Activation of bacterial ceramidase by anionic glycerophospholipids: possible involvement in ceramide hydrolysis on atopic skin by *Pseudomonas* ceramidase

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We have reported previously that the ceramidase from *Pseudomonas aeruginosa* AN17 isolated from a patient with atopic dermatitis requires detergents for hydrolysis of ceramide (Cer) [Okino, Tani, Imayama and Ito (1998) J. Biol. Chem. **273**, 14368–14373]. In the present study, we report that some glycerophospholipids strongly activated the hydrolysis of Cer by *Pseudomonas* ceramidase in the absence of detergents. Among the glycerophospholipids tested, cardiolipin was most effective in stimulating hydrolysis of Cer followed by phosphatidic acid, phosphatidylethanolamine and phosphatidylglycerol, whereas phosphatidylcholine, lysophosphatidic acid and diacylglycerol were less effective. Interestingly, *Staphylococcus aureus*-derived lipids, which contain cardiolipin and phosphatidylglycerol as major lipid components, also strongly enhanced the hydrolysis of normal Cer, as well as the human skin-specific ω-hydroxyacyl Cer, by the enzyme in the absence of detergents. It was confirmed that several strains of *P. aeruginosa*, including AN17, secrete a significant amount of staphylolytic proteases to lyse *S. aureus* cells, resulting in the release of cardiolipin and phosphatidylglycerol. Since both *P. aeruginosa* and *S. aureus* are suspected of being present in microflora of atopic skin, we speculate that *S. aureus*-derived glycerophospholipids stimulate the hydrolysis of Cer in atopic skin by bacterial ceramidase.

Key words: anionic phospholipids, atopic dermatitis, *Pseudomonas aeruginosa*, staphylolytic protease.

INTRODUCTION

Ceramide (Cer; *N*-acylsphingosine) functions as a water retainer as well as a permeability barrier by forming a multi-lamellae structure in the stratum corneum of the mammalian epidermis [1]. These lamellae arrays are derived from the extruded lipid contents of lamellae bodies after fusion of these organelles with the plasma membrane of the outermost granular cells [1]. Cer is produced from glucosylCer [2] and sphingomyelin [3] by the action of β-glucosidase (glucosylcerebrosidase, E.C. 3.2.1.45) and sphingomyelinase (E.C. 3.1.4.12) respectively, and is accumulated in the stratum corneum [2,4]. The typical skin Cer is a ω-hydroxylated Cer, which is covalently bound to structural proteins of the cornified cell envelope, forming a hydrophobic surface on terminally differentiated keratinocytes [1].

In lesions of atopic skin, Cer content was found to be decreased [5]. This symptom has been thought to be significantly related to some etiological aspect of atopic dermatitis. A change in epidermal permeability caused by a decrease of Cer would permit the invasion of allergens or irritants, which is associated with the pathogenesis of atopic dermatitis [6].

Recently, a ceramidase (CDase)-producing bacterium, *Pseudomonas aeruginosa* AN17, was isolated from the skin of patients with atopic dermatitis [7], and the gene encoding its CDase was cloned [8]. Interestingly, CDase-producing bacteria were found with significant frequency in atopic skin [9]. However, Cer was not hydrolysed by the CDase in the absence of detergents that are unlikely to be present on human skin. In the present study, we report that a number of anionic glycerophospholipids, which could be released from *Staphylococcus aureus* by *Pseudomonas* proteases [10,11], stimulated the hydrolysis of Cer by bacterial CDase in the absence of detergents. This observation would explain, in part, how bacterial CDase hydrolyses Cer in atopic skin. This present study also describes a new candidate for inhibition of bacterial CDase.

EXPERIMENTAL

Materials

D-*erythro*-Sphingosine (Sph) was purchased from Wako Pure Chemicals (Osaka, Japan). [1-^{14}C]palmitic acid was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). All glycerophospholipids, Azocoll and *S. aureus* strain Newman D_2C were purchased from Sigma (St. Louis, MO, U.S.A.). Cer ($C_{18:0}$, $C_{d16:0}$; where d is dihydroxylated) and *N*-(27-stearoyloxyheptacosanoyl)phytoSph were generously given by Dr T. Sugai (Department of Chemistry, Keio University, Yokohama, Japan) and Dr S. Hamanaka (Sphingolipid Expression Laboratory of Suprabiomolecular System Research Group, RIKEN Frontier

Abbreviations used: Cer, ceramide; CDase, ceramidase; CL, cardiolipin; FAB–MS, fast atom bombardment–MS; NBD, 7-nitrobenz-2-oxa-1,3-diazole; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PSL, photostimulated luminescence; OPA, *o*-phthalaldehyde; NOE, *N*-oleoylethanolamine; D-MAPP, D-*erythro*-*N*-myristoylamino-1-phenyl-1-propanol; L-MAPP, L-*erythro*-*N*-myristoylamino-1-phenyl-1propanol, Sph, sphingosine.

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Research System, Saimata, Japan) respectively. Cer analogues (GCAS-3, -4, -7 and -10) were chemically synthesized (H. Ishida, T. Izawa, K. Matsumoto and M. Kiso, unpublished work). Precoated silica gel 60 TLC plates were obtained from Merck (Darmstadt, Germany). 7-Nitrobenz-2-oxa-1,3-diazole (NBD)-C_{12}-Cer [12] and [^{14}C]Cer ($C_{16:0}$, $C_{d18:1}$) [13] were prepared by the reverse hydrolysis reaction of sphingolipid Cer *N*-deacylase ('SCDase') [14]. Lysophosphatidylglycerol and lysophosphatidic acid were prepared as described previously [15].

Preparation of recombinant CDase

Escherichia coli JM109 cells were transfected with pTCD11, containing the full-length DNA of *Pseudomonas* CDase, and cultured at 37 °C using isopropyl β-D-thiogalactoside as an inducer [8]. Recombinant CDase was purified from cell lysates by chromatography as described previously [7].

Bacterial strains and culture conditions

P. aeruginosa AN17 was isolated from the skin of patients with atopic dermatitis [7]. Type strain K was kindly donated by Dr A. Umeda (Laboratory of Microbiology, Faculty of Medicine, Kyushu University, Fukuoka, Japan), and type cultures IFO 12689, 13275, and 13736 were from the Institute for Fermentation (IFO), Osaka, Japan. Each strain of *P. aeruginosa* was grown at 37 °C on heart-infusion agar or liquid medium (Difco, Detroit, MI, U.S.A.). *S. aureus* was obtained from Dr A. Umeda (Kyushu University, Japan) and cultured at 37 °C in a nutrient broth (Eiken Co. Ltd., Tokyo, Japan). For the measurement of staphylolytic and protease activities, bacteria were cultured at 37 °C for 2 days, and the supernatant obtained following centrifugation (5600 ***g*** at 4 °C for 5 min) was used as a crude enzyme solution.

