Sphingosylphosphorylcholine is upregulated in the stratum corneum of patients with atopic dermatitis

Reiko Okamoto,* Junko Arikawa,* Mutsumi Ishibashi,* Makoto Kawashima,* Yutaka Takagi,† and Genji Imokawa[1,*,†]

Department of Dermatology,* Tokyo Women's Medical University, Tokyo; and Kao Biological Science Laboratories,† Tochigi, Japan

Abstract To clarify the functional relevance of sphingomyelin (SM) deacylase to the ceramide deficiency seen in atopic dermatitis (AD), we developed a new highly sensitive method and measured the metabolic intermediate sphingosylphosphorylcholine (SPC) that accumulates in the stratum corneum. SPC in intercellular lipids extracted from stratum corneum was reacted with [^{14}C]acetic anhydride to yield [^{14}C-C_2]SM, which was then analyzed by TLC. In both the lesional and non-lesional stratum corneum obtained from patients with AD, there was a significant increase in the content of SPC over that of healthy control subjects. There was a reciprocal relationship between increases in SPC and decreases in ceramide levels of stratum corneum obtained from healthy controls, and from lesional and non-lesional skin from patients with AD. Comparison with other sphingolipids present in the stratum corneum demonstrated that there is a significant positive correlation between SPC and glucosylsphingosine, another lysosphingolipid derived from glucosylceramide by another novel epidermal enzyme, termed glucosylceramide deacylase. In contrast, there was no correlation between SPC and sphingosine, a degradative product generated from ceramide by ceramidase. These findings strongly suggest the physiological relevance of SM deacylase function in vivo to the ceramide deficiency found in the skin of patients with AD.—Okamoto, R., J. Arikawa, M. Ishibashi, M. Kawashima, Y. Takagi, and G. Imokawa. **Sphingosylphosphorylcholine is upregulated in the stratum corneum of patients with atopic dermatitis. *J. Lipid Res.* 2003.** 44: **93–102.**

Supplementary key words sphingomyelin deacylase • barrier disruption • ceramide deficiency

Intercellular lipids in the stratum corneum have been implicated as important determinants in the water-retaining properties (1, 2) and in the barrier function (3, 4) of the skin. Ceramides are major constituents of intercellular lipids, comprising more than 50% of them. Previously, we demonstrated that there is a marked reduction in the amount of ceramides in the stratum corneum in lesional and in non-lesional forearm skin of patients with atopic dermatitis (AD) (5), which suggested that the ceramide deficiency is an important etiologic factor for the barrier-disrupted and dry skin seen in AD. Subsequently, we found that the causative factor behind the ceramide deficiency in the stratum corneum of patients with AD is an abnormal expression of sphingomyelin (SM) deacylase in their epidermis (6). This enzyme hydrolyzes SM at the acyl site to yield free fatty acid and sphingosylphosphorylcholine (SPC) instead of the formation of ceramide and phosphorylcholine (PC) by sphingomyelinase (SMase). Direct enzymatic analysis of the stratum corneum or of the epidermis of patients with AD revealed that there are 9-fold or 3-fold increases, respectively, in the activity of SM deacylase in patients with AD compared with healthy normal controls (7). The sum of those findings demonstrates that the novel epidermal enzyme SM deacylase is expressed at high levels in the epidermis of patients with AD. The competition of this enzyme with SMase for the common substrate SM leads to the ceramide deficiency in the stratum corneum of patients with AD. Thus in this study, in order to clarify the physiologic and functional relevance of SM deacylase to the ceramide deficiency in the epidermis of patients with AD, we have determined whether the major metabolic intermediate, SPC, that is produced actually accumulates in the stratum corneum of patients with AD as a result of SM deacylase activity.

MATERIALS AND METHODS

Materials

SPC was purchased from Matreya Inc. (Pleasant Gap, PA). Sphingosine (Sph), glucosylsphingosine (GS), and ceramides

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Abbreviations: AD, atopic dermatitis; Cdase, ceramidase; GlCdase, β-glucocerebrosidase; PC, phosphorylcholine; SM, sphingomyelin; SMase, sphingomyelinase; SPC, sphingosylphosphorylcholine; Sph, sphingosine; TLC, thin layer chromatography.

[1] To whom correspondence should be addressed.
e-mail: imokawag@dream.ocn.ne.jp

were obtained from Sigma Chemical Co. (Saint Louis, MO). [Methyl-^{14}C]acetic anhydride was purchased from Amersham. All other chemicals were of reagent grade.

Preparation of stratum corneum

Diagnoses of AD were made according to the criteria of Hanifin and Rajka (8). Specimens of stratum corneum were obtained from normal forearm skin of healthy individuals (aged from 18 to 31 years old) and from uninvolved and from involved skin of AD patients (aged from 14 to 33 years old) by tape stripping three times with adhesive tape (P.P.S. Nichiban, Tokyo Japan) in the same region. Such tapes were stored frozen at −80°C until use. In this study, erythematous lesions without exudation and swelling were chosen as the involved skin of patients with AD for the preparation of the stratum corneum. The study was approved by the Ethics Committee in Tokyo Women's Medical University. Informed consent was obtained from each patient prior to sampling.

Purification of SPC from stratum corneum lipids

After extraction by acetone from human forearm skin, 10 mg of stratum corneum lipids were solubilized in chloroform-methanol (2:1, v/v) and loaded on a 1 ml Silica column (Sep-Pak). After a 4 ml wash with chloroform following a 3 ml wash with acetone, SPC was eluted with 5.6 ml 0.1 N NaOH / 80% methanol and was then extracted by the addition of 4.5 ml chloroform and 2.95 ml water. The lower phase was evaporated under N_2 and was then resuspended in small volumes of chloroform-methanol (2:1, v/v) and applied to silica gel 60 HPTLC plates (Merck, KGaA Darmstadt, Germany). The plates were developed in chloroform-methanol-acetic acid-water (50:30:8:5, v/v/v/v) in a horizontal HPLC chamber (CAMAG, Muttenz, Switzerland).

