The skin of atopic dermatitis patients contains a novel enzyme, glucosylceramide sphingomyelin deacylase, which cleaves the N-acyl linkage of sphingomyelin and glucosylceramide

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We have demonstrated previously that there is an abnormal expression of sphingomyelin (SM) deacylase in the epidermis of patients with atopic dermatitis (ADe). In the present study, we have prepared *N*-[palmitic acid-1-¹⁴C]SM and *N*-[palmitic acid-1-¹⁴C]glucosylceramide (GcCer) to use as substrates and have quantified SM deacylase activity by detecting the release of [¹⁴C]palmitic acid in extracts of the stratum corneum or the epidermis of ADe patients. In studies using [palmitic acid-1-¹⁴C]SM as a substrate, a pH dependency of catalytic activity with a peak at pH 5.0 was found. Preparative SDS/PAGE using an extract of ADe epidermis revealed that the molecular mass of SM deacylase is 40000 Da, which is consistent with its apparent molecular mass of 42000 Da estimated by gel-filtration analysis of stratum corneum extracts. Analytical isoelectric focusing (IEF) chromatography demonstrated that the pI values of SM deacylase, β -glucocerebrosidase (GlcCDase), sphingomyelinase (SMase) and acid ceramidase were 4.2, 7.4, 7.0 and 5.7, re-

spectively. In enzymic analysis using pI-4.2 SM deacylase partially purified by IEF, which had no detectable contamination with acid ceramidase, GlcCDase or SMase, radio-TLC analysis revealed that radiolabelled sphingosylphosphocholine or [1-¹⁴C]palmitic acid was enzymically liberated from [choline-methyl-¹⁴C]SM or *N*-[palmitoyl-1-¹⁴C]GcCer, respectively, used as substrates. Further the pI-4.2 protein purified from extracts of the stratum corneum of ADe patients was able to hydrolyse *N*-[palmitoyl-1-¹⁴C]SM and GcCer, but not *N*-[palmitoyl-1-¹⁴C]ceramide. These results indicate that a hitherto undiscovered epidermal enzyme, termed here glucosylceramide sphingomyelin deacylase, is expressed in the skin of ADe patients, which plays an important role in ceramide deficiency (including acylceramides) in the stratum corneum.

Key words: ceramide, sphingomyelinase, sphingosylphosphocholine.

INTRODUCTION

There has been no evidence to date which demonstrates the existence in mammalian tissues of glucosylceramide sphingomyelin (GcSM) deacylase, which catalyses the hydrolysis of the N-acyl linkage of sphingomyelin (SM) or glucosylceramide (GcCer) to yield sphingosylphosphocholine (SPC) or glucosylsphingosine (GSph), respectively, and fatty acid. In actinomyces, however, a lysoglycosphingolipid-generating hydrolase activity has been reported [1]. Ito et al. [2] demonstrated that a novel enzyme, purified to apparent homogeneity from the culture filtrate of a newly isolated bacterium (*Pseudomonas* sp. TK 4), cleaves the N-acyl linkage of ceramides in various glycosphingolipids, as well as SM, to produce their lyso-forms. In addition, this enzyme does not have the potential to degrade ceramides, indicating that it is an enzyme distinct from ceramidase (EC 3.5.1.23). On the other hand, sphingomyelinase (SMase, EC 3.1.4.12) is an acidic lysosomal hydrolase which is regarded as a major ceramide-generating enzyme that catalyses the hydrolysis of the phosphodiester linkage in SM to yield ceramides and phosphorylcholine. Ceramides constitute the core structure of several sphingolipids which play essential roles in cell proliferation and differentiation [3–5] and which have been implicated as inducers of programmed cell death [6]. In the

epidermis, ceramides are an important determinant of the functions of the uppermost layer of epidermis, the stratum corneum [7], as a permeability barrier and water reservoir, and they account for 50 % of the stratum corneum lipids.

We have demonstrated previously that there is a marked decrease in ceramide levels within the stratum corneum of involved and uninvolved skin of atopic dermatitis (ADe) patients compared with age-matched healthy controls [8]. Those results indicated that an insufficiency of ceramides may be an aetiological factor in dry and barrier-disrupted ADe skin. In our previous study to clarify biochemical mechanisms leading to ceramide deficiency in the stratum corneum of ADe patients, we found, using radio-TLC combined with a quantitative assay for SM hydrolysis, that this activity is over-expressed in the epidermis of ADe patients as a result of the high expression of a hitherto undiscovered epidermal enzyme, tentatively termed SM deacylase [9]. SM deacylase degrades SM at the N-acyl linkage to yield SPC rather than ceramide, the authentic SMase reaction product. Those findings suggested that the high expression of SM deacylase competes with the ceramide-generating enzyme SMase for the same substrate SM, which results in decreased levels of ceramides seen in the stratum corneum of ADe patients. In order to clarify biochemical properties of the enzyme, designated GcSM deacylase in this study, we have prepared [fatty acid-¹⁴C]SM and

Abbreviations used: GcSM, glucosylceramide sphingomyelin; ADe, atopic dermatitis; SM, sphingomyelin; SMase, sphingomyelinase; SPC, sphingosylphosphocholine; GlcCDase, β -glucocerebrosidase; IEF, isoelectric focusing; GcCer, glucosylceramide; GSph, glucosylsphingosine.

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[fatty acid- ^{14}C]GcCer as substrates to quantify the activity of GcSM deacylase and to characterize its enzymic properties in extracts of the stratum corneum of ADe patients.

EXPERIMENTAL

Materials

[1- ^{14}C]Palmitic acid (55 mCi/mmol) and [choline-methyl- ^{14}C]SM (52 mCi/mmol) were purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). SPC was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, U.S.A.). Palmitoylsphingosine, glucopsychosine and sphingosine were obtained from Sigma (St. Louis, MO, U.S.A.). The HPLC TSK-3000SW column was obtained from TOSO Industries (Tokyo, Japan). All other chemicals were of reagent grade.