CDase assay

The enzyme activity was determined using NBD-C_{12}-Cer as a substrate as described previously [7]; 1 unit of CDase was defined as the amount of enzyme capable of hydrolysing 1 μmol of NBD-C_{12}-Cer/min. A value of 10^{-3} and 10^{-6} unit of enzyme was expressed as 1 m-unit and 1 μ-unit respectively. Unless otherwise indicated, the effects of phospholipids were estimated as follows: (i) for the hydrolysis reaction, 40 μ-unit of CDase was incubated with [^{14}C]Cer ($C_{16:0}$, $C_{d18:1}$) in 80 μl of 25 mM Tris/HCl buffer (pH 8.5) containing 2.5 mM $CaCl_2$ and an appropriate amount of the glycerophospholipids to be tested; and (ii) for the reverse hydrolysis reaction, 40 μ-unit of CDase was incubated with 2 nmol each of Sph ($C_{d18:1}$) and [1-^{14}C]palmitic acid in 80 μl of 25 mM Tris/HCl buffer (pH 7.5) containing 2.5 mM $CaCl_2$ and an appropriate amount of the glycerophospholipids to be tested. The reactions were terminated by boiling in a water bath for 5 min, and the mixture was dried with a Speed Vac concentrator (Savant, Farmingdale, NY, U.S.A.). Each sample was resuspended in chloroform/methanol (2:1, v/v) and applied on to a TLC plate, which was developed with chloroform/methanol/28 % (v/v) ammonia (180:40:1, by vol.), and analysed using a BAS-1500 image analyser (Fuji Photo Film, Tokyo, Japan). The extent of the reaction was calculated as follows: (i) hydrolysis reaction (%) = PSL for fatty acid produced × 100/(PSL for remaining Cer + PSL for fatty acid produced); and (ii) condensation reaction (%) = PSL for Cer produced × 100/(PSL for remaining fatty acid + PSL for Cer produced); where PSL is photostimulated luminescence.

Preparation of heat-killed *S. aureus*

Freeze-dried *S. aureus* cells (strain Newman D_2C) were suspended in 20 mM Tris/HCl buffer (pH 8.5) at a concentration of 50 mg/ml and heated on a heating block at 100 °C for 10 min.

Staphylolytic activity

The activity was determined by measuring the lysis of heat-killed *S. aureus* as described previously [10]. A portion (50 μl) of the heat-killed cell suspension was incubated at 37 °C for 36 h with 100 μl of crude enzyme and 550 μl of 20 mM Tris/HCl buffer (pH 8.5). Staphylolytic activity was determined by measuring the decrease in absorbance at 595 nm.

Protease assay

The activity was determined using Azocoll as a substrate as described previously [16], with minor modification. Portions (600 μl) of Azocoll solution (10 mg/ml of each buffer solution) were incubated at 37 °C for 3 h with 100 μl of the bacterial culture supernatant. After centrifugation (5600 ***g*** for 5 min at 4 °C), the supernatants were transferred into wells of a 96-well microtitre plate. Released dye was measured as the increase in absorbance at 520 nm.

Extraction of bacterial lipids

Extraction of *S. aureus* lipids was conducted as described previously [17]. Briefly, cells (approx. 4 g of wet weight) were washed with 800 ml of cold Tris-buffered saline (pH 7.2), and extracted with 400 ml of 5 % (w/v) trichloroacetic acid. The precipitates obtained were suspended in 40 ml of distilled water, and 200 ml of methanol and 400 ml of chloroform were added. The mixture was shaken thoroughly at 15-min intervals for 2 h at 25 °C, and 250 ml of saline was added. The organic phase (lower phase) was collected and evaporated using a rotary evaporator, and the residue was dissolved in 100 μl of chloroform/methanol (2:1, v/v). To separate phosphatidylglycerol (PG) and cardiolipin (CL), 50 μl aliquots were applied on to a TLC plate, which was developed with chloroform/methanol/1 M ammonia (12:7:1, by vol.). The portions corresponding to PG and CL were scraped separately from the TLC plate and extracted with 2 ml of chloroform/methanol (1:2, v/v). The concentration of lipids was determined by TLC with Dittmer–Lester reagent using PG as a standard [18].

Extraction of Cer from human skin

Human skin Cer was prepared as described previously [19]. Briefly, epidermis lipids were extracted from peeled skin with successive treatments of the following three solvents: chloroform/methanol/water (2:4:1, by vol.); chloroform/methanol (1:1, v/v); and chloroform/methanol (2:1, v/v). Extracts were dried and redissolved in 1 ml of chloroform/methanol/acetic acid (190:9:1, by vol.). After centrifugation (5800 ***g*** for 5 min), the supernatant was applied to a TLC plate, which was developed twice with the same solvent. The portions corresponding to the R_F of the Cer standard were scraped from the TLC plate and then extracted three times with 3 ml of chloroform/methanol (2:1, v/v). For visualization of various lipids, including Cer, cholesterol and its derivatives, triacylglycerol and non-esterified fatty acid, the TLC plates were air-dried, sprayed with 10 % (w/v) copper sulphate in 8 % (w/v) phosphoric acid and charred on a hot plate at 180 °C for 10 min [20].

Quantification of Cer

Cer content was determined by measuring the amount of free Sph bases after alkaline hydrolysis as reported previously [21]. A portion (10 μl) of sample containing Cer was transferred to a glass screw-topped vial. After evaporation, 0.5 ml of 1 M potassium hydrate in 90 % methanol was added and heated at 90 °C for 1 h to quantitatively convert Cer into free Sph. For measurement of the hydrolysis of Cer by the CDase, the released Sph was determined as follows. After addition of eicosasphinganine ($C_{d20:0}$) to the reaction mixture as an internal standard, sphingoid bases were extracted by the addition of 0.5 ml of 1 M HCl in methanol, 1 ml of chloroform and 0.75 ml of 1 M NaCl. The organic phase was dried under N_2 and redissolved in 50 μl of methanol. Free Sph bases were analysed by HPLC as *o*-phthalaldehyde (OPA) derivatives as described previously [22].

Fast-atom-bombardment (FAB)–MS analysis

Cer (approx. 5–10 nmol) was mixed with 3-nitrobenzylalcohol (Aldrich, Milwaukee, WI, U.S.A.) as a matrix. MS analyses were performed using a JMS-SX/SX102A FAB mass spectrometer (JEOL, Tokyo, Japan), operating at an acceleration voltage of 10 kV with an Xe atom beam (5 kV) in positive-ion mode.