HPLC-mass spectrometry

Spots on HPTLC plates corresponding to the SPC standard were scraped and dissolved in 100 μl water and approximately 3.4 pg lipids (0.68 ppm × 5 μl) were then subjected to HPLC-mass spectrometry, according to the method of Suzuki et al (9). A JMN-HX110 double focusing mass spectrometer equipped with a fast atom bombardment (FAB) 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 ions from 300 *m/z* to 2,200 *m/z* at intervals of 5 s. HPLC was performed with a Model 120A (Applied Biosystems) on a reversed-phase column of SPHERI-5 (RP-18, 250 mm × 1 mm id, Applied Biosystems) with methanol containing 0.1% trifluoroacetic acid (TFA) and 1% glycerol (G) as the elution solvent at a flow rate of 100 ml per min. The column oven temperature was kept at 40°C. A frit interface with a splitter (JEOL) was set up between the HPLC and the MS. The split ratio was 1:19; therefore, one-twentieth of each injected sample was introduced into the FAB/MS and the rest was discarded via a drainage tube.

Quantitative assay for SPC, GS, or Sph by *N*-acetylation with [methyl-^{14}C]acetic anhydride

SPC, GS, or Sph was quantified using the modified method of Yatomi et al (10). Each dried sample (20–50 μg) was dissolved in 20 μl 0.008 N NaOH in dehydrated methanol. The acetylation reaction as depicted in **Fig. 1A** was started by the addition of 20 μl 10 mM [methyl-^{14}C]acetic anhydride in chloroform and proceeded for 2 h at 37°C. The remaining anhydride was hydrolyzed by the addition of 200 μl 0.2 N NaOH in methanol and incubation at room temperature. The [^{14}C]C_2-glucosylceramide and SM or ceramide produced, which were identified using each standard, was extracted by adding 0.78 ml methanol, 0.98 ml chloroform, and 0.9 ml 1N KCl. Carrier lipids were applied to the solvent to increase the efficiency of extraction. The resultant lower phase was evaporated under N_2 and was then resuspended in a small volume of chloroform-methanol (2:1, v/v) and applied to silica gel 60 HPTLC plates (Merck). The plates were developed in chloroform-methanol-acetic acid (190:9:1, v/v/v) for C-2 ceramide, chloroform-methanol-acetone (76:20:4, v/v/v) for C-2 glucosylceramide, and chloroform-methanol-water (65:25:4, v/v/v) for C-2 sphingomyelin in a horizontal HPLC chamber (CAMAG), and were quantitated using a imaging analyzer (BAS-2000, Fuji Film Corp, Tokyo, Japan). A representative chromatogram and the standard SPC curve are shown in Fig. 1B and **Fig. 2**. In this experiment using the stratum corneum samples, SPC appears to exist at 5–50 ng per sample. A representative chromatogram and the standard curve for Sph and GS was described elsewhere (11, 12).

Lipid extraction

To measure ceramide and lysosphingolipids, including SPC, GS and Sph, stratum corneum lipids were extracted with the modified method of Rawlings et al (13). In brief, stratum corneum was removed from the volar side of forearm skin by stripping with adhesive tape (P.P.S. Nichiban: 4 × 2.5 cm) three times. Next, the stratum corneum was separated from the adhesive tape by washing with n-hexane under ultrasonication (Bransonic B3200, Yamato Scientific Co.). The separated stratum corneum was dried and weighted (0.5–1.0 mg per sample) to express SCP amount as ng/mg stratum corneum weight. Sphingolipids, including ceramides, SPC, GS, and Sph, were extracted from the separated stratum corneum with chloroform-methanol (2:1, v/v) and were subjected to quantitation of ceramides, SPC, GS, and Sph.

Thin-layer chromatography for ceramide quantitation

For quatitation of ceramides, approximately 30 μg of the extracted lipids was applied to thin-layer chromatograms (TLC), which was developed twice with chloroform-methanol-acetic acid (190:9:1, v/v/v) to resolve ceramides. After solvent development, the chromatograms were air-dried, sprayed with 10% $CuSO_3$, 8% H_3PO_4 aqueous solution, and were then charred on a 180°C hotplate. The charred lipids were quantitated by photodensitometry (Shimazu CS-9000) and the data were subjected to 2-Dimensional Image Analyzer (Shimazu). Ceramides were quantitated by determining the micrograms of ceramides on a TLC chart from appropriate commercial standards, and are expressed as μg ceramide/mg stratum corneum weight. Ceramides (non-hydroxy fatty acid and hydroxy fatty acid type) (Sigma Chemical Co.) were used as standards for ceramides 1 and 2, and ceramides 3, 4, 5, and 6, respectively. The reproducibility of this method was confirmed using triplicate samples from the same subjects and the deviation of values (total lipids or micrograms ceramide per milligrams stratum corneum) was within 5% of the means.

Statistical analysis

A nonparametric one-way ANOVA (Kruskal-Wallis test) was used to evaluate differences between groups. Where appropriate, a nonparametric post hoc multiple comparison test (Steel-Dwass test) was performed to evaluate differences between the groups. $P < 0.05$ was considered statistically significant.

