Epidermal sphingomyelins are precursors for selected stratum corneum ceramides

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Abstract **Epidermal ceramides (Cer) comprise a heterogeneous family of seven species, including two unique ω-hydroxylated Cer, that are key components of the stratum corneum (SC) intercellular lamellar membranes responsible for the epidermal permeability barrier. Although both glucosylceramide (GlcCer) and the phospho-sphingolipid sphingomyelin (SM) are potential precursors of SC Cer, based on reported chemical structures of epidermal GlcCer and SC Cer, it is assumed that all major subfractions of SC Cer are generated from lamellar body-derived GlcCer. Yet, we and others have shown that SM-derived Cer are required for normal barrier homeostasis. Moreover, two pools of SM, one from plasma membrane, the other from lamellar body-derived contents, are potentially available for Cer production. To clarify the role of SM as a potential precursor of bulk or specific SC Cer, we compared Cer moieties in epidermal SM, Cer generated from epidermal SM by sphingomyelinase treatment, Cer within SC, and Cer that persist in Gaucher SC, where GlcCer cannot generate Cer due to an absence of β-glucocerebrosidase. Using gas chromatography-mass spectrometry, fast atom bombardment-mass spectrometry, and nuclear magnetic resonance for Cer characterization, epidermal SM comprise three major subfractions with distinctive amide-linked (*N*-acyl) fatty acid (FA) compositions: that is, either long-chain FA (SM-1; C_{22-26}), short-chain FA (SM-2; primarily C_{16}), and short-chain α-hydroxy FA (SM-3; C_{16-18}). In contrast, only trace quantities of ω-hydroxy FA were present. For each SM subfraction, the sphingoid base was either sphingosine or sphinganine, but phytosphingosine was not detected. Comparison of these SM with corresponding sphingomyelinase-generated epidermal Cer and SC Cer revealed that the Cer moieties of SM-1 and SM-3 are equivalent to Cer 2 (NS) and Cer 5 (AS), respectively. Moreover, both Cer 2 and Cer 5 occurred in Gaucher SC, whereas other Cer subfractions did not occur. These results indicate that two epidermal SM, that is, SM-1 and SM-3, are important precursors of two corresponding Cer in mammalian SC, that is, Cer 2 and Cer 5, but other Cer species, including the ω-hydroxy Cer species, do not derive from SM.** —Uchida, Y., M. Hara, H. Nishio, E. Sidransky, S. Inoue, F. Otsuka, A. Suzuki, P. M. Elias, W. M. Holleran, and S. Hamanaka. **Epidermal sphingomyelins are precursors for selected stratum corneum ceramides. *J. Lipid Res.* 2000.** 41: **2071–2082.**

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Ceramides (Cer) are biologically active lipid molecules associated with inhibition of cellular proliferation and induction of cellular differentiation and programmed cell death (1, 2). In the epidermis, a large, additional pool of secreted Cer, in combination with cholesterol and free fatty acids (FA), form extracellular lamellar membrane structures within the stratum corneum (SC) that subserve the permeability barrier to water loss, necessary for the survival of mammals in a terrestrial environment (3). These extracellular Cer comprise ~50% of the SC lipids (4, 5), and represent a heterogeneous family of at least seven molecules, with variations in the long-chain sphingoid base structure, as well as in the chain length and α-hydroxylation of constituent amide-linked FA (6–8). In addition, two of these seven Cer species, Cer 1 (EOS)[2] and Cer 4 (EOH), also contain *N*-acylated ω-hydroxy FA (ω-OH Cer) that are unique to epidermis (6, 9, 10). These

Abbreviations: Cer, ceramide; FA, fatty acid; GlcCer, glucosylceramide; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; non-OH, nonhydroxy; α-OH, α-hydroxy; ω-OH, ω-hydroxy; SC, stratum corneum; SM, sphingomyelin.

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[2] Abbreviations for Cer structures are according to Motta et al. (50) and Robson et al. (10): EOS (Cer 1), esterified ω-hydroxy (OH) FA with sphingosine base; NA (Cer 2), non-OH FA, sphingosine; NP (Cer 3), non-OH FA, phytosphingosine; EOH (Cer 4), esterified ω-OH FA, 6-OH-sphingosine; AS (Cer 5), α-OH FA, sphingosine; AP (Cer 6), α-OH FA, phytosphingosine; AH (Cer 7), α-OH FA, 6-hydroxysphingosine.

ω-OH Cer are not only key constituents of the extracellular lamellae, but also major components of the corneocyte lipid envelope (CLE), a covalently attached bilayer, bound to the external aspect of the cornified envelope (11–13a).

Both non-OH FA-containing Cer (non-OH Cer) and α/ω-OH FA-containing Cer can be generated by β-glucocerebrosidase-dependent hydrolysis of epidermal glucosylceramides (GlcCer) (14). Studies of patients with type 2 Gaucher disease, in which severe β-glucocerebrosidase deficiency is a hallmark, as well as of β-glucocerebrosidase-deficient null allele mice, demonstrate that conversion of GlcCer to Cer by β-glucocerebrosidase is required for the formation of the highly organized SC membrane structures essential for normal barrier function (15, 16). Moreover, because of the similarity of the chemical structures of epidermal GlcCer, and the major SC Cer subfractions, it is generally assumed that Cer are generated from their corresponding, glucosylated lamellar body precursors (6, 8, 17–20). Deglucosylation of the entire family of GlcCer occurs after the extrusion of lamellar body contents at the interface of the stratum granulosum (SG) and SC (3, 15).

However, some or all of the SC Cer could also originate from hydrolysis of the epidermal sphingophospholipid sphingomyelin (SM). SM is present in large quantities both in the plasma membrane of SG cells (21, 22), where it is degraded along with other phospholipids during terminal differentiation, and in the lipid-rich epidermal lamellar bodies (23, 24). Moreover, epidermis contains abundant sphingomyelinase, which is localized in the outer epidermis (25–29). Two studies demonstrate that normal barrier function requires sphingomyelinase activity either to generate a pool of SC Cer (30), and/or to remove the hydrophilic phosphorylcholine residue (31). Furthermore, the decreased Cer levels reported in SC of atopic dermatitis patients, which demonstrate a defective barrier (32–34), have been attributed to enhanced Cer degradation within epidermal SM due to increased activity of epidermal SM deacylase (35). Together, these studies suggest that SM-derived Cer are important for permeability barrier homeostasis.

Yet, whether SM is a precursor of bulk or specific Cer subfractions remains unresolved. Human, murine, and porcine epidermal SM contain Cer moieties with either hydroxylated or nonhydroxylated *N*-acyl FA in the SC, with a carbon chain length of C_{14}–C_{28} (36, 37). However, whether epidermal SM species also contain ω-OH-containing amide-linked FA [i.e., *N*-(ω-OH)-acyl-sphingosylphosphorylcholine, or acylsphingomyelin (*N*-ω-*O*-linoleoyl)-acylsphingosyl-phosphorylcholine)] is not known. Moreover, the sphingoid base composition of epidermal SM has not been analyzed or compared with SC Cer. We first analyzed the Cer moieties in epidermal SM isolated from both hairless mouse and human epidermis. The resultant Cer structures generated by sphingomyelinase treatment of epidermal SM were then compared with those of Cer within the SC. Second, we assessed the composition of Cer in Gaucher epidermis. Our results indicate, first, that the Cer moieties of epidermal SM correspond to only two of the seven Cer in SC, suggesting a precursor-product relationship. In contrast, we show that epidermal SM do not contain ω-OH Cer moieties, indicating that these unique species are produced solely from GlcCer precursors.