RESULTS

Enhancement of bacterial CDase activity by glycerophospholipids

The CDase of *P. aeruginosa* AN17 was found to require a detergent for hydrolysis of Cer [7]. The highest activity of the CDase was observed in the presence of 0.25 % Triton X-100 [7].

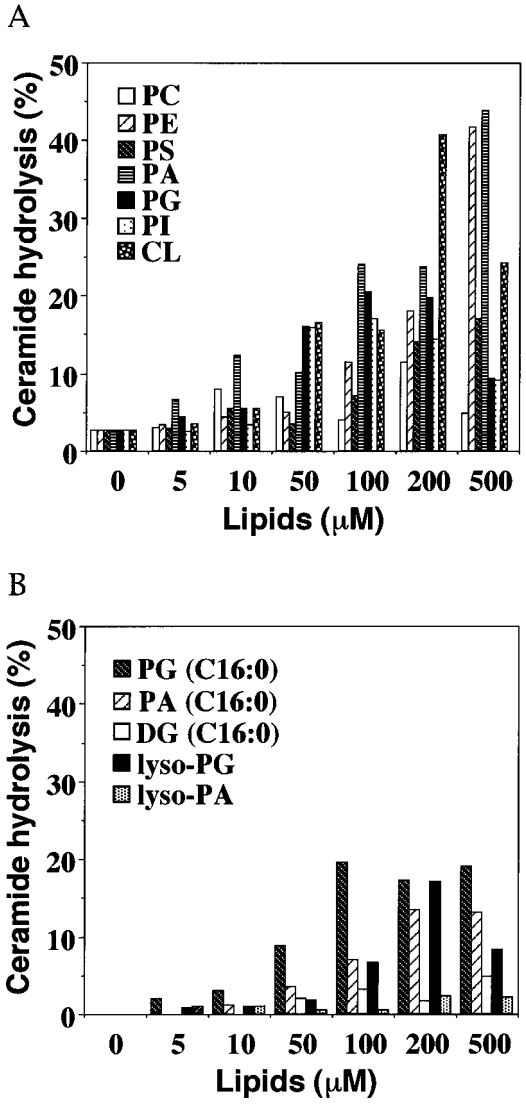

Figure 1 Effects of various glycerophospholipids (A) and their derivatives (B) on the hydrolysis of Cer by *Pseudomonas* CDase in the absence of detergents

[^{14}C]Cer ($C_{16:0}$, $C_{d18:1}$; 2 nmol) was incubated with 20 μ-unit of CDase in the presence of lipids at the concentrations indicated, as described in the Materials and methods section. Values are means from duplicate determinations. DG, diacylglycerol; lyso-PA, lysophosphatidic acid; lyso-PG, lysophosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidyl-L-serine.

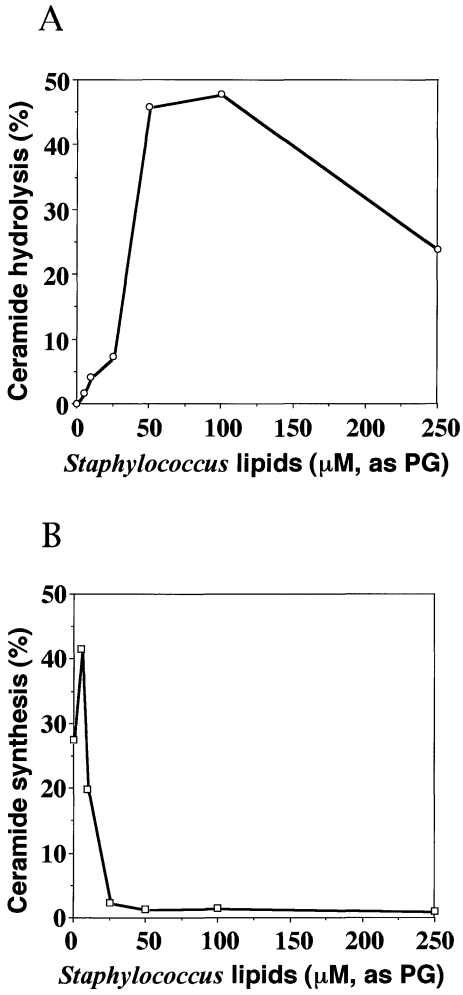

Figure 2 Effect of *Staphylococcus*-derived lipids on the hydrolysis (A) and reverse hydrolysis (B) of *Pseudomonas* CDase

[^{14}C]-Cer ($C_{16:0}$, $C_{d18:1}$; 2 nmol) (**A**) or 2 nmol each of [1-^{14}C]palmitic acid and D-*erythro*-Sph (**B**) were incubated with 20 μ-unit of CDase in the presence of *Staphylococcus*-derived lipids at the concentrations indicated (μM, as PG indicates that the phospholipid content was determined by TLC using PG as a standard). The experiments were performed as described in the Materials and methods section. Values are means from duplicate determinations.

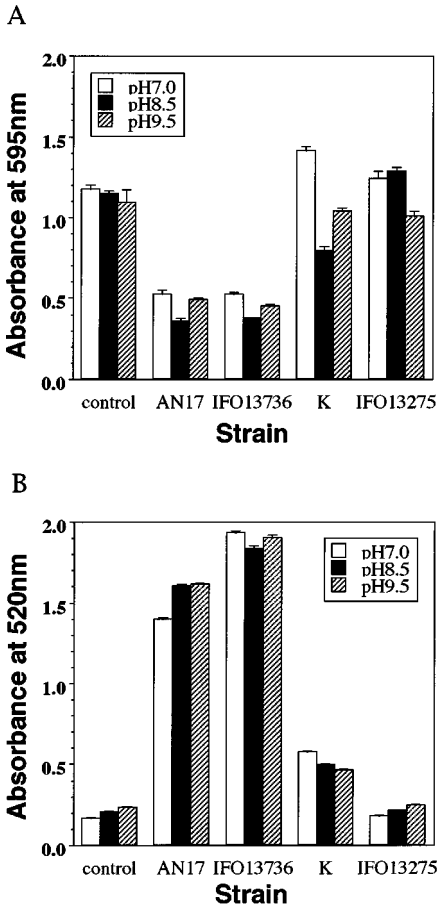

Figure 3 Staphylolytic activity (A) and protease activity (B) of several strains of *P. aeruginosa* at various pHs

(**A**) Staphylolytic activity was measured by the decrease in absorbance at 595 nm with the heat-killed *S. aureus* strain Newman D_2C as a substrate. (**B**) Protease activity was measured at an absorbance of 520 nm using Azocoll as the substrate. The experiments were performed as described in the Materials and methods section. Values are means ± S.D. from triplicate determinations.

