Human Epidermal Glucosylceramides are Major Precursors of Stratum Corneum Ceramides

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Ceramides are the major component of the stratum corneum, accounting for 30%–40% of stratum corneum lipids by weight, and are composed of at least seven molecular groups (designated ceramides 1–7). Stratum corneum ceramides, together with cholesterol and fatty acids, form extracellular lamellae that are responsible for the epidermal permeability barrier. Previous studies indicated that β-glucocerebrosidase- and sphingomyelinase-dependent ceramide production from glucosylceramides and sphingomyelins, respectively, is important for epidermal permeability barrier homeostasis. A recent study indicated that sphingomyelins are precursors of two stratum corneum ceramide molecular groups (ceramides 2 and 5). In this study, we have examined the role of glucosylceramides in the generation of each of the seven stratum corneum ceramide molecular groups. First, the structures of various glucosylceramide species in human epidermis were determined by gas chromatography–mass spectrometry, fast atom bombardment–mass spectrometry, and nuclear magnetic resonance. The results indicate that total epidermal glucosylceramides are composed of six distinct molecular groups, glucosylceramides 1–6. Glucosylceramide 1 contains sphingenine and nonhydroxy fatty acids, glucosylceramide 2, phytosphingosine and nonhydroxy fatty acids, glucosylceramide 3, phytosphingosine with one double bond and nonhydroxy fatty acids, glucosylceramide 4, sphingenine and α-hydroxy fatty acids, glucosylceramide 5, phytosphingosine and α-hydroxy fatty acids, and glucosylceramide 6, phytosphingosine with one double bond and α-hydroxy fatty acids. The nonhydroxy fatty acids typically have 16–24-carbon-length chains, whereas α-hydroxy fatty acids are limited to 24-, 25-, and 26-carbon chains. The sphingosine bases are C18 or C20 chains. Next, acylglucosylceramides and glucosylceramides were treated with β-glucocerebrosidase and the ceramides released were compared with stratum corneum ceramides. Ceramide moieties of acylglucosylceramides and glucosylceramides 1, 2, 4–6 correspond to stratum corneum ceramides 1–7. These results, together with those of our previous reports characterizing epidermal sphingomyelins, indicate that all ceramide species, including ω-hydroxy fatty-acid-containing ceramides, are derived from glucosylceramides, and fractions of ceramides 2 and 5 are from sphingomyelins. Furthermore, structural analysis of glucosylceramides revealed that human epidermal glycosphingolipids display a unique lipid profile that is rich in very long chain hydroxylated (α- and ω-hydroxy) fatty acids and phytosphingosine.

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Abbreviations: Cer, ceramide; CLE, corneocyte lipid envelope; FA, fatty acid; GlcCer, glucosylceramide; β-GlcCer'ase, β-glucocerebrosidase; GSL, glycosphingolipid; α-OH, α-hydroxy; ω-OH, ω-hydroxy; SC, stratum corneum; SM, sphingomyelin. Abbreviations for Cer structures are according to Wertz and Downing (1983c) and Robson *et al* (1994). The shortened designations h, d, and t used in the paper indicate hydroxy, dihydroxy, and trihydroxy, respectively. The numbers before and after the colon delineate carbon chain length and the number of double bonds, respectively.

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Glucosylceramide (GlcCer) is a simple glycosphingolipid (GSL) composed of one mole each of glucose and ceramide (Cer). Cer is composed of a sphingosine base and an amide-linked fatty acid (FA). GlcCer is distributed widely in mammalian tissues and is the major precursor for more complex GSLs. In the epidermis, however, GlcCers were reported to be the only GSL (Gray and Yardley, 1975), until unique acylated GlcCers (acylglucosylceramides, acylGlcCers) were found later in pig and human epidermis (Gray *et al*, 1978). The structures of GlcCers in pig and fetal rat epidermis (Wertz and Downing, 1983b; Wertz *et al*, 1984) and cultured human keratinocytes, as well as acylGlcCers in human (Hamanaka *et al*, 1989, 1990, 1993), pig (Wertz and Downing 1983a; Abraham *et al*, 1985; Bowser *et al*, 1985), and guinea pig (Uchida *et al*, 1988), have been determined, but the structures of

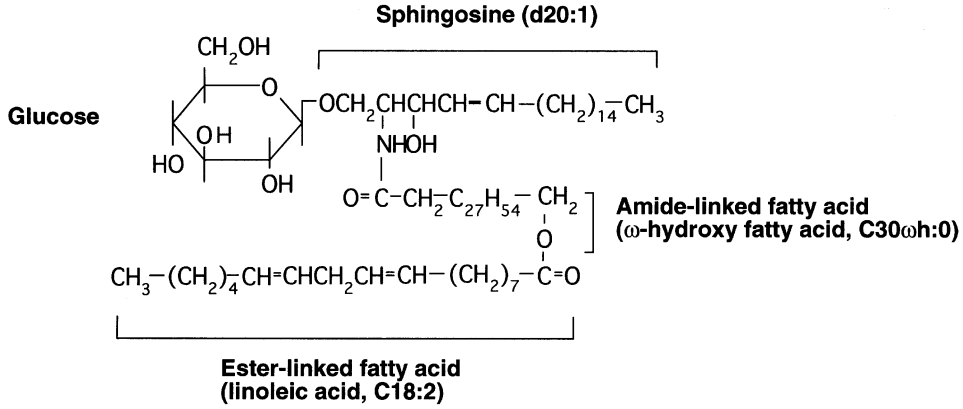

Figure 1. Structure of epidermoside I. Epidermoside I is composed of glucose, a sphingosine base, an amide-linked ω-hydroxy FA, and an ester-linked FA (linoleic acid). d20:1 denotes a 20-carbon dihydroxysphingosine with one double bond. C30 ω-h:0 denotes a 30-carbon ω-hydroxy fatty acid without a double bond.

