Abnormal Expression of Sphingomyelin Acylase in Atopic Dermatitis: An Etiologic Factor for Ceramide Deficiency?

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Previously, we demonstrated that there is a marked reduction in the amount of ceramide in the stratum corneum of both lesional and nonlesional forearms in atopic dermatitis (AD), suggesting that an insufficiency of ceramides in the stratum corneum is an etiologic factor in atopic dry and barrier-disrupted skin. In this study, we investigated, as a possible mechanism involved in the ceramide deficiency, whether sphingomyelin (SM) metabolism is altered in AD as compared to normal controls. In stripped stratum corneum and biopsied whole epidermis of patients with AD, SM hydrolysis as measured at pH 4.7 using [*choline-methyl*-^{14}C]sphingomyelin as a substrate were markedly increased by 27- and 7-fold, respectively. Radio–thin-layer chromatography of the reaction products revealed that, whereas the SM hydrolysis in age-matched normal controls were associated with sphingomyelinase (SMase) that degrades SM to yield ceramides and phosphorylcholine (PC), most of the SM hydrolysis detected in AD were attributable not to the SMase but to a hitherto undiscovered epidermal enzyme, SM acylase, which releases free fatty acid and sphingosyl-PC (Sph-PC) instead of ceramides. The potential of this acylase-like enzyme to generate Sph-PC through SM hydrolysis was corroborated by thin-layer chromatographic analysis of the reaction products obtained using porcine kidney acylase, followed by high-performance liquid chromatography-mass spectrometry. Furthermore, Sph-PC was also detected by high-performance liquid chromatography-mass spectrometry after incubation of SM with atopic stratum corneum samples. On the other hand, the stratum corneum of patients with contact dermatitis or chronic eczema exhibited neither increased SM hydrolysis nor the generation of Sph-PC upon radio–thin-layer chromatographic analysis. These findings suggest that SM metabolism is altered in AD, resulting in a decrease in levels of ceramides, which could be an etiologic factor in the continuous generation of atopic dry and barrier disrupted skin observed in AD. ***Key words: atopic dermatitis/ceramide/sphingomyelin/sphingomyelinase/sphingomyelin acylase. J Invest Dermatol 106:1242–1249, 1996***

Previously, we demonstrated that there is a marked decrease in ceramide level within the stratum corneum of both uninvolved and involved skin of patients with atopic dermatitis (Imokawa *et al*, 1991a). A different group of investigators also demonstrated a similar ceramide abnormality in atopic dermatitis (Yamamoto *et al*, 1991). As ceramides are an important determinant involved in water-holding properties (Imokawa and Hattori, 1985; Imokawa *et al*, 1986; Imokawa *et al*, 1991b) and stratum corneum barrier function (Lampe *et al*, 1983; Grubauer *et al*, 1989; Holleran *et al*, 1991a; Imokawa *et al*, 1994), the insufficiency of ceramides may provide an etiological basis for atopic dry and barrier-disrupted skin. The impaired function of the stratum corneum may trigger subsequent inflammatory reactions evoked by mites or foreign substances in atopic dermatitis, and therefore, clarification of the biological mechanisms involved in the ceramide deficiency would provide substantial insight into the pathogenesis of atopic dermatitis.

Because the amount of ceramide in the stratum corneum is regulated by the balance among the rate-limiting enzymes of sphingolipid base synthesis serine-palmitoyl transferase (Holleran *et al*, 1991b), ceramide-generating enzyme sphingomyelinase (SMase) (Menon *et al*, 1986; Yamamura and Tezuka, 1990), and β-glucocerebrosidase (GCase) (Holleran *et al*, 1993), and the degradative enzyme ceramidase (CDase) (Yada *et al*, 1995), it is of considerable interest to determine whether the enzyme balance may be altered in the skin of patients with atopic dermatitis. In aged skin, in which a similar deficiency in the mass of ceramides in the stratum corneum was identified with the appearance of dry, itchy skin (Akimoto *et al*, 1993), there was a significant increase in the activity of CDase, which hydrolyzes ceramides to yield sphingosine and free fatty acid,

Manuscript received October 5, 1995; revised January 26, 1996; accepted for publication February 7, 1996.

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Abbreviations: SM, sphingomyelin; SMase, sphingomyelinase; Sph-PC, sphingosyl-phosphorylcholine; CDase, ceramidase; TLC, thin-layer chromatography; GCase, β-glucocerebrosidase; PMSF, phenylmethylsulfonyl fluoride.

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probably contributing to the decreased quantity of ceramides in the stratum corneum of aged skin (Jin *et al*, 1994).

In our previous study, in which we assessed enzyme balance, there were no significant differences in the activities of either GCase or CDase in stratum corneum extracts from patients with atopic dermatitis in comparison with those of age-matched normal controls (Jin *et al*, 1994). Because their activities in the stratum corneum may reflect the epidermal level of the same enzymes (Holleran *et al*, 1992), the absence of any abnormality in either enzyme activity in the stratum corneum of atopic patients suggests the alternative possibility that upstream metabolism of sphingolipids, namely sphingomyelin (SM) hydrolysis, is altered, resulting in the ceramide deficiency observed in atopic dermatitis. In the current study, we determined whether SM metabolism is altered in atopic dermatitis in comparison with normal controls and patients with other types of eczema.

MATERIALS AND METHODS

Materials [*choline-methyl*-^{14}C]Sphingomyelin (52 mCi/mmol) was purchased from New England Nuclear (Boston, MA). Ceramide (type III), sphingosine, and sphingosylphosphorylcholine (Sph-PC) were obtained from Sigma Chemical Co. (Saint Louis, MO). Porcine kidney acylase I and II was purchased from Seikagaku Co Ltd (Tokyo, Japan). All other chemicals were of reagent grade.

