, and artificial barrier restoration inhibits, these effects remain unknown, although changes in SC water content (Denda *et al*, 1998; Fluhr *et al*, 1999), ion content and distribution (particularly calcium) (Lee *et al*, 1998), or cytokine production (Jensen *et al*, 1999) may be involved in the signaling pathways.

Although a recent study showed an increase in serine palmitoyltransferase (the rate-limiting enzyme in sphingolipid synthesis) mRNA following tape stripping of human skin (Stachowitz *et al*, 2002), as rodent epidermis is different from human, including having poorer barrier function, which of the findings in hairless mouse models will translate to human skin needs further study (Rigg and Barry, 1990, and references therein). Several studies of human skin *in vivo* have shown no effect of occlusion with membranes of varying permeability on barrier recovery following tape stripping, detergent-induced damage, or wounding (Silverman *et al*, 1989; Van de Kerkhof *et al*, 1995; Welzel *et al*, 1995, 1996; Fluhr *et al*, 1999) and a study in premature infants showed that the use of semipermeable membranes improved barrier function in treated compared with untreated sites (Mancini *et al*, 1994). Under physiologic conditions, barrier lipid synthesis, LG formation, and lipid extrusion take place continuously under a competent barrier. Whether barrier abrogation results in additional stimulation of all of these processes, as has been demonstrated in mouse skin, needs additional study in human skin.

LAMELLAR GRANULE ASSEMBLY

An unanswered question in the field of epidermal barrier formation is how keratinocyte LG assembly is orchestrated. Containing both lipid membranes and acid hydrolases destined for extrusion into the extracellular environment, the LG is something of a cross between a secretory granule and a lysosome. A large body of evidence now supports the concept that LG

originate from the Golgi apparatus and the very active and rapidly advancing field of Golgi research is ripe for application to keratinocyte biology. Of particular interest is recent work on the *trans*-Golgi network, which is the highly tubulated sorting and delivery portion of the Golgi apparatus. It is now thought that Golgi to plasma membrane transport is mediated by pleiomorphic tubulovesicular structures (sometimes referred to as "post-Golgi carriers") that are formed by maturation of the *trans*-Golgi compartment, rather than by vesicles (Hirschberg *et al*, 1998; Mironov *et al*, 1998). In this paradigm, secretory organelles are the remnants of Golgi cisternae that have already exported all of the components not destined for secretion; thus they are formed by terminal maturation of *trans*-Golgi network cisternae.

Careful examination of high magnification electron microscopy images of epidermis clearly shows that LG do not constitute a uniform vesicular population. There are numerous highly irregular shapes, including ovals, dumbbells, and elongated tubular structures filled with the characteristic stacked lamellae. These images are consistent with sections through a tubular network (Madison and Howard, 1996; Elias *et al*, 1998; Madison *et al*, 1998) suggesting that keratinocyte lamellar "granules" are *trans*-Golgi network structures and that the secretion of their contents may be an excellent example of the current Golgi paradigm. Further studies are needed to determine the validity of this paradigm and/or whether the specific mechanisms may be unique to keratinizing epithelia. Norlen (2001a) has recently proposed a "membrane folding model" of barrier lipid delivery that does not require membrane trafficking or fusion as classically described.

Even if we accept the Golgi origin of lamellar "granules", how all of the enzymes involved in barrier lipid synthesis are regulated, how the internal membranes are formed, how lysosomal enzymes are incorporated into the membrane structure, what stimulates fusion (if classic membrane fusion does occur) with the keratinocyte plasma membrane, and how the whole process is coordinated with the many other events occurring during terminal epidermal differentiation are questions that remain to be answered. Clearly, disruptions in any of these processes could have a significant effect on SC barrier function.

FLAKY SKIN

Flaky skin, often called "dry" skin, is a cutaneous reaction pattern reflecting abnormal desquamation of diverse etiologies. Corneocytes are normally shed in small enough groups that they are not visible on the skin surface; when this process is disturbed in any way, corneocytes collect in visible clumps (scales) that produce a rough texture and appearance.

