Impaired Sphingomyelinase Activity and Epidermal Differentiation in Atopic Dermatitis

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A defective permeability barrier leads to the penetration of environmental allergens into the skin and initiates immunological reactions and inflammation crucially involved in the pathogenesis of atopic dermatitis (AD). Decreased stratum corneum ceramide content may cause the defect in permeability barrier function consistently found in AD. Acid and neutral sphingomyelinase (A- and N-SMase) generate ceramides with structural and signal transduction functions in epidermal proliferation and differentiation. We determined epidermal SMase activities, DNA synthesis, involucrin, loricrin, filaggrin, and keratin expression in lesional and non-lesional skin of AD patients. We found decreased epidermal A-SMase activity in lesional and non-lesional skin, correlating with reduced stratum corneum ceramide content and disturbed barrier function. N-SMase activity was reduced in non-lesional skin and more significantly reduced in lesional skin, correlating with impaired expression of cornified envelope proteins and keratins, important for skin barrier function. Changes in involucrin, loricrin, filaggrin, keratin K 5 (basal) and K 16 (proliferation associated) were noticed in non-lesional and lesional skin, whereas changes in K 10 (suprabasal), K 6 (proliferation associated), and K 17 (inflammation associated) were found only in lesional skin. In summary, reduction in SMase-generating ceramides and impaired differentiation are involved in the defective barrier function found in AD.

Key words: atopic dermatitis/filaggrin/involucrin/keratin/loricrin/sphingomyelinase
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The stratum corneum, the main permeability barrier, is formed from extracellular lipids and corneocytes during epidermal differentiation of the skin (Elias and Menon, 1991; Lee *et al*, 1992; Mauro *et al*, 1998a, b). Epidermal lipids are synthesized by keratinocytes and stored in epidermal lamellar bodies, which contain cholesterol, phospholipids, glucosylceramides, and hydrolytic enzymes including β-glucocerebrosidase and acid sphingomyelinase (A-SMase). The hydrolytic enzymes are delivered to stratum corneum interstices where they convert secreted glucosylceramides and phospholipids, including sphingomyelin, into ceramides and free fatty acids (Schürer and Elias, 1991). Ceramides, due to their amphiphilic structure and extremely long-chain, constituent N-acyl fatty acids, are essential for barrier function. In keratinocytes, TNF rapidly activates two distinct types of SMases, the endosomal A-SMase and a cell membrane-associated neutral sphingomyelinase (N-SMase) (Wiegmann *et al*, 1994). The subcellular site of production apparently determines the mode of action of ceramides. We and others have recently shown that A-SMase plays a major role in ceramide generation for epidermal barrier function (Jensen *et al*, 1999, Schmuth *et al*, 2000). Epidermal sphingomyelins of different structures are precursors for two of the seven known stratum corneum ceramides, ceramides 2 and 5. Ceramide 2 is quantitatively the most important ceramide in the epidermis and ceramide 5 is probably exclusively obtained by sphingomyelin hydrolysis (Robson *et al*, 1994; Vicanova *et al*, 1998; Uchida *et al*, 2000). Beyond their structural importance, SMase-produced ceramides have been recognized as an important second messenger in intracellular signaling of various cytokines and growth factors, depending on cell type.

Epidermal ceramides are obtained by a synthetic pathway (Holleran *et al*, 1991a, b) and through hydrolysis from glucosylceramide (Holleran *et al*, 1993, 1994) and sphingomyelin during a step regulated by the enzyme A-SMase (Bowser and Gray, 1978). Atopic dermatitis (AD) samples displayed a decrease in total lipids, sterol esters, and phospholipids including sphingomyelin, as well as an increase in free fatty acids and sterols compared with normal controls (Mustakallio *et al*, 1967; Jakobza *et al*, 1981; Barth *et al*, 1989; Schäfer and Kragballe, 1991). Also, AD samples showed a significant decrease in ceramide content and all ceramide subfractions (Melnik *et al*, 1988, 1989a and b; Imokawa *et al*, 1991, Yamamoto *et al*, 1991). Ceramide content in the stratum corneum correlated with barrier function in AD (Di Nardo *et al*, 1998).

Epidermal proliferation and differentiation are also very important for permeability barrier formation in the stratum corneum (Ekanayake-Mudiyanselage *et al*, 1998; Marekov

Abbreviations: AD, atopic dermatitis; A-SMase, acid sphingomyelinase; N-SMase, neutral sphingomyelinase; TEWL, transepidermal water loss

and Steinert, 1998). Keratinocytes synthesize specific basal (K 5 and K 14) and suprabasal (K 1 and K 10) keratins, as well as cornified envelope associated proteins, in particular involucrin and loricrin (Fuchs and Green, 1980; Cline and Rice, 1983; Watt, 1983; Moll *et al*, 1984; Wertz *et al*, 1989; Mehrel *et al*, 1990; Hohl, 1993; Ekanayake-Mudiyanselage *et al*, 1998).

During cornification, the phospholipid-enriched plasma membrane is replaced by a ceramide-containing membrane bilayer, which attaches covalently to involucrin, envoplakin, and periplakin moieties on the extracellular surface of the cornified envelope by ω-hydroxyester bonds (Marekov and Steinert, 1998). Transepidermal water loss (TEWL, as marker for skin permeability barrier function) levels correlate to the content of covalently bound ceramides (Meguro *et al*, 2000). Levels of covalently bound ceramides have recently been determined in AD. Protein-bound ω-hydroxyceramides represent 50% of protein-bound lipids in healthy skin, but significantly less in non-lesional and lesional skin (Macheleit *et al*, 2002).