Preparation of Stratum Corneum and Epidermis Extracts Diagnosis of atopic dermatitis was made according to Hanifin and Rajka (1989). Specimens of the stratum corneum were obtained from the normal forearm skin of healthy individuals and from atopic uninvolved and involved or cured skin by tape-stripping three times (Protect-label/B type, 40–60 mm; Iuchiseieidou Corporation, Japan) in the same region. Uninvolved skin was normal skin without clinically eczematous changes and had no current topical medicaments including corticosteroid ointments. Cured skin was normal skin of atopic patients that was free of current skin lesions for at least 1 year. These tapes were stored frozen at −20°C until use. For measurement of SM hydrolysis, the three tapes were immersed in 40 ml of 50 mM acetate buffer, pH 4.7, or 50 mM Tris-HCl buffer, pH 7.4, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% Triton X100 for 30 min at 4°C and then sonicated on ice for a total of 3 min with 30-s bursts in a Bioruptor sonicator (Olympus Corporation, Japan). Mixtures containing the three tapes were further incubated in extraction buffer overnight at 4°C to obtain a Triton X100 extract for the subsequent assay. After removing the tapes, extracted solution was concentrated to about 0.5 ml using concentrators (Centriprep 10 and Centricon 10, Amicon, Millipore Corporation, Milford, MA). The concentrated solution was used for SM hydrolysis assay. For SM hydrolysis assay of epidermis, specimens of biopsied skin (4-mm punch) were treated with 1000 units/ml Dispase (Godoshusei Co. Ltd., Tokyo, Japan) at 4°C for 4 h to separate epidermis. The isolated epidermal sheet was homogenized with a glass homogenizer in 300 μl of 50 mM acetate buffer, pH 4.7, containing 0.5 mM PMSF. The homogenate was centrifuged at 10,000 × *g* for 30 min to obtain supernatant and particulate fractions. The former was subjected to SM hydrolysis assay, while the latter was further incubated for 2 h in 300 μl of 50 mM acetate buffer, pH 4.7, containing 0.5 mM PMSF and 0.1% Triton X100, and the mixture was centrifuged at 10,000 × *g* for 30 min to obtain the extracted fraction as the supernatant which was subjected to SM hydrolysis assay.

Assay of SM Hydrolysis SM hydrolysis was assessed by the method of Bowser and Gray (1978). The incubation mixtures contained 50 mM acetate buffer, pH 4.7, or 50 mM Tris-HCl buffer, pH 7.4, 0.1% Triton X100, 20 mM $CaCl_2$, 10 nmol of [*choline-methyl*-^{14}C]sphingomyelin (52 mCi/mmol) and the extracts (250 μl) of the stratum corneum, epidermis, and our stocks of *Staphylococcus aureus* or enzymes in a total volume of 0.5 ml. Incubations were carried out at 37°C for 1.5–16 h and stopped by addition of 0.5 ml of water and 2 ml of chloroform:methanol (2:1, v/v). The mixtures were vortex-mixed and centrifuged for 10 min at 2000 × *g*. The upper phase was counted in a liquid scintillation counter, and the SM hydrolysis was expressed as dpm/ml of the aqueous phase when protein content was not measured. The aqueous phase (1.2 ml) was dried at −80°C and dissolved in 100 μl of water:methanol (1:1, v/v). The sample solution (5–25 μl) was subjected to thin-layer chromatography (TLC) (HPTLC plate silica gel 60; Merck, Darmstad, Germany), and developed twice with methanol:0.5% NaCl:13 N ammonia solution (50:50:1, v/v). The radioactive compounds were quantified using a TLC scanner (System 200 Imaging Scanner; Bioscan Corporation, Washington, DC).

High-Performance Liquid Chromatography-Mass Spectrometry Nonradiolabeled sphingomyelin was incubated for 6 h at 37°C with porcine kidney acylase (Type II) or atopic stratum corneum samples in Tris-HCl buffer, pH 7.4, containing 0.1% Triton X100 and 20 mM $CaCl_2$ and stopped by addition of 0.5 ml of water and 2 ml of chloroform:methanol (2:1, v/v). The mixtures were vortex-mixed and centrifuged for 10 min at 2000 × *g* to obtain the upper phase according to the procedure for the assay of sphingomyelinase. The upper phase was dried at −80°C, dissolved in 100 μl of water, and subjected to high-performance liquid chromatography-mass spectrometry according to the method of Suzuki *et al* (1989). A JMN-HX110 double focusing mass spectrometer equipped with a fast atom bombardment ion source and a JMA-DA5000 data system (JEOL, Tokyo) was used. The accelerating voltage was 6.0 kV, and the primary beam for bombardment was 6.0 kV Xe. The ion source temperature was maintained at 40°C. Liquid nitrogen was introduced into the trap of the oil diffusion pump. Mass chromatography was performed to repeat the detection of the ions from m/z 300 to 2200 at an interval of 5 s. High-performance liquid chromatography was performed with a mode 120A (Applied Biosystems, Foster City, CA) on a reversed-phase column of SPHERI-5 (RP-18; 250- × 1-mm i.d., Applied Biosystems), with methanol containing 0.1% trifluoroacetic acid and 1% glycerol as the elution solvent at a flow rate of 100 ml/min. The column oven temperature was kept at 40°C. A frit interface with a splitter (JEOL) was set up between the high-performance liquid chromatography and the MS. The split ratio was 1:19; therefore, one-twentieth of the injected samples was introduced into the fast atom bombardment/MS, and the rest was discarded via a drainage tube.

Preparation of ^{14}C-Labeled Sph-PC To prepare ^{14}C-labeled Sph-PC as standard for radiochromatography, [^{14}C]sphingomyelin was incubated for 1.5 h at 37°C with porcine kidney acylase (Type II) in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% Triton X100 and 20 mM $CaCl_2$ and stopped by addition of 0.5 ml of water and 2 ml of chloroform:methanol (2:1, v/v). The mixtures were vortex-mixed and centrifuged for 10 min at 2000 × *g* to obtain the upper phase according to the procedure for the assay of sphingomyelinase. The upper phase was dried at −80°C to prepare ^{14}C-labeled Sph-PC. The ^{14}C-labeled Sph-PC was dissolved in 100 μl of water:methanol (1:1, v/v), and 10 μl of the solution was subjected as a standard to TLC analysis.

Column Chromatography To separately determine the generation of phosphorylcholine and choline, we dried the reaction products in the aqueous phase (1.2 ml) at −80°C, dissolved in 100 μl of water and subjected to cation exchange column chromatography using AG50W-X8 resin, according to the method of Cook and Wakelam (1989). After application of the sample, the column was washed three times with distilled water (5 ml) to elute the radiolabeled phosphorylcholine; the radiolabeled choline was then eluted by addition of 1 M KCl. The radioactivity of fractions was measured with a liquid scintillation counter.