The importance of SC water content to "normal" nonflaky skin appearance has long been known, with healthy tissue containing greater than 10% water (Blank, 1952, 1953). Both water soluble intracorneocyte substances (collectively referred to as natural moisturizing factor (NMF)) (reviewed in Harding *et al*, 2000) and the intercellular lipid membranes (Imokawa *et al*, 1991) contribute to the water binding properties of the SC and the barrier properties of the intercellular membranes maintain hydration by limiting water loss from the tissue. NMF consists of a mixture of amino acids and their derivatives (pyrrolidone carboxylic acid, urocanic acid), lactic acid, urea, and sugars that is highly hygroscopic and acts as an endogenous humectant. The amino acid portion of NMF derives from proteolysis of filaggrin (from keratohyalin granules) in the mid to outer SC (Harding *et al*, 2000). One of the critical functions of water in the SC is participating in the many hydrolytic enzymatic processes required for normal desquamation (discussed above) and for the generation of NMF.

Knowing this, we can predict that any endogenous defect (primary or secondary) or exogenous insult that decreases SC NMF content, alters the composition or physical properties of the intercellular lipids, or disrupts epidermal differentiation may lead to improper desquamation and clinical scaling. This is a simplification of a very complex situation, but helps in thinking about some mechanisms that can lead to "flaky" skin. Examples include ichthyosis vulgaris, where there is a profound deficiency in filaggrin (Sybert *et al*, 1985) (and thus NMF), aged skin where lipid synthesis (particularly cholesterol) is decreased leading to poor barrier repair after insults (Ghadially *et al*, 1995, 1996a), and so-called "winter xerosis" where low environmental humidity decreases SC water content. Whether "flaky skin" will have impaired barrier function depends on the underlying pathophysiology as well as the effect of any compensatory response. Patients with lamellar ichthyosis due to transglutaminase 1 deficiency have abnormal cornified envelope structure and dramatically scaly skin. Their impaired barrier function has been reported to be due to defects in the composition and organization of the SC lipid lamellae (Lavrijsen *et al*, 1995; Pilgram *et al*, 2001; Elias *et al*, 2002) directly related to the underlying disturbance in the cornified envelope (Elias *et al*, 2002). In epidermolytic hyperkeratosis, which is caused by genetic defects in the suprabasal keratins 1 and 10, keratinocytes are fragile and patients have both blistering and severe scaling. The barrier defect, however, has been shown to be due to abnormal LG secretion and the resulting decrease in SC lipid lamellae (Schmuth *et al*, 2001). In many inflammatory skin diseases where overall epidermal differentiation is disturbed, there are likely secondary effects on corneocyte structure and NMF generation as well as on the composition and function of the intercellular lipids that ultimately result in scaling. It should be noted here that sebaceous gland secretions (sebum) are unlikely to play a significant part in epidermal moisturization in humans; prepubertal children, who have essentially no sebum production, have enviable skin qualities and certainly no particular difficulties with xerosis.

SKIN DISEASES WITH ABNORMALITIES IN BARRIER FUNCTION AND IN LIPID METABOLISM

There are several genetic skin diseases with known defects in lipid metabolism that have scaly or ichthyotic skin as part of the clinical picture (reviewed in Williams and Elias, 2000). RXLI, discussed above, was the first of these diseases to be described. Others include Sjögren–Larsson syndrome (defect in fatty aldehyde dehydrogenase; De Laurenzi *et al*, 1996), Refsum's disease (defect in phytanoyl-coenzyme A hydroxylase; Jansen *et al*, 1997), and X-linked dominant Conradi–Hunermann as well as CHILD syndrome (*C*ongenital *H*emidysplasia with *I*chthyosiform erythroderma and *L*imb *D*efects; defects in 3β-hydroxysteroid-Δ 8, Δ7-isomerase, an enzyme in the sterol biosynthetic pathway; Braverman *et al*, 1999; Grange *et al*, 2000). CHILD syndrome can also be caused by mutations in the gene encoding 3β-hydroxysteroid dehydrogenase (Konig *et al*, 2000). Very recently, Chanarin–Dorfman syndrome (neutral lipid storage disease with ichthyosis), which is characterized by nonbullous congenital ichthyosiform erythroderma, was found to be caused by mutations in a gene (CGI-58) that encodes a new protein of unknown function in the esterase/lipase/thioesterase family (Lefevre *et al*, 2001). Mutations in lipoxygenase-3 (ALOXE3) and 12R-lipoxygenase (ALOX12B) have now been reported in nonbullous congenital ichthyosiform erythroderma linked to chromosome 17p13.1 (Jobard *et al*, 2002). Although the substrates and products of these lipoxygenases are not yet known, further investigation should shed considerable light on the functional role of lipoxygenase pathways in epidermal differentiation. For the majority of these diseases, even when the defect is known, the precise mechanism by which SC structure and function are altered has not been determined. Harlequin ichthyosis is characterized by an absence of LG and SC lipid lamellae (Milner *et al*, 1992). Some subtypes of congenital ichthyosiform erythroderma show abnormalities in lipid structures (Arnold *et al*, 1988) and there are