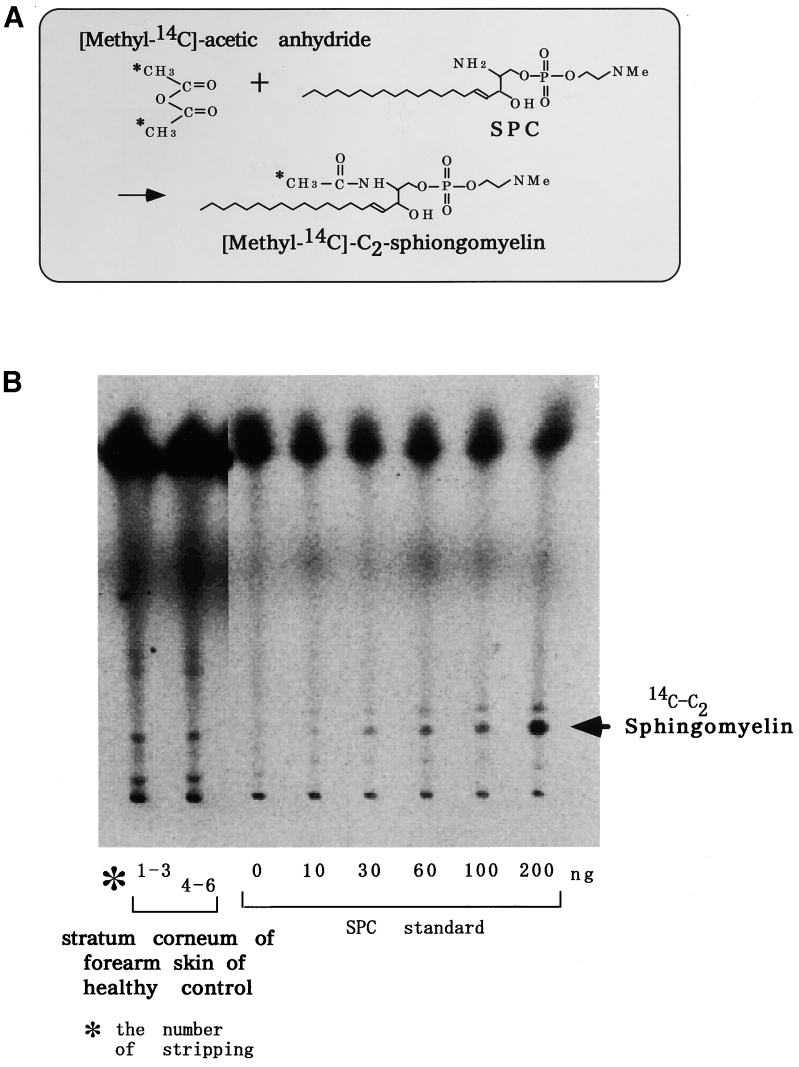

Fig. 1. TLC separation of [^{14}C-C$_2$]sphingomyelin following reaction of SPC with [^{14}C]acetic anhydride. A: chemical reaction of SPC with [^{14}C]acetic anhydride. B: TLC separation of [^{14}C-C$_2$]sphingomyelin produced.

RESULTS

SPC levels in the stratum corneum of healthy subjects

To determine whether SPC exists in the stratum corneum of healthy subjects at substantial levels, HPLC-MS spectrometry was carried out using lipids extracted from the stratum corneum following their separation by TLC (**Fig. 3A**). Figure 3B shows mass chromatograms of purified Sph-PC (20 ng) injected into the LC-MS, and peaks selected as the pseudo-molecular ions ([M-H]$^-$) of the molecular species of Sph-PC at *m/z* 184 and *m/z* 465 were observed clearly. As shown in Fig. 3C, the mass spectrum of each of these molecular species was obtained and pseudo-molecular ions were detected with strong intensity at *m/z* 180 and *m/z* 456. Furthermore, ions due to the elimination of sphingosine from the molecules were also detected at *m/z* 184, although the intensity of those ions was rather weak. Mass chromatograms of the TLC-separated lipids (Fig. 3D) revealed that there are two peaks at 184 *m/z* and 465 *m/z* that correspond in molecular weight to PC and SPC, respectively. This chromatographic pattern is in agreement with that observed using the SPC standard (Fig. 3B) in which there are two similar peaks at 184 *m/z* and 465 *m/z*, which correspond in molecular weight to PC and SPC, respectively.

SPC is significantly increased in the stratum corneum of patients with AD compared with healthy controls

Quantitative analysis of SPC in the stratum corneum of patients with AD revealed that there is a significant in-

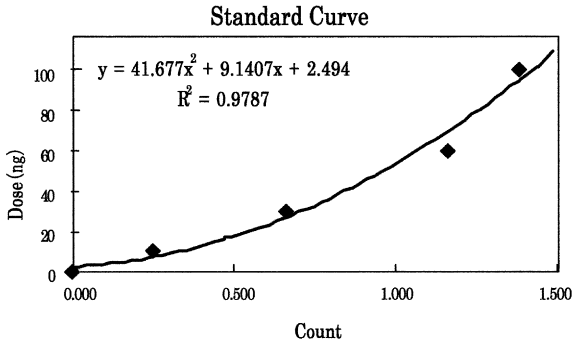

Fig. 2. The standard curve of SPC.