Although defective skin permeability barrier function is an accepted element of AD, abnormality of the epidermis is regarded as a consequence of inflammation (Bos *et al*, 1994). Even in the asymptomatic skin of atopics, however, disturbance of epidermal barrier function through scratching promotes penetration of antigens as well as eczematous dermatitis (Ogawa and Yoshiike, 1993). Lifestyle modifications such as excessive use of detergents and shampoos remove skin lipids and irritate the skin (McNally *et al*, 1998). Finally, psychological stress has been shown to increase skin irritability and abnormal barrier function. (Elias *et al*, 1997, 1999; Altemus *et al*, 2001; Garg *et al*, 2001). Atopic patients, however, display abnormal barrier function in both symptomatic and asymptomatic skin, leading to the assumption that a barrier-initiated cytokine cascade maintains the homeostasis of the skin barrier (Wood *et al*, 1992, 1996; Gfesser *et al*, 1996).

Disturbances in barrier function enable aeroallergens to penetrate the skin more easily, perpetuating the cycle of eczematous lesions (Ghadially *et al*, 1992). Additional studies have shown a 2–5-fold increase in basal TEWL in non-lesional skin in AD (Yoshiike *et al*, 1993). In contrast, TEWL levels and stratum corneum water content became normal in patients free from AD symptoms for more than 5 y (Matsumoto *et al*, 2000).

AD patients showed a thinner horny layer as well as a reduced mean corneocyte area in different types of clinically asymptomatic skin (Al-Jaberi and Marks, 1984). Only AD patients showed an increased sensitivity to chemical irritants and increased epidermal thickness (Nassif *et al*, 1994). In forced desquamation, atopic patients released more corneocyte clumps from the epidermis than controls (Watanabe *et al*, 1991). These results suggest that, in addition to the known changes in lipid content, changes in epidermal differentiation may occur in AD.

In this study, we found a decrease in the activities of ceramide-generating SMases important for structural function and lipid signaling. SMases may not only generate ceramides for structural function in the stratum corneum lipid bilayers but are involved in the regulation of proliferation and differentiation in AD.

Results

Patients with AD showed changes in TEWL and stratum corneum hydration To examine whether our patients exhibited changes in barrier function and skin hydration, we determined TEWL and stratum corneum conductance by biophysical methods (Pinnagoda *et al*, 1990; Hashimoto-Kumasaka *et al*, 1993). TEWL was 2-fold increased in non-lesional and 4-fold increased in lesional skin of patients with AD compared with the skin of normal controls ($p < 0.05$, $n = 8$, respectively) (Fig 1*A*). Hydration was significantly reduced in non-lesional (−24.5%) as well as lesional (−39.1%) epidermis of AD compared with controls (both $p < 0.005$, $n = 8$) (Fig 1*B*).

A- and N-SMase are reduced in AD In several previous publications, a reduced or changed ceramide content in the stratum corneum has been described (Melnik *et al*, 1988, 1989; Melnik and Plewig, 1989; Imokawa *et al*, 1991; Yamamoto *et al*, 1991; Di Nardo *et al*, 1998), but the mechanisms leading to this disturbance are largely unknown. Therefore, we examined whether SMase activity, which releases ceramides through sphingomyelin-hydrolysis, is impaired in AD. We directly assayed enzyme activity in epidermal samples obtained from lesional and non-lesional skin in AD and in healthy controls. In healthy skin, the activity of A-SMase was approximately 4.2 nmol per h per mg protein, while the activity of N-SMase was approximately 1.7 nmol per h per mg. Both A- and N-SMase showed reduced levels of activity in AD. A-SMase showed 63% reduction in non-lesional and 48% reduction in lesional epidermis, corresponding to 2.6 nmol per h per mg and 2.0 nmol per h per mg, respectively (both $p < 0.05$, $n = 6$) in patients with AD compared with the skin of healthy volunteers (Fig 2*A*). N-SMase activity was reduced by 50% in non-lesional skin and by 70% in lesional skin, corresponding to 0.85 nmol per h per mg and 1.20 nmol per h per mg, respectively (both: $p < 0.05$, $n = 5$) (Fig 2*B*). These

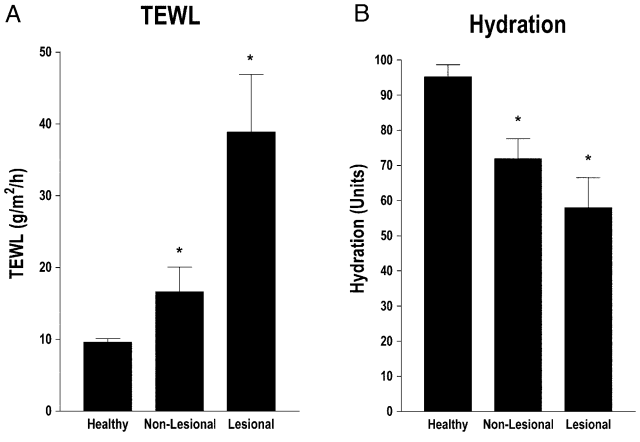


Figure 1
Reduced barrier function and skin hydration in AD. TEWL and skin hydration were measured in lesional and non-lesional skin of patients with AD and in healthy controls, using a Tewameter TM210 and Corneometer. TEWL (*A*) was increased in non-lesional and lesional skin of patients with AD compared to the skin of normal controls ($p < 0.05$, $n = 8$, respectively). Hydration (*B*) was significantly reduced in non-lesional as well as lesional epidermis of AD compared with controls (both $p < 0.005$, $n = 8$).

results suggest that a disturbed hydrolysis of epidermal sphingomyelin may account for the reduced ceramide levels in AD.