Preparation of *N*-[palmitoyl-1- ^{14}C]SM

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

Preparation of *N*-[palmitoyl-1- ^{14}C]GcCer

[1- ^{14}C]Palmitic acid (55 mCi/mmol, Amersham Pharmacia Biotech) and palmitic acid were treated with 1 ml of thionyl chloride and a catalytic amount of *N,N*-dimethylformamide. The mixture was heated at 95 °C for 2 h, then cooled to room temperature. The solvent was removed on a rotary evaporator to give [1- ^{14}C]palmitoyl chloride. Glucopsychosine (0.076 mmol) was also treated with anhydrous tetrahydrofuran (1 ml) and 50 μl of water. The solution was stirred at room temperature and treated simultaneously with saturated aqueous sodium acetate (1 ml) and with [1- ^{14}C]palmitoyl chloride in anhydrous tetrahydrofuran. The mixture was stirred for 2 h at room temperature and 15 ml of saturated aqueous sodium chloride was added. The mixture was extracted three times with 50 ml of dichloromethane. The organic solution was dried with MgSO_4 , and the solvent was removed on a rotary evaporator. The crude residue was subjected to Flash column chromatography on silica and eluted with dichloromethane/methanol (87:3, v/v). The radiochemical purity of synthetic *N*-[palmitoyl-1- ^{14}C]GcCer (47 mCi/mmol) was checked by TLC on silica gel with three solvent systems, chloroform/ethanol (4:1, v/v), dichloromethane/methanol (85:15, v/v) and chloroform/methanol/water (65:25:4, by vol.), with a radioscanner and was proven to be at least 95% pure. The mass spectrum was consistent with the proposed structure.

Preparation of stratum corneum extracts from patients with ADe

Diagnosis of ADe was made according to the criteria by Hanifin and Rajka [10]. Specimens of stratum corneum were obtained from involved ADe skin by three tape-strippings (Protect-label/B type, 40 mm $\times$ 60 mm; Iuchiseidou Corp., Osaka, Japan) in the same region. These tapes were stored frozen at -80 °C until

used. The three tapes were immersed in 40 ml of 50 mM acetate buffer, pH 4.7, containing 0.1% (v/v) Triton X-100 and 0.5 mM PMSF for 30 min at 4 °C, and were then sonicated (Bioruptor, Olympus, Tokyo, Japan) on ice for a total of 3 min with 30-s bursts. Mixtures containing the three tapes were further incubated in the same buffer overnight at 4 °C. After removing the tapes, each extract from the stratum corneum was concentrated to about 1.0 ml using a Centriprep10 (Amicon, Millipore, Beverly, MA, U.S.A.), and the concentrated solutions were used for biochemical characterization of GcSM deacylase. The protein concentrations of stratum corneum extracts were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, U.S.A.) with BSA as a standard.

Preparation of epidermis extract from patients with ADe

Ten specimens (4-mm skin-punch biopsies) were incubated with 1000 units/ml of dispase at 4 °C for 4 h to separate the epidermis. Each isolated epidermal sheet was homogenized with a glass homogenizer in 0.3 ml of 50 mM acetate buffer, pH 4.7, containing 0.1% (v/v) Triton X-100 and 0.5 mM PMSF. The homogenates were centrifuged at 10000 *g* for 30 min to obtain a supernatant that was subjected to SDS/PAGE.

Preparative electrophoresis

Concentrated epidermis extract (0.1 ml) was separated by preparative SDS/PAGE under non-reducing conditions and electrophoresed using 125 V at 4 °C. After electrophoresis, the gel was immersed in five changes of 2.5% (v/v) Triton X-100 with gentle shaking for 1 h at room temperature. The gel was divided into 20 pieces 3.0 mm wide and 1.5 mm thick. Each piece was homogenized in 0.5 ml of 50 mM acetate buffer, pH 4.7, containing 0.1% (v/v) Triton X-100 and 0.5 mM PMSF and was then subjected to measurement of SM hydrolysis activity using [choline-methyl- ^{14}C]SM as a substrate. To confirm the reaction products, the aqueous phase was dried at -80 °C and dissolved in 0.5 ml of distilled water. The sample solution was subjected to a Sep-Pak Plus C_{18} cartridge (Waters, Milford, MA, U.S.A.) to remove salts. After washing with 10 ml of distilled water, the radiolabelled metabolite was eluted from the cartridge with 10 ml of methanol. The eluate was dried, dissolved in 50 μl of methanol, 40 μl of which was subjected to TLC developed with *n*-butyl alcohol/acetic acid/water (3:1:1, by vol.). Radioactivity was visualized using a bio-imaging analyser (BAS-2000, Fuji, Tokyo, Japan). SMase and GcSM deacylase were also assayed by measuring the amount of radioactive ceramide and palmitic acid, respectively, using *N*-[palmitoyl-1- ^{14}C]SM as a substrate.

Assay of SM hydrolysis

SM hydrolysis was assayed by the method of Bowser and Gray [11]. The incubation mixture contained 50 mM acetate buffer, pH 4.7, 0.1% (v/v) Triton X-100, 20 mM CaCl_2 , 10 nmol of [choline-methyl- ^{14}C]SM (52 mCi/mmol) and 0.25 ml of the epidermal extracts after preparative SDS/PAGE in a total volume of 0.5 ml. Incubations were carried out at 37 °C for 15 h and stopped by the addition of 0.5 ml of water and 2 ml of chloroform/methanol (2:1, v/v). The mixtures were vortex-mixed and centrifuged for 10 min at 2000 *g*; 0.25 ml of the upper phase was then counted in a liquid-scintillation counter.