MATERIALS AND METHODS

Preparation of epidermal fractions and lipid extraction

Hairless male mice (Skh: hr-1) were purchased from Japan SLC (Hamamatsu, Japan). Six- to-20-week-old animals were used in this study. Gaucher mice were generated by targeted disruption of the murine β-glucocerebrosidase gene, as previously described (38). Both neonatal β-glucocerebrosidase null allele mice and normal wild-type littermates were used. Human skin was obtained after informed consent from the amputated tissues of 17- to-86 year-old male and female patients, with no prior history of cutaneous disorders. Human and murine epidermal sheets were obtained by incubation in a phosphate-buffered saline (PBS) solution containing 10 mM ethylenediaminetetraacetic acid or 0.1% trypsin (37°C for 30 min). SC was then isolated from the underlying nucleated layers of the epidermis by incubation with 0.5% trypsin in PBS, as previously described (39). Alternatively, the outer epidermal layers (i.e., rich in SC and stratum granulosum) were obtained after incubation with staphylococcal epidermolytic toxin (Toxin Technology, Sarasota, FL), as previously described (39). The remaining inner epidermal layers (i.e., rich in stratum spinosum and stratum basale) were then separated from the underlying dermis after incubation in PBS at 60°C for 30 sec.

Total lipids were isolated from epidermal or SC samples by the method of Bligh and Dyer (40), as previously described (41).

Thin-layer chromatography

The major epidermal and SC lipid species were separated first by high performance thin-layer chromatography (HPTLC; Merck, Darmstadt, Germany), as previously described (42). SM were separated from other polar lipids with chloroform–methanol–acetic acid–water 50:30:8:4 (v/v) or chloroform–methanol–28% NH_4OH 65:35:4 (v/v). GlcCer and Cer species were separated with chloroform–methanol–water 40:10:1 (v/v) to 2.0 cm, and then to 5.0 cm; chloroform–methanol–acetic acid 94:4:1.5 (v/v) to the top of the plate; and finally, *n*-hexane–diethyl ether–acetic acid 65:35:1 (v/v) to the top of the plate. Individual Cer species were separated by HPTLC with the following solvent sequence: *1*) chloroform to 1.5 cm; *2*) chloroform–methanol–acetone 76:16:8 (v/v) to 1.0 cm; *3*) chloroform–methanol–hexyl acetate–acetone 86:4:1:10 (v/v) to 7.0 cm; *4*) chloroform–methanol–acetone 76:20:4 (v/v) to 2.0 cm; *5*) chloroform–methanol–diethyl ether–ethylacetate–hexyl acetate–acetone 72:4:4:1:4:16 (v/v) to 7.5 cm; *6*) *n*-hexane–diethyl ether–ethyl acetate 80:16:4 (v/v) to the top of the plate (43, 44); and finally, *7*) *n*-hexane–diethyl ether–acetic acid 65:35:1 (v/v) to the top of the plate. Lipids were visualized after treatment with cupric acetate-phosphoric acid, and heating to 160°C for 15 min. The quantity of each lipid separated by TLC was determined by spectrodensitometry (CS-9000; Shimadzu, Kyoto, Japan), as previously described (8, 45).

Fractionation of sphingolipids by HPLC

Total lipid extracts were first applied to an aminopropyl silica gel column (Varian, Harbor City, CA) equilibrated with *n*-hexane. After washing the column with six volumes of *n*-hexane, the fraction containing Cer was eluted with chloroform–isopropanol 2:1 (v/v). The SM-containing phospholipid fraction was then eluted

with chloroform–methanol 1:1 (v/v) and 1:2 (v/v), after washing of the column with diethyl ether containing 2% acetic acid. Fractions were monitored for individual SM or Cer by HPTLC, as described above. The combined SM fractions were further separated by high performance liquid chromatography (HPLC) by gradient elution, using an Iatrobeads column (6RSP-8010; 10.0 × 250 mm; Iatron Laboratories, Tokyo, Japan); that is, chloroform–methanol–water 83:16:0.5 (v/v) to 20:80:8 (v/v) over 210 min. Ceramides were further separated by HPLC by gradient elution, using combined Aquasil SS-1251 (60; 4.6 × 250 mm; Senshu, Tokyo, Japan) and Develosil (100 Diol-5; 4.6 × 250 mm; Nomura Chemical, Seto, Japan) columns with *n*-hexane–isopropanol 97:3 (v/v) to 80:20 (v/v) over 55 min. The lipid content of eluate samples was monitored by HPTLC, as described above, and samples were combined, as appropriate, for further analysis (see below).

Preparation of ceramides from SM by sphingomyelinase treatment

To further determine the Cer structures derived from both human and murine epidermal SM, the combined SM eluate fraction(s), obtained by HPLC separation, were treated with recombinant *Bacillus cereus* sphingomyelinase (Funakoshi, Tokyo, Japan) to remove the phosphorylcholine group, as described previously (46). Briefly, the total phospholipid fraction was suspended into a small volume of diethyl ether–ethanol 98:2 (v/v) and incubated in 0.1 M Tris-HCl buffer (pH 7.2), containing 0.02 mM $CaCl_2$ and sphingomyelinase (4.5 U/ml), for 3 h (30°C). Lipids were then extracted from the reaction mixture, as described above (40), and fractionated by HPTLC, as described above. Using standard amounts of SM (Sigma, St. Louis, MO), we determined that more than 90% of extracted SM was converted to free Cer species under these experimental conditions.

GC-MS analysis of amide-linked FA methyl esters and long-chain bases of ceramides

Fractions of both Cer isolated from SC, and Cer resulting from epidermal SM hydrolysis, were further methanolyzed to their corresponding FA methyl esters with aqueous methanolic HCl (70°C for 20 h) (47). The FA methyl esters were then extracted with *n*-hexane and silaylated for gas chromatography-mass spectrometry (GC-MS); that is, incubated with a mixture of 1,1,1,3,3,3-hexamethyldisilazane and trimethylchlorosilane in pyridine (3:1, v/v; GL Sciences, Tokyo, Japan). The long-chain bases also were recovered from the methanol phase after methanolysis, and likewise were converted to their corresponding *O*-trimethylsilyl derivatives. The derivatives of both FA methyl esters and long-chain bases were then analyzed by GC-MS (HP 5972; Hewlett-Packard, Palo Alto, CA) on a 30 cm × 0.32 μm DB-1 capillary column (J&W Scientific, Folsom, CA). For amide-linked FA analyses, the initial temperature was 100°C (5 min), with 5°C/min increases to 300°C; for long-chain base analyses, the starting temperature was 180°C (5 min), increasing at 5°C intervals per minute to 230°C.