GlcCers *in vivo* in human epidermis have not yet been completed. These prior studies indicate that the epidermal GlcCers display molecular heterogeneity. In particular, acylGlcCers, i.e., the ω-OH GlcCer carrying an ester-linked FA, have been reported only in the epidermis and not in other tissues, including the dermis. We found that acylGlcCers isolated from human epidermis predominantly carry linoleic acid as their ester-linked FA (Hamanaka *et al*, 1989), whereas pig (Wertz and Downing, 1983a; Bowser *et al*, 1985), guinea pig (Uchida *et al*, 1988), and cultured human keratinocytes (Hamanaka *et al*, 1990, 1993) contain other FAs, as well as linoleic acid. We have named epidermis-specific, linoleic-acid-containing ω-OH GlcCers as epidermosides (**Fig 1**) (Hamanaka *et al*, 1989), because these essential-fatty-acid-containing lipids are involved in the physiologic maintenance of the epidermal permeability barrier (Hansen, 1986). GlcCer and acylGlcCer expression (Ponec *et al*, 1988), GlcCer synthase activity (Sando *et al*, 1996; Chujor *et al*, 1998; Watanabe *et al*, 1998), and GlcCer structural heterogeneity (Ponec *et al*, 1988) increase with keratinocyte differentiation. GlcCers and acylGlcCers are thought to be located primarily in lamellar bodies (Wertz and Downing, 1982). The lamellar body membrane fuses with the plasma membrane on the apical surface of granular cells, at which point its contents are extruded into the interface between the stratum granulosum and the stratum corneum (SC) (Elias and Menon, 1991). Coincident with the stratum granulosum–SC transition, the glucose moiety of acylGlcCers and GlcCers is hydrolyzed by β-glucocerebrosidase (β-GlcCer'ase), resulting in the production of Cers in the SC (Holleran *et al*, 1993; 1994). Cers play a critical role in the formation of the permeability barrier, together with cholesterol and FAs in the SC.

SC Cers are composed of at least seven molecular groups (Wertz and Downing, 1983a; Robson *et al*, 1994; Vietzke *et al*, 1999; 2001). Deficiency of β-GlcCer'ase or inhibition of its activity in the epidermis decreases the amount of SC Cers and creates an incompetent epidermal permeability barrier (Holleran *et al*, 1993, 1994), suggesting that enzymatic hydrolysis of GlcCer is required for epidermal barrier formation. Sphingomyelinase-mediated hydrolysis of sphingomyelin (SM) is also involved in epidermal permeability barrier homeostasis (Jensen *et al*, 1999; Schmuth *et al*, 2000). These prior studies indicate that both GlcCers and SMs are important precursors for SC Cers. Furthermore, previous studies demonstrated that not only the total amount of SC Cers but also the distribution of each Cer molecular species is important for the formation of lamellar bilayer structures in the SC (Bouwstra *et al*, 1998). Indeed, alteration of Cer composition has been reported in several skin diseases that display epidermal barrier abnormalities (Imokawa *et al*, 1991; Motta *et al*, 1993; Paige *et al*, 1994; Bleck *et al*, 1999; Schreiner *et al*, 2000). Little is known, however, about the mechanism that is responsible for these alterations. Thus, characterization of the specific synthetic pathways for each Cer species is important to elucidate the regulation of SC Cer synthesis. Recently, we analyzed the structures of epidermal SMs and demonstrated that SC Cer 2 and 5 were derived in part from these precursors (Uchida *et al*, 2000). The role of GlcCers in SC Cer generation has not been explored, however.

The esterified, but not the nonesterified, form of ω-OH Cer (acylCer; Cer 1) has been observed in human and pig SC, where it is an important component of the extracellular lamellar bilayer structures (Wertz and Downing, 1985; Robson *et al*, 1994; Bouwstra *et al*, 1998). In addition, ω-OH Cer is covalently bound to corneocyte envelope proteins and forms the corneocyte lipid envelope (CLE) (Wertz and Downing, 1987; Wertz *et al*, 1989; Marekov and Steinert, 1998; Doering *et al*, 1999a; Elias *et al*, 2000). The CLE has been suggested to be a scaffold upon which extracellular lamellar bilayer structures form in the SC (Downing, 1992). Previous studies indicated that ω-OH GlcCers (GlcCer with nonesterified ω-OH FA) are found in pig and fetal rat epidermis (Gray and White, 1978; Wertz and Downing, 1983b; Wertz *et al*, 1984; Hamanaka *et al*, 1988). We could not detect ω-OH GlcCers in human cultured keratinocytes, however (Hamanaka *et al*, 1993). Two explanations are possible; first, the absence of ω-OH GlcCers may be due simply to culture conditions; second, this putative intermediate may not be detectable *in vivo* in human epidermis. Neither of these possibilities has been examined adequately, as ω-OH GlcCers in human epidermis have not been characterized.

In this study, we have examined the structure of normal human epidermal GlcCers in order to clarify the role of GlcCers as a potential precursor of specific SC Cer moieties. In addition to acylGlcCers described previously (Hamanaka *et al*, 1989), six groups of GlcCers were identified. This variation is due to the combination of different sphingosine bases and amide-linked FAs. We did not detect the presence of GlcCer with free ω-OH FAs, however (ω-OH GlcCers). Finally, a comparison between Cers generated from acylGlcCers and GlcCers by β-GlcCer'ase with Cers isolated directly from the SC revealed that the GlcCers are direct precursors of SC Cer 1–7.