TSK-3000SW column chromatography

Each concentrated extract (0.2 ml) was applied to a TSK-3000SW column (0.6 cm $\times$ 60 cm) equilibrated with 50 mM acetate buffer, pH 4.7, containing 0.1% (v/v) Triton X-100, 0.5 mM PMSF

and 0.15 M NaCl. Proteins were eluted isocratically at a flow rate of 1.0 ml/min using a total volume of 50 ml and 0.5-ml fractions were collected. Gel Filtration Standards (Bio-Rad, Hercules, CA, U.S.A., catalogue no. 151-1901) were used as molecular markers.

Isoelectric focusing (IEF)

Each concentrated extract was dialysed for at least 20 h against 10 mM acetate buffer, pH 4.7, containing 0.1 % (v/v) Triton X-100 and 0.5 mM PMSF. The dialysed solution (10 ml) was diluted with 10 ml of dialysis buffer containing 4 % (v/v) Bio-Lyte ampholytes, pH 3.0–10.0 (Bio-Rad). This solution was applied to a preparative IEF chamber (Rotofor Cell, Bio-Rad), and resolved at 12 W constant power for 4 h at 4 °C. The focusing chamber was divided into 20 discrete compartments, each of which was collected simultaneously into a different tube at the end of the run. The pH of each fraction was measured and neutralized prior to measuring enzymic activities.

Hydrolysis of SM, GCer and palmitoylsphingosine by pI-4.2 protein fraction purified by IEF

N-[palmitoyl-1-¹⁴C]Sphingolipids (10 nmol; each 45 mCi/mmol) were incubated at 37 °C for 15 h with pI-4.2 protein purified from ADe stratum corneum in 300 µl of 50 mM acetate buffer, pH 4.7, containing 0.1 % (v/v) Triton X-100 and 20 mM CaCl₂. That sample contained GcSM deacylase that had been partially purified by IEF to exclude SMase, acid ceramidase and β-glucocerebrosidase (GlcCDase). The incubation was stopped by addition of 1 ml of methanol and dried under a stream of nitrogen gas. The dried lipids were dissolved in 1 ml of hexane/diethyl ether/acetic acid (50:50:1, by vol.; solvent A), and applied to a Sep-Pak Plus Silica cartridge (Waters) previously equilibrated with solvent A. Unreacted SM, GCer and palmitoylsphingosine were adsorbed on the cartridge, while free [¹⁴C]palmitic acid was eluted with 10 ml of solvent A [12]. The eluate was dried and counted in a liquid-scintillation counter.

Measurement of GcSM deacylase and SMase activities

GcSM deacylase and SMase activities were assayed by measuring the amount of radioactive palmitic acid and ceramide released from *N*-[palmitoyl-1-¹⁴C]SM, respectively. The standard reaction mixture (final volume, 300 µl) contained 50 mM acetate buffer, pH 4.7, 10 nmol of *N*-[palmitoyl-1-¹⁴C]SM, 0.1 % (v/v) Triton X-100, 20 mM CaCl₂ and enzyme solution. The reaction mixture was incubated for 15 h at 37 °C and terminated by the addition of 50 µl of 2 mg/ml carrier palmitic acid, followed by 3.0 ml of Dole's reagent (2-propanol/heptane/1 M NaOH, 40:10:1, by vol.) [13]. After 1.8 ml of heptane and 1.6 ml of water were added, the mixture was vortex-mixed and centrifuged for 5 min at 2000 *g*. Then 1 ml of the upper heptane phase, which had the enzymically 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 of heptane. Thereafter, 1 ml of 0.5 M H₂SO₄ and 2.4 ml of heptane were added, and the mixture was vortex-mixed and then centrifuged for 10 min at 2000 *g*. The upper phase (1 ml) was washed with an equal volume of 0.5 M H₂SO₄ to remove any remaining unreacted SM. Following mixing and centrifugation, 0.8 ml of the washed upper phase was counted as above for measurement of GcSM deacylase activity. To establish background, blank tubes were carried through without the enzyme

Figure 1 Identification of reaction products using radio-TLC

After the 15 h of incubation of stratum corneum samples (0.3 ml) at pH 4.7 with 10 nmol of *N*-[palmitoyl-1-¹⁴C]SM, the reaction products in the upper heptane-rich phase, which partitioned in the biphasic system, and in the final upper phase, which had been washed with H₂SO₄, were dried under nitrogen and dissolved in 0.1 ml of chloroform/methanol (2:1, v/v). Sample solutions (approx. 1000 d.p.m.) were subjected to TLC analysis. Radioactivities on TLC plates were quantified utilizing a TLC scanner and are expressed as a colour density correlating with radioactive intensity. Lane 1, reaction product in heptane-rich phase; lane 2, radiolabelled metabolite in final upper phase after washing with H₂SO₄; lanes 3–5, standards of radio-labelled SM (*N*-[palmitoyl-1-¹⁴C]SM), ceramide (Cer; *N*-[1-¹⁴C]palmitoylsphingosine) and free fatty acids (FFA; palmitic acid), respectively.

source. The radioactive reaction products from SMase and GcSM deacylase activities were then identified by TLC on silica gel with the solvent system chloroform/methanol/acetic acid (94:1:5, by vol.). These products were compared with radioactive standards, SM, ceramide (i.e. palmitoylsphingosine which was prepared as described previously [14]) and palmitic acid. Radio-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 H₂SO₄) were ceramide and palmitic acid, respectively. These products had mobilities on TLC plates nearly identical with radiolabelled standards (Figure 1), indicating an effective separation of palmitoylsphingosine and palmitic acid using this partition method.