RESULTS

SM Hydrolysis Is Extremely Elevated in Atopic Dermatitis

The stratum corneum from lesional forearm skin (volar side) of patients with atopic dermatitis showed extremely high SM hydrolysis when assayed at pH 4.7 and was expressed as an amount of dpm liberated over a 1.5-h period into the aqueous phase/mg protein, with a magnitude 27 times higher than that found in age-matched normal controls **(Fig 1)**. In the nonlesional skin of patients or normal skin of patients without current skin lesions, SM hydrolysis was still 6–11 times higher than that found in age-matched controls. In contrast, the stratum corneum from contact dermatitis or chronic eczema patients showed levels similar in SM hydrolysis to normal controls. There was a general tendency for the enzyme activity to increase with increasing depth of the stratum corneum layer **(Fig 2)**. To determine whether the SM hydrolysis represents epidermal enzyme activity, we assayed punch-biopsied epidermis from atopic dermatitis patients **(Fig 3)**. Whereas there was no significant difference in SM hydrolysis in the supernatant fraction between control and atopic dermatitis, similar elevated levels were observed in atopic nonlesional and lesional skin in the particulate fraction of epidermal homogenates containing the majority of the activity. To rule out a possibility that the high SM hydrolysis in the stratum corneum from atopic dermatitis is derived from bacterial contamination, we assessed the SM hydrolysis in our stocks of *S. aureus* and in the stratum corneum of patients with impetigo contagiosa. We found that our stocks of *S. aureus* and the

stratum corneum from patients with impetigo contagiosa showed very low SM hydrolysis under our experimental conditions **(Table I)**.

High SM Hydrolysis Is Attributable to Acylase-Like Enzyme Chromatographic separation of reaction products after incubation at pH 4.7 with [*choline-methyl*-^{14}C]sphingomyelin showed an unknown radiolabeled spot distinct from the authentic SMase reaction product, i.e., radiolabeled phosphorylcholine, which became marked with increasing incubation time **(Fig 4*A*)**. There are three possible ways that radiolabeled materials could be released enzymatically from [*choline-methyl*-^{14}C]SM: 1) the formation of [^{14}C]PC by SMase; 2) the formation of [^{14}C]Sph-PC by acylase-like enzyme; and 3) the formation of [^{14}C]choline by a phospholipase D-like enzyme. Based on its TLC mobility, the unknown spot was thought most likely to be Sph-PC. Therefore, to assess the potential of acylase-like enzyme to generate the unknown spot, we determined whether acylase derived from porcine kidney acts on SM to yield Sph-PC **(Fig 4*B*)**. Porcine kidney acylase was found to have the potential to degrade SM at pH 4.7 to produce materials identical in TLC mobility to the unknown spot observed for atopic stratum corneum samples. There was no definite degradative activity at pH 7.4.

To confirm that the reaction product with porcine kidney acylase was Sph-PC, high-performance liquid chromatography-mass (LC-MS) spectrometry was carried out using the reaction products extracted into the aqueous phase according to the assay for SM hydrolysis. **Figure 5*A*** shows mass chromatograms of purified Sph-PC in which pseudo-molecular ions of the molecular species of targeted chemicals are detected after chromatographic separation. Sph-PC (20 ng) was injected into the LC-MS, and the peaks selected as to the pseudo-molecular ions ([M-H]$^-$) of the molecular species of Sph-PC at m/z 184 and 465 were observed clearly. As shown in **Fig 5*B***, the mass spectrum of each of these molecular species was obtained, and pseudo-molecular ions were detected

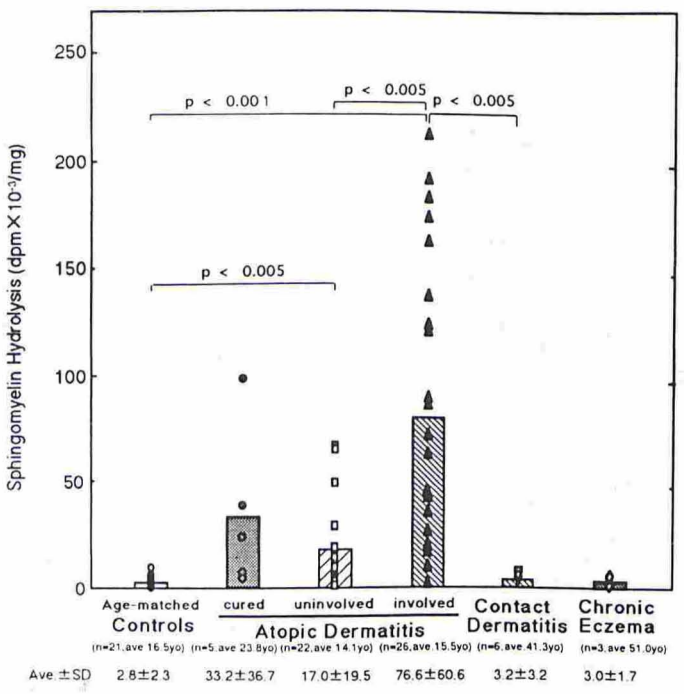

Figure 1. SM hydrolysis is extremely elevated in the stratum corneum from patients with atopic dermatitis in comparison with those from contact dermatitis, chronic eczema, and age-matched normal control subjects. SM hydrolysis are expressed as liberated dpm into the aqueous phase per milligram of protein after 1.5-h incubation at pH 4.7 with 0.5 μCi of [*choline-methyl*-^{14}C]sphingomyelin. *ave.*, average; *yo*, years old.