^{1}H NMR spectrometry analysis

^{1}H NMR spectra were recorded in chloroform–DL-methanol-d_4 1:1 (v/v), using a 400-MHz nuclear magnetic resonance (NMR) spectrometer (JEOL-LA 400; JEOL, Tokyo, Japan).

Positive-ion fast atom bombardment-mass spectrometry

SM were further analyzed by fast atom bombardment (FAB)-MS (JMS-MS 303 mass spectrometer; JEOL), using the positive ion-mode with 2-nitrobenzylalcohol as the matrix.

RESULTS

Epidermal SM of hairless mice

We first characterized the epidermal phospholipids, including SM species, obtained from isolated mouse epidermis. Epidermal phospholipids contained three HPTLC bands; that is, with R_f values of 0.19 (SM-3), 0.23 (SM-2), and 0.27 (SM-1) (**Fig. 1A**, lane 2) that correspond to authentic SM (from bovine brain; Fig. 1A, lane 7). To confirm further that these three species were SMs, the whole phospholipid fraction was treated with bacterial sphingomyelinase to selectively remove the phosphorylcholine residue from all SM species, leaving other phospholipid fractions intact. Whereas the three SM components in the phospholipid fraction were hydrolyzed to three Cer species (i.e., Cer-A, Cer-B, and Cer-C; Fig. 1B, lane 3), other phospholipid components [e.g., phosphatidylethanolamine (PE) and phosphatidylcholine (PC)] remained unaltered by sphingomyelinase treatment (Fig. 1A, lane 3). The relative amounts of each fraction, as determined by spectrodensitometry, were as follows: murine SM-1 (mSM-1) and mCer-A: 25.7% and 27.1%, respectively; mSM-2 and mCer-B: 30.1% and 27.8%, respectively; and mSM-3 and mCer-C: 44.2% and 45.1%, respectively. These results show that murine epidermis contains three distinct SM species, with variations in HPTLC mobility due to variations in their respective Cer structures.

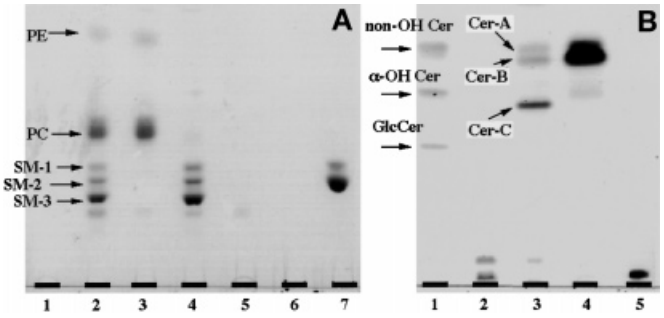

Fig. 1. Murine epidermal SM and Cer fractions. HPTLC separation of phospholipids (A) and ceramides (B) contained in lipid extracts from murine epidermis (see Materials and Methods for details). Lane 1 (A and B): standard GlcCer, α-OH ceramide (α-OH Cer), and non-OH ceramide (non-OH Cer) as indicated by arrows; lane 2 (A and B): epidermal phospholipid fraction, including phosphatidylethanolamine (PE), phosphatidylcholine (PC), and three major sphingomyelin species (i.e., SM-1 through SM-3), as indicated; note that the most polar band (i.e., below SM-3) is unknown, and is unaffected by sphingomyelinase or saponification treatments. Lane 3, lipid fractions remaining/obtained after sphingomyelinase: (A) shows loss of SM species, while PE and PC are unaffected by sphingomyelinase; (B) shows the corresponding resultant Cer species (Cer-A, Cer-B, and Cer-C). Lane 4 (A): saponified phospholipids, showing SM species to be resistant, while both PE and PC species are sensitive to saponification; lane 4 (B): resultant ceramide bands after treatment of standard SM (Sigma) with sphingomyelinase. Lane 5 (A): saponified phospholipid after sphingomyelinase treatment; lane 5 (B): standard SM. Lane 6 (A): lipid extract of the sphingomyelinase enzyme preparation. Lane 7 (A): standard SM.

TABLE 1. Amide-linked fatty acid composition of murine ceramide moieties of corresponding epidermal sphingomyelin

	Percentage of Total		
	Cer-A (mSM-1)*	Cer-B (mSM-2)*	Cer-C (mSM-3)*
16:0	2.74	64.47	2.09
17:0	—	0.37	—
18:2	0.43	0.23	—
18:1	—	—	0.52
18:0	1.05	2.96	—
20:0	2.87	2.70	—
21:0	0.26	0.15	—
22:1	—	0.16	—
22:0	11.76	4.13	—
23:0	1.56	0.44	—
24:1	16.49	4.88	—
24:0	41.02	16.65	6.80
25:0	1.29	0.44	—
26:0	20.17	2.10	—
27:0	—	0.17	—
16:0 (α-OH)	—	—	85.33
18:0 (α-OH)	—	—	5.26
24:0 (α-OH)	—	—	Trace
32:0 (ω-OH)	0.36	—	—

Corresponding SM are denoted by asterisks.

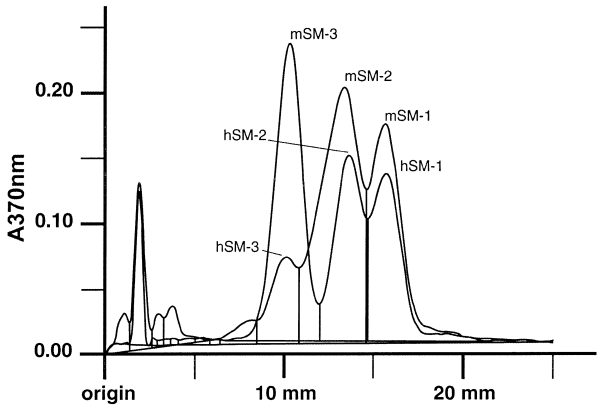

Fig. 2. Densitometric scan of both human (h) and murine (m) epidermal SM. Lipids were separated by HPTLC, visualized with cupric acetate reagent, and scanned by spectrodensitometry (see Materials and Methods for details). The respective species are labeled for both human (hSM) and murine (mSM) sphingomyelin, and demonstrate nearly identical R_f values for the three respective SM species from each.

To further define the Cer structures contained within murine epidermal SM, we next saponified these lipid species, and analyzed the amide-linked FA and long-chain bases by GC-MS. Isolated SMs were converted to their respective Cer species by sphingomyelinase (see Materials and Methods), and again fractionated into mCer-A, mCer-B, and mCer-C, corresponding to mSM-1, -2, and -3, respectively (Fig. 1B, lane 3). The composition of the amide-linked FA revealed that both mCer-A (primarily C_{22} to C_{26}) and mCer-B (primarily C_{16}) contained non-OH FA as their amide-linked acyl residues (**Table 1**). In contrast, a large proportion of the major *N*-acyl species of mCer-C (primarily C_{16}) contained α-OH FA. In all three murine SM species, d_{18}-sphingosine was the predominant long-chain base in the Cer moiety (i.e., 80–90%), while phytosphingosine base was not detected (**Table 2**). Because Cer 1 (acylCer) is the most hydrophobic epidermal Cer (6), acyl-SM, if present, also would be expected to be a highly hydrophobic SM species. However, neither more hydrophobic mSM species, nor significant quantities of ω-OH FA-containing molecules, were detected; that is, only a negligible amount (i.e., <0.4%) of the amide FA of SM-derived Cer-A was ω-hydroxylated (Table 1). These studies show that the Cer moieties in murine epidermal SMs are composed primarily of both non-OH- and α-OH-containing FA moieties that are amide linked to a sphingosine base.