Measurement of acid ceramidase activity

Acid ceramidase was assayed at pH 4.7 by measuring the amount of radioactive palmitic acid released from [1-¹⁴C]palmitoylsphingosine using a slightly modified assay mixture as described previously [14]. The reaction mixture was identical with that used for measurement of GcSM deacylase activity, except for the substrate.

Measurement of GlcCDase activity

GlcCDase was assayed at pH 4.7 by measuring the amount of radioactive palmitoylsphingosine released from *N*-[palmitoyl-1-¹⁴C]GCer. The reaction mixture was identical with that used for measurement of GcSM deacylase activity, except for the substrate. The incubations were carried out at 37 °C for 15 h and were stopped by addition of 1 ml of solvent A. The sample solution was purified over a Sep-Pak Plus Silica cartridge (Waters) previously equilibrated with solvent A. Unreacted GCer and enzymically released [¹⁴C]palmitoylsphingosine were trapped, while free [¹⁴C]fatty acids were eluted with 10 ml of solvent A. [¹⁴C]Ceramide was then eluted from the cartridge with 10 ml of chloroform/methanol/acetic acid (190:9:1, by vol.; solvent B) [12]. The eluate was dried under a stream of nitrogen gas and counted in a liquid-scintillation counter.

Detection of GcSM deacylase activity based upon the release of SPC from [choline-methyl- 14 C]SM

SM hydrolysis, which leads to the release of SPC, was assessed by the method of Bowser and Gray [11]. The incubation mixtures (total volume of 0.5 ml) contained 50 mM acetate buffer, pH 4.7, 0.1 % (v/v) Triton X-100, 20 mM CaCl_2 , 10 nmol of [choline-methyl- 14 C]SM (52 mCi/mmol) and the pI-4.2 protein fraction which contained GcSM deacylase that had been partially purified from ADe stratum corneum extract by IEF to exclude SMase, GlcCDase and acid ceramidase. Incubations were carried out at 37 °C for 15 h, and were stopped by the addition of 0.5 ml of water and 2 ml of chloroform/methanol (2:1, v/v). The mixtures were then vortex-mixed and centrifuged for 10 min at 2000 g. The upper aqueous phase (1.0 ml) was dried at -80 °C and dissolved in 100 μ l of methanol. The sample solution (80 μ l) was subjected to TLC, and developed with *n*-butyl alcohol/acetic acid/water (3:1:1, by vol.). Radioactivities were visualized using a bio-imaging analyser (BAS-2000). Non-radioactive spots, SM and SPC were also visualized by charring with capric sulphate/phosphoric acid spray. Appropriate blanks containing denatured enzyme (100 °C, 10 min) were also run as background controls.

Detection of 14 C-labelled palmitic acid release from *N*-[palmitoyl-1- 14 C]GcCer upon radio-TLC

Release of 14 C-labelled fatty acid was detected using a modification of an assay method for phospholipase A_2 [15]. The reaction mixture contained 10 nmol of *N*-[palmitoyl-1- 14 C]GcCer in 50 mM acetate buffer, pH 4.7, 0.1 % (v/v) Triton X-100, 20 mM CaCl_2 and the pI-4.2 protein fraction that had been partially purified by IEF to exclude SMase, GlcCDase and acid ceramidase. The mixture was incubated for 15 h at 37 °C. At the end of the incubation period, reaction mixtures were promptly mixed with 1.8 ml of chloroform/methanol (2:1, v/v), 0.2 ml of water and 0.06 ml of 0.5 M NaOH, vortex-mixed and then centrifuged for 10 min at 2000 g. A 250- μ l aliquot of the upper phase was evaporated under nitrogen, and the dried lipids were resuspended in 100 μ l of chloroform/methanol (2:1, v/v). The solution (50 μ l) was then subjected to TLC and developed with chloroform/methanol/acetic acid (190:9:1, by vol.). Appropriate blanks containing denatured enzyme (100 °C, 10 min) were also run to establish background.

RESULTS

Effects of non-radiolabelled palmitoylsphingosine on acid ceramidase activity and GcSM deacylase activity

In assays to evaluate GcSM deacylase activity, we took into consideration that if both acid SMase and acid ceramidase exist in the stratum corneum of ADe patients, radiolabelled palmitic acid released would not necessarily represent the activity of GcSM deacylase. This is because a similar release of radiolabelled palmitic acid can also occur from a two-step reaction where SM would be hydrolysed by acid SMase to yield radiolabelled palmitoylsphingosine, which in turn could be converted by acid ceramidase into radiolabelled palmitic acid. Therefore, to assess the contribution of acid ceramidase activity to GcSM deacylase activity in extracts of the stratum corneum of ADe patients, we added unlabelled palmitoylsphingosine to the reaction mixture which should, as a result of dilution, inhibit the enzymic release of radiolabelled palmitic acid from radiolabelled palmitoylsphingosine (generated by SMase) by any acid ceramidase that might contaminate the fraction. In almost all cases, addition of

Figure 2 Effects of non-radiolabelled palmitoylsphingosine on acid ceramidase and GcSM deacylase activities

Unlabelled palmitoylsphingosine (1 mM) was added where indicated (+) to the reaction mixtures for acid ceramidase (using *N*-[1- 14 C]palmitoylsphingosine as a substrate) or GcSM deacylase (using *N*-[palmitoyl-1- 14 C]SM as a substrate). Following 15 h of incubation with stratum corneum extracts from ADe patients (A) or healthy controls (B), radioactive reaction products were measured according to assay procedures as described in the Experimental section to measure acid ceramidase and GcSM deacylase activities. Means $\pm$ S.D. are shown.