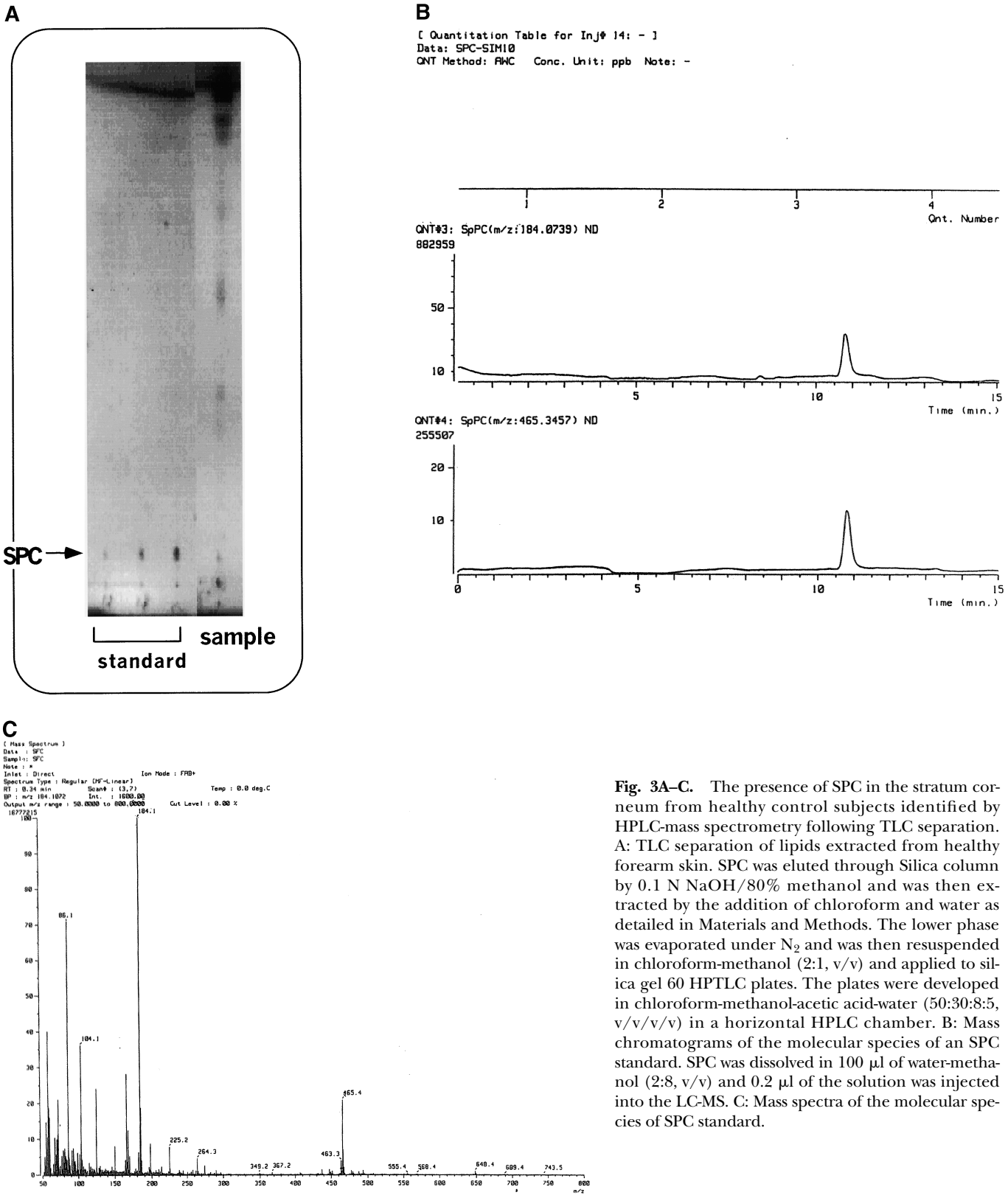

Fig. 3A–C. The presence of SPC in the stratum corneum from healthy control subjects identified by HPLC-mass spectrometry following TLC separation. A: TLC separation of lipids extracted from healthy forearm skin. SPC was eluted through Silica column by 0.1 N NaOH/80% methanol and was then extracted by the addition of chloroform and water as detailed in Materials and Methods. The lower phase was evaporated under N_2 and was then resuspended in chloroform-methanol (2:1, v/v) and applied to silica gel 60 HPTLC plates. The plates were developed in chloroform-methanol-acetic acid-water (50:30:8:5, v/v/v/v) in a horizontal HPLC chamber. B: Mass chromatograms of the molecular species of an SPC standard. SPC was dissolved in 100 μl of water-methanol (2:8, v/v) and 0.2 μl of the solution was injected into the LC-MS. C: Mass spectra of the molecular species of SPC standard.

crease (148% for non-lesion and 297% for lesion, n = 40–47, $P < 0.01$)) in the content of SPC/mg stratum corneum in the uninvolved and in the involved stratum corneum compared with age-matched healthy controls (**Fig. 4A**). In contrast, there was no increase in the SPC content in the involved stratum corneum of patients with chronic eczema, which suggests that SPC is not upregulated as a simple result of the inflammation seen in AD. In a parallel study in which quantitative analysis of ceramides was also carried out using stratum corneum samples of a similar group of

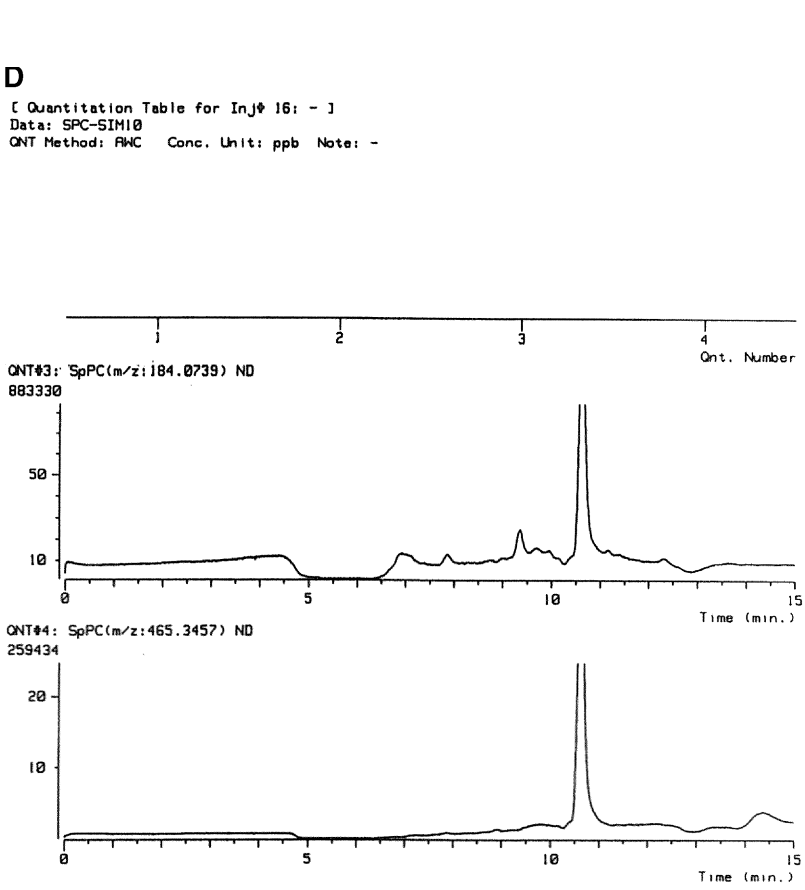

Fig. 3D: Mass chromatograms of the molecular species of SPC separated by TLC from lipids that had been extracted from healthy forearm skin.

