High-Expression of Sphingomyelin Deacylase is an Important Determinant of Ceramide Deficiency Leading to Barrier Disruption in Atopic Dermatitis[1]

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We have previously demonstrated that there is abnormal expression of sphingomyelin (SM) deacylase-like enzyme in the epidermis of patients with atopic dermatitis (AD), which results in decreased levels of ceramides in their involved and uninvolved stratum corneum. For quantitation of the expression of SM deacylase in AD, we synthesized 16-(9-anthroyloxy) hexadecanoylsphingosylphosphorylcholine or [palmitic acid-^{14}C] SM and used them as substrates to directly measure the activity of SM deacylase by detecting the release of labeled free fatty acid. Direct enzymatic measurements demonstrated that stratum corneum from lesional forearm skin (volar side) of AD patients has an extremely high SM deacylase activity that is at least five times higher than in the stratum corneum from healthy controls. In stratum corneum from nonlesional skin of AD patients, SM deacylase activity is still at least three times higher than in healthy controls. In contrast, stratum corneum from contact dermatitis patients shows levels of SM deacylase similar to healthy controls. In extracts of whole epidermis biopsies from AD patients, SM deacylase activities are significantly (3-fold) increased over healthy controls in the particulate fraction, whereas there is no significant difference in the activity of sphingomyelinase between AD and healthy control. In peripheral blood lymphocytes of AD patients, there is no increase in activity compared with healthy controls, indicating a possibility that the high expression of SM deacylase is highly associated with the skin of AD patients. These findings suggest that, in contrast to changes in sphingolipid metabolism due to aging, the hitherto undiscovered enzyme SM deacylase, is highly expressed in the epidermis of AD patients, and competes with sphingomyelinase or β-glucocerebrosidase for the common substrate SM or glucosylceramide, which leads to the ceramide deficiency of the stratum corneum in AD. ***Keywords: atopic dermatitis/ceramide/sphingomyelin/sphingomyelinase/sphingomyelin deacylase. J Invest Dermatol 115:406–413, 2000***

We have previously demonstrated that, as an etiologic factor in dry and barrier-disrupted skin of patients with atopic dermatitis (AD), there is a marked reduction in the amount of ceramides in the stratum corneum of their lesional and nonlesional skin (Imokawa *et al*, 1991a). A similar ceramide deficiency in the stratum corneum was subsequently reported using a different extraction method in AD patients (Yamamoto *et al*, 1991). As ceramides are an important determinant involved in the water-holding property (Imokawa and Hattori, 1985; Imokawa *et al*, 1986, 1991b) and barrier function (Lampe *et al*, 1983; Grubauer *et al*, 1989; Holleran *et al*, 1991a; Imokawa *et al*, 1994) of the stratum corneum, the insufficiency of ceramides may provide an etiologic basis for the dry and barrier-disrupted skin of AD patients. It is possible that atopic dry skin accompanying barrier disruption and water-deficiency is an etiologic factor for eliciting dermatitis following scratching of the skin due to dry-derived itching, and the easy penetration of irritants and high molecular weight mite antigens, the latter of which can never penetrate normal stratum corneum. Therefore, the above implication provides a strong basis that explains some parts of pathogenesis of atopic dermatitis, in terms of biochemical mechanisms involved in the ceramide deficiency. Since the amount of ceramide in the stratum corneum is regulated by a balance of the rate-limiting enzymes of sphingolipid base synthesis, including serine-palmitoyl transferase (SPT) (Holleran *et al*, 1991b), the ceramide-generating enzymes sphingomyelinase (SMase) (Bowser and Gray, 1978; 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 intriguing to determine whether that enzyme balance might be altered in the skin of AD patients. In our previous study, we reported that there were no significant differences in the activities of GCase or CDase in extracts of stratum corneum from AD patients compared with age-matched normal controls (Jin *et al*,

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Abbreviations: CDase, Ceramidase; β-GCase, β-glucocerebrosidase; IEF, isoelectric focusing; PMSF, phenylmethylsulfonyl fluoride; SM, sphingomyelin; SMase, sphingomyelinase; SPC, sphingosylphosphorylcholine; SPT, serine-palmitoyl transferase; TLC, thin layer chromatography.

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1994). Because the activities of sphingolipid metabolic enzymes in the stratum corneum reflect the epidermal levels of those enzymes (Holleran *et al*, 1992; Takagi *et al*, 1999), the absence of any detectable abnormality in activities of those enzymes in the stratum corneum of AD patients suggested an alternative possibility that the upstream metabolism of sphingolipids, namely at the level of sphingomyelin (SM) hydrolysis, might be altered, which would result in the ceramide deficiency observed in AD. In an extensive study (Murata *et al*, 1996) in which we tested that possibility using radio-thin layer chromatography (TLC) and a quantitative assay of SM hydrolysis, we found that in the epidermis of AD patients, SM hydrolysis is over-expressed due to the abnormal expression of a hitherto undiscovered epidermal enzyme, termed SM deacylase. The radio-assay of SM hydrolysis, however, has a potential limitation for assessing accurately the activity of SM deacylase because it includes multiple breakdowns of SM to yield water-soluble radio-labeled phosphorylcholine, choline, or sphingosyl-phosphorylcholine (SPC), which is relevant to the activities of SMase, phosphodiesterase, or SM deacylase, respectively: to what extent the activity of SM deacylase is eventually highly expressed in the skin of AD patients compared with healthy controls remains to be elucidated before a direct quantitative assay for SM deacylase can be established. This quantification of SM deacylase activity is also essential for understanding the estimated release of the reaction product, SPC, in the epidermis of AD because we have already found that SPC is a potent pro-inflammatory molecule that would modulate inflammation in AD by releasing prostaglandins and by expressing intermolecular adhesion molecules-1 (ICAM-1) in human keratinocytes (Imokawa *et al*, 1999). In this study, on possible mechanisms involved in ceramide deficiency, we have established a new assay of SM deacylase and examined whether SM deacylase activity is upregulated in the skin of AD patients compared with normal controls or with patients with contact dermatitis. To this end, we synthesized a fluorescent derivative of SPC, 16-(9-anthroyloxy) hexadecanoyl SPC or [palmitic acid-^{14}C] SM as substrates and directly measured the activity of SM deacylase in tape stripped stratum corneum and in whole epidermis biopsies from AD patients as well as in normal control subjects and in patients with contact dermatitis.