Figure 3 Effect of pH on GcSM deacylase activity

Assays contained 50 mM acetate (○, pH 3–6) or phosphate (●, pH 6–8) buffers, and reactions were performed as described in the Experimental section, except for the elimination of SMase activity by preparative IEF. Results are expressed as means from two separate experiments.

unlabelled palmitoylsphingosine to the reaction mixture did not decrease the activity of GcSM deacylase, as determined by released radiolabelled palmitic acid (Figure 2A), suggesting that

Figure 4 SDS/PAGE using the epidermis extract for GcSM deacylase

Concentrated epidermis extract (0.1 ml) was separated by preparative SDS/PAGE under non-reducing conditions and electrophoresed with 125 V at 4 °C. After electrophoresis, the gel was immersed in five changes of 2.5% (v/v) Triton X-100 with gentle shaking for 1 h at room temperature. The gel was sliced into 20 pieces (3.0 mm wide $\times$ 1.5 mm thick). Each piece was homogenized in 0.5 ml of 50 mM acetate buffer, pH 4.7, containing 0.1% (v/v) Triton X-100 and 0.5 mM PMSF and subjected to assay for GcSM deacylase (A) and SMase (B) activities using N -[palmitoyl- 14 C]SM, and for SM hydrolysis (C) activity using [choline-methyl- 14 C]SM. For TLC preparation, the aqueous phase (as described in the assay procedure) containing the reaction products liberated from [choline-methyl- 14 C]SM were dried and subjected to Sep-Pak Plus C_{18} cartridge to remove salts. After washing with 10 ml of distilled water, radiolabelled metabolites were eluted from the cartridge with 10 ml of methanol. The eluate was dried, dissolved in 50 μ l of methanol, 40 μ l of which was subjected to TLC analysis (D). Molecular-mass markers: myosin, 207 000 Da; β -galactosidase, 123 000 Da; BSA, 86 000 Da (Kaleidoscope Prestained Standard, Bio-Rad; catalogue no. 161-0324, control 82336); phosphorylase *b*, 97 400 Da; catalase, 58 100 Da; and alcohol dehydrogenase, 39 800 Da (Biotinylated SDS Molecular Weight Standard Mixture, Sigma; catalogue no. MW-SDS-6B).

there was no contribution of acid ceramidase to GcSM deacylase activity observed in extracts from ADe patients. In contrast, the addition of unlabelled palmitoylsphingosine to the reaction mixture significantly suppressed the activity of acid ceramidase in extracts of healthy skin measured by the release of radiolabelled

Figure 5 Determination of molecular masses of GcSM deacylase and SMase

The molecular masses of GcSM deacylase ($\square$) and SMase ($\circ$) were determined by SDS/PAGE. Protein standards ($\bullet$) used were phosphorylase *b* (97 400 Da), catalase (58 100 Da) and alcohol dehydrogenase (39 800 Da).

palmitic acid (Figure 2B). These findings indicate that the release of radiolabelled palmitic acid in extracts of the stratum corneum from ADe patients can be ascribed to direct deacylation of SM that results from GcSM deacylase activity, since there were negligible levels of acid ceramidase in those extracts.

Effects of pH

Figure 3 demonstrates GcSM deacylase activity at various pH values in extracts from the stratum corneum of ADe patients after purification by preparative IEF to remove SMase activity. The highest activity was observed at pH 5.0.

Estimation of molecular mass of GcSM deacylase

Preparative SDS/PAGE was performed using the epidermal extracts to estimate the molecular mass of GcSM deacylase. Following SDS/PAGE, the gel was sliced into 20 pieces which were homogenized and subjected to measurements for SM hydrolysis and for the activities of SMase and GcSM deacylase. Enzymic assay with N -[palmitoyl- 14 C]SM as a substrate revealed that the activity of gel-purified GcSM deacylase, detectable by the release of [14 C]free fatty acid, migrates with a molecular mass of 40 000 Da (Figures 4A and 5), while SMase activity, detectable by the release of [14 C]palmitoylsphingosine, migrates at a molecular mass of 100 000 Da (Figures 4B and 5). In the assay for SM hydrolysis with [choline-methyl- 14 C]SM (Figure 4C), there are two separated peaks (peaked fractions 6 and 14) of radioactivity distributed in the aqueous phase (as described in the assay procedure), each corresponding in electrophoretic mobility to the molecular masses estimated for GcSM deacylase and SMase. Furthermore, radio-TLC analysis of reaction products generated by fraction 14 demonstrated that the enzyme possesses activity that can release radiolabelled SPC (Figure 4D).

Figure 6 Comparison of elution profiles of GcSM deacylase and acid ceramidase on TSK-3000SW column

Concentrated extracts (0.2 ml) of stratum corneum from ADe patients were filtered through a TSK-3000SW (0.6 cm $\times$ 60 cm) column previously equilibrated with 50 mM acetate buffer, pH 4.7, containing 0.1% (v/v) Triton X-100, 0.5 mM PMSF and 0.15 M NaCl. The column was eluted isocratically, 0.5-ml fractions were collected and GcSM deacylase and acid ceramidase activities were assayed as described in the Experimental section. Insert: protein standards (●) used for the estimation of the apparent molecular mass were γ -globulin (158 000 Da), ovalbumin (44 000 Da), myoglobin (17 000 Da) and vitamin B12 (1350 Da).

Gel chromatography of GcSM deacylase and acid ceramidase

To compare the elution profiles of GcSM deacylase and acid ceramidase by gel filtration, concentrated extracts from the stratum corneum of ADe patients were eluted through a TSK-3000SW column. There was a much lower activity of acid ceramidase than of GcSM deacylase in fractions that contained GcSM deacylase activity (Figure 6). The elution pattern of acid ceramidase activity was distinct from that of GcSM deacylase, indicating that the activity of acid ceramidase does not contribute to the GcSM deacylase activity measured here. A plot of the elution peak against a molecular-mass standard curve (Figure 6, insert) revealed that the apparent mass of GcSM deacylase was approx. 42 000 Da, which is consistent with that estimated by SDS/PAGE.