patients with AD, there was a significant decrease in the content of ceramide/mg stratum corneum (n = 53–62, $P < 0.01$) in both the uninvolved and in the involved stratum corneum compared with age-matched healthy controls (Fig. 4B). Thus, there is a reciprocal relationship between SPC and ceramide levels among healthy controls, uninvolved and involved skin of patients with AD.

SPC correlates negatively with ceramide and positively with GS but not with Sph

Comparison between the amounts of ceramide and SPC in the same individuals (**Fig. 5A**) demonstrated that there is a weak inverse relationship (n = 51, $r = -0.44$, $P = 0.0012$) between levels of ceramides and SPC that accumulate in the stratum corneum. Among control, uninvolved, and involved groups, there is the inverse relationship (n = 13, $r = -0.641$, $P = 0.018$) only in the involved AD group (**Table 1**). In this group used for correlation analysis, there is a significant decrease in the amounts of ceramide in the uninvolved and involved stratum corneum from patients with AD compared with healthy control (**Table 2**). In comparison with another lysosphingolipid, GS, which is derived from glucosylceramide by another novel epidermal enzyme termed glucosylceramide deacylase, which is significantly increased in the stratum corneum of patients with AD (12), there is a significant positive correlation (n = 30, $r = 0.703$, $P < 0.01$) between levels of SPC and GS (Fig. 5B). Among control, uninvolved, and involved groups, there is a significant positive correlation (n = 11, $r = 0.687$, $P = 0.020$) only in the involved AD group (Table 1). In this group used for correlation analysis there is a significant increase in the amounts of glucosylsphingosine in the uninvolved and involved stratum corneum from patients with AD compared with healthy control (Table 2). In contrast, comparison with Sph, which is a degradative product from ceramide by ceramidase, demonstrated that there is no correlation (n = 32, $r = -0.182$, $P = 0.319$.) between levels of SPC and Sph in the overall group (Fig. 5C) as well as the individual groups (Table 1). In this group used for correlation analysis, there is a significant decrease in the amounts of sphingosine in the uninvolved and involved stratum corneum from patients with AD compared with healthy control (Table 2).

DISCUSSION

To characterize the physiological role of SM deacylase in the epidermis of patients with AD, it was important to determine whether its enzymatic reaction product, SPC, is released into the epidermis and accumulates in the stratum corneum. By means of HPLC-mass chromatography, we were able to demonstrate that SPC exists in the superficial stratum corneum of healthy control skin. Since there are no known biological pathways that might lead to the generation of SPC other than SM deacylase (14), this suggests that SM deacylase functions to some extent even in the epidermis of healthy controls. This is also substantiated by our previous report (7), which described a low but significant level of SM deacylase activity in the epidermis of healthy controls. The existence of SPC in the stratum