***In vitro* A-SMase assays has a 94% specificity for A-SMase** Since not only A-SMase, but also the recently described sphingomyelin deacylase uses the same substrate (sphingomyelin) to produce lyso-sphingomyelin, we had to verify that measuring the liberation of [^{14}C]-phosphorylcholine in the aqueous phase of the *in vitro* assays using N-methyl [^{14}C]-sphingomyelin as substrate is not contaminated with [^{14}C]-lyso-sphingomyelin derived from sphingomyelin deacylase activity. For this, we assayed purified A-SMase and purified sphingomyelin deacylase at conditions optimal for A-SMase in the presence of EDTA and optimal for sphingomyelin deacylase with Ca^{2+} instead of EDTA. As depicted in Fig 3*A*, Ca^{2+} only slightly reduced the production of phosphorylcholine derived from A-SMase activity, while the production of lyso-sphingomyelin by sphingomyelin deacylase was strictly dependent on Ca^{2+}. Since the assays used before to determine A-SMase in the skin biopsies contained EDTA and no $CaCl_2$, a cross-activity of sphingomyelin deacylase in the assay appears unlikely. We repeated the assays using skin biopsies from healthy controls, and as shown in Fig 3*B*, in the presence of Ca^{2+} only lyso-sphingomyelin is detected, while in the absence of Ca^{2+} and presence of EDTA, the prominent band corresponds to phosphorylcholine and only a minor band (less than 6% of the total radioactivity) occurred at the position of lyso-sphingomyelin. Thus, measuring radioactivity by scintillation counting of the aqueous phase of the *in vitro* A-SMase assays is specific for A-SMase at least by 94%.

Increased proliferation in epidermal samples of patients with AD We have previously shown that epidermal DNA synthesis, a marker for proliferation, increases during permeability barrier repair after experimental barrier disruption (Proksch *et al*, 1991). In this study using the Ki-67 marker, we found that lesional skin exhibited 5-fold higher amounts of labeled cell nuclei in the basal and the lower spinous layers (Fig 4). Even in non-lesional skin epidermis, however, the number of labeled cells was significantly increased (2-fold increased compared with controls). This demonstrates increased proliferation in non-lesional and more pronounced in lesional skin of AD.

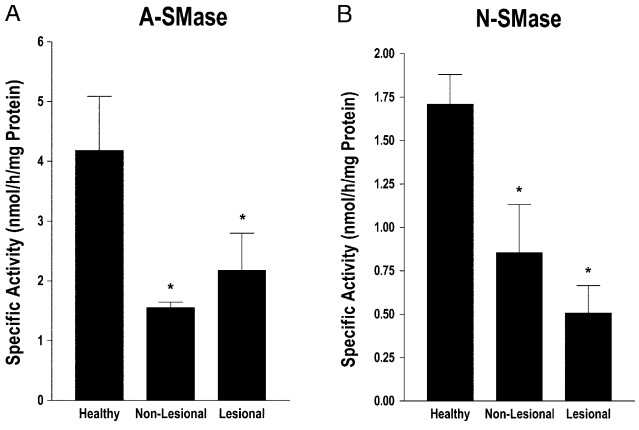


Figure 2
Reduced A- and N-SMase activities in AD. The enzymatic activities of A-SMase and N-SMase were determined after extraction from punch biopsies by mixed micellar *in vitro* assays using [^{14}C] sphingomyelin as substrate. A-SMase (*A*) showed a significant reduction in lesional and non-lesional skin in patients with AD compared with the skin of healthy volunteers (both $p < 0.05$, $n = 6$). Also, N-SMase (*B*) activity was reduced in non-lesional and in lesional skin (both $p < 0.05$, $n = 5$).