MATERIALS AND METHODS

Materials GlcCers from the spleen of a patient with Gaucher disease were kindly provided by Dr. M. Oshima (Tohoku University of Community and Science, Yamagata, Japan) to be used as a standard. Other sphingolipids were purchased from Sigma (St. Louis, MO). Human recombinant β-GlcCer'ase was purchased from Genzyme (Cambridge, MA). Human skin was obtained from amputated tissue from Japanese patients, 17–86 y old, with no prior history of skin disorders under the condition of informed consent. Amputation was performed within 2–3 h after the accidents. No skin abnormalities were observed in the skin samples. Split-thickness samples of cutaneous sheets

were prepared using a free hand dermatome (Keisei Medical Industrial, Tokyo, Japan). The epidermal sheets were obtained by incubation in a phosphate-buffered saline solution with 0.1% trypsin, pH 7.2, at 37°C for 1 h.

Thin-layer chromatography (TLC) TLC was performed on silica-gel 60 high-performance TLC plates (Merck, Darmstadt). GlcCers were separated with chloroform/methanol/water (40:12:1 vol/vol/vol). The solvent system for the separation of Cers was as previously described (Ponec and Weerheim, 1990; Uchida *et al*, 2000): chloroform to 1.5 cm; chloroform/methanol/acetone (76:16:8 vol/vol/vol) to 1.0 cm; chloroform/methanol/hexylacetate/acetone (86:4:1:10 vol/vol/vol) to 7.0 cm; chloroform/methanol/acetone (76:20:4 vol/vol/vol) to 2.0 cm; chloroform/methanol/diethyl ether/ethylacetate/hexylacetate/acetone (72:4:4:1:4:16 vol/vol/vol) to 7.5 cm; and n-hexane/diethyl ether/acetic acid (65:35:1 vol/vol/vol) to the top of the plate. GlcCer species were visualized by spraying with orcinol reagent, following by heating of the plates at 110°C for 15 min. Cer species were visualized after spraying with cupric acetate–phosphoric acid, and heating to 160°C for 15 min.

Extraction and isolation of GlcCers Lipids were extracted and GSLs were purified by DEAE-Toyopearl (Tosoh, Tokyo, Japan) and Unisil (Clarkson Chemical, Williamsport, Sweden) column chromatography, as described previously (Hamanaka *et al*, 1989). The GlcCer species were obtained in the chloroform/methanol (9:1 vol/vol) fraction following Unisil column chromatography. This fraction was further separated by high-performance liquid chromatography (HPLC) using a silica gel column (Aquasil Senshu Pack 4301-N; 10 mm i.d. × 30 cm, Senshu Scientific, Tokyo, Japan) using the following programmed gradient elution: chloroform for the first 60 min, a linear gradient of 0%–20% methanol in chloroform for the next 120 min, and 20% methanol in chloroform for the last 60 min, at a flow rate of 1.5 ml per min. The eluted GlcCer samples were monitored by TLC. This HPLC step allowed for the separation of four GlcCer fractions.

Gas–liquid chromatographic analysis The GlcCer fractions were subjected to methanolysis and the resulting FA methylesters were silylated as trimethylsilylated derivatives as described previously (Karlsson and Martensson, 1968; Uchida *et al*, 2000). The sphingosine bases were also prepared by the methods of Ando and Yu and were converted to their corresponding trimethylsilylated derivatives (Ando and Yu, 1979). The derivatives of both FA methylesters and sphingosine bases were then analyzed by gas–liquid chromatography. Peaks were detected by flame ionization detector and identified by mass spectrometry (HP 5972; Hewlett Packard, Palo Alto, CA) on a 0.32 mm i.d. × 30 m DB-1 capillary column (J & W Scientific, Folsom, CA), constant helium flow pressure 10.5 psi. The electron energy was 70 eV, the ionizing current was 60 μA, and the accelerator voltage was 3.5 kV (HP 5972 series Mass Selective Detector). For FA analyses, samples were held at an initial temperature of 100°C for 5 min, after which the temperature was increased to 300°C at a rate of 5°C per min; for the sphingosine bases, the initial temperature was 180°C and it was increased to 230°C at the same rate.

Negative ion fast atom bombardment–mass spectrometry The four GlcCer fractions were analyzed with a JEOL HX-110 mass spectrometer (JEOL, Tokyo, Japan) in a liquid matrix of triethylenetetramine/m-nitrobenzylalcohol (1:1 vol/vol). The accelerating voltage was 8 kV and the primary beam for bombardment was 6 kV XeO.

^{1}H nuclear magentic resonance (NMR) spectroscopy The isolated GlcCer samples were analyzed in 0.4 ml of chloroform-d_1/methanol-d_4 (8:2 vol/vol), with a JEOL JNM-GX 500 ^{1}H 500 MHz NMR spectrometer at 40°C. Tetramethylsilane was used as an internal chemical shift standard.

Beta-GlcCer'ase treatment The four GlcCer fractions, and an epidermoside I- and II-enriched fraction previously isolated from human epidermis (Hamanaka *et al*, 1989), were treated with human recombinant β-GlcCer'ase. Briefly, samples were suspended in a citrate–phosphate buffer (pH 5.6) containing 5 mM sodium taurocholate and incubated with β-GlcCer'ase (80 U per ml) at 37°C for 1 h. Cers produced by this treatment were extracted from the lower phase of Folch's partition (Folch *et al*, 1957).

RESULTS

Human epidermal GlcCers GlcCers extracted from human epidermis gave four bands showing specific color reactions to glycolipids upon orcinol treatment (**Fig 2**, *lane 2*). The two GlcCer bands with the highest Rf values represented epidermosides I and II. The structure of epidermoside I is shown in **Fig 1**. The other two GlcCer bands with lower Rf values were further separated into four fractions, Fr-1 to Fr-4, by HPLC (**Fig 2**, *lanes 3–6*). The relative amounts of each fraction, as determined by TLC densitometry, were as follows: Fr-1, 25.3%; Fr-2, 14.0%; Fr-3, 26.0%; and Fr-4, 34.7%.