TABLE 2. Long-chain sphingoid base composition of the ceramide moieties of murine epidermal sphingomyelin

	Percentage of Total		
	Cer-A (mSM-1)*	Cer-B (mSM-2)*	Cer-C (mSM-3)*
d17:1	0.95	5.84	11.36
d17:0	7.79	—	—
d18:1	81.40	93.42	86.85
d18:0	9.87	0.74	1.79

Corresponding SM are denoted by asterisks.

Human epidermal SM

We next examined the structures of SM species isolated from normal human epidermis. Similar to murine epidermis, three distinct SM bands, denoted hSM-1, hSM-2, and hSM-3, with R_f values (HPTLC) identical to those of murine sphingomyelins; that is, mSM-1, mSM-2, and mSM-3, respectively, were identified (**Fig. 2**). However, hSM-2, rather than hSM-3, was the predominant component of epidermal hSM (i.e., 45%) (Fig. 2 and **Table 3**).

To further determine the structures of the major human SM species, the three hSM bands were separated by HPLC, and analyzed separately both by ^{1}H NMR (**Fig. 3** and **Table 4**) and by FAB-MS (**Fig. 4**). As in mouse epidermis, hSM-1 and hSM-2 contained only non-OH FA (C_{16-26}), amide linked to sphingosine base. Moreover, the most hydrophobic species, hSM-3, again was an α-OH-containing, but not an ω-OH-containing, SM species. The predicted structures of the major murine and human epidermal SM are presented in **Fig. 5**. These results show that the three major Cer moieties of human epidermal SMs are nearly identical to those in murine epidermis.

TABLE 3. Composition of epidermal sphingomyelin of human and hairless mouse

	Human	Hairless Mouse
	%	
SM-1	34.56	27.28
SM-2	53.85	28.03
SM-3	11.59	44.69

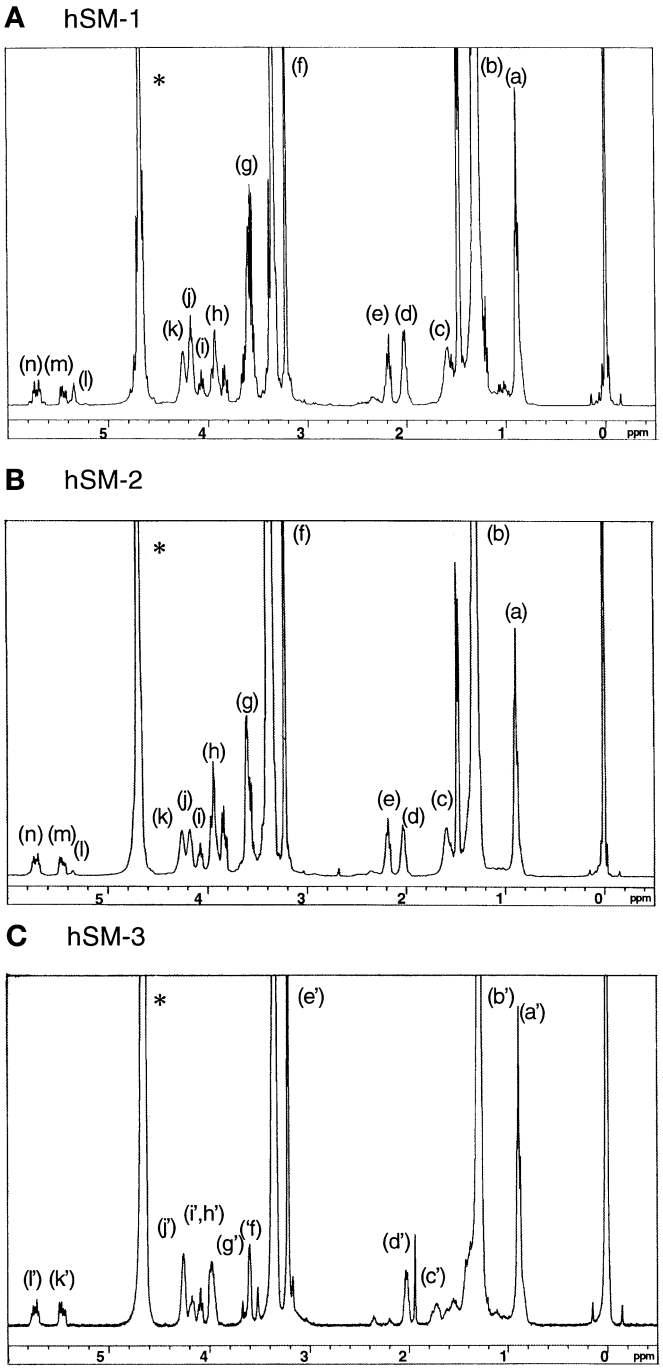

Fig. 3. ^{1}H NMR spectra of human epidermal sphingomyelin. Major ^{1}H NMR chemical shift peaks are shown and labeled (i.e., a–n for hSM-1 and hSM-2; and a′ – l′ for hSM-3). Details for each corresponding peak are summarized in Table 4, using the identical nomenclature. (A) ^{1}H NMR spectrum for hSM-1; (B) ^{1}H NMR spectrum for hSM-2; (C) ^{1}H NMR spectrum for hSM-3. Peak labeled with an asterisk (*) corresponds to the chemical shift for the solvent (CD_3OH).

Identity of epidermal SM and SC ceramides

A comparison of the Cer species in the SC with those released by sphingomyelinase from murine epidermal SM revealed that mCer-A and mCer-C (i.e., from murine SM-1 and SM-3) displayed the same HPTLC mobilities as mCer 2 and mCer 5, respectively, isolated from murine SC (**Fig. 6**). In contrast, no band corresponding to mCer-B (i.e., from epidermal mSM-2) was detected in the Cer isolated from murine SC. To further confirm that SM-1 and SM-3 are hydrolyzed to Cer 2 and Cer 5, respectively, the FA composition of both the amide-linked FA and the long-chain bases of these two mCer species were compared with these moieties in their respective epidermal mSMs (**Tables 5** and **6**). mCer 2 contained long chain ($\geq C_{18}$), non-OH FA(s) amide linked with either d_{18} sphingosine or d_{18} sphinganine (Table 6), while α-OH palmitic acid ($C_{16:0}$) was the major amide-linked FA of Cer 5 (Tables 5 and 6). Thus, the FA and base composition of mCer 2 and 5 are similar to the FA composition of mSM-1 and mSM-3 (cf. Tables 1 and 2), respectively, providing futher support for a precursor-product relationship.