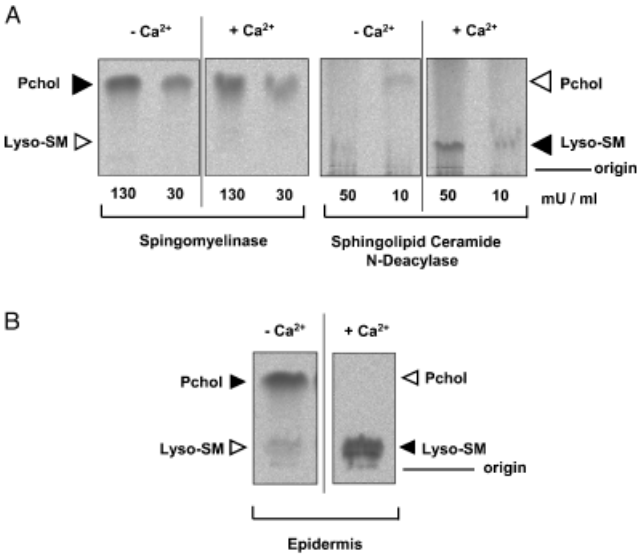
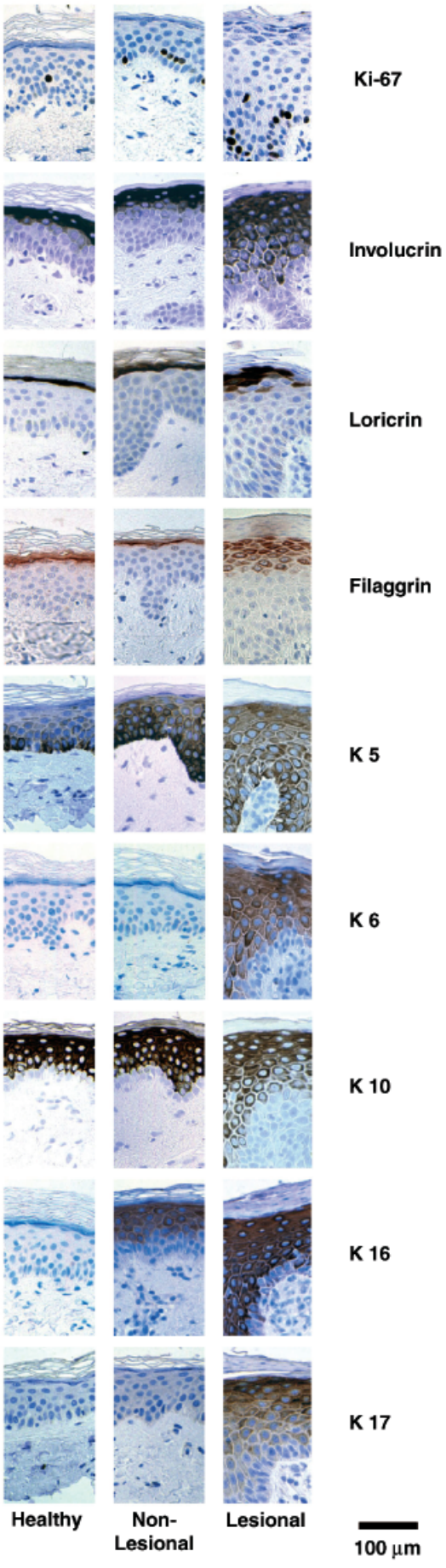


Figure 3
Determination of A-SMase and sphingomyelin deacylase activity depending on Ca^{2+}. As not only the ceramide-generating A-SMase, but also sphingomyelin deacylase uses sphingomyelin as substrate to produce lyso-sphingomyelin, we needed to verify that measurements of the liberation of [^{14}C]-phosphorylcholine in the aqueous phase of the *in vitro* assays using [^{14}C]-N-methyl sphingomyelin as substrate were not contaminated with [^{14}C]-lyso-sphingomyelin derived from sphingomyelin deacylase activity. Exogenous A-SMase and sphingomyelin deacylase were assayed in either buffer D containing EDTA or in buffer E, containing $CaCl_2$. As depicted in (*A*), Ca^{2+} only slightly reduced the production of phosphorylcholine derived from A-SMase (left two blots), whereas the production of lyso-sphingomyelin by sphingomyelin deacylase (right two blots) was strictly dependent on Ca^{2+}. Using skin biopsies from healthy controls in the presence of Ca^{2+} only lyso-sphingomyelin is detected, whereas in the absence of Ca^{2+} and presence of EDTA, the prominent band corresponds to phosphorylcholine and only a minor band (less than 6% of the total radioactivity) occurred at the position of lyso-sphingomyelin (*B*).

Altered distribution of involucrin, loricrin, filaggrin, and keratins in the epidermis Immunohistochemistry with several differentiation-associated proteins is shown in Fig 4. In healthy epidermis during normal differentiation, involucrin is expressed only in the upper spinous layers and the stratum granulosum. In non-lesional atopic skin, the band of involucrin staining broadened and was already found in the middle part of the spinous layers. In lesional atopic skin, we found premature expression of involucrin already in the lower spinous layers. The intensity of the staining reduced, however, with dark brown staining appearing in healthy control skin and light brown staining in diseased skin.

The amount of loricrin was enhanced in non-lesional as well as in lesional atopic skin. Whereas loricrin staining in healthy skin was limited to the upper stratum granulosum, staining appeared in the entire granular cell layer of non-lesional skin. Loricrin staining extended to the upper spinous layers in an uneven pattern in lesional skin. The



thickness of the stained layers increased 2-fold as compared with non-lesional skin.

In healthy and in non-lesional skin, filaggrin is primarily expressed in the granular and the transition zone to the lower horny layers of the skin. The filaggrin staining appears thinned in non-lesional skin. In lesional skin, the thickness of the stained band increased 5-fold. This was primarily due to increased thickness of the granular layer in concert with an overall increase of epidermal thickness, but filaggrin does not reach the horny layers in lesional epidermis and stains primarily to the granules in the stratum granulosum cells. Remarkably, staining intensity for filaggrin in non-lesional and lesional skin of AD was significantly reduced.

Immunostaining for basal K 5 was primarily restricted to the basal epidermal layer in healthy skin. In non-lesional skin, extension to suprabasal epidermal layers occurred, but staining intensity remained most pronounced in the basal layer. In lesional atopic skin, we found equal distribution of K 5 throughout the entire nucleated epidermal layers.