some cases that share the microscopic findings reported for harlequin ichthyosis (Virolainen *et al*, 2001), but the underlying defects in these genetic diseases remain unknown.

Netherton syndrome and Papillon–Lefevre syndrome are caused by defects in proteolysis: mutations in SPINK5, a serine protease inhibitor (Chavanas *et al*, 2000), and cathepsin C (Hart *et al*, 1999), respectively. Abnormalities in SC lipid structure have been described in Netherton syndrome (Fartasch *et al*, 1999), but how SPINK5 defects result in these alterations is not known. A recent paper reported that SC hydrolytic activity is increased in Netherton syndrome and the authors suggest that SPINK5 is necessary to regulate the enzymes involved in desquamation (Komatsu *et al*, 2002). Type 2 Gaucher disease, with severe defects in β-glucocerebrosidase, discussed above, results in failure to metabolize LG glucosylceramides to ceramides. The recent finding of delayed barrier repair in Niemann–Pick disease (acid sphingomyelinase deficiency) (Schmuth *et al*, 2000) suggests these patients might be more susceptible to exogenous barrier insults. Many genetic skin diseases with defects in a variety of epidermal protein structures, as discussed above for lamellar ichthyosis and epidermolytic hyperkeratosis, have associated changes in SC lipid structure and function and these are currently being investigated.

Of the inflammatory skin diseases, atopic dermatitis and psoriasis have been the most studied with respect to epidermal barrier function and SC lipid alterations. A decrease in ceramides has been the most consistent finding in atopic dermatitis and this has been suggested to result from increased sphingomyelin deacylase activity (Hara *et al*, 2000). Macheleidt *et al* (2002) have recently demonstrated decreased free very long chain fatty acids and lipid envelope ω-hydroxyceramides in atopic skin as well as decreased very long chain fatty acid, ceramide, and glucosylceramide synthesis by atopic epidermis *in vitro*. Although the mechanisms underlying these changes are not known, the findings offer an explanation for the decreased barrier function of atopic skin. In addition, gene polymorphisms in SPINK5, the gene mutated in Netherton syndrome (which has atopy as part of the clinical picture), have been found to show significant association with common atopic disease, including atopic dermatitis (Walley *et al*, 2001). Combined with the recent demonstration that mice overexpressing SC chymotryptic enzyme develop a chronic itchy dermatitis (Hansson *et al*, 2002), this suggests tantalizing links between excess protease activity, barrier function, epidermal differentiation, and inflammation. In psoriasis, alterations in ceramide content have been demonstrated (Motta *et al*, 1994) and abnormal lipid structures reported (Ghadially *et al*, 1996b). More work is needed to determine whether these changes are primary or secondary and/or are specific for the disease.