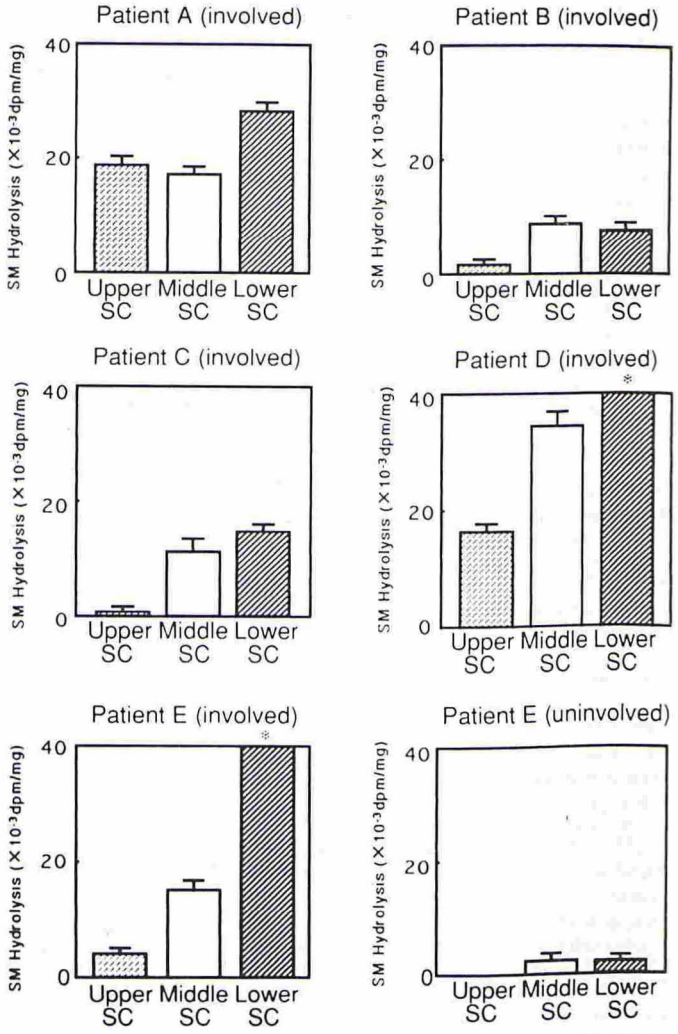

Figure 2. SM hydrolysis is localized through whole stratum corneum layers in atopic dermatitis. The stratum corneum from atopic dermatitis was stripped three times with adhesive tape to obtain the upper stratum corneum. The stratum corneum was further stripped ten times with adhesive tape, followed by another three strips to obtain the middle stratum corneum. Following application of another ten strips, the lower stratum corneum was obtained on three subsequent strips. SM hydrolysis are expressed as dpm liberated into the aqueous phase/mg protein after 1.5-h incubation at pH 4.7 with 0.5 μCi of [*choline-methyl*-^{14}C]sphingomyelin. *, experimental values exceed 4×10^3 dpm/mg protein; *SC*, stratum corneum. The data are expressed as means ± SD of triplicate determinations.

with strong intensity at m/z 180 and 456. Furthermore, ions due to the elimination of sphingosine from the molecules were also detected at m/z 184, although the intensity of the ions was rather weak. **Figure 5*C*** shows mass chromatograms of the reaction product extracted into the aqueous phase after incubation of SM for 6 h at 37°C with porcine kidney acylase. The peaks at m/z 184 and 465 were observed clearly in the mass chromatograms. As shown in **Fig 5*D***, the mass spectrum of each of these molecular species was obtained, and pseudo-molecular ions were detected with a definite intensity at m/z 180 and 456. This mass spectrometric comparison between purified Sph-PC and the reaction product confirmed that the material generated from SM by the enzymic action of porcine kidney acylase was identical to Sph-PC. Therefore, we used the radiolabeled reaction product extracted into the aqueous phase after 6 h incubation of [^{14}C]sphingomyelin with porcine kidney acylase as a radiolabeled Sph-PC standard for subsequent TLC experiments.

To further confirm that the atopic stratum corneum acts on SM

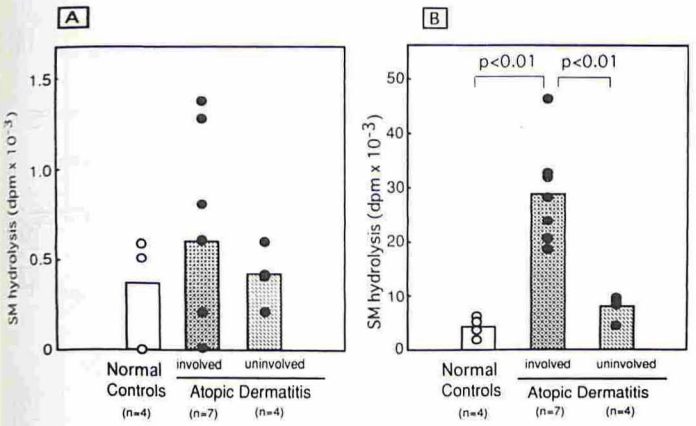

Figure 3. SM hydrolysis is highly elevated in biopsied epidermis from patients with atopic dermatitis in comparison with those from normal controls. *A*, $10^4 \times g$ supernatant fraction of epidermis homogenate. *B*, $10^4 \times g$ particulate fraction. SM hydrolysis are expressed as dpm liberated into 1 ml of the aqueous phase after 1.5-h incubation of epidermis samples (250 μl) prepared from 4-mm, punch-biopsied skin, at pH 4.7 with 0.5 μCi of [*choline-methyl*-^{14}C]sphingomyelin.