Proliferation-associated K 6 was not expressed in healthy control skin or in non-lesional AD skin. In contrast, pronounced staining in the suprabasal layers appeared in lesional AD skin.

Suprabasal/differentiation-related K 10 expression was found in the entire suprabasal compartment in healthy control and in non-lesional AD skin. In lesional AD, staining for K 10 was concentrated in the granular and upper spinous layers, but reduced in the lower spinous layers of the epidermis.

The proliferation-associated cytokeratin K 16 was not expressed in healthy skin. In non-lesional skin, intense staining was already noticeable in the middle and upper parts of the spinous layers. In lesional AD, intense staining occurred in all the suprabasal layers.

The inflammation-associated K 17 was not expressed in healthy skin or in non-lesional skin. In lesional skin, staining was found in all suprabasal layers with continually increasing intensity toward the stratum granulosum.

Quantitative analysis of involucrin and loricrin as markers of terminal differentiation using western blotting Using immunoblotting analysis, we demonstrated that extracts of epidermal proteins contain involucrin as well as loricrin, both in the lesional and non-lesional skin of patients with AD and healthy volunteers. We found reduced levels of mature involucrin (65 kDa) in non-lesional skin (−35%, NS,

Figure 4
Impaired epidermal proliferation and differentiation in non-lesional and lesional skin of patients with AD as shown by immunohistochemistry. Increased labeling of proliferating keratinocytes is seen in non-lesional and more pronounced in lesional epidermis (Ki-67 antibody staining). Premature expression of involucrin was found in non-lesional and more pronounced in lesional skin. Staining intensity, however, was reduced. The loricrin staining band broadened in lesional skin and broadened more in non-lesional AD. Basal K 5 expression was restricted to the basal layer in normal skin, but extended to the stratum spinosum in non-lesional and even to the entire nucleated epidermis in lesional AD. K 6, known to be proliferation associated, appeared in lesional AD epidermis. The expression of differentiation related/suprabasal K 10 was found in the entire suprabasal epidermis in normal and in non-lesional skin of AD, but staining was reduced in diseased skin. K 16 labeling was already found in non-lesional skin and more pronounced in lesional skin of AD, whereas K 17 showed immunostaining only in lesional epidermis.

the differences did not reach significance due to the small sample size of human biopsies, n = 6) and even more reduced levels in lesional epidermis (−52%, $p < 0.05$, n = 6) of AD (Fig 5*A*).

With immunohistochemistry, the broadening of the stained band with unchanged intensity already suggested an increase in the total amount of loricrin. In western blot analysis, loricrin levels showed a 3-fold increase in non-lesional (+233%, $p < 0.01$, n = 6), and lesional epidermis (+341%, $p < 0.01$, n = 6) compared with control samples (Fig 5*B*).

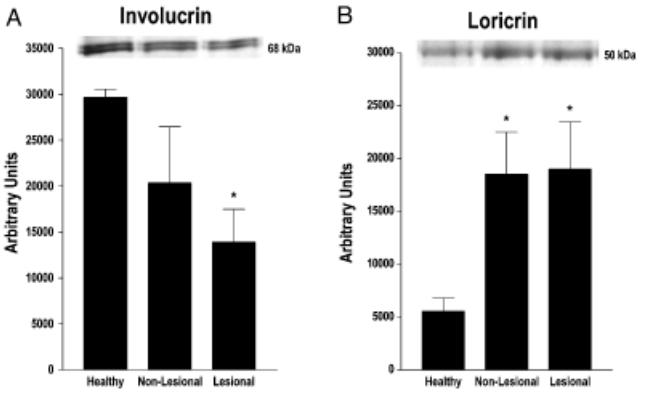


Figure 5
Reduced involucrin and increased loricrin in AD as shown by western blotting. Epidermal proteins from punch biopsies were extracted and analyzed by western blotting. Specific bands for involucrin (*A*) and loricrin (*B*) were identified with immunostaining and quantified by 2D laser scanning densitometry and given in arbitrary units. Involucrin levels decreased, whereas loricrin levels increased significantly in lesional and non-lesional skin of AD (involucrin: non-lesional NS, n = 6, the differences did not reach significance due to the small sample size of human biopsies, lesional $p < 0.01$, n = 6, loricrin: $p < 0.01$, n = 6).

Discussion

In this study, we found a significant reduction of A-SMase activity in lesional as well as in non-lesional epidermal samples from patients with AD compared with normal controls. The reduced A-SMase activity is most probably responsible in part for the reduced stratum corneum ceramide content, which manifests in AD (Melnik *et al*, 1988, 1989; Melnik and Plewig, 1989; Imokawa *et al*, 1991; Yamamoto *et al*, 1991; Di Nardo *et al*, 1998). Previously, A-SMase protein immunostaining extending from the upper spinous cell layers to the upper stratum corneum and a slight increase in lesional *versus* non-lesional stratum corneum in AD has been described (Kusuda *et al*, 1998). The immunostaining result does not necessarily contradict our findings, as it shows the localization and content of the protein, but not the level of activity of the enzyme. In contrast, we directly measured A-SMase activity with a specific *in vitro* enzyme assay. Our results correlate reduced A-SMase activity with reduced ceramide content in AD.