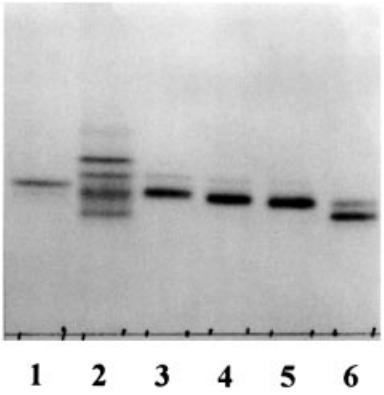

Figure 2. Thin layer chromatogram of human epidermal GlcCers. *Lanes 1* and *2* contain GlcCers as a reference and a human epidermal glycolipid extract, respectively. *Lanes 2–6* show glycolipid fractions isolated by HPLC; *lane 3*, Fr-1; *lane 4*, Fr-2; *lane 5*, Fr-3; *lane 6*, Fr-4.

Structural characterization of human epidermal GlcCers ^{1}H-NMR analysis indicated the presence of the glucose residue protons including the anomeric proton of β-glucoside as well as the protons of methyl, methylene, and amide linkage in Cer moieties in all GlcCer fractions (**Table I**).

FA compositions analyzed by gas chromatography–mass spectrometry are shown in **Table II**. Major components include saturated normal FAs in Fr-1 (primarily C24–C28), long chain α-OH FAs (C26) in Fr-2, a mixture of saturated normal FAs (primarily C24–C28) and long chain α-OH FAs (C24 and C26) in Fr-3, and long chain α-OH FAs (C24–C26) in Fr-4. In addition to the major FA species described above, all fractions contained a minor FA population composed of saturated nonhydroxy FAs from C16 to C18, but not C20–C22. As for the sphingosine bases, Fr-1 contained 18-phytosphingosine (t18:0), 18- and 20-phytosphingosine with one double bond (t18:1 and t20:1), and sphingenines (d18:1 and d20:1); Fr-2 and Fr-3 were composed of 18- and 20-sphingenine (d18:1 and d20:1); and Fr-4 contained 18- and 20-phytosphingosine (t18:0 and t20:0), phytosphingosines with one double bond (t18:1 and t20:1), and sphingenine (d18:1).

The major GlcCer species of each fraction was analyzed by fast atom bombardment–mass spectrometry (**Fig 3**) and their structures were characterized by the detection of pseudo-molecular ions. The characterized structures are as follows: Glc-C28:0-t20:1, giving an *m/z*-value of 910, Glc-C26:0-t20:1 and/or Glc-C28:0-t18:1 for *m/z* 882, and Glc-C24:0-t18:0 for *m/z* 828 (**Fig 3*A***) in Fr-1; Glc-C26 αh:0-d20:1 for *m/z* 882 and Glc-C26 αh:0-d18:1 for *m/z* 854 (**Fig 3*B***) in Fr-2; Glc-C24 αh:0-d20:1 and/or Glc-C26 αh:0-d18:1 for *m/z* 854 and Glc-C24 αh:0-d18:1 for *m/z* 826 (**Fig 3*C***) in Fr-3; and Glc-C26 αh:0-t20:1 for *m/z* 898, Glc-C24 αh:0-t20:1 for *m/z* 870, and Glc-C24 αh:0-t18:0 for *m/z* 844 (**Fig 3*D***) in Fr-4. Ions representing fragment species produced from GlcCer molecules after the elimination of hexose (glucose in this case) are as follows: *m/z* 748 from 910, 720 from 882, and 666 from 828 in Fr-1 (**Fig 3*A***); *m/z* 720 from 882 and 692 from 854 in Fr-2 (**Fig 3*B***); *m/z* 692 from 854 and 664 from 826 in Fr-3 (**Fig 3*C***); and *m/z* 736 from 898, 708 from 870, and 682 from 844 in Fr-4 (**Fig 3*D***). Matrix adduct ions detected in these mass spectra, as indicated by italic figures in **Fig 3**, are as follows: *m/z* 1064, 1036, and 982, which are 144 adducts to *m/z* 910, 882, and

Table I. Summary of ^{1}H-NMR analysis of GlcCers

Peaks and chemical shift (ppm)/Coupling constants *J* (Hz)					
Fr-1	Fr-2	Fr-3	Fr-4	Multiplicity	Assigned structures
	0.85/ 7.0		0.86/6.5	2	(-C<u>H</u>$_3$) x 2
	1.23-1.24			1	-(C<u>H</u>$_2$) x-
4.09/ 7.70	4.11/ 7.70	4.08/ 7.70	4.14/ 7.70	m	glucose (C<u>H</u>, 1-position)
2.94-3.00	2.93-3.00	2.92-2.99	2.90-3.00	m	glucose (C<u>H</u>, 2-position)
3.03-3.22	3.01-3.18	3.03-3.16	3.03-3.12	m	glucose (C<u>H</u>, 3, 4, 5-position)
3.47-3.53	3.46-3.53	3.43-3.50	3.62-3.70	m	glucose (C<u>H</u>, 6-position)
3.64-3.69	3.64-3.69	3.64-3.69			
7.37/ 9.00	7.39/ 9.80	7.39/ 9.40	7.49/ 9.40	d	-N<u>H</u>-CO-(CH$_2$)y-
7.48/ 9.00 and	7.47/9.00 and	7.50/ 8.60			
7.61/ 9.00	7.49/ 9.50				