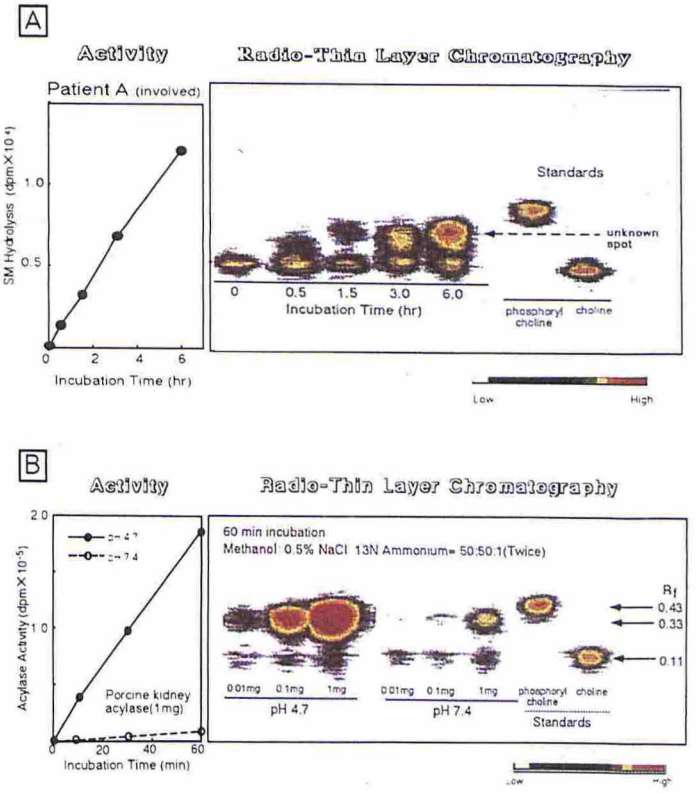

Figure 4. SM hydrolysis after incubation with atopic stratum corneum sample (*A*) and porcine kidney acylase (*B*) and their radio-TLC of the reaction products. *A*, SM hydrolysis are expressed as dpm liberated into the aqueous phase after the indicated periods of incubation of stratum corneum samples (250 μl) at pH 4.7 with 0.5 μCi of [*choline-methyl*-^{14}C]sphingomyelin. For TLC preparation, reaction products in the aqueous phase were dried at −80°C and dissolved in 100 μl of water:methanol (1:1, v/v). The sample solutions (5–25 μl) with the same dpm counts (approximately 10^3 dpm) were subjected to TLC analysis. *B*, 0.01–1.0 mg (0.65–65 unit) of acylase II (derived from porcine kidney) was incubated with 0.5 μCi of [*choline-methyl*-^{14}C]sphingomyelin in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.4, or 50 mM acetate buffer, pH 4.7, both containing 0.5 mM PMSF and 0.1% Triton X100. SM hydrolysis are expressed as dpm liberated into 1 ml of the aqueous phase after the indicated periods of incubation. The reaction products in the aqueous phase (1.2 ml) were dried at −80°C and dissolved in 100 μl of water:methanol (1:1, v/v), 5 μl of which was subjected to TLC analysis. The radioactivities on TLC plates were quantified using a TLC scanner and expressed as color density responsible for dpm intensity.

to yield Sph-PC, LC-MS spectrometry was also carried out using the reaction products after 1.5-h incubation of SM with atopic stratum corneum samples. **Figure 6** shows mass chromatograms of the reaction products extracted into the aqueous phase after incubation of SM for 1.5 h at 37°C with atopic stratum corneum samples. The peaks at m/z 465 and 184 were observed clearly with the same retention times in the mass chromatograms. This mass chromatographic comparison between purified Sph-PC and the reaction product confirmed that Sph-PC was generated from SM by the enzymic action of atopic stratum corneum.

Almost All Atopic Individuals Exhibit Acylase Reaction Product, Sph-PC Comparison of the reaction products on TLC separation among atopic dermatitis, contact dermatitis, and normal control subjects indicated that atopic-involved stratum corneum samples definitely generated a material identical to Sph-PC, whereas the stratum corneum samples of patients with contact dermatitis and of normal control subjects yielded only small amounts of materials identical to PC without the emergence of a spot identical to Sph-PC **(Fig 7*A*)**, suggesting that the stratum corneum in atopic dermatitis contains SM acylase-like activity. Because the site of SM hydrolysis by SM acylase is the same as that by CDase, which is also available in the stratum corneum in atopic dermatitis (Yamamura and Tezuka, 1990; Holleran *et al*, 1993), we determined whether SM can be utilized as the substrate for CDase at acidic pH to yield Sph-PC. We found that purified CDase derived from guinea pig epidermis (Yada *et al*, 1995) possessed no SM hydrolysis at both acidic and alkaline pH levels (data not shown). Furthermore, ceramidase activity in stratum corneum samples from atopic dermatitis patients, as measured at pH 4.7 using

Table I. SM Hydrolysis Is Very Low in Stocks of *Staphylococcus aureus* and in the Stratum Corneum from Patients with Impetigo Contagiosa

	Activity (dpm)	Protein (μg/ml)	SM Hydrolysis[a] (dpm/mg)
Staphylococcus aureus	6.2	17.5	1.42×10^3
Impetigo contagiosa (n = 2)	23.5	73.3	1.28×10^3

[a] SM hydrolysis is expressed as liberated dpm into the aqueous phase per milligram of protein after 1.5-h incubation at pH 4.7 with 0.5 μCi of [*choline-methyl*-^{14}C]sphingomyelin.

[^{14}C]palmitoyl sphingosine, was not accentuated relative to that found with normal control subjects (data not shown). Taken together, these findings suggest that there is no involvement of ceramidases in the increased SM acylase-like activity in atopic dermatitis.

To see clearly the variation of Sph-PC generation among atopic individuals, their reaction products obtained using stratum corneum samples from several patients with atopic dermatitis after 1.5-h incubation were subjected to radio-TLC analysis. The radiolabeled unknown spot was detected in almost all stratum corneum samples from atopic-involved skin with a mobility on TLC plates identical to that of radiolabeled Sph-PC prepared from the enzymatic reaction of radiolabeled SM with porcine kidney acylase **(Fig 7*B*)**. There were a few cases in which the authentic SMase reaction product, i.e., radiolabeled phosphorylcholine, was generated without the Sph-PC spots after 1.5-h incubation with atopic stratum corneum samples. Among 21 atopic stratum corneum samples assayed for 1.5-h incubation, 16 samples showed only spots identical in mobility to Sph-PC, whereas 5 samples produced the spots