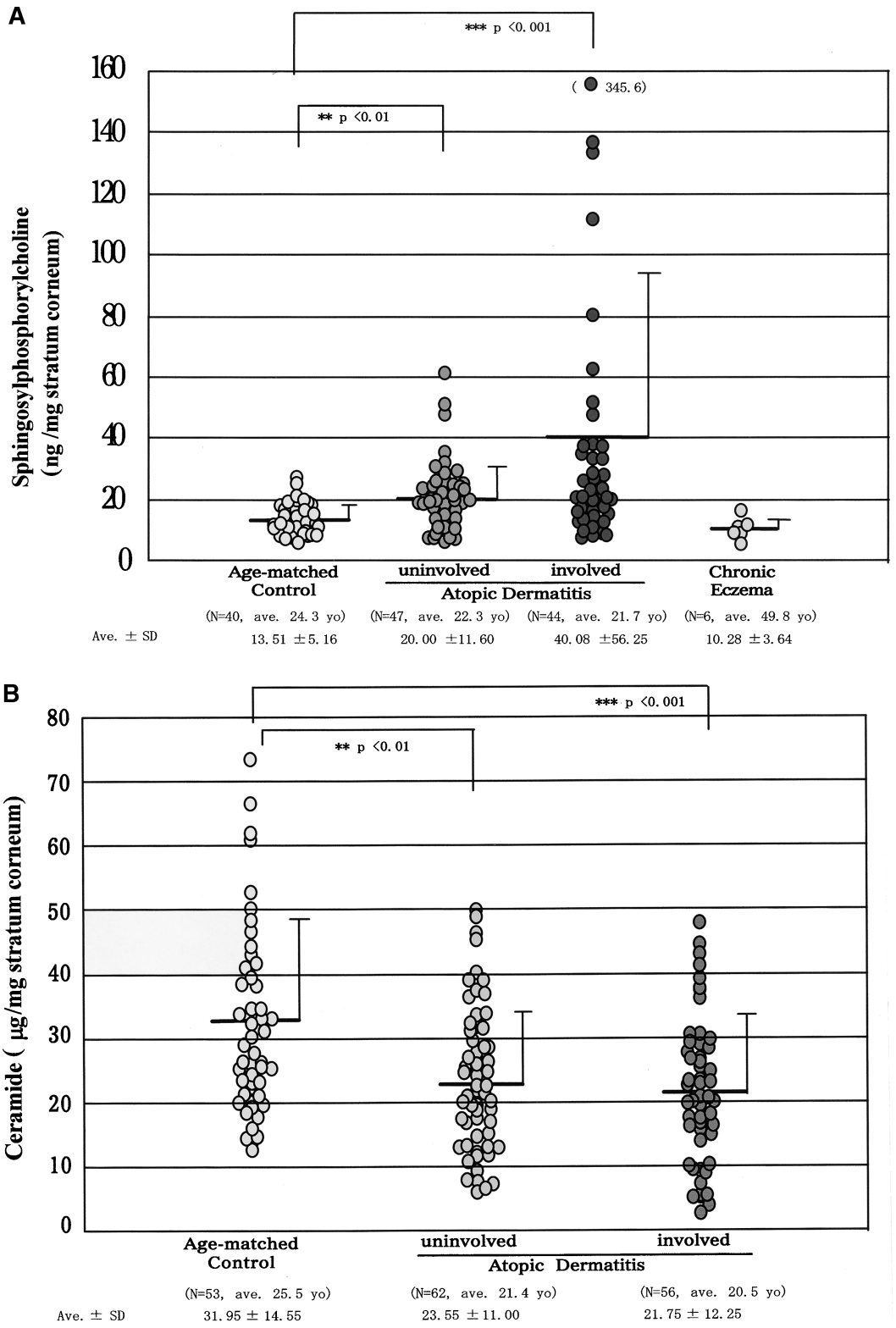

Fig. 4. The quantitation of SPC (A) and ceramides (B) in the stratum corneum from healthy controls and from patients with atopic dermatitis (AD) or chronic eczema. A: SPC measurement: age-matched control, n = 40, average 24.3 years old; uninvolved skin of AD, n = 47, average 22.3 years old; involved skin of AD, n = 44, average 21.7 years old; chronic eczema, n = 6, average 49.8 years old. B: Ceramide measurements: Age-matched control, n = 53, average 25.5 years old; uninvolved skin of AD, n = 62, average 21.4 years old; involved skin of AD, n = 56, average 20.5 years old.

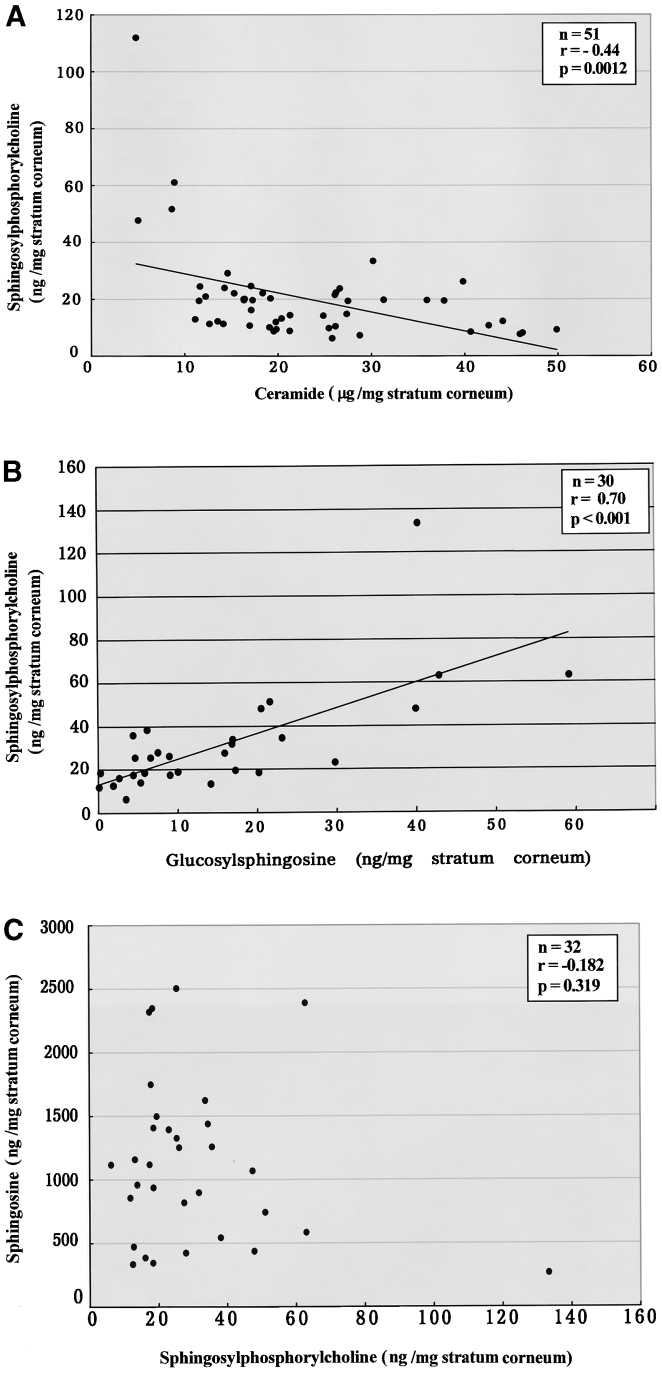

Fig. 5. The relationship between SPC and other sphingolipids in the stratum corneum of some of the patients shown in Fig. 4. A: SPC and ceramide. n = 51, The levels of SPC and ceramide were simultaneously measured as described in the Materials and Methods using the stratum corneum from healthy control skin (n = 20), or from uninvolved skin (n = 18) or involved skin (n = 13) of patients with AD. B: SPC and GS, n = 30. The levels of SPC and GS were simultaneously measured as described in the Materials and Methods using the stratum corneum from healthy control skin (n = 8), or from uninvolved skin (n = 11) or involved skin (n = 11) of patients with AD. C: SPC and Sph, n = 32. The levels of SPC and Sph were simultaneously measured as described in the Materials and Methods using the stratum corneum from healthy control skin (n = 9), or from uninvolved skin (n = 12) or involved skin (n = 11) of patients with AD.

corneum further suggests that SPC generated from SM by the action of SM deacylase (probably in the interface between the granular and the stratum corneum layers) is transferred to and eventually accumulates in the stratum corneum during the keratinization process.