NEONATAL SKIN

Current dogma holds that the SC is not functionally mature until 32 to 34 wk estimated gestational age and that the skin of premature infants develops competent barrier function within 2 to 4 wk of birth regardless of gestational age. A recent longitudinal study in premature infants suggests that the barrier may be mature at about 30 wk, but that for neonates younger than 25 wk estimated gestational age, postnatal barrier maturation may take as long as 5 to 7 wk (Kali *et al*, 1998). The high TEWL from premature infant skin can lead to multiple complications and a mainstay of therapy is to prevent this loss by maintaining a humidified environment or using petrolatum-based topical preparations until a competent barrier develops (Siegfried, 1998). In the future, this problem may be approached by stimulating the normal development of barrier function (either *in utero*, if possible, or postnatally) and/or providing a more physiologic temporary artificial barrier. Studies of fetal rat barrier development suggest that acceleration of this process is possible (Williams *et al*, 1998; Hanley *et al*, 1999), but this has not yet been shown in humans. In fact, a recent study of barrier maturation in preterm infants showed no stimulation by the administration of antenatal steroids and no difference between the sexes (Jain *et al*, 2000), although steroids stimulate, and testosterone inhibits, barrier development in the rat model. If topically applied physiologic lipids must enter LG and be extruded to improve barrier function (see below), this approach may not work in premature skin where LG assembly and secretion might be relatively undeveloped. If a mix of appropriate lipids could be prepared such that they would function as they do *in vivo*, however, repeated topical application should provide a physiologic barrier until normal epidermal maturation occurs. Any improvement in the current management of these fragile infants would be a valuable clinical contribution from basic work on epidermal barrier development and function.

"MOISTURIZERS" AND BARRIER CREAMS

The use of topically applied materials to improve the appearance, function, and "feel" of skin is as old as human life. Our knowledge about the mechanisms by which all of these agents work continues to evolve along with our understanding of SC structure and function and at present is incomplete. As discussed above, flaky "dry" skin is a heterogeneous condition with varied pathogenetic mechanisms that may or may not be associated with altered barrier function. It follows that controlled studies in homogeneous populations of patients would be necessary to establish the efficacy of an agent or product for a given condition.

The term moisturizer implies that the substance applied adds water and/or retains water in the SC. This is true for many of the products in use today, although the mechanism by which this is accomplished may vary. In addition, "moisturizing" substances are known to have a variety of less well defined effects on SC function separate from their effects on water content. Urea, propylene glycol, glycerin, and hydroxy acids (especially lactic acid) are humectants (water holding) and are used in many moisturizing formulations; however, they all also function as exfoliants, i.e., they promote desquamation. Although some of these substances are referred to as "keratolytics", true protein denaturation only occurs at high concentrations that are not used in moisturizing formulations. Whether the effect of these agents on desquamation is due solely to the increased water content or whether they have other effects on the desquamation process has not been completely worked out.

Another mechanism for moisturizing skin is to provide an exogenous barrier to water loss (TEWL) so that more water is retained in the SC, a "barrier cream". This is the mechanism by which petrolatum works, but rather than simply forming a film on the skin surface, it has been shown in hairless mice that petrolatum penetrates into the intercellular spaces of the SC to provide this function (Ghadially *et al*, 1992). Studies in hairless mice have also shown that certain combinations of SC lipids in optimal ratios can accelerate restoration of barrier function following tape stripping or acetone abrogation of the barrier (Mao-Quiang *et al*, 1995, 1996). Importantly, certain lipid mixes actually inhibited restoration of barrier function, which has implications for the design of topical products. There are relatively few studies on human skin of products containing the appropriate mix of SC lipids, with varying results (Loden and Barany, 2000; Chamlin *et al*, 2002). Additional well-controlled trials in defined human populations are needed to determine if mixtures of SC lipids are superior to other formulations.

In mouse skin, it is argued that topically applied lipids can permeate to the granular layer where they become part of LG membranes and are then extruded into the intercellular space to form the intercellular lamellae (Mao-Quiang *et al*, 1995). These findings, however, need to be confirmed in additional animal models and extended to human skin. It has also been shown that a mixture of SC lipids applied to lipid-extracted SC sheets can restore barrier function (Onken and Moyer, 1963; Imokawa *et al*, 1991) and reform intercellular lamellae (Imokawa *et al*, 1991) and

that mixtures of SC lipids can form bilayer structures *in vitro* (Kuempel *et al*, 1998). This suggests that *in situ* formation of barrier lipid membranes by topically applied lipids is another possible mechanism of barrier restoration. Ultimately, the goal is to be able to tailor topical products to the needs of patients based on specific knowledge of their underlying epidermal defects and a more complete understanding of how these products work at the molecular level.