MATERIALS AND METHODS

Materials [1–^{14}C] palmitic acid was purchased from Amersham (Bucks, U.K.). SPC was purchased from BIOMOL Research Laboratories (PA). Phospholipase A_2 (PLA_2) (p-8913 from bovine pancreas) and inhibitors of PLA_2, bromophenacyl bromide (BPB), and quinacrine were obtained from Sigma (St Louis, MO).

Preparation of 16-(9-anthroyloxy) hexadecanoyl-SPC As a fluoro-sphingomyelin to be used as a substrate of SM deacylase, 16-(9-anthroyloxy) hexadecanoyl-SPC was synthesized by condensing 16-(9-anthroyloxy) hexadecanoic acid with SPC according to the procedure of Hammastrom (1971). Fluorosphingomyelin was then purified by silicic acid chromatography or by preparative thin-layer chromatography (TLC) on 0.5 mm thick silica gel plates HR (Merck, Darmstadt, Germany), with redistilled analytical-grade solvents. 16-(9-anthroyloxy) hexadecanoyl-SPC synthesized here, when chromatographed on thin-layer plates of silica gel G, migrated with R_f values as described by Kates (1972), in the following solvent system: $CHCl_3$/MeOH/H_2O, 65/25/4; $CHCl_3$/MeOH/NH_4OH/H_2O, 65/35/2.5/2.5; $CHCl_3$/MeOH/acetic acid/H_2O, 25/15/4/2; $CHCl_3$/acetone/MeOH/acetic acid/H_2O, 6/8/2/2/1; and butanol/acetic acid/H_2O, 6/2/2.

Preparation of [1–^{14}C] palmitoyl SPC To a mixture of 0.65 mmol SPC and 37.5 mCi [1–^{14}C] palmitic acid in a silanized flask under nitrogen, 37.5 ml anhydrous tetrahydrofuran, 0.74 mmol o-benzotriazolyl-N,N,N,N-tetramethyluronium hexafluorophosphate, 60 ml anhydrous acetonitrile, 19.5 ml anhydrous N,N-dimethylformamide, 37.5 ml anhydrous toluene, and 0.36 ml triethylamine was added. The reaction mixture was stirred for 5 h at room temperature. It was evaporated to dryness under vacuum and purified by high performance liquid chromatography to yield (after removal of solvent), pure [1–^{14}C] palmitoyl SPC with a gravimetric specific activity of 59 mCi per mmol. The radiochemical purity of the synthetic SM checked by TLC on silica gel in chloroform:methanol:water (60:35:8) was at least 99%.

Preparation of stratum corneum and epidermis extracts Diagnoses of AD were made according to the criteria of Hanifin and Rajka (1989). Specimens of stratum corneum were obtained from normal forearm skin of healthy individuals (aged from 18 to 31 y old, mean age = 28.2 y old) and from uninvolved and involved skin of AD patients (aged from 14 to 33 y old; mean age, 21.7 y old) by tape-stripping three times (Protect-label/B type, 40–60 mm, Iuchiseieidou, Osaka, Japan) in the same region. Such tapes were stored frozen at –80°C until use.

For measurement of SM deacylase activity, the three tapes were immersed in 40 ml of 50 mM acetate buffer, pH 4.7, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% Triton X-100 for 30 min at 4°C, and were then sonicated on ice for a total of 3 min with 30 s bursts in a Bioruptor sonicator (Olympus, Tokyo, Japan). Mixtures containing the three tapes were then further incubated in extraction buffer overnight at 4°C to obtain Triton X-100 extracts for subsequent assay. After removing tapes, the extract solutions were concentrated to about 0.5 ml using a Centriprep 10 and Centricon 10 (Amicon Millipore, Beverley, MA), and concentrated solutions were used for SM deacylase assay.

For SM deacylase assay using epidermis, 4 mm skin punch biopsy specimens were treated with 1000 unit per ml dispase at 4°C for 4 h to separate the epidermis. Each 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 homogenates were centrifuged at $10000 \times g$ for 30 min to obtain supernatant and particulate fractions. The latter was further solubilized for 2 h in 300 μl of 50 mM acetate buffer, pH 4.7, containing 0.5 mM PMSF and 0.1% Triton X-100. The solubilized mixture was then centrifuged at $10000 \times g$ for 30 min to obtain a supernatant of the extracted fraction that was used for the SM deacylase assay.

Preparation of peripheral blood lymphocytes Blood was separated by Hypaque-Ficoll gradient centrifugation at $400 \times g$ for 30 min, and peripheral blood lymphocytes (PBL) were harvested from the interface of plasma and separation fluid. The cells were washed three times with saline and sequentially centrifuged at 400, 300, and $250 \times g$ for 10 min to eliminate platelet contamination. PBL were homogenized in PBS(–) and centrifuged at $10000 \times g$ for 30 min to obtain the supernatant fraction that was used for the SM deacylase assay.