Distinct distribution of SM-1, SM-2, and SM-3 generation within murine epidermis

As described above, only two of the three epidermal mSM species are reflected in corresponding Cer species in murine SC. During keratinocyte differentiation both GlcCer production (48) and heterogeneity increase; that is, at least seven distinct species are present in the mammalian SC (6, 7, 10, 49, 50). Therefore, we next examined whether the distinctive fate of SM-2 versus SM-1 and -3 is linked to epidermal differentiation. As shown in **Table 7**, the percentage of mSM-3, versus other sphingolipid species, increases in the outer (i.e., SG/SC) more than in the inner (i.e., stratum basale and stratum spinosum) epidermal fraction, consistent with increased production of SM-3 during keratinocyte differentiation. In contrast, the quantities of both SM-1 and SM-2, which are commonly encountered in the plasma membranes of most cells (51, 52), did not change with differentiation. As noted above, only SM-1- and SM-3-derived Cer are retained as SC Cer.

Cer species formed in the SC of β-glucocerebrosidase-deficient epidermis

GlcCer are considered the predominant precursor pool for the generation of SC Cer. To determine further whether GlcCer and SM are precursors of specific Cer species in the SC, we next examined the profile of epidermal Cer in Gaucher mouse epidermis, which accumulates GlcCer species in the SC because of a deficiency of β-glucocerebrosidase (14, 15). As described previously (15, 53, 54), GlcCer, including acylGlcCer, again increase in Gaucher mouse SC, while each Cer species, excluding Cer 5, is decreased (**Fig. 7**, lane 2). Pertinently, the major Cer components present in Gaucher mouse epidermis were identical to those prepared by sphingomyelinase treatment of both Gaucher and normal mouse epidermal phospholipids (Fig. 7, lane 2 vs. lanes 3 and 4). Again, two of these species corresponded with Cer 2 and Cer 5 of normal SC (Fig. 7, lane 5). Because Gaucher mouse epidermis cannot generate Cer from GlcCer, these studies provide further evidence that Cer 2 and 5 in the SC are generated from SM precursors, by sphingomyelinase-dependent hydrolysis of SM-1 and SM-3, respectively.

TABLE 4. Summary of ^{1}H NMR analysis of human epidermal sphingomyelin

Peak	Chemical Shift	Coupling Constant J	Multiplicity	Assigned Structures (Corresponding Proton Underlined)
	ppm	*Hz*		
hSM-1 and hSM-2				
a	0.89	6.1	3	(-CH_3) × 2
	1.27			
	1.54–1.66			
b			l	-$(CH_2)_x$-
c			m	-CO-CH_2-C$\underline{H}_2$-
d	1.97–2.07		m	-CH(OH)-CH=CH-C$\underline{H}_2$-
e	2.18	7.5	3	-NH-CO-C$\underline{H}_2$-
f	3.22		l	($C\underline{H}_3$)$_3$N-
g	3.58	7.3	3	($C\underline{H}_3$)$_3$N-C$\underline{H}_2$-CH_2-
h	3.90–3.98		m	-O-C$\underline{H}_2$-C$\underline{H}$(NH)-, b position
i	4.04–4.12	8.4 and 7.5	2 × 2	-CH-C$\underline{H}$ (OH)-CH=CH-
j	4.12–4.22		m	-O-C$\underline{H}_2$-CH(NH-)-, a-position
k	4.26			($C\underline{H}_3$)$_3$N-CH_2-C$\underline{H}_2$-
l	5.32–5.40		m	-C$\underline{H}$=C$\underline{H}$-
m	5.45	15.3 and 7.5	2 × 2	-C$\underline{H}$=CH-
n	5.72	15.3 and 7.3	2 × 3	-CH=C$\underline{H}$-
hSM-3				
a	0.89	6.9	3	(-CH_3) × 2
b	1.27		l	-$(CH_2)_x$-
c	1.70–1.79		m	-CO-CH(OH)-C$\underline{H}_2$-
d	2.00–2.07		m	-CH(OH)-CH=CH-C$\underline{H}_2$-
e	3.22		l	($C\underline{H}_3$)$_3$N-
f	3.58–3.64		3	(CH_3)$_3$N-C$\underline{H}_2$-CH_2-
g	3.92–4.02		m	-O-C$\underline{H}_2$-C$\underline{H}$(NH-)-, b-position -NH-CO-C$\underline{H}$(OC)-
h	4.07–4.12		2 × 2	-CH-C$\underline{H}$(OH)-CH=CH-
i	4.14–4.21		m	-O-C$\underline{H}_2$-CH(NH-)-, a-position
j	4.26			(CH_3)$_3$N-CH_2-C$\underline{H}_2$-
k	5.46	15.5 and 7.3	2 × 2	-C$\underline{H}$=CH-
l	5.73	15.5 and 6.5	2 × 3	-CH=C$\underline{H}$-

DISCUSSION

Cer are key components of the extracellular lamellar membrane structures of the SC, which provide the epidermal permeability barrier (3). Not only their bulk content, but also the molecular heterogeneity of these Cer (i.e., representing at least seven subfractions) (6, 7, 10, 49), appear to be critical for the formation of membrane bilayer structures, which subserve the barrier (55). Although these Cer appear to be generated primarily from GlcCer precursors by the activity of β-glucocerebrosidase (14, 15), the contribution of one or more epidermal SM as precursors is not known. Studies from this (30) and other laboratories (31) demonstrate a requirement for bulk Cer generation from SM for normal permeability barrier homeostasis. However, the relative contribution of GlcCer and SMs to both total and individual SC Cer species remains unresolved. Specifically, which subfraction(s) of Cer is (are) generated from sphingomyelinase-mediated hydrolysis during terminal differentiation is not known. To address this question, we compared the chemical structures of Cer moieties in epidermal SMs (36, 37), isolated both from hairless mouse and human epidermis, with individual Cer species in SC, and with Cer generated by sphingomyelinase treatment of epidermal SM. In both human and hairless murine epidermis, SMs comprise three major subfractions, containing long-chain FA (SM-1), short-chain FA (SM-2), and short-chain α-OH FA (SM-3) in amide linkage(s) with either a sphingosine or sphinganine base. These results are consistent with a previous study of Cer in whole SENCAR mouse epidermis, in which a similar SM distribution was reported (37). A further comparison of the Cer moieties in individual epidermal SMs with SC Cer species described here revealed that two of the SM Cer moieties correlated structurally with Cer 2 (NS)[1] and Cer 5 (AS) [using the current nomenclature (10, 49)]. These results suggest that only two of the three epidermal SM, that is, SM-1 and SM-3, are likely precursors of SC Cer; that is, Cer 2 and 5. The third epidermal SM, SM-2, although present in all epidermal nucleated cell layers, does not appear to generate Cer species in either mouse or human SC.