Isoelectric point determination

Extracts of the stratum corneum from ADe patients were applied directly to IEF. In separate experiments (results not shown), we found that enzyme activity was not inhibited by ampholytes used in IEF. In those extracts, GcSM deacylase activity was observed in a peak at pH 4.2 (pI-4.2 protein fraction, Figure 7B), whereas broad peaks for SMase and GlcCDase activities were found between pH 5.0 and 8.0 (Figures 7C and 7D). Further, the pI of acid ceramidase was 5.7 (Figure 7E) which is in close agreement with a previous study [14]. These findings indicate that the isoelectric properties of GcSM deacylase are completely distinct from those of SMase, GlcCDase and acid ceramidase.

Comparison of isoelectric points of GcSM deacylase in the stratum corneum of ADe patients and healthy control subjects

Since we have previously reported that low levels of GcSM deacylase activity are present in the stratum corneum and epidermis of normal control subjects [9], we next determined

Figure 7 IEF of GcSM deacylase and other sphingolipid-hydrolysis enzymes

Enzymes were isoelectrically focused at 12 W constant power for 4 h at 4 °C. The 20 compartments were collected simultaneously in 20 tubes at the end of the run. pH values were determined in each fraction (A), which were then subjected to assay for GcSM deacylase (B) and SMase (C) using *N*-[palmitoyl-1-¹⁴C]SM, and for GlcCDase (D) and acid ceramidase (E), as described in the Experimental section.

Figure 8 Comparison of isoelectric points of GcSM deacylase in the stratum corneum of ADe patients (A) and control subjects (B)

Approx. 30 strips ($n = 10$) from ADe patients and 90 strips ($n = 30$) from control subjects were used for IEF chromatography. Enzymes were isoelectrically focused at 12 W constant power for 4 h at 4 °C. The 20 compartments were collected simultaneously in 20 tubes at the end of the run, neutralized and assayed using *N*-[palmitoyl-1-¹⁴C]SM as a substrate, as described in the Experimental section. ○, GcSM deacylase activity; □, pH.

Figure 9 Identification of GcSM deacylase activity based upon the release of SPC from [choline-methyl-¹⁴C]SM using radio-TLC

Each incubation mixture contained 50 mM acetate buffer, pH 4.7, 0.1% (v/v) Triton X-100, 20 mM CaCl₂, 10 nmol of [choline-methyl-¹⁴C]SM (52 mCi/mmol) and a pooled fraction from the extract of the stratum corneum from ADe patients. The extracts contained GcSM deacylase that had been partially purified by IEF to exclude SMase, acid ceramidase and GlcCDase, and were further 4–5-fold-concentrated in a total volume of 0.5 ml before enzymic reaction. Incubations were carried out at 37 °C for 15 h, and were stopped by the addition of 0.5 ml of water and 2 ml of chloroform/methanol (2:1, v/v). Mixtures were vortex-mixed and centrifuged for 10 min at 2000 *g*. The upper aqueous phase (1.0 ml) was dried at -80 °C and dissolved in 100 μ l of methanol. The sample solution (80 μ l) was subjected to TLC developed with *n*-butyl alcohol/acetic acid/water (3:1:1, by vol.). Radioactive compounds were analysed using a bio-imaging analyser. SC, stratum corneum; St., standard; PC, phosphocholine.

Figure 10 Detection of [¹⁴C]palmitic acid release from *N*-[palmitoyl-1-¹⁴C]GcCer by radio-TLC

Each reaction mixture contained 10 nmol of *N*-[palmitoyl-1-¹⁴C]GcCer in 50 mM acetate buffer, pH 4.7, 0.1% (v/v) Triton X-100, 20 mM CaCl₂ and a pooled fraction from stratum corneum extract from ADe patients, which contained GcSM deacylase partially purified by IEF to remove SMase, acid ceramidase and GlcCDase. Following incubation for 15 h at 37 °C, the mixtures were mixed with 1.8 ml of chloroform/methanol (2:1, v/v), 0.2 ml of water and 0.06 ml of 0.5 M NaOH, vortex-mixed and centrifuged for 10 min at 2000 *g*. The upper phase was evaporated under nitrogen and re-suspended in 100 μ l of chloroform/methanol (2:1, v/v). Then 50 μ l of the solution was subjected to TLC and developed with chloroform/methanol/acetic acid (190:9:1, by vol.). FFA, free fatty acids, palmitic acid; SC, stratum corneum; St., standard.

Table 1 Comparison of SM, GcCer and palmitoylsphingosine as substrates

Sphingolipids (10 nmol; 45 mCi/mmol each) were incubated at 37 °C for 15 h with partially purified pl-4.2 protein fraction as described in the Experimental section. Following incubation, the reaction products were dried and dissolved in hexane/diethyl ether/acetic acid (50:50:1, by vol.) and applied to a Sep-Pak Plus Silica cartridge. The amount of radioactive palmitic acid released from each sphingolipid was measured with a liquid-scintillation counter.

Sphingolipid	Substrate structure	Hydrolysis (d.p.m.)
SM	Choline phosphate-ceramide	30525.3
GcCer	Glc β 1'-ceramide	7344.8
Palmitoylsphingosine	Palmitoylsphingosine	0

whether GcSM deacylase in normal stratum corneum was similar to that seen in ADe stratum corneum by IEF chromatography. A similar acidic pI value for GcSM deacylase was observed in samples from healthy control subjects, although more highly concentrated extracts of that stratum corneum were needed for assay (Figure 8).