Table II. Components of GlcCers

Carbohydrate	Fr-1	Fr-2	Glucose	Fr-3	Fr-4
Sphingosines	d18:1, d20:1	d18:1, d20:1	d18:1, d20:1	d18:1	
	t18:0				t18:0, t20:0
	t18:1, t20:1				t18:1, t20:1
Fatty acids (%)					
16:0	7.2	5.0		3.1	
17:0	6.9	4.8			
18:0	11.6	7.8		4.5	4.2
24:0	16.3	3.9		12.7	3.6
25:0	5.4	5.3			
26:0	22.0	9.4		23.3	3.7
27:0	3.9			3.9	
28:0	19.0	11.4		15.3	
29:0		4.5			
30:0	5.1				
24:0 (α-OH)				15.4	27.4
25:0 (α-OH)				8.1	
26:0 (α-OH)		44.6		14.0	43.6
Others	2.8	8.6		0	9.4

828, respectively, in Fr-1 (**Fig 3*A***); *m/z* 1036 and 1008, adducts to 882 and 854, respectively, in Fr-2 (**Fig 3*B***); *m/z* 1008 and 980 from 854 and 826, respectively, in Fr-3 (**Fig 3*C***); and *m/z* 1052, 1024, and 998 from 898, 870, and 844, respectively, in Fr-4 (**Fig 3*D***). Based on these results, human epidermal GlcCers were classified into six molecular groups (GlcCer-1 to GlcCer-6) (**Table III**).

Cers produced by β-GlcCer'ase treatment **Figure 4** shows Cers derived from recombinant human β-GlcCer'ase treatment of an enriched epidermoside sample. The derivative of epidermoside I, Glc-[C(26–34) ω-h-C18:2]-d18:1/20:1, as shown in *lane 3* of **Fig 4**, displayed the same mobility as SC Cer 1 in *lane 8*. The epidermoside II derivative, Glc-[C(26–34) ω-h-C18:2]-t18:1/20:1, indicated by an arrow in *lane 5*, corresponded to SC Cer 4 in *lane 8*. AcylCer obtained by β-GlcCer'ase treatment from epidermoside II was then subjected to mild alkaline treatment, yielding a product (*lane 6*) migrating between Cer 5 and Cer 6 in *lane 8*. This finding suggests that the alkaline treatment released linoleic acid by breaking the ester bond between the ω-OH amide-linked FAs and ester-linked FAs.

All GlcCer fractions, Fr-1 to Fr-4, were treated with β-GlcCer'ase (**Fig 5**). The comparison of the derived Cer species with those endogenous to the SC revealed that the constituents of Fr-1 exhibited similar mobility to Cer 2, 3, and 4 (*lane 3*), those of Fr-2 to Cer 3, 4, and 5 with Cer 2 as a minor component (*lane 4*), those of Fr-3 to Cer 4 and 5 with Cer 2 and 3 as minor components (*lane 5*), and those of Fr-4 to Cer 6 and 7 with Cer 2, 4, and 5 as minor components (*lanes 6, 7*).

DISCUSSION

Much attention has been paid to two characteristics of epidermal sphingolipids. First, they are required for the formation of the epidermal barrier to water loss, and second, they are putative regulators of keratinocyte growth and differentiation (Wakita *et al*, 1994; Marsh *et al*, 1995). Although sphingolipids in the epidermis are limited to five classes – acylGlcCer, GlcCer, SM, acylCer and Cer – their structures and metabolism are not completely understood.

In 1978, Gray and Yardley analyzed human epidermal GSLs and detected three bands of GlcCers on TLC (Gray and Yardley, 1975). In this study, we isolated four human epidermal GlcCer bands using HPLC and identified six molecular groups (summarized in **Table III**). GlcCers are heterogeneous in their Cer components, which are composed of combinations of at least six different sphingosine bases (d18:1, d20:1, t18:0, t20:0, t18:1, and t20:1) and 13 amide-linked FAs (C16:0, C17:0, C18:0, C24:0, C25:0, C26:0, C27:0, C28:0, C29:0, C30:0, C24 αh:0, C25 αh:0, and C26 αh:0). In addition to these GlcCers, we previously determined two major molecular groups of human epidermal acylGlcCers, epidermosides I and II. The former is defined by its dihydroxy-sphingosine base, whereas the second is characterized by its trihydroxysphingosine moiety. In epidermosides I and II, at least