The present quantitative analysis of SPC in the stratum corneum of patients with AD demonstrated that there is a significant up-regulation in the amount of SPC in uninvolved and involved stratum corneum from patients with AD compared with age-matched healthy controls. This contrasts with no increase in SPC in the involved stratum corneum of patients with chronic eczema, which suggests that the up-regulation of SPC in AD does not result from ordinary inflammation, but is associated with the altered lipid metabolism characteristic for AD. Parallel analysis of ceramides revealed that while there is a significant decrease in the content of ceramides in uninvolved and in involved stratum corneum of patients with AD, which is consistent with results previously reported (5), there is a reciprocal relationship between the increase in SPC and the decrease in ceramide in the stratum corneum of healthy controls, and of lesional and non-lesional skin from patients with AD. This inverse relationship between SPC and ceramides allows us to assume that the ceramide deficiency in AD can mainly be attributed to physiological factors responsible for the increased generation of SPC, which so far is thought to result only from the action of the recently discovered epidermal enzyme, SM deacylase (6).

As novel properties of SM deacylase that are distinct from other deacylase enzymes reported by Ito et al (15), we have already characterized several enzymatic characteristics of this enzyme as follows (16); *1*) using [palmitic acid-1-^{14}C]SM as a substrate, a sharp pH dependency was found for its catalytic activity, with a peak at pH 5.0; *2*) in contrast to the molecular weight of sphingolipid ceramide *N*-deacylase (52,000) found in bacteria (15), preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis using AD epidermis extracts revealed that the molecular weight of SM deacylase is 40,000 (16); *3*) analytical isoelectric focusing (IEF) chromatography demonstrated that the pI values of SM deacylase, β-glucocerebrosidase (GlCdase), SMase, and acid ceramidase (CDase) were 4.2, 7.4, 7.0, and 5.7, respectively, again suggesting that these enzymes and sphingolipid ceramide *N*-deacylase (with a pI = 6.9–7.4) are different from each other; *4*) enzymatic analysis using the pI 4.2 protein partially purified by IEF, which contained no contamination with acid CDase, GlCdase, or SMase, showed that radio-labeled SPC was enzymatically liberated from [choline-methyl-^{14}C]SM used as a substrate; and *5*) the pI 4.2 protein purified from the stratum corneum extract of AD patients could hydrolyze *N*-[palmitoyl-1-^{14}C]SM, but not *N*-[palmitoyl-1-^{14}C]Cer, thus indicating that the SM deacylase is distinct from any known CDases or N-deacylases. These unique enzymatic properties allow us to assume that there is a new identity of this SM deacylase enzyme in the epidermis of patients with AD and that the expression and subsequent function of this novel enzyme is eventually responsible for the bio-

TABLE 1. Correlation of SPC with ceramide, glucosylsphingosine and sphingosine in the same groups

Counterpart to Sphingosylphosphorylcholine	Control	AD Uninvolved	AD Involved	Overall
Ceramide	n = 20	n = 18	n = 13	n = 51
	$r = -0.275$	$r = -0.303$	$r = -0.641$	$r = -0.44$
	$P = 0.210$	$P = 0.222$	$P = 0.018$	$P = 0.0012$
Glucosylsphingosine	n = 8	n = 11	n = 11	n = 30
	$r = 0.397$	$r = 0.427$	$r = 0.687$	$r = 0.703$
	$P = 0.330$	$P = 0.190$	$P = 0.020$	$P = 0.0000$
Sphingosine	n = 9	n = 12	n = 11	n = 32
	$r = 0.293$	$r = -0.216$	$r = -0.050$	$r = -0.182$
	$P = 0.444$	$P = 0.499$	$P = 0.882$	$P = 0.319$

chemical events leading to the ceramide deficiency in the stratum corneum of patients with AD.

A similar accumulation of SPC has been observed in Niemann-Pick disease, which is associated with defects of SMase and results in the lipidosis for SM (17), although no data suggest that this SPC accumulation is linked to the expression of an SM deacylase-like enzyme. Although SPC accumulation has been speculated to result from a defect of SMase [because SPC is a substrate for SMase (17)], it would be intriguing to determine whether there is a similar up-regulated expression of SM deacylase in Niemann-Pick disease as that could also provide a mechanism for the production of SPC in that disease as it does for AD (6, 7, 16). A similar accumulation of substrates and reaction products by corresponding *N*-deacylase enzymes has been found in Gaucher disease, in which there is an accumulation of glucosylceramide and GS due to a defect of GlCdase activity (18). In Gaucher disease, there has been no explanation for the accumulation of GS in terms of the action of the corresponding *N*-deacylase, namely glucosylceramide deacylase. Thus, GS accumulation has also been speculated to result from a defect of GCDase since GS can serve as a substrate for that enzyme (18). Similarly, the possible existence and expression of glucoslyceramide deacylase in Gaucher disease would provide a reasonable mechanism for the upregulation of GS. Another similar relevance of *N*-deacylase for the generation of pychosine has been reported in globoid cell leukodystrophy (GLD) or Krabbe's disease (19). The primary defect of GLD is a deficiency in galactosylceramidase activity, which leads to the accumulation of galactosylceramide and its metabolic intermediate galactosylsphingosine. This was speculated to be produced by deacylation of galactosylceramide (19), although there was no evidence for the expression of galactosylceramide deacylase in GLD. Such altered lipid metabolisms associated with genetic defects, which lead to the accumulation of lipid substrates and deacylated metabolic intermediates, strongly suggest the principle that defects of metabolic enzymes might induce corresponding alternative pathways in which those substrates are converted to corresponding lysoforms by deacylation. Such a possible induction of an alternative pathway following the loss of metabolic enzymes has been reported in a Gaucher-like mouse induced by a glucosylceramidase inhibitor that shows the accumulation of GS in tissue (20).