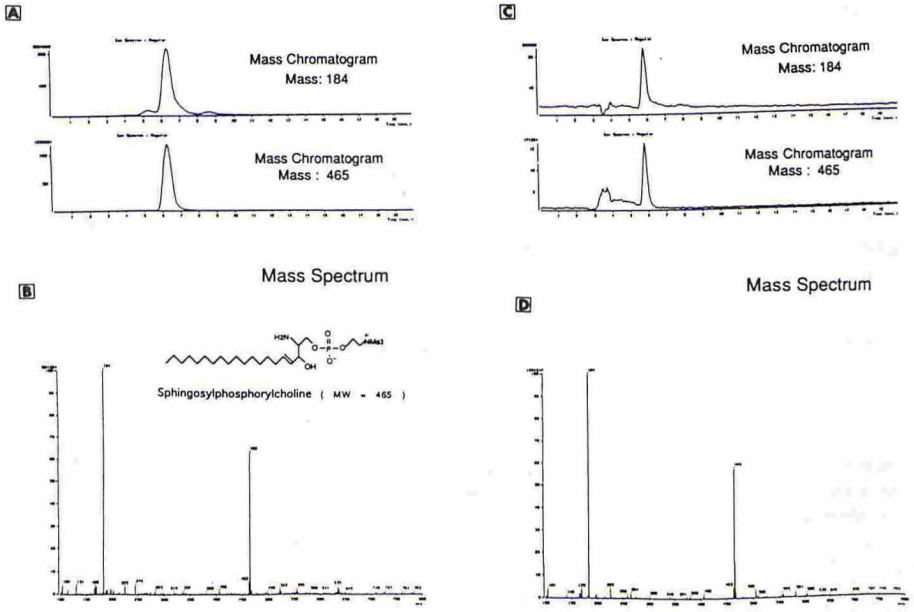

Figure 5. The reaction product generated by porcine kidney acylase is identified as sphingosylphosphorylcholine upon high-performance liquid chromatography-mass spectrometry. *A*, mass chromatograms of the molecular species of Sph-PC. *B*, mass spectra of the molecular species of Sph-PC. Sph-PC was dissolved in 100 μl of water:methanol (2:8, v/v), and 0.2 μl of the solution was injected into LC-MS. *C*, mass chromatograms of the molecular species of the reaction product extracted into the aqueous phase after 6-h incubation of sphingomyelin (100 nM) with porcine kidney acylase (325 unit). *D*, mass spectra of the molecular species of the same reaction product.

identical to PC without the emergence of Sph-PC spots (data not shown). The radiolabeled Sph-PC spot appeared dependent on low SM hydrolysis **(Fig 7*C*)**. To determine the cause of the generation of PC spots in a few atopic samples, radio-TLC analysis of the reaction products obtained after different incubation periods was carried out using atopic stratum corneum samples that had shown the production of a PC spot after 6-h incubation. Time course analysis on radio-TLC of the reaction products from the atopic samples **(Fig 8)** revealed that, before the emergence of radiolabeled PC at 6 h, the spots identical to Sph-PC appeared and became intense with increasing incubation time until 3 h later, when the PC spot began to appear. This result suggests that the generation of PC is mediated through the degradation of Sph-PC produced, and not by the action of SMase. To further confirm the contribution of truncated Sph-PC to the generation of PC, we examined the inhibitory effect of nonradiolabeled Sph-PC on the generation of PC during enzymatic reaction by column chromatography. When Sph-PC, the SM acylase reaction product, was added to the reaction mixture of SM and stratum corneum samples from atopic dermatitis patients, the synthesis of PC as detected by column chromatography separation was reduced by 50%, whereas the addition of SMase reaction product, ceramide, did not affect the production of PC **(Fig 9*A*)**, suggesting that the reaction product had an inhibiting or diluting effect on radiolabeled Sph-PC during the enzymatic reaction. **Figure 9*B*** shows average percentages of the inhibitory effect by the addition of Sph-PC in a series of experiments, indicating that the generation of PC during enzymatic reaction of the mixture of SM and atopic stratum corneum samples was mediated through the decomposition of Sph-PC.

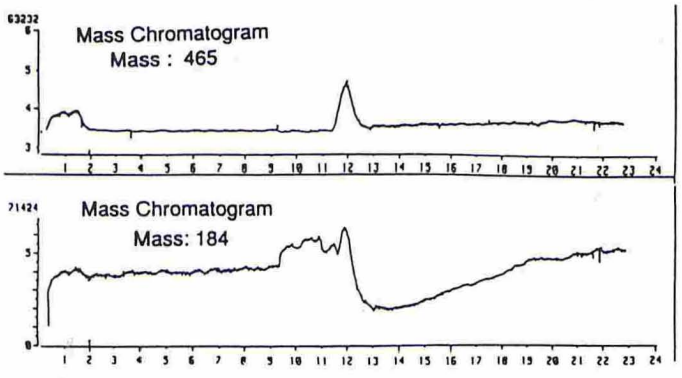

Figure 6. The reaction product by atopic stratum corneum sample is identified as sphingosylphosphorylcholine upon high-performance liquid chromatography-mass spectrometry. Mass chromatograms of the molecular species of the reaction product extracted into the aqueous phase after 1.5-h incubation of sphingomyelin (10 nM) with atopic stratum corneum sample (250 μl).

DISCUSSION

This study demonstrated that apparent SM hydrolysis is enhanced by more than 25-fold in involved skin and more than 5-fold in uninvolved skin in patients with atopic dermatitis as compared to age-matched normal controls. In contrast, the stratum corneum of patients with contact dermatitis or chronic eczema showed no increases in SM hydrolysis as compared to that of normal controls, suggesting that changes in SM hydrolysis are unlikely to be involved in the etiology of cutaneous inflammation. To exclude the possibility that the observed high SM hydrolysis was due to contamination by bacteria such as *S. aureus* for which patients with atopic dermatitis have a high carriage rate on the surface of the stratum corneum (Leyden *et al*, 1974; Dahl, 1983), we examined whether the high SM hydrolysis was present only in the upper layer of the stratum corneum. The enzyme activity showed, rather, an increase depending on the depth of the stratum corneum layer, suggesting that the high SM hydrolysis in the stratum corneum from atopic dermatitis is not derived from bacterial contamination. Consistent with this observation, we found that our stocks of *S. aureus* and the stratum corneum from patients with impetigo contagiosa showed no substantial SM hydrolysis activity under our experimental conditions.