Measurement of activities of SM deacylase and SMase and identification of enzymatic hydrolysis products SM deacylase and SMase activities were assayed by measuring the amount of 16-(9-anthroyloxy) hexadecanoic acid or radioactive palmitic acid and ceramide released from 16-(9-anthroyloxy) hexadecanoyl SPC or [1–^{14}C] palmitoyl SPC, respectively. The standard reaction mixture (final volume, 300 μl) contained 50 mM acetate buffer, pH 4.7, 10 nmol [1–^{14}C] palmitoyl SPC, 0.1% Triton X-100, 20 mM $CaCl_2$ and enzyme. The reaction mixture was incubated for 15 h at 37°C and terminated by the addition of 50 μl of 2 mg per ml carrier palmitic acid, followed by 3.0 ml of Dolels reagent (2-propanol-heptane-1N NaOH 40:10:1 vol/vol). After 1.8 ml heptane and 1.6 ml water were added, the mixture was vortex-mixed and centrifuged for 5 min at $2000 \times g$. One milliliter of the upper heptane phase, which had the enzymatically released radioactive ceramide, was transferred to a vial and mixed with scintillation fluid. The radioactivity was determined in a liquid scintillation counter to assess SMase activity. The remainder of the heptane phase was carefully aspirated, and the lower phase was washed twice with 2 ml heptane. Thereafter, 1 ml 1N H_2SO_4 and 2.4 ml heptane was added, and the mixture was vortex-mixed, then centrifuged for 10 min at $2000 \times g$. One milliliter of the upper phase was washed with an equal volume of 1N H_2SO_4 to remove the small amount of the unreacted SM. Following mixing and centrifugation, 0.8 ml of the washed upper phase was counted as above for measurement of SM deacylase activity. To establish background, blank tubes were carried through without the enzyme source. The labeled reaction products from SMase and SM deacylase activities were then identified by TLC on silica gel with the solvent system chloroform-methanol-acetic acid 94:1:5 (vol/vol). These were compared with radioactive standards, SM, ceramide, i.e., palmitoylsphingosine, which was prepared as described previously (Yada *et al*, 1995), and fluorescent standards or palmitic acid. Radio or normal TLC analysis demonstrated that reaction products in the first upper heptane phase (which partitioned in the biphasic system of Dole) and in the final upper phase (after washes with H_2SO_4) were ceramide and fluorescent hexadecanoic acid or palmitic acid, respectively, with mobilities on TLC plates nearly identical to labeled standards (**Fig 1*a*, *b***), indicating an effective separation of palmitoyl-

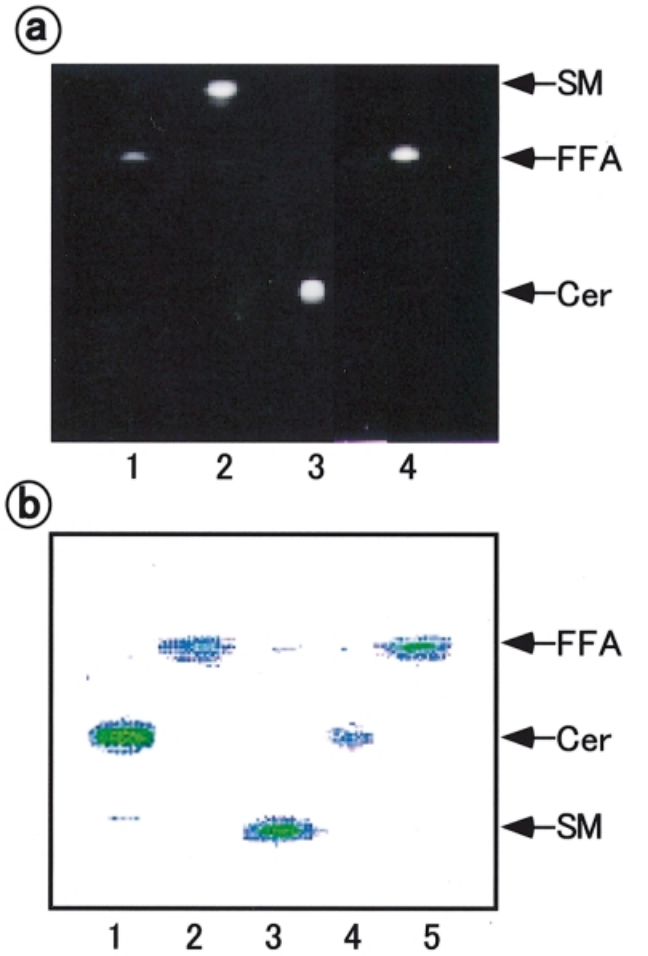

Figure 1. Identification of reaction products as fatty acid by TLC. After the indicated periods of incubation of stratum corneum sample (0.3 ml) at pH 4.7 with 10 nmol SM, the reaction products in the final upper phase, after washing with H_2SO_4, were dried under nitrogen and dissolved in 0.1 ml of chloroform:methanol (2:1 vol/vol) for TLC. Sample solutions with approximately 1000 dpm or 2000 fluorescent intensity were subjected to TLC analysis. Radioactivities or fluorescent intensity on TLC plates were quantified utilizing a TLC scanner and are expressed as a color density responsible for dpm or fluorescent intensity. (*a*) *Lane 1*, fluorescent metabolite in final upper phase after washing with H_2SO_4; *lanes 2*, *3*, and *4*, standards of fluorescent SM, ceramide, and fatty acid, respectively. (*b*) *Lane 1*, radio-labeled metabolite in the first upper phase; *lane 2*, radio-labeled metabolite in final upper phase after washing with H_2SO_4; *lanes 3*, *4*, and *5*, standards of radio-labeled SM (palmitoyl SPC), ceramide (palmitoyl-sphingosine), and palmitic acid (FFA), respectively.

sphingosine (ceramide) and 16-(9-anthroyloxy) hexadecanoic acid or palmitic acid using this partition method.