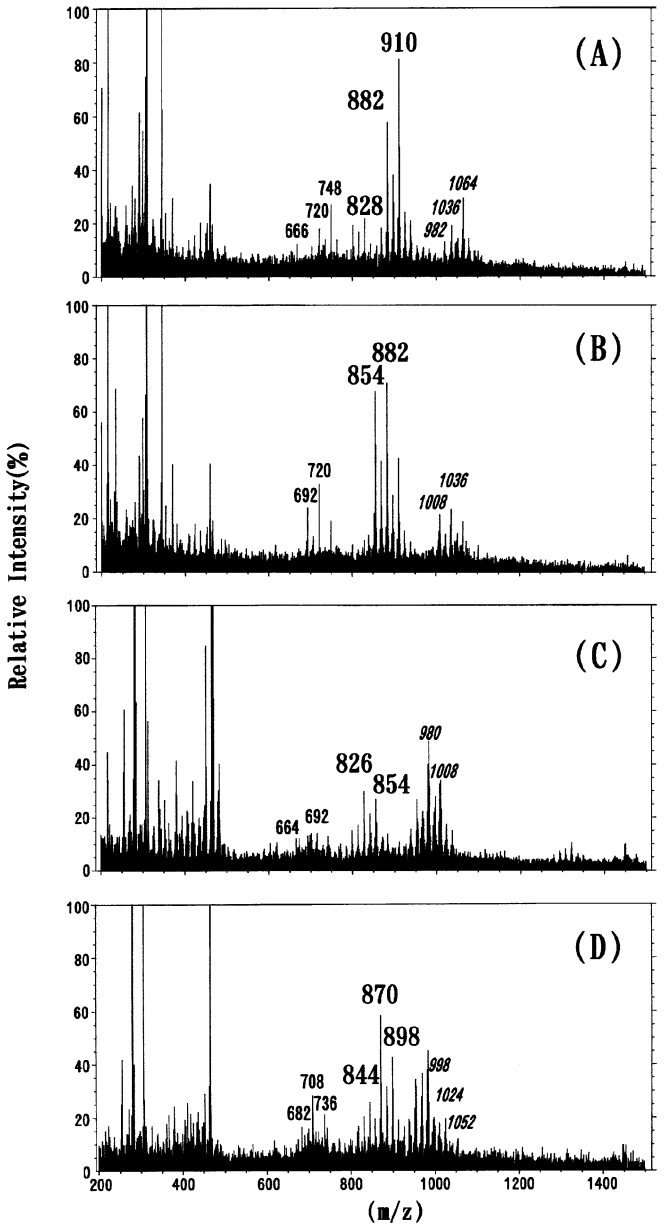

Figure 3. Negative-ion fast atom bombardment mass spectra of Fr-1, Fr-2, Fr-3, and Fr-4. (*A*)Fr-1, (*B*)Fr-2, (*C*)Fr-3, (*D*)Fr-4. Pseudo-molecular ions are described in bold, fragment ions due to the elimination of hexose in normal type, and adduct ions in italics.

10 different ω-OH FAs (C26 ωh:0, C28 ωh:0, C30 ωh:0, C32 ωh:0, C34 ωh:0, C26 ωh:1, C28 ωh:1, C30 ωh:1, C32 ωh:1, and C34 ωh:1) represent the amide-linked FA component (Hamanaka *et al*, 1989). This molecular heterogeneity of GlcCers and acylGlcCers could be the source of the similarly diverse Cer population (Cer 1–7) in the SC. To further investigate whether epidermal GlcCers are indeed precursors of specific SC Cers, we have taken a new approach to represent *in vivo* SC Cer production from GlcCers. Isolated human epidermal GlcCers were hydrolyzed by recombinant human β-GlcCer'ase *in vitro* and the resultant Cers were compared with SC Cers. As summarized in **Table III**, epidermal GlcCers contain Cer back-bone structures that are equivalent to SC Cers, indicating that SC Cer 1–7 are generated from GlcCers and acylGlcCers (**Fig 6**). In contrast to GlcCers, epidermal SMs are composed of primary sphingenine (d18:1) and 10 molecules of amide-linked FAs (C16:0, C17:0, C18:0, C20:0, C24:0, C24:1, C25:0, C26:0, C16 αh:0, and C18 αh:0), resulting

Table III. Structures of Cer moieties of human epidermal GlcCers

GSLs	Ceramide moieties	SC Cers[a]
Epidermoside I	C30:0ωh-C18:2/d20:1	Cer 1 (EOS)
	C32:1ωh-C18:2/d20:1	
Epidermoside II	C30:0ωh-C18:2/t20:1	Cer 4 (EOH)
GlcCer-1	C24:0/d20:1	Cer 2 (NS)
	C26:0/d18:1	
GlcCer-2	C24:0/t18:0	Cer 3 (NP)
GlcCer-3	C26:0/t20:1	Cer Y?[b]
	C28:0/t20:1	
	C28:0/t18:1	
GlcCer-4	C24:0αh/d18:1	Cer 5 (AS)
	C24:0αh/d20:1	
	C26:0αh/d18:1	
	C26:0αh/d20:1	
GlcCer-5	C24:0αh/t18:0	Cer 6 (AP)
GlcCer-6	C24:0αh/t20:1	Cer 7 (AH)
	C26:0αh/t18:1	
	C26:0αh/t20:1	

[a]Robson *et al*, 1994; [b]Vietzke *et al*, 1999.

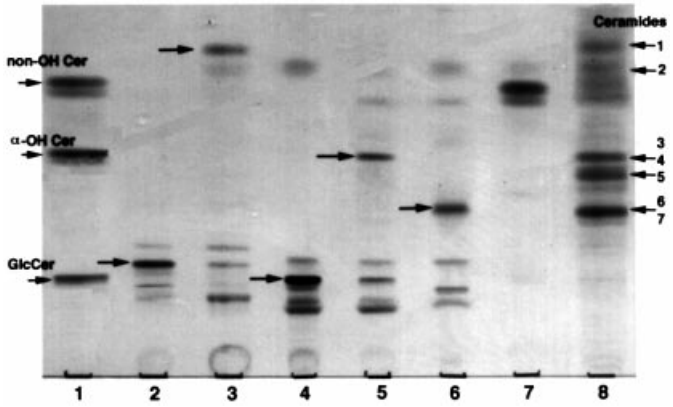

Figure 4. Cers generated by β-GlcCer'ase treatment of epidermosides. *Lanes 2* and *4* contain epidermosides I and II, respectively, indicated by *arrows*. *Lanes 3* and *5* contain Cers obtained by β-GlcCer'ase treatment of epidermoside I or II, respectively. *Lane 6* contains Cers with ω-OH FAs obtained from acylCers in *lane 5* by saponification. *Lane 1* contains standard nonhydroxy Cers, α-OH Cers, and GlcCers. *Lane 8* shows the spectrum of Cer species obtained from human SC. *Lane 7* contains nonhydroxy Cers as a standard.