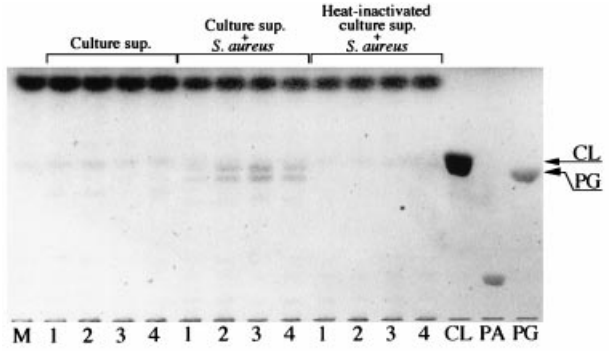

Figure 4 Detachment of glycerophospholipids from *S. aureus* cells by the culture supernatant of *P. aeruginosa*

P. aeruginosa culture supernatants (100 μl) were incubated at 37 °C for 18 h in the absence (culture sup.) or presence of heat-killed *S. aureus* cell suspension [(2.5 mg in 600 μl of 20 mM Tris/HCl buffer (pH 8.5)] (culture sup. + *S. aureus*), and heat-inactivated *P. aeruginosa* culture supernatants (heat-inactivated culture sup. + *S. aureus*) were used as a negative control. After centrifugation at 5600 ***g*** for 5 min, 3.5 ml of chloroform/methanol (2:1, v/v) was added to the supernatant, shaken, and allowed to stand at 25 °C. The lower phase was removed, concentrated under N_2 and applied to a TLC plate, which was developed with chloroform/methanol/1 M ammonia (12:7:1, by vol.). Glycerophospholipids were visualized with 10% (w/v) copper sulphate in 8% (w/v) phosphoric acid [20]. Lane 1, *P. aeruginosa* K; lane 2, AN17; lane 3, IFO13736; lane 4, IFO 12689. M, heart-infusion medium. The positions of CL and PG are indicated on the right.

In the present study, we found that a number of anionic glycerophospholipids strongly activated the hydrolysis of [^{14}C]Cer ($C_{16:0}$, $C_{d18:1}$) by *Pseudomonas* CDase in the absence of detergents (Figure 1A). The activity of the CDase increased on addition of various glycerophospholipids in a concentration-dependent manner up to 200 μM; CL was most effective followed by phosphatidic acid (PA), phosphatidylethanolamine (PE) and PG (Figure 1A). At higher concentrations (500 μM), PA and PE were more effective than CL. Phosphatidylcholine was less effective than other glycerophospholipids. Next, we examined the effects of chemically synthesized glycerophospholipids and their derivatives on the hydrolysis of Cer by the enzyme. Consistent with the results in Figure 1(A), PG (di-$C_{16:0}$) and PA (di-$C_{16:0}$) stimulated the activity of the CDase in a concentration-dependent manner up to 200 μM. Lysophosphatidylglycerol also stimulated Cer hydrolysis, but to a lesser extent than PG, whereas lysophosphatidic acid and diacylglycerol had little effect on enzyme activity (Figure 1B). In summary, a number of anionic glycerophospholipids appeared to be effective in stimulating the activity of bacterial CDase in the absence of detergents.

Effects of *Staphylococcus*-derived lipids on the hydrolysis of Cer by the CDase

PG, CL and PA, which are capable of stimulating CDase activity (Figure 1), are known to be major glycerophospholipids of Gram-positive bacteria, including *S. aureus* [23]. This bacterium is the dominant species of microbial flora on atopic skin [24–26] from which the CDase-producing *P. aeruginosa* AN17 was isolated [7]. Thus we examined whether *Staphylococcus*-derived lipids stimulated the hydrolysis of Cer by the CDase instead of detergents. As expected, *Staphylococcus*-derived lipids at a concentration of 50–100 μM (as determined by TLC using PG as a standard) strongly enhanced the hydrolysis of Cer by the CDase in the absence of detergents (Figure 2A). Interestingly, the stimulation of CDase activity by *Staphylococcus*-derived lipids was stronger than that by the authentic PG or CL alone (Figure 1), suggesting that other lipid components of the bacteria enhanced the hydrolysis of Cer by the CDase. *Pseudomonas* CDase catalyses a reversible reaction in which the amide linkage of Cer is hydrolysed or synthesized [27]. Even in the absence of detergents, the reverse hydrolysis reaction proceeded reasonably well (Figure 2B). *Staphylococcus*-derived lipids at a concentration of more than 10 μM strongly suppressed the reverse hydrolysis reaction, indicating that glycerophospholipids tend to make the reaction proceed forward (Figure 2). In addition, we examined the molecular species of *Staphylococcus*-derived lipids by FAB–MS in positive-ion mode. As a result, the major pseudomolecular ions, $[M+H]^+$, corresponding to PG (di-$C_{16:0}$) and CL, were detected at m/z 721 and 1465 respectively (results not shown).

Staphylolytic activity of several strains of *P. aeruginosa*

P. aeruginosa, a well-known opportunistic pathogen possessing the CDase gene, was found to produce specific proteases capable

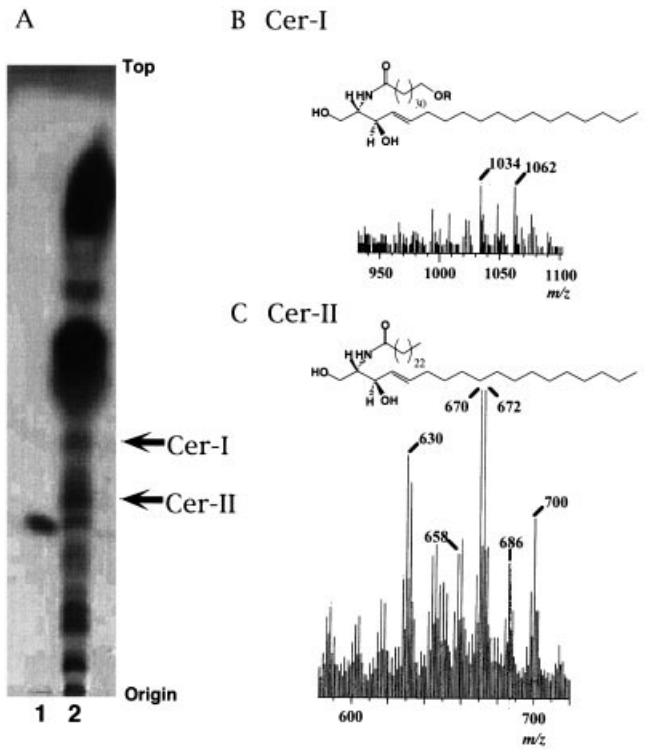

Figure 5 Structural analyses of human skin Cer

(**A**) TLC of human skin-derived lipids. The TLC plate was visualized with 10% (w/v) copper sulphate in 8% (w/v) phosphoric acid [20]. Lane 1, Cer standard ($C_{16:0}$, $C_{d18:1}$); lane 2, crude lipids from human skin. Arrows indicate Cer-I and Cer-II. Predicted chemical structure and FAB–MS spectra of Cer-I (**B**) and Cer-II (**C**) are shown. The experiments were performed as described in Materials and methods section.