Measurement of acid ceramidase assay Acid ceramidase was assayed at pH 4.7 by measuring the amount of radioactive palmitic acid released from [1–^{14}C] palmitoylsphingosine (50 mCi per mmol) using a slightly modified assay mixture as described previously (Yada *et al*, 1995). The reaction mixture was identical to that used for measurement of SM deacylase activity described above, except for the substrate.

Other methods The protein concentration of tissue extracts was determined using a BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Statistics The level of significance of the difference was calculated by Student t test.

RESULTS

Acid ceramidase activity in the stratum corneum of AD patients Assays using ^{14}C-palmitoylsphingosine as a substrate demonstrated that acid CDase activity in stratum corneum extracts from AD patients is significantly decreased to a negligible level compared with that from healthy control subjects, particularly in involved skin (**Fig 2**).

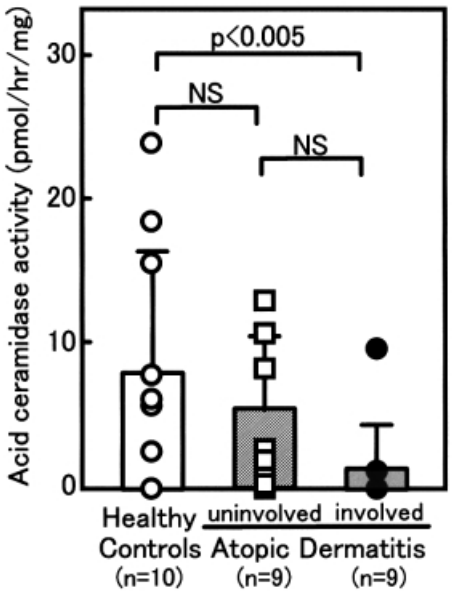

Figure 2. Acid ceramidase activities in the stratum corneum from AD patients and from healthy controls. Acid ceramidase activities are expressed as liberated pmol of radioactive FFA into the final upper phase per h per mg protein after 15 h incubation at pH 4.7 with 0.5 μCi ^{14}C-palmitoylsphingosine.

Table I. SM deacylase activity and PLA_2[a]

PLA_2 (units)	Release of FFA (pmol per h)
0.001	0.18
0.01	0.22
0.1	0.04
1	0
10	0

[a]PLA_2 was incubated with [palmitic acid ^{14}C-] SM at pH 4.7 for 15 h in the same assay buffer as that used for SM deacylase and the release of ^{14}C-palmitic acid in the final upper phase used was counted in a liquid scintillation counter. PLA_2, phospholipase A_2; FFA, free fatty acid.

Table II. Lack of effect of PLA_2 inhibitors on SM deacylase activity in extracts from the stratum corneum of patients with AD[a]

	SM deacylase (pmol per h per mg)
Control	180.5
BPB 0.1 mM	175.4
1.0 mM	182.3
Control (DMSO)	169.4
quinacrine 0.1 mM	172.8
1.0 mM	179.2

[a]Extracts from the stratum corneum of patients with AD were incubated in the presence of PLA_2 inhibitors with [palmitic acid ^{14}C-] SM at pH 4.7 for 15 h in the same assay buffer as that for SM deacylase. The release of ^{14}C-palmitic acid in the final upper phase of the assay procedure for SM deacylase was counted in a liquid scintillation counter. BPB, bromophenacyl bromide; SM, sphingomyelin; DMSO, dimethylsulfoxide; AD, atopic dermatitis.

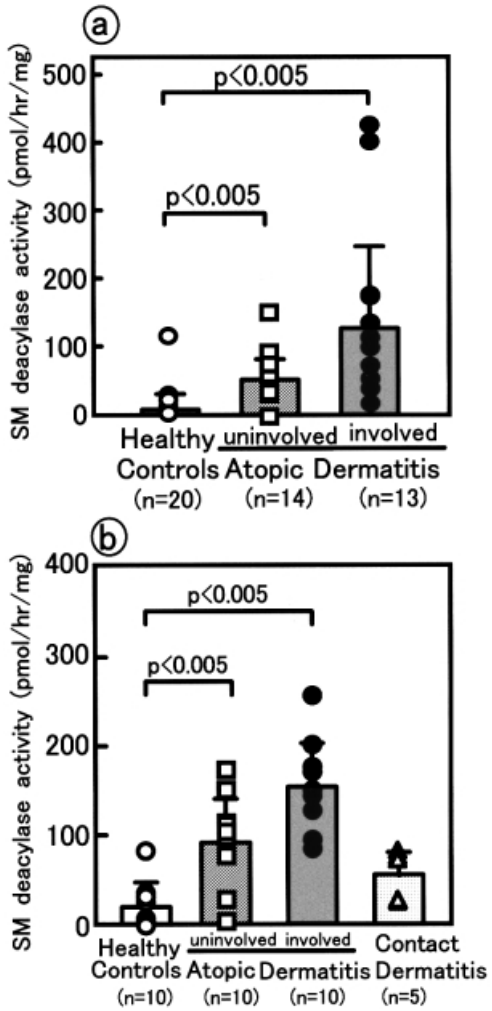

Figure 3. SM deacylase activities in the stratum corneum from AD patients in comparison with those from contact dermatitis and age-matched healthy control subjects. SM deacylase activities are expressed as liberated pmol of fluorescent FFA (*a*) or radioactive FFA (*b*) into the final upper phase per h per mg protein after 15 h incubation at pH 4.7 with fluorescent SM or 0.5 μCi [palmitic acid-^{14}C] SM.