Recently, it was found that the AD epidermis contains elevated levels of a new enzyme glucosylceramide/sphingomyelin deacylase, which cleaves the N-acyl linkage of both sphingomyelins and glucosylceramides. It was claimed that this may be the cause of the reduced ceramide content in AD, because sphingomyelin deacylase reduces ceramides by releasing free fatty acids and sphingosyl-phosphorylcholine (Murata *et al*, 1996; Hara *et al*, 2000; Higuchi *et al*, 2000; Imokawa, 2001). Sphingomyelin deacylase uses the same substrate sphingomyelin as A-SMase, but in contrast to A-SMase, its activity strictly depends on Ca^{2+}. Since our in-vitro A-SMase assays contain EDTA instead of $CaCl_2$, we can exclude enzymatic activity of sphingomyelin deacylase (Fig 3*A*). In the skin biopsies we found a residual amount of radiolabeled lysosphingomyelin of less than 6% (Fig 3*B*). The activity of acid sphingomyelin deacylase was reportedly enhanced in atopic skin (Hara *et al*, 2000), and we measured a strong reduction in A-SMase activity, indicating that the two assays indeed measure both enzymes independently. Our data show that SMases (which are downregulated in AD) do not contribute to the reduced sphingomyelin levels. Given the possibility that sphingomyelin deacylase and SMases compete for the same substrate, the high sphingomyelin deacylase activity resulting in reduced sphingomyelin levels may even be a reason for the downregulated SMases observed in our study. The decreased SMases shown in our report will, in concert with enhanced sphingomyelin deacylase activity, further reduce the availability of ceramides in the stratum corneum of atopic skin.

Jin *et al* examined β-glucocerebrosidase and ceramidase activities in the stratum corneum of AD and age-related dry skin. As they did not find differences in either β-glucocerebrosidase or ceramidase activities in uninvolved stratum corneum of AD, the decrease of ceramides in AD could not be attributed to enhanced ceramide degradation (Jin *et al*, 1994). Likewise, the presence of unchanged β-glucocerebrosidase in stratum corneum from non-eczematous dry skin of AD was confirmed by Redoules (Redoules *et al*, 1999; reviewed in Proksch *et al*, 2003).

We found that N-SMase activity was also reduced, but based on the comparatively lower activity of this enzyme to A-SMase, the contribution of A-SMase appears to be more important. The role of N-SMase in the skin is only partially known. It has been described that stress-induced changes in the immune system and in keratinocyte cell growth are mediated by the SMase signal transduction pathway leading to changes in membrane-bound N-SMase activity (Buisson-Legendre *et al*, 1999; Korneva *et al*, 2001). Clinical evidence confirms stress-induced aggravation of AD. These changes correlate to our results of impaired N-SMase activity and epidermal differentiation.

We found a several-fold increase in epidermal proliferation in AD after staining with Ki-67 antibody. Similar results have been previously described after *in vitro* incorporation with tritiated thymidine (Van Neste *et al*, 1979). Increased proliferation may be an attempt to reconstitute normal barrier function and to remove invaded antigens. We have previously shown that epidermal DNA synthesis increases during permeability barrier repair after experimental skin injury (Proksch *et al*, 1991). Here we show that epidermal proliferation is already increased in uninvolved AD skin. An increase in epidermal proliferation was found previously in young and aged dry skin (Engelke *et al*, 1997). AD skin is well known for dry skin conditions; reduced stratum corneum hydration was also noted in our patients. The

increase in epidermal proliferation may also be related to dry skin in AD.

Increased epidermal proliferation is often linked to disturbed differentiation, as there may not be sufficient time for proper differentiation during exaggerated epidermal proliferation. Epidermal differentiation is of crucial importance for the integrity of the permeability barrier (Ekanayake-Mudiyanselage *et al*, 1998). As filaggrin expression is linked to stratum corneum hydration and filaggrin content is reduced in atopic skin, it has been suggested that frequently seen skin dryness in atopic patients may derive from these filaggrin changes (Scott and Harding, 1986). A decreased staining for involucrin and filaggrin has been described previously in non-lesional skin of AD and decreased filaggrin was confirmed by the ELISA method (Seguchi *et al*, 1996). In our studies, total staining density of involucrin and filaggrin were reduced, thereby suggesting a reduced protein content in diseased skin. The remainder of the staining extended over the normal localization for involucrin and filaggrin. A significantly reduced involucrin protein content was found by western blot analysis in lesional skin and even more pronouncedly in non-lesional skin of AD. Involucrin serves as a substrate for the covalent attachment of ceramides to the cornified envelope (Wertz *et al*, 1986; Marekov and Steinert, 1998). Recently, it was found that the amount of protein-bound ω-hydroxyceramides was significantly decreased in non-lesional skin and drastically reduced in lesional skin of AD (Macheleidt *et al*, 2002). According to our results, reduced involucrin content may cause the reduced amount of ω-hydroxyceramides in AD by failing to provide a sufficient amount of substrate for the attachment of ceramides.

In contrast to involucrin and filaggrin, loricrin expression was significantly increased in non-lesional and lesional epidermis of AD, as shown by western blotting. Immunohistology also revealed increased staining in the upper epidermis. Though the genes for filaggrin, involucrin, and loricrin are localized on the same gene 1q21, also known as the epidermal differentiation complex, the loricrin gene does not necessarily share the same regulatory elements with the filaggrin and involucrin genes (Yoneda *et al*, 1992). The reason for the increase in loricrin expression in AD is unclear. Hohl described overexpression of loricrin and involucrin in orthokeratotic acanthosis, such as lichen planus. In unspecified dermatitis, however, he found reduced loricrin expression (Hohl, 1993). The altered expression of involucrin, filaggrin, and loricrin is in agreement with recently published work on genome screening for AD. Cookson *et al* found genetic linkage to AD on chromosome 1q21 (Cookson *et al*, 2001).