in the generation of Cer 2 and Cer 5. The proportion of Cer 2 and Cer 5 in the total SC Cers is 17.6% and 20.5%, respectively (Uchida *et al*, 2000). Therefore, GlcCers must be responsible for producing more than 61.9% of the total SC Cer population, as well as at least five of the seven Cer subtypes. To support the importance of GlcCer for SC Cer formation, Gaucher type 2 patients, who have a β-GlcCer'ase deficiency, have significant epidermal permeability barrier abnormalities, whereas Niemann–Pick patients, whose acidic sphingomyelinase activities are decreased, have normal barrier function at basal conditions (Holleran *et al*, 1994; Schmuth *et al*, 2000). Moreover, transgenic mice that lack β-GlcCer'ase activity accumulate GlcCers, decrease Cers in the epidermis, and display abnormality of the epidermal permeability barrier function (Holleran *et al*, 1994). β-GlcCer'ase-deficient transgenic mice demonstrate altering the SC Cer composition, i.e., acylCers, phytosphingosine containing Cer and C24/26 α-OH amide-linked FA containing Cer and not changing C16 α-OH amide-linked FA containing Cer, as well as decreasing bulk of the SC Cer (Doering *et al*, 1999b; Uchida *et al*, 2000). These prior studies and our study provide evidence of the physiologic relevance

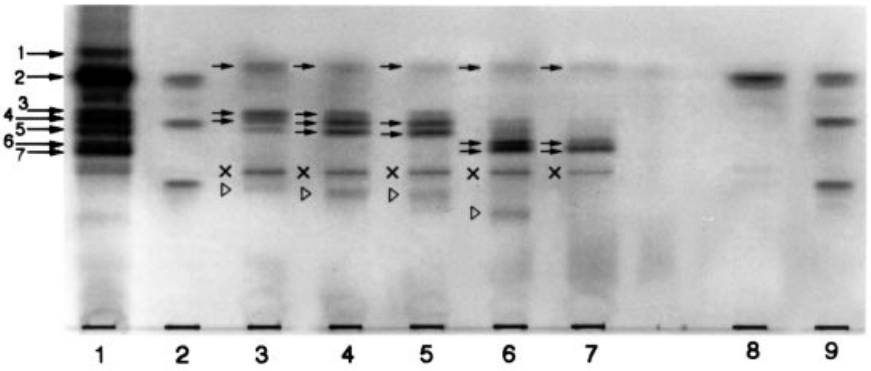

Figure 5. Cers generated by β-GlcCer'ase treatment of human epidermal GlcCers. *Lanes 3*, *4*, *5*, and *6* contain Cers obtained by β-GlcCer'ase treatment of human GlcCer fractions: Cers from Fr-1 in *lane 3*, Cers from Fr-2 in *lane 4*, Cers from Fr-3 in *lane 5*, and Cers from Fr-4 in *lane 6*. *Lane 7* contains Cers from Fr-4 lipids but smaller contents. The symbol X indicates byproducts that derive from the enzyme solution containing detergent and stabilizer(s), and triangles indicate GlcCers that were not hydrolyzed. *Lanes 2* and *9* contain standard nonhydroxy Cers, α-OH Cers, and GlcCers. *Lane 1* shows Cer species obtained from human SC. *Lane 8* contains nonhydroxy Cers as a standard.