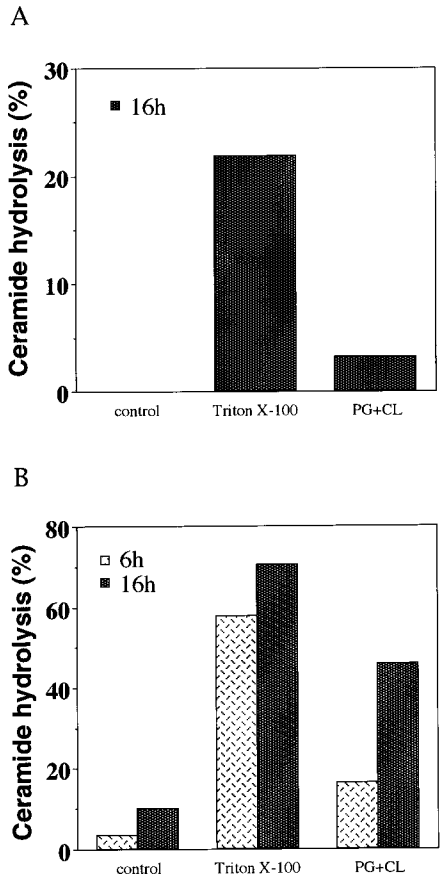

Figure 6 Hydrolysis of human skin-derived Cer by *Pseudomonas* CDase

Skin Cer (1 μmol) was incubated with 20 μ-unit of CDase in the presence of 4 nmol PG and 1 nmol CL (PG + CL) or 0.25% Triton X-100 in 40 μl of 25 mM Tris/HCl buffer (pH 8.5) containing 2.5 mM $CaCl_2$. After incubation at 37 °C for 16 h with Cer-I (**A**) or 6 and 16 h with Cer-II (**B**), the reaction mixtures were dried and the amount of Sph released was determined by HPLC as described in the Materials and methods section. Values are means from duplicate determinations.

of decomposing *S. aureus* cells. These staphylolytic proteases were named LasA [10] and LasD [11]. As shown in Figure 3(A), *P. aeruginosa* AN17 and the authentic-type strain IFO13736 of *P. aeruginosa* (where authentic refers to a bacterial strain obtained from an official culture collection) showed significant staphylolytic activity, whereas type strain K showed only weak activity at pH 8.5, and the strain IFO13275 no activity. The protease activities of these *P. aeruginosa* strains were also determined using Azocoll, a synthetic chromogenic substrate for the proteases. Strong activities were detected in strains AN17 and IFO13736 and weak activity in strain K, but no activity in strain IFO13275 (Figure 3B). These results were consistent with those obtained using heat-killed *S. aureus* as a substrate, i.e. staphylolytic activity (Figure 3A). It is noteworthy that CL and PG were detached from *S. aureus* cells after treatment with culture supernatants of several *P. aeruginosa*-type strains and AN17 (Figure 4). The detachment of these glycerophospholipids from *S. aureus* cells appears to depend on the staphylolytic proteases, as the amount of release was proportional to their activities and detachment was not observed if heat-inactivated supernatants were used as an enzyme source (Figure 4). On the basis of these observations, we speculate that *S. aureus*-derived glycerophospholipids could stimulate the hydrolysis of Cer on atopic skin by bacterial CDase. This would explain, in part, how Cer is hydrolysed on atopic skin by bacterial CDase in the absence of detergents.

Isolation and degradation of human skin Cer

Cer from human skin, which plays a role in maintaining the water-barrier function [1,3], has a somewhat unique structure [1]. We thus examined whether *Pseudomonas* CDase hydrolysed human Cer. Firstly, we isolated Cer from peeled human skin and examined its structure. Figure 5(A) shows the TLC of total lipid extracts derived from human skin. Two major Cer species (Cer-I and Cer-II) were detected (Figure 5A, lane 2). The R_Fs of Cer-I and Cer-II coincided with those of ω-hydroxyacyl Cer and Cer composed of $C_{24:1}$ and $C_{d18:1}$ Sph respectively. By OPA analysis after alkaline hydrolysis [22], long-chain bases of both Cer-I and Cer-II were found to consist mainly of octadecyl-Sph ($C_{d18:1}$). FAB–MS analysis showed that Cer-I contained ω-hydroxylated fatty acids, having various carbon chain lengths (C_{28}–C_{34}), among which $C_{32:0}$ (m/z 1062 $[M+Na]^+$) and $C_{30:0}$ (m/z 1034 $[M+Na]^+$) were predominant (Figure 5B). The spectrum of Cer-II also showed the presence of various fatty acids having carbon chain lengths from C_{21}–C_{26} (Figure 5C), among which $C_{24:0}$ (m/z 672 $[M+Na]^+$) and $C_{24:1}$ (m/z 670 $[M+Na]^+$) were most dominant. Significant amounts of odd-chain fatty acids, $C_{21:0}$ (m/z 630 $[M+Na]^+$) and $C_{23:0}$ and

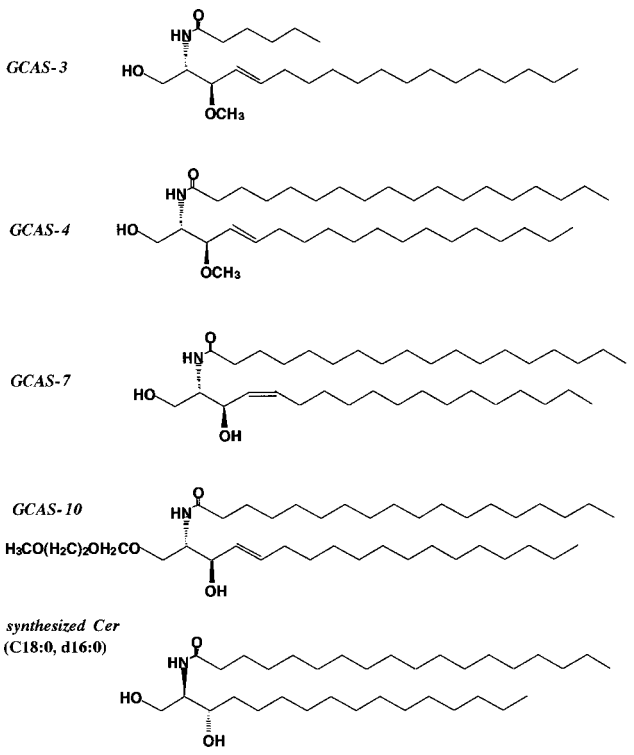

Figure 7 Chemical structures of Cer analogues

$C_{25:0}$ (m/z 644 and 686 $[M+Na]^+$ respectively) were also found in Cer-II, consistent with previous reports [28,29].