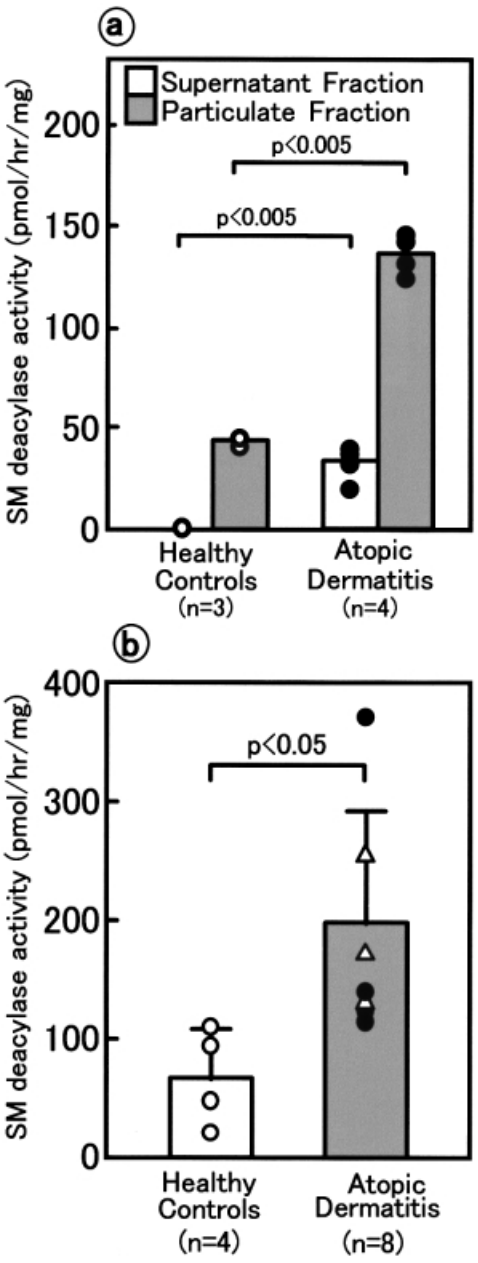

Figure 4. SM deacylase activities in the biopsied epidermis from AD patients and from age-matched healthy controls. SM deacylase activities are expressed as liberated pmol of fluorescent FFA (*a*) or radioactive FFA (*b*) into the final upper phase per h per mg protein after 15 h incubation of epidermal samples ($10^4 \times$ g particulate fraction; 200 μl) prepared from 4 mm skin punch biopsies, at pH 4.7 with fluorescent SM or 0.5 μCi [palmitic acid-^{14}C] SM.

SM deacylase activity is not derived from phospholipase A_2 In order to rule out the possibility that the SM deacylase activity is derived from phospholipase A_2, we determined whether phospholipase A_2 has the ability to hydrolyze [palmitic acid-^{14}C] SM at the acyl site to yield ^{14}C-palmitic acid. Measurement of radioactivity in the final upper layer according to the assay method used for SM deacylase activity revealed that there was no substantial release of ^{14}C-free fatty acid following incubation with PLA_2 at concentrations ranging from 0.001 to 10 units (**Table I**). This shows that PLA_2 is not able to hydrolyze SM at the acyl site to yield free fatty acid. In other experiments using inhibitors of PLA_2 (Mao-Qiang *et al*, 1995), we found that the activity of SM deacylase in extracts of the stratum corneum from patients with AD was not affected by the PLA_2 inhibitors, bromophenacyl bromide or quinacrine (**Table II**). The sum of these data rule out the possibility that the activity of SM deacylase noted in this study could be ascribed to the activity of PLA_2.

SM deacylase in the stratum corneum Assays using fluorescent free fatty acid bearing SM as a substrate for SM deacylase (**Fig 3*a***) demonstrate that the stratum corneum from AD lesional forearm skin (volar side) has an extremely high level of SM deacylase activity with a magnitude 17 times higher than that in healthy controls. In nonlesional AD skin, SM deacylase activity is still nine times higher than that found in healthy controls. Assays using [palmitic acid-^{14}C] SM as a substrate for SM deacylase (**Fig 3*b***) demonstrate that the stratum corneum from lesional forearm skin (volar side) of patients with AD has an extremely high level of SM deacylase activity, with a magnitude five times higher than that present in healthy controls. In nonlesional AD skin, SM deacylase activity is still three times higher than that detected in healthy controls. In contrast, the stratum corneum from contact dermatitis patients shows levels similar to healthy controls.

Activities of SM deacylase and SMase in biopsied epidermis In biopsies of whole epidermis of patients with AD, SM deacylase activity (as measured using fluorescent SM as a substrate) is significantly increased by 3-fold over healthy control

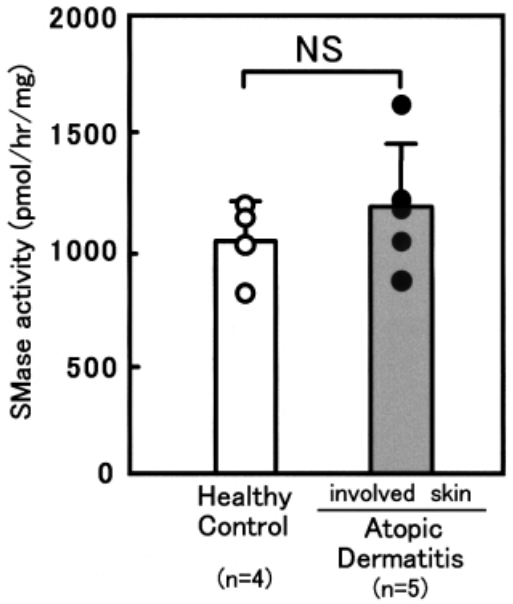

Figure 5. SMase activities in the biopsied epidermis from AD patients and from age-matched healthy control. SMase activities are expressed as liberated pmol of radioactive ceramide into the first upper phase per h per mg protein after 15 h incubation of epidermal samples ($10^4 \times$ g particulate fraction; 200 μl) prepared from 4 mm skin punch biopsies, at pH 4.7 with 0.5 μCi [palmitic acid-^{14}C] SM.

levels in the particulate fraction of epidermal homogenates (**Fig 4*a***). When measured using [palmitic acid-^{14}C] SM as a substrate, SM deacylase activity is again seen to be significantly increased by 3-fold compared with healthy controls (**Fig 4*b***). As depicted by open triangles, which report data obtained from AD epidermis tape-stripped of stratum corneum that show higher SM deacylase activities, we can rule out the possibility that the high activity of SM deacylase is due to contamination of bacterial enzymes in the AD stratum corneum. In contrast, there is no significant difference in SMase activity measured using [palmitic acid-^{14}C] SM as a substrate between AD and healthy controls (**Fig 5**).