To further confirm the role of specific SM as precursors of SC Cer species, we also assessed Cer profiles in β-glucocerebrosidase-deficient Gaucher mice (15, 38). Epidermal Cer in the Gaucher mouse again matched the pattern of the Cer moieties generated by sphingomyelinase hydrolysis of SM in normal mouse epidermis. This finding provides further evidence of the generation of non-OH Cer (i.e., Cer 2) and α-OH palmitic acid-containing Cer (Cer 5), from SM (**Fig. 8**). Similar Cer species have been noted in the epidermis of another Gaucher mouse model (54), and in the epidermis of the prosaposin (precursor of sphingolipid activator protein)-deficient mouse, in which the enzymatic hydrolysis of sphingolipids, particularly GlcCer,

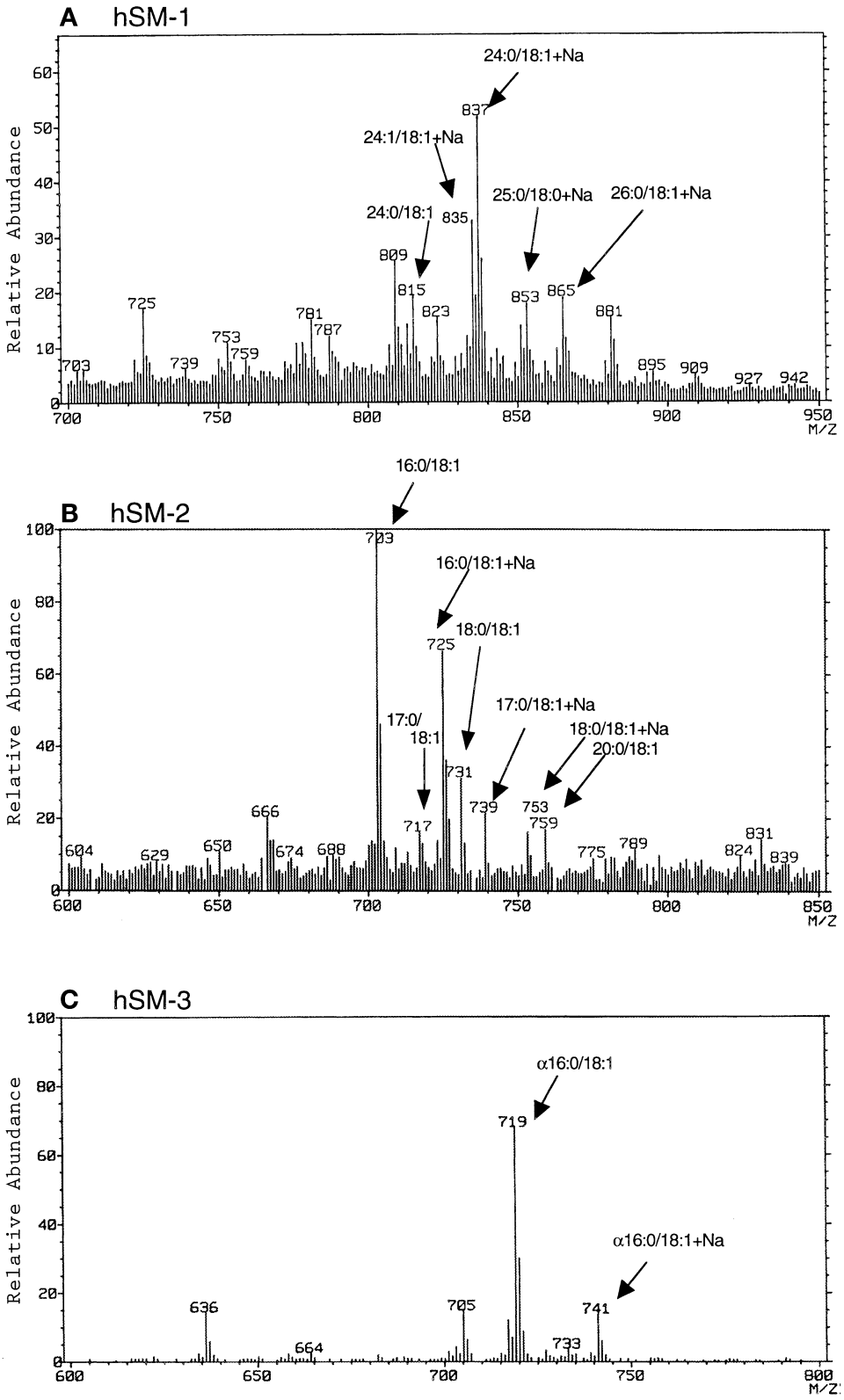

Fig. 4. Positive-ion FAB mass spectra of human sphingomyelin (hSM) species. Mass specta for each of the three hSM species are shown. Individual ions with the largest relative abundance, with their respective *N*-acyl FA and sphingoid base composition (i.e., 24:0/18:1 for *N*-tetracosanoic acid with sphingosylphosphorylcholine, respectively), are delineated (arrows). (A) hSM-1; (B) hSM-2; (C) hSM-3.

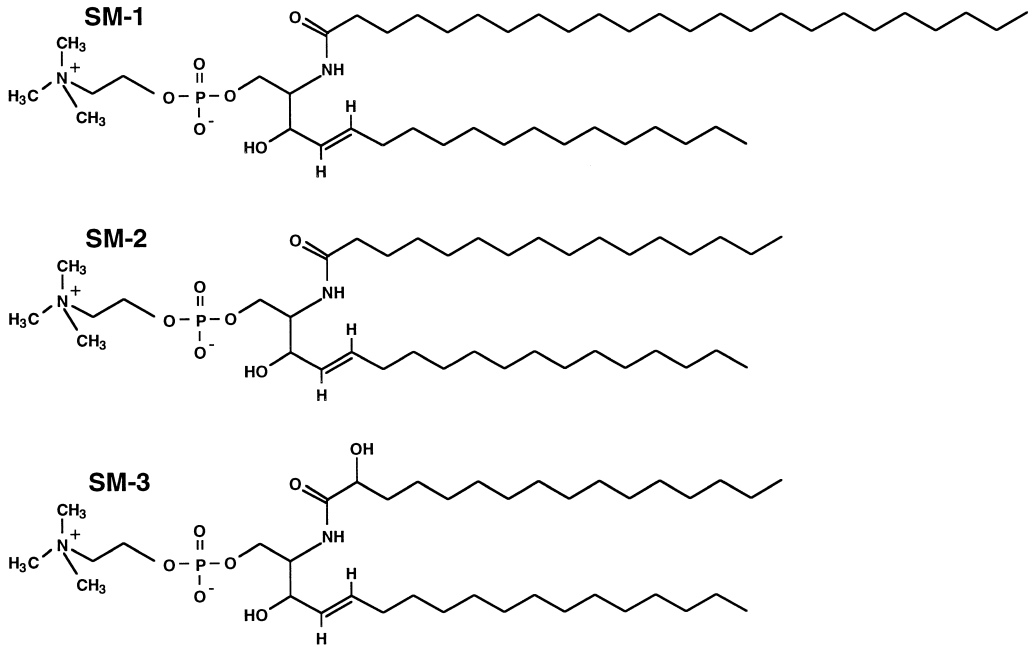

Fig. 5. Deduced structures of most abundant SM species from both murine and human epidermis. Shown are SM-1 (24:0/18:1), SM-2 (16:0/18:1), and SM-3 (α-OH 16:0/18:1) species.

is decreased (56). In fact, the content of both the α-OH palmitic acid-containing Cer (i.e., corresponding to Cer 5) and epidermal SM actually increase in this latter model, suggesting that SM represents an obligate precursor and/or is increased as a possible compensatory response by this pathway. However, the fact that Cer-2 content decreases in both Gaucher and prosaposin-deficient mice suggests that not only SM, but also GlcCer, serve as precursors for this SC Cer species. Together, these results are consistent with SM-3 as a significant precursor for Cer 5, while either or both SM-1 and GlcCer contribute to the Cer 2 pool in normal SC (Fig. 8).