Figure 6 shows that *Pseudomonas* CDase hydrolysed not only normal Cer (Cer-II, Figure 6B), but also human skin-specific ω-hydroxyacyl Cer (Cer-I, Figure 6A), which usually contains linoleic acid at the ω-position of the fatty acid moiety [30] in the presence of detergents or glycerophospholipids. The hydrolysis of both Cer-I and-II by the CDase was strongly stimulated by addition of Triton X-100 or glycerophospholipids (PG+CL).

Effects of Cer analogues on CDase activity

From a clinical point of view, it is important to find inhibitors of bacterial CDase. Therefore we attempted to explore the effects of several Cer analogues on CDase activity. The chemical structures of the analogues used in this study are shown in Figure 7. Among these analogues, GCAS-4 and -7 were found to be effective in inhibiting the activity of *Pseudomonas* CDase (Figure 8A). It should be noted that these two analogues were not hydrolysed by bacterial CDase (results not shown). We also examined the authentic CDase inhibitors, *N*-oleoylethanolamine (NOE) [31], D-*erythro*-2-*N*-myristoylamino-1-phenyl-1-propanol (D-MAPP), and its stereoisomer, L-*erythro*-2-*N*-myristoylamino-1-phenyl-1-propanol (L-MAPP) [32]. NOE, an inhibitor of mammalian acidic CDase, had no inhibitory effect on bacterial CDase. D-MAPP and L-MAPP had inhibitory effects on bacterial CDase, but their effective concentrations were relatively high compared with those of GCAS-4 and -7 (Figure 8B). Although L-MAPP was more effective than D-MAPP in inhibiting bacterial CDase, the former does not appear to be suitable for clinical application, as it is hydrolysed by CDase to produce the cytotoxic compound 2-amino-1-phenyl-1-propanol. In summary, GCAS-4 and -7 appear to be candidates for clinical use as inhibitors of bacterial CDase.

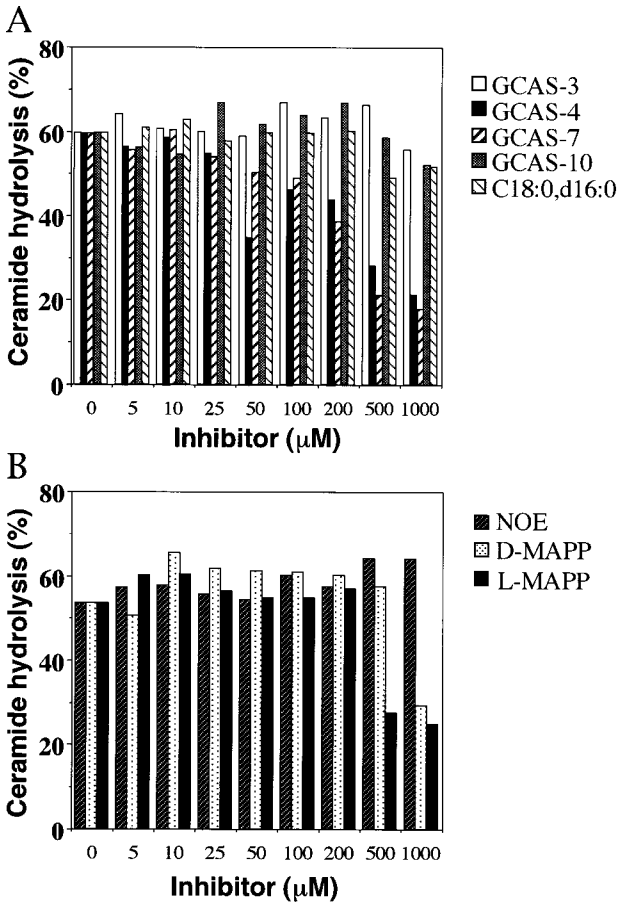

Figure 8 Effects of Cer analogues on Cer hydrolysis by *Pseudomonas* CDase

Inhibitory effects of newly synthesized Cer analogues (**A**) and authentic CDase inhibitors (**B**) were measured using the standard assay with [^{14}C]Cer ($C_{16:0}$, $C_{d18:1}$) as a substrate, as described in the Materials and methods section. Triton X-100 (0.05%, v/v) was used as a detergent. NOE [31] and D-MAPP [32] are inhibitors of acidic and neutral CDases respectively. Values are means from duplicate determinations.

DISCUSSION

Our recent findings [9] have suggested that bacterial CDase may cause the deficiency of Cer in the stratum corneum of atopic skin, since the lesions of atopic skin were often infected with CDase-producing bacteria. However, it has remained unclear how Cer in atopic skin is hydrolysed by bacterial CDase. Hydrolysis of Cer on the skin by the bacterial enzyme is unlikely because, in the absence of detergents, almost no hydrolysis of Cer occurs by the CDase [7], and the presence of detergents is unlikely on the skin. The findings of the present study appear to resolve this contradiction. We found that anionic glycerophospholipids derived from *S. aureus* could function to stimulate the activity of bacterial CDase in place of detergents. Furthermore, it was found that some strains of *P. aeruginosa*, including AN17, produced staphylolytic proteases (Figure 3) which decomposed *S. aureus* cells to generate these anionic glycerolipids (Figure 4). The present study also shows that normal Cer as well as esterified ω-hydroxyCer was degraded by the action of *Pseudomonas* CDase in the presence of *Staphylococcus*-derived glycerophospholipids instead of detergents (Figure 6). It should be noted that *S. aureus* is dominant in microflora of the atopic skin [24–26] and that

CDase-producing *P. aeruginosa* was originally isolated from atopic skin [7,9].

CDases are classified into three groups on the basis of their catalytic pH optima, i.e. acid, neutral and alkaline enzymes. Recently, it has been reported that the hydrolysis reaction of rat neutral CDase is activated by anionic phospholipids, especially CL [33]. On the other hand, extensive studies by Sandhoff and co-workers [34] revealed that the lysosomal acid CDase is activated by bis(monoacylglycero)phosphate, phosphatidylinositol and dolichol phosphate. The primary structure of *Pseudomonas* alkaline CDase is very close to those of murine neutral CDases [35], but is completely different from that of acid CDase [36]. These results indicate that CDases are activated by acidic phospholipids in general regardless of their catalytic pH optima.