Characterization of GcSM deacylase activity based upon the release of SPC

To further characterize GcSM deacylase activity in extracts of the stratum corneum from ADe patients using [choline-methyl-¹⁴C]SM as another substrate, we used IEF chromatography to purify the pI-4.2 protein fraction which contains GcSM deacylase

activity but not SMase, GlcCDase or acid ceramidase. We then determined whether radiolabelled SPC is generated by the action of GcSM deacylase following incubation of the enzyme with [choline-methyl- ^{14}C]SM. Radio-TLC of reaction products in the aqueous phase demonstrated that GcSM deacylase is a heat-sensitive enzyme that can release radiolabelled SPC following a 15-h incubation with [choline-methyl- ^{14}C]SM (Figure 9), suggesting that stratum corneum of ADe patients contains a distinct activity of GcSM deacylase. The TLC analysis also revealed that there is an increase in the release of SMase product, phosphocholine. One possibility for the release of phosphocholine would be that SPC generated by SM deacylase is broken down non-enzymically to yield phosphocholine during overnight incubation, although we cannot rule out another possibility that there is contamination of unknown enzymes that can accelerate the breakdown of SPC to phosphocholine.

Identification of GCer deacylase activity based upon the release of ^{14}C -labelled palmitic acid from *N*-[palmitoyl-1- ^{14}C]GCer

To determine whether GcSM deacylase has the potential to cleave GCer at the acyl site to yield free fatty acid and GSph, *N*-[palmitoyl-1- ^{14}C]GCer was incubated overnight at 37 °C with the pI-4.2 protein fraction derived from the stratum corneum of ADe patients. Radio-TLC of the reaction products demonstrated that the fraction has a heat-sensitive activity that can release ^{14}C -labelled palmitic acid (Figure 10), suggesting that GcSM deacylase prepared from stratum corneum of ADe patients also possesses GCer deacylase activity.

Hydrolysis of SM, GCer and palmitoylsphingosine by the pI-4.2 protein fraction derived from the stratum corneum of ADe patients

The substrate specificity of the pI-4.2 protein partially purified by IEF was examined using SM, palmitoylsphingosine or GCer as substrates. Although the pI-4.2 protein fraction can definitely liberate ^{14}C -free fatty acid from *N*-[palmitoyl-1- ^{14}C]GCer, the hydrolysis rate for GCer was found to be slower than that for *N*-[palmitoyl-1- ^{14}C]SM. *N*-[1- ^{14}C]Palmitoylsphingosine was not hydrolysed by the enzymic action of the pI-4.2 protein fraction under these assay conditions (Table 1).

DISCUSSION

Few sphingolipid-hydrolysing enzymes have ever been reported in bacterial cells. In cells of *Nocardia* sp. Hirabayashi et al. [1] have demonstrated enzymes which release fatty acids from G_{M3} , G_{M2} , G_{M1} and G_{D1a} , and from neutral glycosphingolipids, but not from sphingolipids such as ceramide, Gal-ceramide, Glc-ceramide or Lac-ceramide. The optimum pH for that enzyme was 5.8 although it is not clear whether it cleaves SM. That enzyme was designated as a glycosphingolipid ceramide deacylase. Another enzyme that hydrolyses the N-acyl linkage of the ceramide framework in some glycosphingolipids, including SM but not ceramide, has been purified from the culture filtrate of the bacterium *Pseudomonas* sp. TK4; it has been tentatively named sphingolipid ceramide N-deacylase [2]. The optimal activity of sphingolipid ceramide N-deacylase from bacteria was at pH 5.0–6.0 and its molecular mass was estimated to be 52 000 Da by SDS/PAGE. Further, this sphingolipid ceramide N-deacylase showed very wide substrate specificity. Other relevant enzymes include ceramidases which hydrolyse the N-acyl linkage of ceramides, and which were first purified from guinea pig epidermis and characterized by our group [14]. One species (termed CDase-I) migrated upon SDS/PAGE as a single band

with an apparent molecular mass of 60 000 Da and its optimal pH for enzyme activity was approx. 7.0–9.0.

We have recently found that there is an unusually high activity of GcSM deacylase in the stratum corneum and the epidermis of ADe patients, and that expression of GcSM deacylase occurs in ADe, but not in contact dermatitis or chronic eczema [9]. In peripheral blood lymphocytes of ADe patients, a very low level of this enzyme activity was found which was similar in concentration to healthy controls. The high expression of GcSM deacylase in the epidermis of ADe could be the mechanism by which there is a ceramide deficiency in the stratum corneum, which is an important aetiological factor in the dry and barrier-disrupted skin found in ADe patients. Since there is no available report on other properties of GcSM deacylase in mammalian tissue, we have now characterized the properties of this hitherto undiscovered epidermal enzyme using *N*-[palmitoyl-1- ^{14}C]SM, [choline-methyl- ^{14}C]SM or [palmitoyl-1- ^{14}C]GCer as substrates after incubation with a partially purified fraction derived from extracts of the stratum corneum of ADe patients.

In an experiment examining the use of [choline-methyl- ^{14}C]SM as a substrate, we found by radio-TLC analysis that radiolabelled SPC was enzymically released following incubation with GcSM deacylase-containing fractions that had been partially purified from extracts of ADe stratum corneum by IEF. Those fractions did not have activities of acid ceramidase, acid SMase or GlcCDase. This finding strongly indicates that GcSM deacylase enzymes exist that are distinct from acid ceramidase or other enzymes in the stratum corneum of ADe patients. In another experiment using *N*-[palmitoyl-1- ^{14}C]SM as a substrate for GcSM deacylase, since there were negligible levels of acid ceramidase, the release of [^{14}C]palmitic acid from *N*-[palmitoyl-1- ^{14}C]SM could be ascribed to the direct deacylation of SM, reflecting GcSM deacylase activity in the stratum corneum of ADe patients.