OTHER COMPONENTS OF EPIDERMAL BARRIER FUNCTION

Although tight junctions have been sporadically observed in epidermis for many years, and tight junction proteins are expressed in epidermis (Morita *et al*, 1998), it was not until the recent development of a claudin-1 knockout mouse (Furuse *et al*, 2002) that the functional role of epidermal tight junctions was examined. The neonatal knockout mice showed wrinkled skin with markedly increased TEWL and died within 1 d of birth. Expression of loricrin, involucrin, and transglutaminase were normal, and lamellar lipid structures in the LG extrusion zone and the SC appeared normal. Whereas it is not surprising, given the importance of the epidermal barrier, that there should be more than one mechanism contributing to barrier function, it is interesting that neither system is able to compensate for defects in the other. As claudin-1-deficient SC appears different on histology and electron microscopy (more compact and without a "basketweave" artifact), it will be important to conduct more detailed studies of the lipid composition and structure in these mice to determine whether or not lipid alterations contribute to the diminished barrier function.

CHALLENGES

Relatively few mechanistic studies of barrier repair or moisturizer effects on human skin have been performed. Short-term acetone treatment, a commonly used "barrier disrupter" in many models, does not extract significant amounts of barrier lipids from normal human skin (Onken and Moyer, 1963; Adams *et al*, 1993) and the mechanism of its effect on barrier function has not been sufficiently investigated. A variety of solvents, including acetone, may give spurious TEWL readings (Morrison, 1992; Adams *et al*, 1993) suggesting that solvent-induced barrier dysfunction may be a less desirable model. Other models of barrier disruption, both experimentally induced and natural (due to disease, genetic manipulation, or environmental conditions) are available, but there remains a need for more sophisticated and controlled methods to disrupt barrier function. Because TEWL data are affected by a number of variables, including varying by up to about 20% based on circadian rhythms alone (Le Fur *et al*, 2001), measurements obtained after experimental manipulation of the skin require very careful interpretation (McCallion and Li Wan Po, 1995; Orth and Appa, 2000) with attention to physiologic and clinical *versus* statistical significance. In fact, recent studies by Chilcott *et al* (2002) showed no correlation between measured TEWL and skin barrier function. Although these rather startling findings need to be duplicated by other investigators and in other models, the authors correctly conclude that further work needs to be done on the interpretation of TEWL. It is best for studies of SC function to measure multiple parameters including clinical appearance, TEWL, SC water content, permeability to exogenous substances, lipid content and composition (and ideally synthesis), and morphology. Although the use of ruthenium tetroxide postfixation in electron microscopy allows visualization of the SC lipid lamellae and is now widely used to assess changes in lamellar structure under a variety of conditions, this technique is far from optimal for overall preservation of tissue architecture and is known to result in numerous artifacts (Swartzendruber *et al*, 1995). For this reason, establishing true differences between normal *versus* abnormal tissue or treated *versus* untreated tissue using this technique can be problematic. For both osmium- and ruthenium-fixed samples, it is best to have the microscopist blinded to the status of the tissues if possible, multiple samples and sections must be examined, and nondramatic findings of difference quantitated wherever possible.

FUTURE DIRECTIONS

Studies of epidermal differentiation in keratinocyte culture models and in fetal development models will continue to improve our understanding of the mechanisms underlying SC barrier formation and how they are regulated. Biophysical studies of SC lipids will delineate the molecular basis for their barrier properties. Sophisticated cell-free *in vitro* systems will be developed to study lamellar "granule" assembly and the membrane dynamics involved in the formation of SC lamellae. Transgenic and knockout mice with unexpected barrier defects will be helpful in delineating new regulatory pathways in barrier formation. The development of rodent models with defects in specific aspects of barrier lipid metabolism will improve our understanding of barrier disruption, as we have already seen with β-glucocerebrosidase-deficient mice and acid sphingomyelinase-deficient mice. Careful interpretation and clinical application of all of these findings will greatly improve the specificity and efficacy of treatments for human skin with abnormal SC structure and function.

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