We also found that changes in keratins occurred in AD. Extended expression of basal keratin K 5 was already noted in non-lesional skin and was even more pronounced in lesional skin of AD. Previously, we have found increased K 5 immunostaining in young as well as aged dry skin (Engelke *et al*, 1997). The expression of the suprabasal/differentiation related K 10 was reduced in lesional AD only. Staining for K 10 was concentrated in the granular and upper spinous layers, but reduced in the lower spinous layers of the epidermis. Reduced K 10 was also noted in dry skin in our previous studies (Engelke *et al*, 1997). We have also found in previous studies that expression of differentiation marker K 10 is impaired after acute experimental barrier disruption (Ekanayake-Mudiyanselage *et al*, 1998). In K 10 deficient mice, a defect in permeability barrier function has been found (Jensen *et al*, 2000). Therefore, reduced K 10 in lesional skin of AD may be related to dry skin conditions and to disturbed barrier function. But K 10 may not be a very sensitive marker for these conditions, as staining in non-lesional skin was unchanged when compared with normal skin.

Proliferation-associated cytokeratins were expressed in AD, a reasonable outcome with the increased proliferation already shown above. Staining with the cytokeratins K 6 and K 16, however, produced different results. K 6 was not expressed in healthy control and in non-lesional AD skin. In contrast, pronounced staining in the suprabasal levels occurred in lesional AD. While K 16 was not expressed in healthy skin, intense staining was already noted in the middle and upper parts of the spinous layers in non-lesional skin. In lesional AD, intense staining in all suprabasal layers occurred. The reason for these differences in K 6 and K 16 protein expression are unknown. The inflammation-associated K 17 was not expressed in healthy and non-lesional skin. In lesional skin, staining was found in all suprabasal layers and the intensity continuously increased toward the stratum granulosum. Clinically, redness as a sign of inflammation is present in lesional but not in non-lesional skin of AD.

In conclusion, our study shows that AD exhibits reduced A- and N-SMase activities. Reduced A-SMase activity may be partially responsible for the reduced content of stratum corneum ceramides. Reduced N-SMase activity may lead to changes in signal transduction, thereby regulating differentiation. The resulting reduction in involucrin could cause the reduced number of covalently bound ceramides in AD. Changes in epidermal lipids and differentiation lead to the disturbed barrier function present in AD, which consequently allow for the entry of harmful environmental substances into the skin, thereby triggering immunological reactions and inflammation.

Materials and Methods

Patients Twenty-five adult patients with AD (17 male and eight female) according to the criteria of Hanifin and Rajka, and 18 age and sex-matched non-atopic controls (nine male and nine female) were included in the study (Hanifin and Rajka, 1980). Skin biopsies were obtained from lesional and non-lesional skin on the backs or thighs of each patient. Individuals were selected for participation when they showed a severe form of AD (mean SCORAD of 54.2 SEM 3.2 and a IgE of 6301.1 kU per L SEM 1050.7 kU per L; SCORAD index combines objective (extent and intensity) with subjective (loss of sleep, pruritus) parameters. In addition, skin hydration and TEWL were also measured on patients with lesional and non-lesional AD and healthy controls, using a Tewameter TM210 and Corneometer (Courage + Khazaka, Germany). The local ethical commission approved the study protocol.

Isolation of epidermal samples Five mm punch biopsies were obtained from the backs of patients with confirmed AD, shock frozen, and stored at -70°C or fixed with 2.5% formaldehyde for immunohistochemistry. After thawing, the samples were immediately placed epidermal-side downward onto a covered petri dish

containing crushed ice. The skin pieces were scraped with a scalpel blade to remove excess subcutaneous fat and immersed at 37°C for 30 min. in 10 mM EDTA in Dulbecco's phosphate-buffered saline, calcium (Ca^{+})- and magnesium (Mg^{2+})-free (0.16 M NaCl, 0.01 M Na_2HPO_4, 0.03 M KCl, 0.01 M KH_2PO_4, pH 7.4). Thereafter, the epidermis was peeled away from the dermis in one piece by gentle scraping with a scalpel blade and dried on paper towels. Epidermal sheets were shredded into small pieces (<1 mm^3) with scissors and stored at -70°C.

***In vitro* assay for A- and N-SMase-activity** The epidermis samples were homogenized, respectively, in buffer A (for the A-SMase extraction: 0.2% Triton-X 100) or B (for the N-SMase extraction 20 mM hepes, 10 mM NaF, 2 mM EDTA, 10 mM $MgCl_2$, 30 mM p-nitrophenyl-phosphate, 100 μM NaVanadat, 10 mM α-glycero-phosphate, 1 mM phenyl-methyl-sulfonyl-fluoride, 1 mM PLA (pepstatin A, leupeptin, antipain), 750 μM ATP, and 0.2% NP-40) using a glass homogenizator (Potter S, Braun, Melsungen, Germany) on ice at 600 rpm in 400 μL. The cell debris and nuclei were removed by low-speed centrifugation at 420 × *g* for 10 min. The supernatants were used for the *in vitro* assay as described by Freinkel and Traczyk (1983): 20 μg protein from the supernatants were incubated for 2 h at 37°C in a reaction buffer C (250 mM sodium acetate and 1 mM EDTA at pH 5.0) or D (20 mM hepes and 10 mM $MgCl_2$ at pH 7.4) for A- or N-SMase (50 μg final volume), respectively, containing 2.25 μL of N-methyl-[^{14}C]-sphingomyelin (0.2 μCi per mL, specific activity 56.6 mCi per Mol, Amersham, Freiburg, Germany) as a substrate. The reaction was stopped by adding 800 μL chloroform:methanol (2:1, vol/vol) and 250 μL H_2O. [^{14}C]-phosphorylcholine, product from [^{14}C]-sphingomyelin, was extracted from the aqueous phase, identified by thin layer chromatography and routinely determined by liquid scintillation counting. The results are presented as nmol per h per mg protein.