SM is a common membrane component of all mammalian cells, where it is concentrated primarily in the outer leaflet of the plasma membrane (57, 58). SMs generally contain non-OH FA with a carbon chain length of C_{16-24} (21, 37, 51), with palmitic acid (C_{16}) as the predominant *N*-acyl FA component. Conversely, although SM contain-

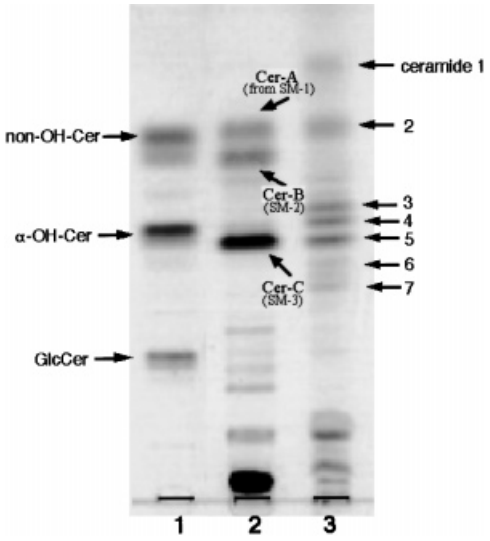

Fig. 6. Comparison of ceramides prepared from murine epidermal sphingomyelin with complete SC Cer species. Representative HPTLC of standard GlcCer, α-OH ceramide (α-OH Cer), and non-OH ceramide (non-OH Cer) is shown in lane 1. Ceramides obtained after treatment of murine epidermal phospholipid-enriched, Cer-free fraction with sphingomyelinase (see Materials and Methods) (lane 2); Cer from lipid extracts of hairless mouse SC (lane 3), showing the spectrum of Cer species present in vivo, including Cer-1 through Cer-7. Note the absence of Cer-B band; that is, corresponding to SM-2 in SC Cer fraction (lane 3).

TABLE 5. Composition of amide-linked fatty acid in ceramides 2 and 5 in murine stratum corneum

	Cer 2	Cer 5
	%	
16:0	1.74	2.81
17:0	0.12	—
18:0	0.59	5.36
20:0	0.66	—
21:0	0.24	—
23:0	1.68	—
24:0	38.11	—
25:0	7.29	—
26:0	47.33	—
27:0	0.57	—
28:0	1.53	—
30:0	0.11	—
14:0 (α-OH)	—	Trace
15:0 (α-OH)	—	Trace
16:0 (α-OH)	—	98.49
17:0 (α-OH)	—	1.51

TABLE 6. Long-chain sphingoid base composition of ceramide generated from human epidermal sphingomyelin

	Percentage of Total	
	Cer 2	Cer 5
d17:1	4.03	41.38
Unknown (d18:2?)	1.29	8.11
d18:1	58.89	50.54
d18:0	35.79	9.98

ing α-OH FA have been detected in bovine stomach (59), guinea pig harderian gland (60), mammalian testes/spermatozoa (61), and murine epidermis (37), this relatively hydrophilic SM is not widely distributed in mammalian tissues. The present study now demonstrates the presence of significant quantities of C_{16} α-OH *N*-acyl FA-containing SM in both hairless mouse and human epidermis. In contrast to epidermal SM species, both epidermal GlcCer and SC Cer also contain very long-chain α-OH *N*-acyl FA ($C_{\geqslant 24}$) (19, 62), further supporting divergent metabolic pathways for the generation of specific Cer from epidermal SM versus GlcCer precursors.

It is interesting to speculate that specific Cer species, destined for different functions within the epidermis, may be directed toward either SM or GlcCer precursor pools. Whereas α-hydroxylation of *N*-acyl FA in brain galactosylceramide(s) occurs prior to Cer synthesis (63), α-hydroxylation of (mannosylinositolphosphoryl)-Cer in *Saccharomyces cerevisiae* occurs after Cer synthesis (64). Thus, if α-OH Cer are synthesized after α-hydroxylation of the FA has occurred in mammalian epidermis, then the substrate preferences for SM synthase and/or GlcCer synthase could be restricted solely by selection for short- versus long-chain amide-linked FAs, respectively. Divergence of the pathways of SM and GlcCer synthesis is also implicit from their differing subcellular sites of synthesis: Cer glucosylation occurs on the cytosolic aspect of the ER and proximal Golgi, while SM synthesis is localized to the lumenal side (65), while trafficking of the precursor Cer for GlcCer versus SM appears distinct (66). Together, these studies suggest that the transport pathways for de novo-synthesized GlcCer versus SM may be dependent on their molecular structures; that is, the pathway for SM synthesis may select for short-chain, α-OH Cer with a sphingosine base, while that for GlcCer may select for long-chain, α-OH-containing or ω-OH-containing Cer, independent of base composition. Further studies will be required to clarify the structural determinants for de novo generation of SM and GlcCer within mammalian epidermis.

TABLE 7. Distribution of epidermal sphingomyelin in hairless mouse epidermis

	Percentage of Total		
	Whole Epidermis	Inner Epidermis	Outer Epidermis
mSM-1	25.7	27.2	13.7
mSM-2	30.1	33.3	16.0
mSM-3	44.2	39.5	70.3

Inner epidermis, rich in stratum basale and stratum spinosum; outer epidermis, rich in stratum granulosum and stratum corneum.

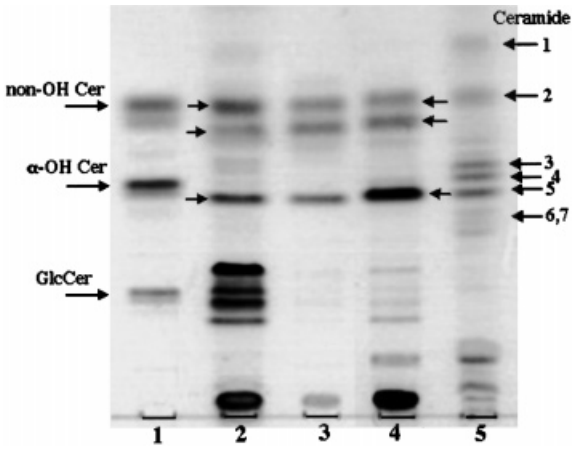

Fig. 7. Ceramides generated by sphingomyelinase treatment of β-glucocerebrosidase-deficient (Gaucher) epidermal lipid extracts. Lane 2 contains Gaucher mouse epidermal lipid. Lanes 3 and 4 contains Cer obtained by sphingomyelinase treatment of either Gaucher (lane 3) or hairless (lane 4) mouse epidermal phospholipid-enriched, Cer-free fraction. Lane 1 contains standard GlcCer, α-OH Cer, and non-OH Cer, while lane 5 shows the spectrum of Cer species obtained from hairless mouse SC.