In studies characterizing the properties of GcSM deacylase, its activity showed a pH dependency, with a peak at pH 5.0. SDS/PAGE analysis revealed that the molecular mass of GcSM deacylase was approx. 40 000 Da, while that of SMase is approx. 100 000 Da. Analytical IEF chromatography demonstrated that the pI values of GcSM deacylase and of SMase are 4.2 and 6.9, respectively, and similar values were obtained with extracts of the stratum corneum from healthy controls. Since GcSM deacylase is similar in enzymic properties to sphingolipid ceramide N-deacylase, it is intriguing to consider that GcSM deacylase may in fact be identical with sphingolipid ceramide N-deacylase. However, in contrast with the molecular mass of sphingolipid ceramide N-deacylase (52 000 Da), the lower molecular mass of GcSM deacylase suggests that GcSM deacylase is distinct from the sphingolipid ceramide N-deacylase purified from bacteria. With regard to enzyme origin, it is possible that hydrolysis of the N-acyl linkage of SM is due to deacylating enzymes from bacteria (such as *Staphylococcus aureus*), which are abundant on the skin surface (i.e. the stratum corneum) of ADe patients. However, GcSM deacylase activity was also demonstrable in germ-free epidermis even after the stratum corneum had been completely removed from the epidermis by excessive tape-strippings before biopsy. Further, very low levels of activity were found in the stratum corneum of patients with impetigo contagiosa [9], which also suggests that GcSM deacylase activity of the stratum corneum from ADe patients is not derived from bacterial contamination. The question of whether ceramidase (N-acylsphingosine deacylase) is involved in the hydrolysis of the N-acyl linkage of SM remains to be elucidated.

In connection with this, at least two different ceramidases have been described in mammalian tissues. In porcine epidermis, the

ceramidase activity is maximal at pH 8.0–9.0 [16]. We have purified two forms of ceramidase from guinea pig epidermis that have alkaline pH optima and the apparent molecular masses of these two ceramidases, as estimated by gel filtration, were 148 000 and 60 000 Da [14]. Thus alkaline ceramidases are enriched in the epidermis compared with the acidic type. Acid ceramidase has been purified and cloned by Li et al. [17]. In our parallel study which assessed acid ceramidase, there was no increase in that activity in extracts of the stratum corneum from ADe patients compared with age-matched normal controls (J. Hara, K. Higuchi, R. Okamoto, M. Kawashima and G. Imokawa, unpublished work). In addition, when GcSM deacylase of the stratum corneum from ADe patients was partially purified by TSK-3000SW column chromatography and assessed at pH 4.7 using [1-¹⁴C]palmitoylsphingosine as a substrate, negligible levels of acid ceramidase activity were found in the partially purified fractions which contained significant levels of GcSM deacylase activity. Further, the pI-4.2 protein fraction, which is rich in GcSM deacylase activity, has no capacity to hydrolyse ceramide even under incubation conditions where SM and GCer can be hydrolysed. Therefore, it is unlikely that acid ceramidase is involved in the activity of GcSM deacylase in the stratum corneum of ADe patients.

Sphingolipid metabolism plays an important role in many cellular events involved in the regulation of cell proliferation and differentiation [3,4]. Above all, ceramide is the basic unit and serves as an intercellular effector molecule [18,19]. Ceramides, which are the immediate product of SM hydrolysis by SMase, play several roles in various signal-transduction pathways. In mammalian epidermis, where ceramides are a major product of terminal differentiation, ceramides comprise more than 50 % of the stratum corneum lipids and are major components involved in water-retention function [20,21] and barrier function [22,23] between stratum corneum cells. Ceramide concentrations are biochemically regulated by the balance of three sphingolipid-hydrolysis enzymes, GlcCDase, SMase and ceramidase. Whereas GlcCDase and alkaline ceramidase were not altered in the stratum corneum of ADe patients compared with age-matched controls [24], Kusuda et al. [25] used an antibody for acid SMase to demonstrate that there was no deficiency of acid SMase in the epidermis of ADe patients. Since the optimal pH for GcSM deacylase activity is approx. 5.0 and SMases in porcine and human epidermis have optimum pHs of 4.5–5.0 [11], it is quite possible that GcSM deacylase and SMase may act competitively upon the same substrate, SM, under acidic conditions such as occurs in lysosomes. As the generation of SPC by GcSM deacylase can competitively inhibit SMase activity [26], it is likely that the expression or activation of GcSM deacylase exacerbates the ceramide deficiency in ADe patients. Further, the finding that partially purified GcSM deacylase (which contains no SMase, acid ceramidase or GlcCDase activity) was able to degrade GCer at the N-acyl linkage of ceramide to yield free fatty acid suggests that GcSM deacylase can compete with GlcCDase for the same substrates, GCer or acylglucosylceramide. This would yield GSph rather than ceramide, which would in turn lead to a deficiency of ceramides, including acylceramides, since acylceramides are generated through deglycosylation of acylglucosylceramides by GlcCDase [27].

Taken together, it is likely that the enzymic characteristics of GcSM deacylase would account for deficiencies in species of ceramides seen in the stratum corneum of ADe patients [8], providing an aetiological basis for dry and barrier-disrupted skin of these ADe patients. Based on IEF studies, GcSM deacylases, which exist in extracts of stratum corneum from ADe patients and control subjects, seem to have similar enzymic properties.

Therefore, up-regulation of GcSM deacylase may be induced in the epidermis of ADe patients, although triggering factors are still unknown.

In summary, this study provides evidence that GcSM deacylase functions in the skin of ADe patients to exert several unique biochemical activities that result in a ceramide deficiency in the stratum corneum. Further characterization of GcSM deacylase should provide valuable insights into the relationship of this enzyme with a large number of important cellular processes, including proliferation, differentiation and signal transduction, as well as into the nature of the metabolic abnormality of SM underlying ADe.

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