SM deacylase activity in peripheral blood lymphocytes In contrast, in peripheral blood lymphocytes from patients with AD, assays for SM deacylase using fluorescent SM as a substrate demonstrate that there is no increase in SM deacylase activity compared with healthy controls (**Fig 6*a***). When measured using [palmitic acid-^{14}C] SM as a substrate, there is no increase in SM deacylase activity in peripheral blood lymphocytes from AD patients relative to healthy controls (**Fig 6*b***), suggesting a possibility that the high expression of SM deacylase is associated with the skin of AD patients.

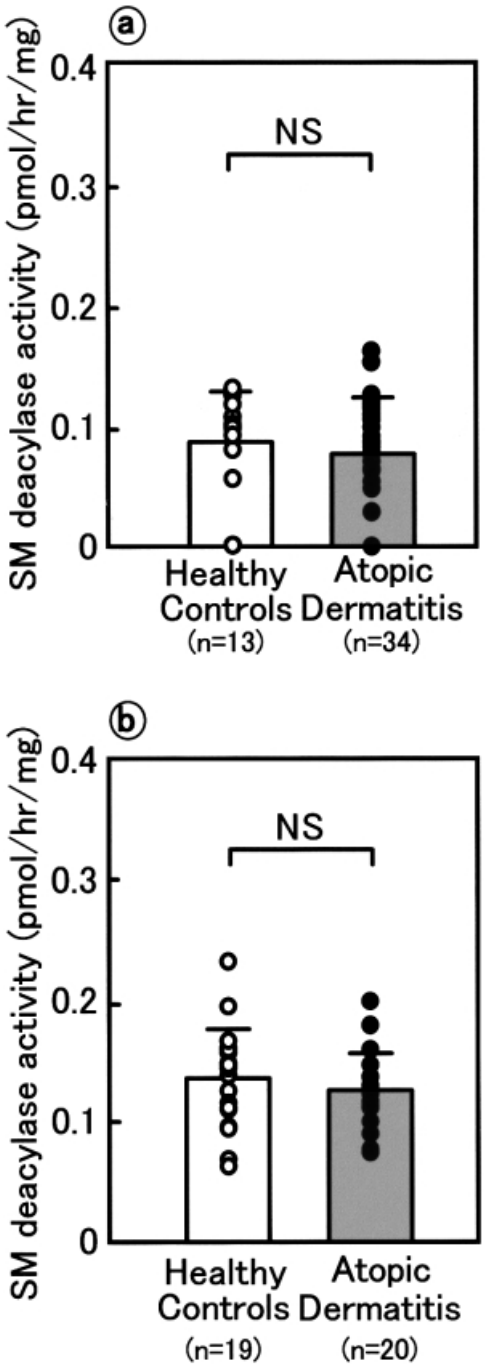

Figure 6. SM deacylase activities in peripheral blood lymphocytes from AD patients and from age-matched healthy controls. SM deacylase activities are expressed as liberated pmol of fluorescent FFA (*a*) or radioactive FFA (*b*) into the final upper phase per h per mg protein after 15 h incubation at pH 4.7 with fluorescent SM or 0.5 μCi [palmitic acid-^{14}C] SM.

DISCUSSION

In assays to measure the release of [1–^{14}C] palmitic acid as SM deacylase activity, the released amounts of palmitic acid would not necessarily represent the activity of SM deacylase if both acid SMase and CDase are present in stratum corneum extracts from AD patients. This is due to the fact that the release of radiolabeled palmitic acid could also result from a two step reaction where SM would be initially hydrolyzed by acid SMase to yield radiolabeled palmitoylsphingosine (ceramide), which in turn could be converted by acid CDase to radiolabeled palmitic acid. The negligible levels of acid CDase in AD stratum corneum, however, allow us to conclude that the released amounts of radiolabeled palmitic acid reflect direct cleavage of [palmitic acid-^{14}C] SM at the acyl site. Consistent with this, in our parallel study (Higuchi *et al*, 2000) on the enzymatic properties of SM deacylase, the addition of nonradiolabeled palmitoylsphingosine (ceramide) to the reaction mixture for SM deacylase (as a dilution effect on radiolabeled ceramides possibly generated by SMase) did not suppress SM deacylase activity measured using stratum corneum extracts from AD patients. In contrast, similar additions to the reaction mixture for acid CDase significantly suppressed the activities of acid CDase for the release of radiolabeled palmitic acid, suggesting that there was no contribution of acid CDase to the SM deacylase activity. Thus, in our assays of AD stratum corneum, it is probable that released amounts of [1–^{14}C] palmitic acid are due to the direct deacylation of [palmitic acid-1–^{14}C] SM, corresponding to the