It is noteworthy that atopic skin tends to have a neutral pH, whereas healthy skin is normally acidic [37,38]. The pH shift for atopic skin optimizes the action of both bacterial CDase [7] and staphylolytic proteases (LasA and D) [11], since both enzymes exhibit alkaline pH optima.

The effects of *Staphylococcus*-derived glycerophospholipids on endogenous CDase activity in the atopic skin remains to be elucidated, since an endogeneous CDase is involved in Cer catabolism in stratum corneum [39,40]. Recently, Hara et al. [41] reported the presence of sphingomyelin deacylase in atopic skin, an enzyme which hydrolyses sphingomyelin to generate sphingosylphosphorylcholine and fatty acid. They argued that the enzyme activity was significantly up-regulated, which evoked the decrease in Cer on atopic skin [41], as sphingomyelin is also a precursor of Cer [4]. Interestingly, a similar enzyme has been found in bacteria [42]. Further study is necessary to elucidate the molecular mechanism underlying the loss of barrier function in atopic skin.

In cultured human keratinocytes, exogenously added Cer promoted cellular differentiation, whereas Sph stimulated cellular proliferation [43]. Sph, a potent inhibitor of protein kinase C, is implicated in the dysfunction of intracellular signalling in sphingolipidoses [44]. Presumably, Sph, produced by the action of bacterial CDase, may disturb the terminal differentiation of keratinocytes or induce cytotoxic effects.

Sallusto et al. [45] also reported that Cer functions as a down-regulator for antigen uptake in mature dendritic cells, which are a kind of antigen-presenting cell. Hence, the decrease in the level of Cer in skin induced by the action of bacterial CDase would cause the increase in antigen uptake, concomitantly with antibody production. This may trigger the subsequent allergic response, as mast cells, which are known to participate in the IgE-mediated immediate hypersensitivity reaction, are present in the lesions on atopic skin [46].

In the present study, we observed the inhibitory effects of several Cer analogues on the activity of bacterial CDase. GCAS-4 is *O*-methylated and GCAS-7 has an unnatural *cis* double bond in the sphingoid moiety of Cer. These analogues were weakly hydrolysed by bacterial CDase, indicating that the hydroxy residue at the C-3 position of the sphingoid base is essential for the action of bacterial CDase. These results provide further information with which to synthesize more potential inhibitors of the bacterial enzyme.

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REFERENCES

1 Elias, P. M. and Menon, G. K. (1991) Structural and lipid biochemical correlates of the epidermal permeability barrier. Adv. Lipid Res. **24**, 1–26
2 Holleran, W. M., Takagi, Y., Menon, G. K., Legler, G., Feingold, K. R. and Elias, P. M. (1993) Processing of epidermal glucosylceramides is required for optimal mammalian cutaneous permeability barrier function. J. Clin. Invest. **91**, 1656–1664
3 Uchida, Y., Hara, M., Nishio, H., Sidransky, E., Inoue, S., Otsuka, F., Suzuki, A., Elias, P. M., Holleran, W. M. and Hamanaka, S. (2000) Epidermal sphigomyelins are precursors for selected stratum corneum ceramides. J. Lipid Res. **41**, 2071–2082
4 Schmuth, M., Man, M.-Q., Weber, F., Gao, W., Feingold, K. R., Fritsch, P., Elias, P. M. and Holleran, W. M. (2000) Permability barrier disorder in Niemann-Pick disease: sphingomyelin: ceramide processing required for normal barrier homeostasis. J. Invest. Dermatol. **115**, 459–466
5 Imokawa, G., Abe, A., Jin, K., Higaki, Y., Kawashima, M. and Hidano, A. (1991) Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin?. J. Invest. Dermatol. **96**, 523–526
6 Bos, J. D., Kapsenberg, M. L. and Smitt, J. H. (1994) Pathogenesis of atopic eczema. Lancet **343**, 1338–1341
7 Okino, N., Tani, M., Imayama, S. and Ito, M. (1998) Purification and characterization of a novel ceramidase from *Pseudomonas aeruginosa*. J. Biol. Chem. **273**, 14368–14373
8 Okino, N., Ichinose, S., Omori, A., Imayama, S., Nakamura, T. and Ito, M. (1999) Molecular cloning, sequencing, and expression of the gene encoding alkaline ceramidase from *Pseudomonas aeruginosa*: cloning of a ceramidase homologue from *Mycobacterium tuberculosis*. J. Biol. Chem. **274**, 36616–36622
9 Ohnishi, Y., Okino, N., Ito, M. and Imayama, S. (1999) Ceramidase activity in bacterial skin flora as a possible cause of ceramide deficiency in atopic dermatitis. Clin. Diagn. Lab. Immunol. **6**, 101–104
10 Kessler, E., Safrin, M., Olson, J. C. and Ohman, D. E. (1993) Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. J. Biol. Chem. **268**, 7503–7508
11 Park, S. and Galloway, D. R. (1995) Purification and characterization of LasD: a second staphylolytic proteinase produced by *Pseudomonas aeruginosa*. Mol. Microbiol. **16**, 263–270
12 Tani, M., Kita, K., Komori, H., Nakagawa, T. and Ito, M. (1998) Enzymatic synthesis of ω-amino-ceramide: preparation of a sensitive fluorescent substrate for ceramidase. Anal. Biochem. **263**, 183–188
13 Mitsutake, S., Kita, K., Okino, N. and Ito, M. (1997) [^{14}C]Ceramide synthesis by sphingolipid ceramide *N*-deacylase: new assay for ceramidase activity detection. Anal. Biochem. **247**, 52–57
14 Kita, K., Kurita, T. and Ito, M. (2001) Characterization of the reversible nature of the reaction catalyzed by sphingolipid ceramide *N*-deacylase: a novel form of reverse hydrolysis reaction. Eur. J. Biochem. **268**, 592–602
15 Mason, J. T., Broccoli, A. V. and Huang, C.-H. (1981) A method for the synthesis of isomerically pure saturated mixed-chain phosphatidylcholines. Anal. Biochem. **113**, 96–101
16 Peters, J. E. and Galloway, D. R. (1990) Purification and characterization of an active fragment of the LasA protein from *Pseudomonas aeruginosa*: enhancement of elastase activity. J. Bacteriol. **172**, 2236–2240
17 Rozgonyi, F., Kiss, J. and Szitha, K. (1980) Lipid extraction and phospholipid phosphorus determination in Gram-positive bacteria. Acta Microbiol. Acad. Sci. Hung. **27**, 23–29
18 Dittmer, J. C. and Lester, R. L. (1964) A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J. Lipid Res. **5**, 126–127
19 Doering, T., Holleran, W. M., Potratz, A., Vielhaber, G., Elias, P. M., Suzuki, K. and Sandhoff, K. (1999) Sphingolipid activator proteins are required for epidermal permeability barrier formation. J. Biol. Chem. **274**, 11038–11045
20 Touchstone, J. C., Levin, S. S., Dobbins, M. F. and Beers, P. C. (1983) Analysis of saturated and unsaturated phospholipids in biological fluids. J. Liq. Chromatogr. **6**, 179–192
21 Bose, R., Chen, P., Loconti, A., Grüllich, C., Abrams, J. M. and Kolesnick, R. N. (1998) Ceramide generation by the Reaper protein is not blocked by the caspase inhibitor, p35. J. Biol. Chem. **273**, 28852–28859
22 Merrill Jr, A. H., Wang, E., Mullins, R. E., Jamison, W. C. L., Nimkar, S. and Liotta, D. C. (1988) Quantitation of free sphingosine in liver by high-performance liquid chromatography. Anal. Biochem. **171**, 373–381
23 Hayami, M., Okabe, A., Kariyama, R., Abe, M. and Kanemasa, Y. (1979) Lipid composition of *Staphylococcus aureus* and its derived L-forms. Microbiol. Immunol. **23**, 435–442
24 Leyden, J. J., Marples, R. R. and Kligman, A. M. (1974) *Staphylococcus aureus* in the lesions of atopic dermatitis. Br. J. Dermatol. **90**, 525–530
25 Aly, R., Maibach, H. I. and Shinefield, H. R. (1977) Microbial flora of atopic dermatitis. Arch. Dermatol. **113**, 780–782
26 Goodyear, H. M., Watson, P. J., Egan, S. A., Price, E. H., Kenny, P. A. and Harper, J. I. (1993) Skin microflora of atopic eczema in first time hospital attenders. Clin. Exp. Dermatol. **18**, 300–304