As a physiological and biochemical factor leading to the ceramide deficiency, we have found that SM deacylase is expressed at high levels (3–5-fold higher than in controls) in the epidermis of patients with AD, which results in the generation of SPC rather than ceramide. Consistent with the upregulation of SM deacylase activity in AD, there is a significant decrease (0.73–0.68-fold) in ceramide as well as a significant increase (1.5–3.0-fold) in SPC. Since increased or decreased ratios of SM deacylase activity and SPC or ceramide levels in healthy controls and patients

TABLE 2. Comparison in the amounts of ceramide, glucosylsphingosine and sphingosine in the same groups used for correlation analysis

	Control	AD Uninvolved	AD Involved
		μg/mg stratum corneum	
Ceramide	27.94 ± 11.90 n = 20 (100%)	21.03 ± 10.85[a] n = 18 (75.3%)	17.44 ± 7.91[c] n = 13 (63.4%)
		ng/mg stratum corneum	
Glucosylsphingosine	8.05 ± 6.86 n = 8 (100%)	13.37 ± 8.87[a] n = 11 (166.1%)	22.74 ± 19.78[a] n = 11 (282.5%)
Sphingosine	1578.91 ± 679.01 n = 9 (100%)	954.47 ± 381.55[b] n = 12 (60.5%)	937.59 ± 678.47[a] n = 11 (59.4%)

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.005$.

with AD occur at similar levels, this suggests the relevance of the ceramide deficiency to the increased SM deacylase activity that results in the accumulation of SPC. On the other hand, the degree of the increased levels of SPC (6.5–26.5 ng/mg) is far less than the degree of the decreased levels of ceramide (~8,400–10,200 ng/mg) in the stratum corneum of AD patients. Such great differences in their contents and the weak inverse relationship observed between SPC and ceramide levels in the same individuals may be explained in terms of the following two biological events that are related to the generation and degradation of SPC in situ. First, the original site of the generation of SPC in the skin is located in the interface between the granular and stratum corneum layers. In contrast to the major ceramide processing toward the stratum corneum, the biosynthesized SPC would be predicted to be translocated to the stratum corneum as well as to the lower epidermis because SPC is water-soluble and would easily spread toward the surrounding tissues. Thus, it seems reasonable to assume that the amounts of SPC found in the upper stratum corneum from patients with AD substantially reflect only a minor proportion of the SPC actually produced. Second, there is at least one other pathway (involving GlCdase) in which ceramide is produced in situ in the epidermis other than the SMase pathway (21). This indicates that altered lipid metabolism related to SM hydrolysis might not necessarily account for all of the decrease in ceramides that is found in the stratum corneum from patients with AD. The possible involvement of another altered lipid metabolism in AD is corroborated by our evidence that glucosylceramide deacylase, which hydrolyzes glucosylceramide at its acyl site to yield GS and free fatty acid, is highly expressed in the stratum corneum of patients with AD (12). Further, its enzymatic reaction product, GS, accumulates in the upper stratum corneum from patients with AD at a level of 10–20 ng/mg stratum corneum to a significantly greater extent than in those of healthy controls (12), though whether SM deacylase and glucosylceramide deacylase are the same enzyme remains to be clarified. Intriguingly, our comparison between SPC and GS in the stratum corneum from the same patients with AD demonstrated a close relationship between these two lysosphingosines derived from possibly different *N*-deacylases, which suggests that both SM deacylase and glucosylceramide deacylase are functioning at similar levels of enzyme activity in the epidermis of patients with AD. Sph is another sphingolipid present in the stratum corneum and is known to serve as an antimicrobial lipid in normal skin (22). We have recently found that the level of Sph in the stratum corneum is significantly downregulated in AD skin, providing a possible predisposition of the skin surface to Staphylococci aureus colonization (11). The decreased level of Sph in the stratum corneum from patients with AD is attributed to the decreased activity of acid ceramidase that degrades ceramide to generate Sph (11), suggesting that the Sph content is an indicator for the functional status of acid CDase. Our evidence that there is no relationship between SPC and Sph present in the stratum corneum from the same patients with AD seems to indicate that the enzymatic pathway responsible for SPC generation, an essential process which leads to the ceramide deficiency in AD, is distinct from that for Sph generation that is mainly attributable to the activity of CDase.

In conclusion, we have elucidated in this study the functional relevance of SM deacylase to the ceramide deficiency that is an essential etiologic factor for the dry and barrier-disrupted skin of patients with AD. Interestingly, the enzymatic reaction product, SPC, which is an essential surrogate to determine whether SM deacylase is functioning in situ in the epidermis, is significantly up-regulated in the uninvolved and in the involved stratum corneum of patients with AD compared with healthy controls, and is reciprocally related to the decreased levels of ceramides in a similar group of patients with AD. SPC has been shown to be a potent biologically active substance capable of stimulating expression of intercellular adhesion molecule-1 (23), of activating transglutaminase in human keratinocytes (24), and of enhancing melanogenesis in human melanocytes (25). These stimulatory effects of SPC reflect the clinical symptoms seen in the skin of patients with AD, which suggests that the abundant generation of SPC in AD epidermis acts as a potent activator in situ.

Taken together, the increased accumulation of SPC in the stratum corneum of patients with AD suggests that the novel epidermal enzyme, SM deacylase, is expressed in situ at significant levels in the epidermis of AD patients. This results in the production and accumulation of SPC, which leads in turn to the ceramide deficiency seen in the stratum corneum of those patients. It is likely that the biogenesis of SM deacylase may be relevant to the pathogenesis of AD, if AD can be considered a ceramide-deficient disease.

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