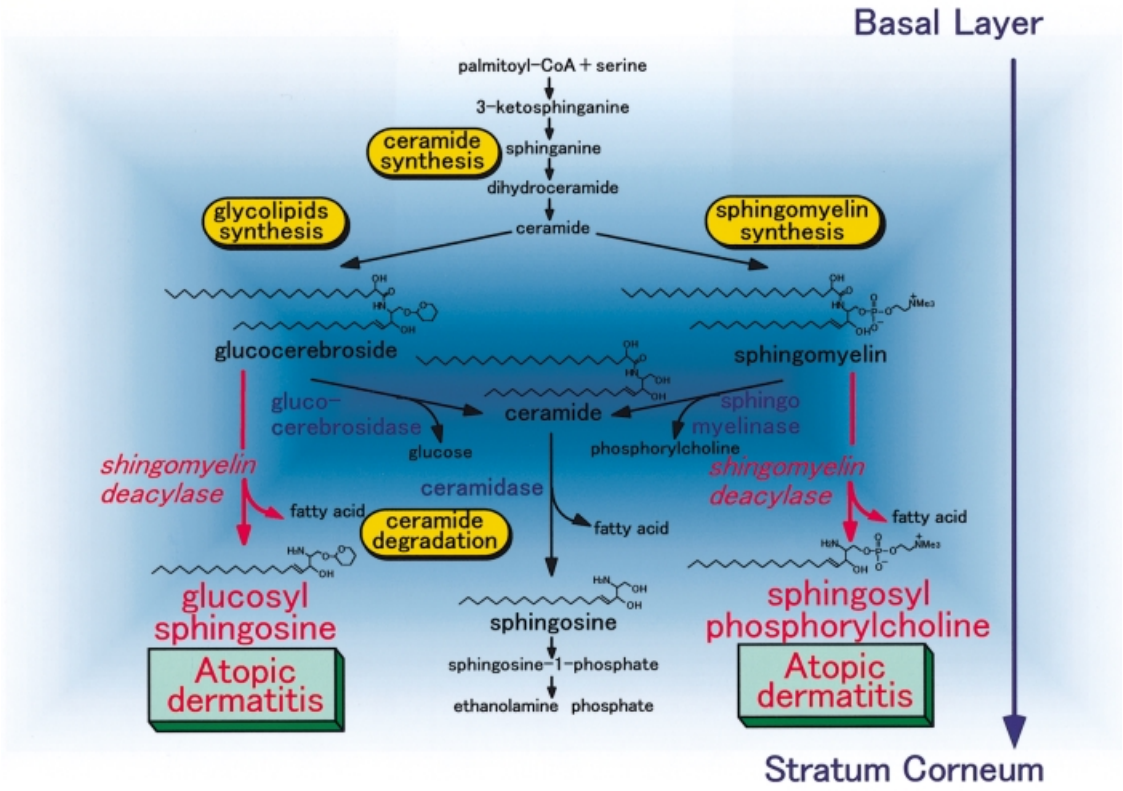

Figure 7. Glucosylceramide and SM hydrolysis pathways in AD.

activity of SM deacylase. Furthermore, the existence and the entity of the SM deacylase in extracts of AD stratum corneum was corroborated by isoelectric focusing chromatography in which SM deacylase activity was eluted with an isoelectric point distinct from SMase activity (Higuchi *et al*, 2000). This study has demonstrated that SM deacylase activity is enhanced more than 5-fold in involved stratum corneum and more than 3-fold in uninvolved stratum corneum of AD patients, compared with normal stratum corneum. In contrast, the stratum corneum from patients with contact dermatitis showed no increases in SM deacylase activity compared with healthy controls, suggesting that changes in SM deacylase activity are unlikely to be involved in the general etiology of cutaneous inflammation. Our previous study on the epidermal localization of CDase and GCase, the latter being a hydrolytic enzyme in intercellular spaces between the stratum corneum and the granular layer, suggested that the activities of ceramide metabolism-related enzymes within the stratum corneum approximately represent the epidermal activities of the same enzymes (Holleran *et al*, 1992; Yada *et al*, 1995; Takagi *et al*, 1999). Consistent with this relationship, similar high levels of SM deacylase activity were detected in the epidermis from AD patients, whereas there was no significant difference in SMase between AD and healthy controls, suggesting that epidermal cells from these patients show abnormal production of a hitherto undiscovered epidermal enzyme, termed SM deacylase. To exclude the possibility that the SM deacylase activity observed was due to contamination by bacteria, such as *Staphylococcus aureus*, which are often present on the surface of the stratum corneum of AD patients (Leyden *et al*, 1974; Dahl, 1983), we examined whether high levels of SM deacylase activity were present in epidermis freed of bacteria by excessive tape-stripping of the stratum corneum before biopsy. The SM deacylase activities of such tape-stripped skin still showed higher levels in AD patients, suggesting that the high SM deacylase activity noted is not derived from bacterial contamination of the stratum corneum.