We also demonstrated that the ratio of individual SM to total SM in the outer versus inner epidermal layers is altered, and that production of SM-3 is enhanced during keratinocyte differentiation. The likely source of this SM-3 precursor pool is the contents of epidermal lamellar bodies, which are known to contain not only GlcCer, but also SM (23, 24). Lamellar bodies first appear in the spinous cell layer, and increase in number from the spinous layer through the SG. The contents of lamellar bodies are then extruded at the interface of the SG and SC, eventually forming the extracellular lamellar membrane system. Thus, it appears likely that SM-3 (but not SM-2) may be stored in lamellar bodies, rather than in plasma membranes, and that it is delivered to the extracellular spaces of SC concurrent with lamellar body secretion. Conversely, Cer 2 may derive both from lamellar body GlcCer precursors, and/or plasma membrane SM-1. In either case, the results of Jensen and colleagues (31) demonstrate a critical role for SM-derived Cer-2 in murine permeability barrier function, as Cer 2 may account for as much as 40% to 50% of murine SC Cer. Whereas the fate of SM-2 remains unresolved, these shorter *N*-acyl FA-containing Cer (i.e., $C_{16:0}$) do not contribute significantly to SC Cer composition. Perhaps these short-chain Cer species are involved in as yet unresolved signaling pathways, or they could be degraded during the latter stages of epidermal differentiation.

Although the functional role of each SC Cer species has not yet been resolved, Cer species appear to be critical for SC membrane structure and function. For example, only ω-OH-containing Cer species are covalently attached to cornified envelope proteins, underscoring their putative

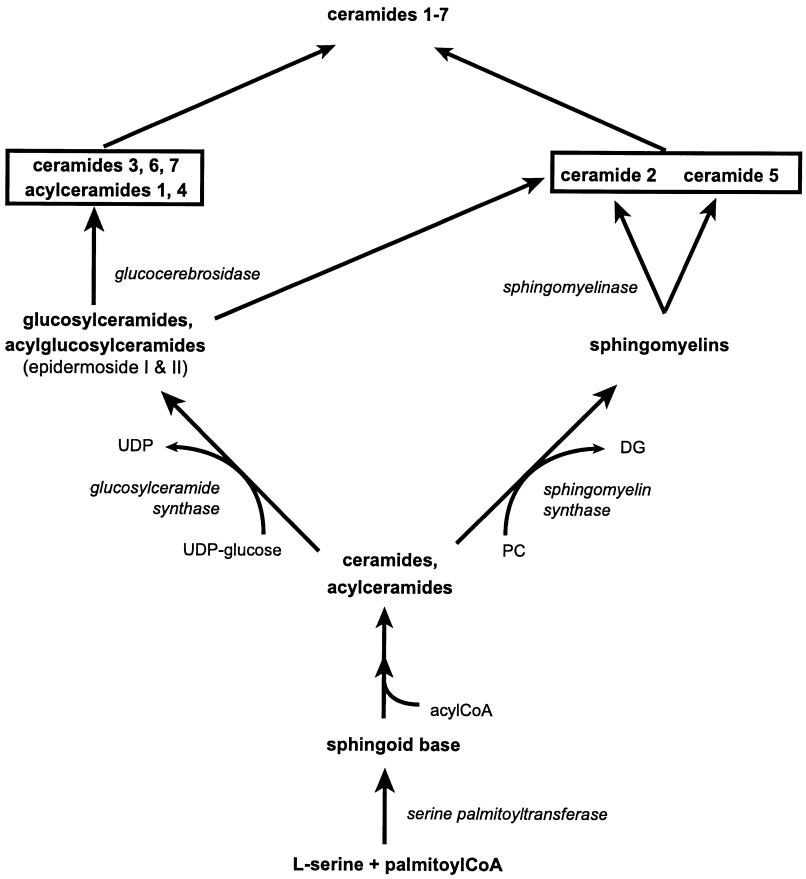

Fig. 8. Proposed pathway for production of SC ceramides. Scheme for the production of the seven SC Cer species (Cer 1–Cer 7) distinguishes between GlcCer- and sphingomyelin-dependent pathways, and is based on the results presented in this article. Abbreviations: PC, phosphatidylcholine; DG, diacylglycerol; UDP, uridine diphosphate.

role in lamellar membrane organization. In addition, one of the ω-acylated Cer species (Cer 1) has been shown to be an essential component in the formation of the broad-narrow-broad sequence of the lamellar membrane unit structure (67). The present study supports the hypothesis that the majority of SC Cer 1 is derived from acylGlcCer hydrolysis, rather than from SM precursors (15, 68, 69). This finding also may explain the fact that significant permeability barrier dysfunction and abnormal SC lamellar structures occur in Gaucher type 2 patients (16), while in contrast, patients with Niemann-Pick disease display lesser barrier abnormalities (30). Because critical SC Cer, such as Cer 1, appear only to be generated from GlcCer precursors, alteration of this pathway may have more severe cutaneous consequences. Moreover, the α-OH-containing Cer (Cer 5), derived all, or at least in part, from the SM-3 precursor pool, is predicted to be localized to the narrow, electron-dense region in the lamellar phase (67), suggesting a role in membrane organization. Interestingly, although the Cer composition of human and mouse SC lipids exhibits certain differences [e.g., Cer 5 comprises approximately 20% of the total Cer in the hairless mouse SC, but only 5.7% of the total Cer in human SC (data not shown)], their lamellar membrane structures are strikingly similar. In addition, because Cer 5 can be partially replaced by cholesterol in lamellar structures in vitro (70), this Cer species may lack an obligate role in cutaneous function. Finally, as SM-2 consists primarily of a sphingosine base with a non-OH $C_{16:0}$ *N*-acyl substituent, the structure resembles "normal" SM observed in the majority of mammalian cells, often localized to the plasma membrane (21, 37, 51, 57, 58). We would propose that this SM species is involved with cell membrane functions that are not unique to the epidermis. For example, it is possible that SM-2 is involved in signal transduction events induced by the activation of SMase activity. In this regard, one study demonstrated that $C_{16:0}$ Cer is specifically increased during Fas, or radiation-induced apoptosis in Jurkat cells (71). Conversely, Jensen and colleagues (31) found no change in C_{16} Cer during the early phase of epidermal barrier repair. Because limited content of shorter chain, saturated, non-α-OH *N*-acyl Cer species (i.e., $C_{16:0}$) is present in mammalian SC [(6–10, 49, 50), and the present study], only the longer chain, non-OH *N*-acyl-containing SM species are utilized for generating SC Cer species that appear to be important for permeability barrier function. Together, these results suggest that certain Cer species, including those derived from SM hydrolysis, have specific structural roles within mammalian SC.

In summary, both SM and GlcCer play roles in the generation of Cer that are critical for epidermal barrier function. The present results indicate, first, that two epidermal SM (i.e., SM-1 and SM-3) are important precursors of Cer 2 and Cer 5, respectively, in the SC. However, the precise contribution of SM versus GlcCer to the content of these two Cer species in normal and diseased SC will require further investigation. Second, our results demonstrate that ω-OH Cer species in mammalian SC do not derive

from SM, indicating that these unique species derive solely from GlcCer precursors.

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