***In vitro* assay for sphingomyelin deacylase-activity** For estimating the activity of endogenous epidermal sphingomyelin-deacylase activity, skin samples were prepared as described for measurements of A-SMase. 20 μg protein from the supernatants were incubated for 2 h at 37°C in reaction buffer E optimal for sphingomyelin deacylase (Murata *et al*, 1996; Hara *et al*, 2000) (250 mM sodium acetate and 20 mM $CaCl_2$ at pH 5.0) containing 2.25 μL of N-methyl-[^{14}C]-sphingomyelin (0.2 μCi per mL, specific activity 56.6 mCi per Mol, Amersham) as a substrate. The reaction was stopped by adding 800 μL chloroform: methanol (2:1, vol/vol) and 250 μL H_2O. [^{14}C]-lyso-sphingomyelin, products from [^{14}C]-sphingomyelin were extracted from the aqueous phase, identified by thin layer chromatography. Exogenous sphingolipid ceramide N-deacylase was purchased from Sigma (Germany), *in vitro* assays were performed in buffer D optimal for A-SMase and E, containing $CaCl_2$ for sphingolipid ceramide N-deacylase activity. Exogenous A-SMase (30 and 130 mU per mL) and sphingomyelin deacylase (10 and 40 mU per mL) were assayed each either in buffer D containing EDTA or in buffer E, containing $CaCl_2$ (A). To determine endogenous activity of epidermal A-SMase and epidermal sphingomyelin deacylase, assays were performed either in buffer D containing EDTA or in buffer E, containing $CaCl_2$ (B). Radioactive metabolites were extracted, the aqueous phases subjectes to thin layer chromatography and [^{14}C]-phosphorylcholine and [^{14}C] lyso-sphingomyelin identified by autoradiography in a FUJI S-2000 bioimager (Raytest, Germany).

Protein determination The protein content in epidermal homogenates was determined by the Bradford method using bovine serum albumin as standard (BCA-protein assay) (Bradford, 1976).

Immunohistochemistry staining Skin samples were fixed in formalin and embedded in paraffin. After deparaffination and rehydration, 5 μm thick sections were incubated with 3% H_2O_2 for 5 min. to block endogenous peroxidase activity and the rinsed sections exposed to 5 × 5 min. microwave irradiation (650 W) for antigen detection according to Hazelbag *et al* (1995). After blocking non-specific antibody binding by incubation with 20% normal pig serum (DAKO, Hamburg, Germany), the primary antibodies (anti-involucrin, anti-loricrin, anti-filaggrin, or anti-keratin 5, 6, 10, 16, and 17) were applied (Rosenthal *et al*, 1992; Hohl, 1993). A strep-AB-complex/HRP (DAKO, Hamburg, Germany) was used as a third antibody, followed by incubation with diaminobenzidine as peroxidase substrate.

Epidermal proliferation assay As described for the previously mentioned histological slides, slides were generated and incubated with Ki-67 antibodies. The stained sections (one per slide) were examined microscopically (× 160) by counting the labeled nuclei of interfollicular keratinocytes in seven microscopic fields/sections. Labeling index in samples of patients with AD was defined by numbers of stained cells per mm epidermal basal membrane.

Western blotting Epidermal samples were homogenized in 400 μL homogenization buffer (H-buffer: 40 mM hepes, 150 mM KCl, and 5 mM NaF, pH 7.4) using a glass homogenizer (Potter S, Braun, Melsungen, Germany) on ice at 600 rpm. The samples were lyzed with SDS-page application buffer (50 mM tris, 4 M urea, 1% SDS, 15% glycin, 0.01% bromphenolblue, and 9% mercaptoethanol, pH 6.8), and boiled for 10 min. Five to 15 mg protein in H-buffer were separated by SDS-page using 7.5%–12.5% polyacrylamide gels, followed by transfer to a nitrocellulose membrane sheet. The membranes were blocked with 2% skim milk in TBST, pH 7.0. After incubation with polyclonal rabbit-anti-human anti-loricrin or anti-involucrin antibody (Jackson Laboratories, Bar Harbor, Maine) the membranes were thoroughly washed with TBST and then incubated with HRP-conjugated second antibody. After incubation with strep-AB-complex/HRP, signals were detected using the ECL system (Amersham). Results are 2D laser scanned and given in arbitrary units representing density minus background. Actin quantification served as inner control of the western blot analysis.

Statistics Statistical significance was determined using two-tailed Student's *t*-test. Each sample was measured in duplicate and the results are presented as the mean ± SEM.

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