In our parallel study (Higuchi *et al*, 2000), we characterized the properties of this hitherto undiscovered epidermal enzyme by measuring the release of [palmitic acid-1–^{14}C] SM as a substrate after incubation with extracts of stratum corneum from AD patients. The pH optimum for SM deacylase showed a sharp pH dependency of catalytic activity, with a peak at pH 5.0. The pI of SM deacylase is 4.2 (determined by analytical isoelectric focusing (IEF) chromatography) and the apparent molecular mass of SM deacylase is 40 000 by SDS-PAGE, which are molecular properties completely distinct from those of SMase (6.9 and 100,000, respectively). It is interesting to note that SM deacylase activity can be detected by IEF, though to a lesser degree, with a similar pI in stratum corneum from healthy control subjects. Because SM deacylase is similar in enzymatic properties to sphingolipid ceramide N-deacylase, a novel enzyme present in *Pseudomonas* sp. *TK4*, which is capable of hydrolyzing the N-acyl linkage in some glycosphingolipids including SM but not ceramide (Ito *et al*, 1995), it is an intriguing possibility that they might be identical. The molecular properties of SM deacylase determined in our previous study (Higuchi *et al*, 2000), however, suggest that SM deacylase is also completely different from the sphingolipid ceramide N-deacylase (with a molecular weight of 50 000). Furthermore, it is unlikely that phospholipase A_1 or A_2 present in the epidermis contributes to SM deacylase activity based upon the facts that (1) there was no inhibitory effect of phospholipase A_2 inhibitors on the SM deacylase activity observed, (2) commercially available phospholipase A_2 is unable to hydrolyze SM, and (3) there was no detectable activity of phospholipase A_1 or A_2 in human epidermis following extraction with 0.1% Triton X-100 because of the inhibitory effect of 0.1% Triton X-100 (Bowser and Gray, 1978; Freinkel and Traczyk, 1980). With regard to the enzymatic properties of SM deacylase, whether CDase (N-acylsphingosine deacylase) is involved in the hydrolysis of the N-acyl linkage of SM remains to be elucidated. In this connection, we have used guinea pig epidermis to purify two forms of CDase with alkaline pH

optima and apparent molecular weights of 148 000 and 60 000 (Yada *et al*, 1995). Thus, alkaline CDases are enriched in the epidermis compared with the acidic type. Furthermore, we had previously found that purified CDase derived from guinea pig epidermis has no SM hydrolysis activity at acidic or alkaline pH (Yada *et al*, 1995). In this study, there is a significant decrease in the acid CDase activity of extracts of involved and uninvolved stratum corneum from AD patients compared with healthy controls. In addition, when SM deacylase from stratum corneum of AD patients was partially purified by column chromatography and assessed at pH 4.7 using [1–^{14}C] palmitoylsphingosine as a substrate, there was a negligible level of acid CDase in the partially purified fractions with SM deacylase activity (Higuchi *et al*, 2000). Therefore, it is unlikely that acid ceramidase is involved in the activity of SM deacylase detected in the stratum corneum of AD patients.

In focusing on SM metabolism rather than on reactions mediated by CDase and GCase as an unresolved mechanism underlying the ceramide deficiency of stratum corneum from AD patients, we found a novel abnormally expressed epidermal enzyme related to SM metabolism, termed SM deacylase. The abnormal expression of SM deacylase in the epidermis of AD patients allows 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 intercellular spaces by normal levels of SMase, and thus there is no residual SM in the stratum corneum. It is therefore likely that the specific expression of SM deacylase in the skin of AD patients provides a strong basis that explains the ceramide deficiency, regardless of whether SMase activity is enhanced or downregulated. This explanation of the alteration of SM metabolism leading to ceramide deficiency, however, is not the complete story when considering metabolism leading to the formation of acylceramides (ceramide-1), as acylceramides are not generated through SM metabolism but are markedly decreased in the stratum corneum of AD patients (Imokawa *et al*, 1991).

In our previous study[2] using specific inhibitors of glucosyltransferase and β-GCase, we reported that glycosylation to ceramides and subsequent deglycosylation of acylglucosylceramide are required to form acylceramides in the stratum corneum, suggesting that acylceramides are eventually generated through deglycosylation of acylglucosylceramides by β-GCase. In our preliminary study using ^{14}C-palmitic acid bearing glucosylceramide, partially purified SM deacylase (excluding β-GCase and acid CDase) was able to degrade glucosylceramide at the N-acyl linkage of ceramide to yield glucosylsphingosine. This suggests that SM deacylase can compete with β-GCase for the same substrate, glucosylceramide or acylglucosylceramide, to yield glucosylsphingosine rather than ceramide, which would lead to a deficiency of ceramides, including acylceramide.

In conclusion, in this study we have elucidated the biologic mechanism involved in ceramide deficiency that may be an important etiologic factor for the dry and barrier-disrupted skin of AD patients. The characteristic of barrier-disrupted skin in AD patients may be involved in the high susceptibility to irritants or allergens and infection, especially to mite components. In a previous study (Kondo *et al*, 1998), we found that allergic reactions induced percutaneously by such high molecular weight antigens were dominated by Th_2 type immunity. In AD, the dominance of Th2 type immunity has been reported (van der Heijden *et al*, 1991). Thus, the expression of SM deacylase may trigger the metabolism of SM, resulting in the ceramide deficiency that is closely associated with barrier disruption and deranged water-holding function in the stratum corneum of AD patients.

Because these dysfunctions of the stratum corneum provide an etiologic basis for eliciting atopic dermatitis through the easy penetration of high molecular weight antigens or irritants and scratching due to dryness-derived itching, it is probable that the mechanism behind the abnormal expression of SM deacylase is closely linked to the pathogenesis of AD.

With regard to the abnormality of SM metabolism as an important etiologic basis for abnormal function of the stratum corneum in AD patients, it would be of considerable interest to determine to what extent SM deacylase is functional *in vivo* in reducing the production of ceramides and/or in releasing the reaction product, SPC, in AD skin. Furthermore, we have recently found that the exogenous addition of SPC at μM concentrations causes human keratinocytes in culture to augment the expression of the inflammatory adhesion molecule, ICAM-1 (Imokawa *et al*, 1999), suggesting that an excessive production of SPC *in vivo* may trigger the inflammatory process in the skin of AD patients. For this reason, measurement of SPC actually generated in epidermal tissue from AD patients *in vivo* is currently under investigation. Taken together, these findings suggest that, as shown in **Fig 7**, SM metabolism is definitely altered in AD, resulting in a reduction in levels of ceramides and a reciprocal increase of a potent inflammatory chemical, SPC (Imokawa *et al*, 1999), which may be important etiologic factors in the atopic dry skin and the genesis of cutaneous inflammation, respectively.

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