27 Kita, K., Okino, N. and Ito, M. (2000) Reverse hydrolysis reaction of a recombinant alkaline ceramidase of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **1485**, 111–120
28 Wertz, P. W. and Downing, D. T. (1983) Ceramides of pig epidermis: structure determination. J. Lipid Res. **24**, 759–765
29 Wertz, P. W., Miethke, M. C., Long, S. A., Strauss, J. S. and Downing, D. T. (1985) The composition of the ceramides from human stratum corneum and from comedones. J. Invest. Dermatol. **84**, 410–412
30 Robson, K. J., Stewart, M. E., Michelsen, S., Lazo, N. D. and Downing, D. T. (1994) 6-Hydroxy-4-sphingenine in human epidermal ceramides. J. Lipid Res. **35**, 2060–2068
31 Sugita, M., Williams, M., Dulaney, J. T. and Moser, H. W. (1975) Ceramidase and ceramide synthesis in human kidney and cerebellum: description of a new alkaline ceramidase. Biochim. Biophys. Acta **398**, 125–131
32 Bielawska, A., Greenberg, M. S., Perry, D., Jayadev, S., Shayman, J. A., McKay, C. and Hannun, Y. A. (1996) (1*S*,2*R*)-D-*erythro*-2-(*N*-Myristoylamino)-1-phenyl-1-propanol as an inhibitor of ceramidase. J. Biol. Chem. **271**, 12646–12654
33 Bawab, S. E., Birbes, H., Roddy, P., Szule, Z. M., Bielawska, A. and Hannun, Y. A. (2001) Biochemical characterization of the reverse activity of rat brain ceramidase: a CoA-independent and fumonisin B_1-insensitive ceramide synthase. J. Biol. Chem. **276**, 16758–16766
34 Linke, T., Wilkening, G., Sadeghlar, F., Mozcall, H., Bernardo, K., Schuchman, E. and Sandhoff, K. (2001) Interfacial regulation of acid ceramidase activity. Stimulation of ceramide degradation by lysosomal lipids and sphingolipid activator protein. J. Biol. Chem. **276**, 5760–5768
35 Tani, M., Okino, N., Mori, K., Tanigawa, T., Izu, H. and Ito, M. (2000) Molecular cloning of the full-length cDNA encoding mouse neutral ceramidase: a novel but highly conserved gene family of neutral/alkaline ceramidase. J. Biol. Chem. **275**, 11229–11234
36 Koch, J., Gärtner, S., Li, C.-M., Quintern, L. E., Bernardo, K., Levran, O., Schnabel, D., Desnick, R. J., Schuchman, E. H. and Sandhoff, K. (1996) Molecular cloning and characterization of a full-length complementary DNA encoding human acid ceramidase: identification of the first molecular lesion causing Farber disease. J. Biol. Chem. **271**, 33110–33115
37 Blank, I. H. (1939) Measurement of pH of the skin surface III: measurements on the arms of children with no apparent skin lesions. J. Invest. Dermatol. **2**, 231–234
38 Anderson, D. S. (1951) The acid-base balance of the skin. Br. J. Dermatol. **63**, 283–296
39 Wertz, P. W. and Downing, D. T. (1989) Free sphingosine in porcine epidermis. Biochim. Biophys. Acta **1002**, 213–217
40 Wertz, P. W. and Downing, D. T. (1990) Free sphingosine in human epidermis. J. Invest. Dermatol. **94**, 159–161
41 Hara, J., Higuchi, K., Okamoto, R., Kawashima, M. and Imokawa, G. (2000) High-expression of sphingomyelin deacylase is an important determinant of ceramide deficiency leading to barrier disruption in atopic dermatitis. J. Invest. Dermatol. **115**, 406–413
42 Ito, M., Kurita, T. and Kita, K. (1995) A novel enzyme that cleaves the *N*-acyl linkage of ceramides in various glycosphingolipids as well as sphingomyelin to produce their lyso forms. J. Biol. Chem. **270**, 24370–24374
43 Wakita, H., Tokura, Y., Yagi, H., Nishimura, K., Furukawa, F. and Takigawa, M. (1994) Keratinocyte differentiation is induced by cell-permeant ceramides and its proliferation is promoted by sphingosine. Arch. Dermatol. Res. **286**, 350–354
44 Hannun, Y. A. and Bell, R. M. (1987) Lysosphingolipids inhibit protein kinase C: implications for the sphingolipidoses. Science (Washington, D.C.) **235**, 670–674
45 Sallusto, F., Nicolo, C., Maria, R. D., Corinti, S. and Testi, R. (1996) Ceramide inhibits antigen uptake and presentation by dendritic cells. J. Exp. Med. **184**, 2411–2416
46 Imayama, S., Shibata, Y. and Hori, Y. (1995) Epidermal mast cells in atopic dermatitis. Lancet **346**, 1559

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