Although the SM hydrolysis was consistent with SMase activity, which converts SM to ceramide and PC in normal control subjects, TLC analysis of enzymatically decomposed materials revealed that most of the high SM hydrolysis in atopic dermatitis was attributed not to the SMase but to SM acylase-like enzyme which releases Sph-PC and free fatty acid. The suggestion that SM can be hydrolyzed by acylase-like enzymes to yield Sph-PC was corroborated by another experiment in which porcine kidney acylase showed the potential to hydrolyze SM to yield materials identical in TLC mobility to the spot generated by the stratum corneum sample from atopic dermatitis. LC-MS spectrometric analysis of the reac-

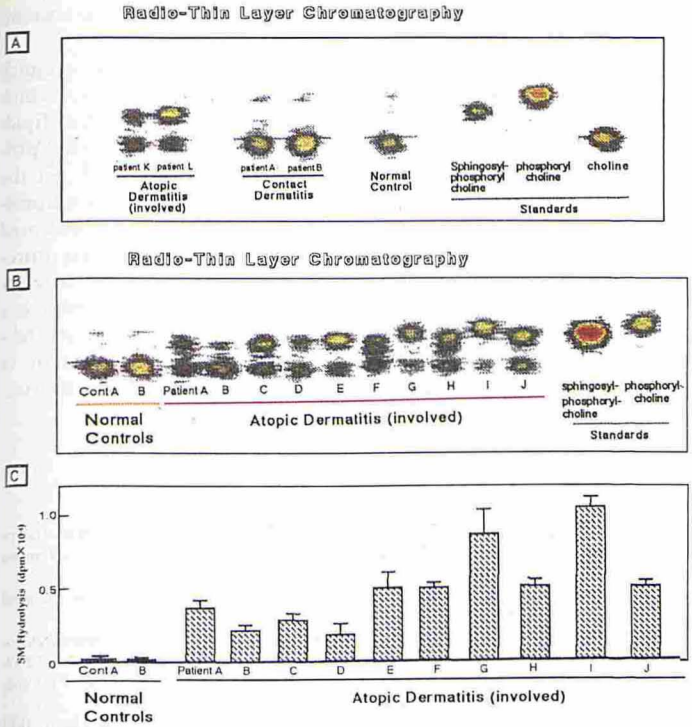

Figure 7. Radio-TLC comparison of the reaction products by stratum corneum samples between atopic dermatitis, contact dermatitis, and normal control subjects (*A*) and among individuals with atopic dermatitis (*B*) and their SM hydrolysis (*C*). *A* and *B*, incubation of the stratum corneum samples (250 μl) at pH 4.7 with [*choline-methyl*-^{14}C]sphingomyelin was carried out for 1.5 h in 0.5 ml of 50 mM acetate buffer, pH 4.7, containing 0.5 mM PMSF and 0.1% Triton X100. The reaction products in the aqueous phase (1.2 ml) were dried at −80°C and dissolved in 100 μl of water:methanol (1:1, v/v). The sample solutions (5–25 μl) with the same dpm counts (approximately 10^3 dpm) were subjected to TLC analysis. The radioactivities on TLC plates were quantified using a TLC scanner and expressed as color density responsible for dpm intensity. *C*, SM hydrolysis activities are expressed as dpm liberated into 1 ml of the aqueous phase after 1.5-h incubation of stratum corneum samples (250 μl) at pH 4.7 with 0.5 μCi of [*choline-methyl*-^{14}C]sphingomyelin. The data are expressed as means ± SD of triplicate determinations.

tion products obtained using porcine kidney acylase and atopic stratum corneum samples revealed that both the reaction products contained materials identical to Sph-PC, strongly suggesting that the activity of SM acylase is highly expressed in the stratum corneum from patients with atopic dermatitis.

Our previous study of the epidermal localization of CDase and GCase, hydrolytic enzymes that work in intercellular spaces between the stratum corneum and granular layer, suggested that the activity of ceramide metabolism-related enzymes within the stratum corneum approximately represents the epidermal activity of the same enzymes (Holleran *et al*, 1992; Yada *et al*, 1995). Consistent with this relationship, a similar high level of SM acylase activity was detected in the epidermis from atopic dermatitis patients, suggesting that epidermal cells from these patients show abnormal production of a hitherto undiscovered epidermal enzyme, SM acylase.

As there were a few cases where the authentic SMase reaction product, i.e., radiolabeled PC, appeared upon radio-TLC plates after 1.5-h incubation with atopic stratum corneum samples, we determined whether the production of PC is directly mediated by SMase or through decomposition of produced Sph-PC. Time course analysis by radio-TLC of the reaction products from the atopic samples, which had shown the generation of PC at 6-h incubation, revealed that the spots identical to Sph-PC first appeared and became intense with increasing incubation time until 3 h later, when the PC spot began to appear. This implies that the generation of PC is mediated through the degradation of Sph-PC produced, with no contribution of the action of SMase. To further confirm the contribution of truncated Sph-PC to the generation of PC, we examined the inhibitory effect of nonradiolabeled Sph-PC on the generation of PC during enzymatic reaction by column chromatography. Upon column chromatography to separate radiolabeled PC and choline, the exogenous addition of nonradioactive Sph-PC during enzymatic reaction significantly abrogated the generation of radiolabeled PC. This inhibitory effect was not induced by exogenously added ceramides, again indicating that the generation of radiolabeled phosphorylcholine was mediated through the production by SM acylase and the subsequent decom-

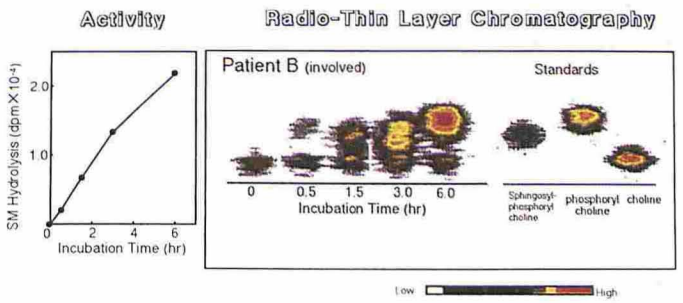

Figure 8. Time course study on radio-TLC of the reaction products after incubation with atopic stratum corneum sample and SM hydrolysis. SM hydrolysis are expressed as dpm liberated into the aqueous phase after indicated period of incubation of stratum corneum samples (250 μl) at pH 4.7 with 0.5 μCi [*choline-methyl*-^{14}C]sphingomyelin. For TLC preparation, reaction products in the aqueous phase were dried at −80°C and dissolved in 100 μl of water:methanol (1:1, v/v). The sample solutions (5–25 μl) with the same dpm counts (approximately 10^3 dpm) were subjected to TLC analysis. The radioactivities on TLC plates were quantified using a TLC scanner and expressed as color density responsible for dpm intensity.

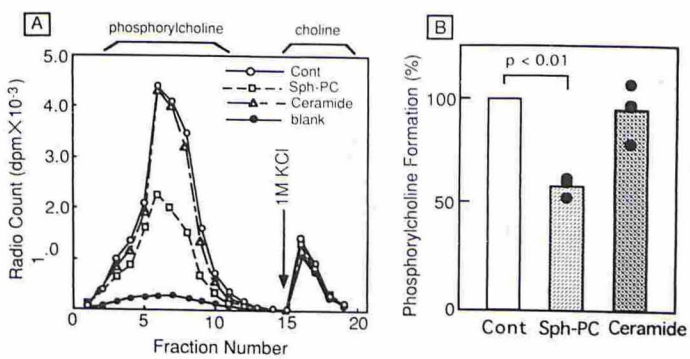

Figure 9. Column chromatography of the reaction products for separating phosphorylcholine and choline, and inhibitory effects of the addition of Sph-PC or ceramide on the formation of radiolabeled phosphorylcholine during the incubation with [*choline-methyl*-^{14}C]sphingomyelin (*A*) and percent inhibition of phosphorylcholine formation (*B*). Incubation of the stratum corneum sample (250 μl) at pH 4.7 with 0.5 μCi of [*choline-methyl*-^{14}C]sphingomyelin was carried out in the presence of Sph-PC (5 mM) or ceramide (5 mM). The reaction products in the aqueous phase (1.2 ml) were dried at −80°C, dissolved in 100 μl of water, and applied to cation exchange column chromatography using AG50W-X8 resin. After application of the sample, the column was washed three times with distilled water (5 ml) to elute the radiolabeled phosphorylcholine, then the radiolabeled choline was eluted by 1 M KCl. The radioactivity of each fraction (1 ml) was measured using a liquid scintillation counter. *Sph-PC*, sphingosylphosphorylcholine.

position of Sph-PC. This suggests that the stratum corneum in atopic dermatitis contains high SM acylase activity, attributed to the abnormally high level of SM hydrolysis, although the partial involvement of SMase in the high SM hydrolysis observed for the stratum corneum from patients with atopic dermatitis could not completely excluded.

To clarify the mechanisms involved in the ceramide deficiency in atopic dermatitis, our previous study demonstrated that there are no abnormalities in the activities of the ceramide-degrading enzyme CDase or the ceramide-producing enzyme GCase in the stratum corneum in atopic dermatitis as compared with those in age-matched normal controls (Jin *et al*, 1994). The enzymatic profile seen in the stratum corneum may provide a convenient model for understanding the status of enzyme function in epidermal tissues with special reference to enzymes acting in the subcorneal layers, because they can remain in the stratum corneum without undergoing further processing following the accomplishment of their function in intercellular spaces between the stratum corneum and granular layers. In focusing on SM metabolism other than that mediated by CDase and GCase enzymes as an unresolved mechanism underlying ceramide deficiency, we noted a novel abnormally expressed epidermal enzyme related to SM metabolism in atopic dermatitis. As a result of this abnormal expression of SM acylase with the deficient function of SMase, because the normal level of acid SMase activity was very low in the stratum corneum, quantitative measurement of the reduced level of the enzyme activity in atopic dermatitis was difficult. The abnormal expression of SM acylase in the epidermis from atopic dermatitis patients, however, led us to speculate that the action of acid SMase becomes deficient at least in terms of substrate utility in that almost all SM is degraded in the intercellular spaces by the normal level of SMase, and thus there is no residual amount in the stratum corneum. In connection to this, Kusuda *et al*[1] reported recently that there was a deficiency of acid SMase in atopic dermatitis when an antibody to acid SMase was used to detect the enzyme localization in the epidermis. Consistent with the deficiency of SMase, which is originally contained in lamellar bodies, an impairment in exocytosis of lamellar bodies from granular cells has been demonstrated in the epidermis of atopic dermatitis patients (Fartasch *et al*, 1992), although how the deficiency of SMase activity is involved in the impairment of exocytosis remains unclear.

The reaction product of SM acylase, Sph-PC, has been reported to be a wide-spectrum, growth-promoting agent for a variety of cell types (Desai and Spiegel, 1991; Desai *et al*, 1993). In fibroblasts, Sph-PC acts as a modulator of cellular proliferation via distinct signaling pathways, where it stimulates the release of arachidonic acid or eicosanoids, both strong proinflammatory mediators and pruritic agents. In our studies,[2] the addition of this metabolite to human keratinocyte cultures induced mobilization of intracellular calcium, an increase in PGE_2 synthesis, and enhanced expression of ICAM-1 on their plasma membrane. In agreement with these biological effects, marked accumulation of the arachidonate metabolites such as prostaglandin E_2 and leukotriene B_4 has been reported in the skin from patients with atopic dermatitis (Ruzicka *et al*, 1986; Fogh *et al*, 1989). In some cases intercellular adhesion molecule-1 can be visualized on epidermal keratinocytes in atopic dermatitis (Griffiths *et al*, 1989; Singer *et al*, 1989). It is, therefore, likely that the high level of expression of SM acylase in atopic dermatitis provides a basis not only to explain the ceramide deficiency, but also more importantly to clarify the marked vulnerability of the atopic skin to irritants or allergens, implying that Sph-PC acts as a potent modulator of epidermal cell function and is, to some extent, involved in inflammation in atopic dermatitis.

In normal skin, disruption of barrier function elicits a quick activation of epidermal lipid metabolisms including SMase, which acts as a driving force to repair deficiency of intercellular lipids including ceramides in the stratum corneum, leading to the programmed barrier recovery (Holleran *et al*, 1991b). Based on the normal barrier repair system, it is likely that the abnormal expression of SM acylase triggers the metabolism of SM in an unfavored way to the restoration of ceramide synthesis, leading to an unrecovery from barrier perturbation. Although it remains unclear whether the barrier disruption observed in atopic dermatitis is a cause or a result, our findings suggest that the altered SM metabolism could be an etiologic factor in the continuous generation of atopic dry and barrier-disrupted skin observed in atopic dermatitis.

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