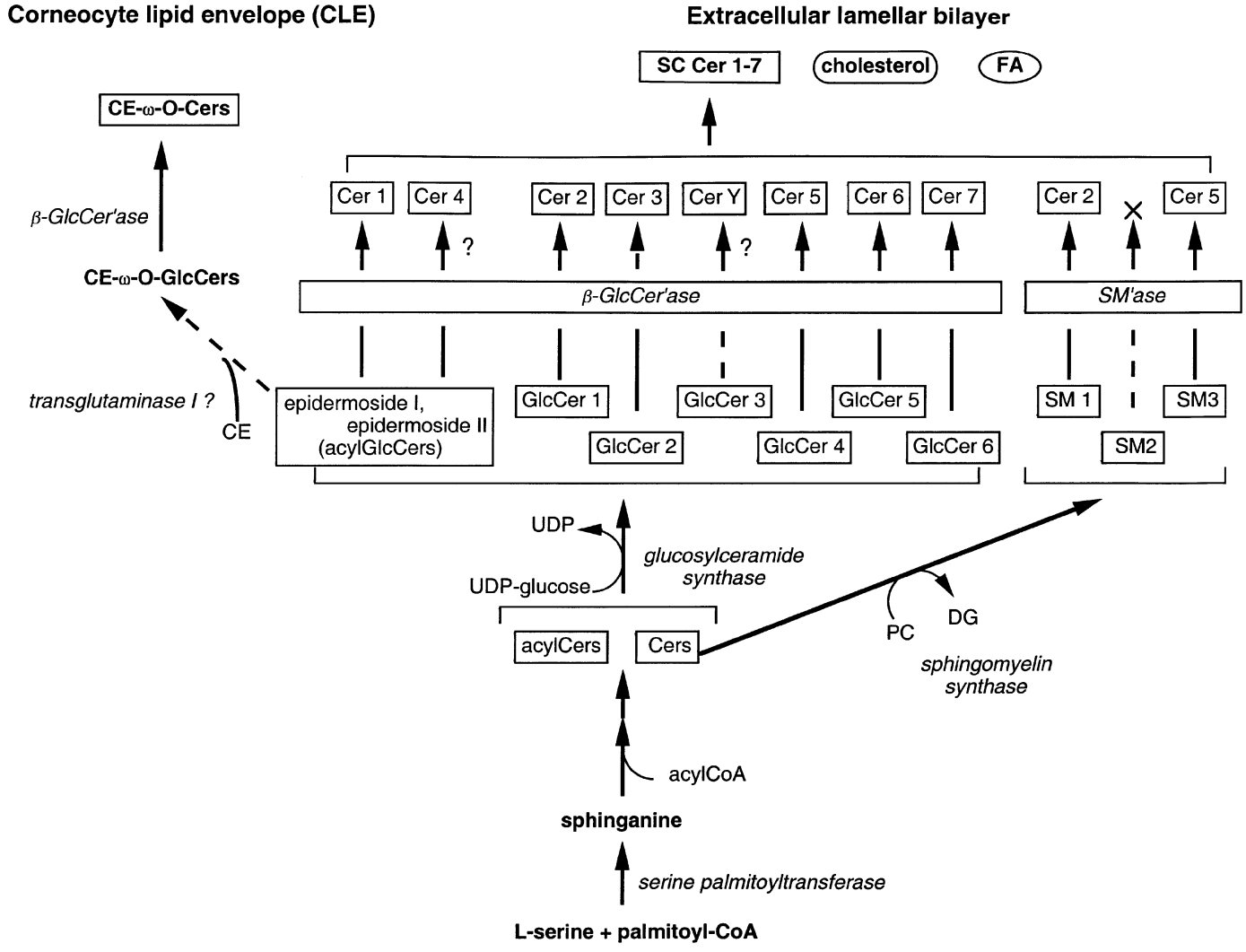

Figure 6. Summary of proposed synthetic pathway of SC Cers. Abbreviations: PC, phosphatidylcholine; DG, diacylglycerol; UDP, uridine diphosphate.

of GlcCers as a precursor of SC Cer. The importance of sphingomyelinase-dependent Cer production, however, has been demonstrated in Niemann–Pick patients following acute barrier disruption (Schmuth *et al*, 2000). The importance of sphingomyelinase-mediated epidermal responses in barrier recovery is also suggested in a murine model upon acute barrier disruption (Jensen *et al*, 1999). Thus, recovery of barrier function following acute and chronic insults, including skin diseases such as atopic dermatitis, psoriasis, and ichthyosis, may require both GlcCer- and SM-dependent pathways to compensate for the reduction in barrier lipids. Finally, it has not yet been elucidated whether *de novo* synthesized Cer is directly delivered to the SC. As described above,

however, the conversion of GlcCer and SM to Cer is required for forming a competent barrier as well as displaying a SC Cer molecular heterogeneity. Therefore, free Cer in the stratum granulosum could not play a main role for SC Cer generation. Based on these findings, the proposed synthetic pathway of the SC Cer is summarized in **Fig 6**.

Our previous studies and this study have defined the structures of acylGlcCers and GlcCers in normal human epidermis. Two questions have been raised, however. First, although Cer produced from epidermoside II by β-GlcCer'ase showed the same Rf value as Cer 4 (**Fig 4**), it should be noted that the structures of Cer 4 (Robson *et al*, 1994) and the Cer portion of epidermoside II (Hamanaka *et al*, 1989) are not entirely the same, i.e., the positions of the hydroxy residues in the sphingosine bases of these two sphingolipids are different (Cer 4 contains 6-hydroxysphingenine, whereas epidermoside II has 4-hydroxysphingenine). Thus, the role of epidermoside II as a precursor of Cer 4 is still in question. Second, an SC Cer potentially derived from GlcCer 3 is not reported in SC Cer (Robson *et al*, 1994). Interestingly, Vietzke *et al* (1999) found Cer X (N-acylsphinganine, C24-33:0/C18:0) and Cer Y (N-acyl-6-OH-sphingosine, C22-32/t18:1) in human SC. Although it is not determined whether the sphingosine base structure of GlcCer 3 is 6-OH sphingosine, GlcCer 3 appears to be a potential precursor of Cer Y.

Although pig and fetal rat contain both acylGlcCers and their nonesterified form, ω-OH GlcCers, in the epidermis (Wertz and Downing, 1983b; Wertz *et al*, 1984; Hamanaka *et al*, 1988), humans only express acylGlcCers. Wertz *et al* proposed that fetal rat epidermal ω-OH GlcCers may be produced by the deacylation of acylGlcCers (Wertz *et al*, 1984). If this were the case in humans, the absence of ω-OH GlcCers suggests that the human epidermis is able to protect acylGlcCers from deacylation. The physiologic meaning of this difference between human and other mammalian epidermis GlcCers is unknown. The absence of ω-OH GlcCers in human epidermis, however, raises hypotheses regarding the synthetic pathway of acylGlcCers and CLE formation. On the synthesis of acylGlcCer, two pathways are postulated. First, the synthesized ω-OH Cers may be immediately acylated (addition of linoleic acid) to acylCers followed by glucosylation to acylGlcCers. Second, the ω-OH FAs could be esterified by linoleic acid and then incorporated into sphingosine bases followed by glucosylation. On the generation of the major lipid component in the CLE, previous studies demonstrated that glucosylated ω-OH Cers first covalently bind to the envelope proteins and then are hydrolyzed by β-GlcCer'ase, based on Gaucher model transgenic mice, which are deficient in β-GlcCer'ase, and prosaposin (sphingolipid activator proteins that stimulate enzymatic hydrolysis of sphingolipids) deficient transgenic mice (Doering *et al*, 1999a; 1999b).[2] Supporting these results, immunohistochemistry using anti-GlcCer antibody revealed that GlcCer is present at the cornified envelopes of the cells only in the first layer of the SC and disappears in the upper SC (Vielhaber *et al*, 2001). Doering *et al* proposed that acylGlcCers are the precursors of binding ω-OH Cers, by releasing linoleic acid and binding to proteins (Doering *et al*, 1999b) (**Fig 6**). Our results, indicating the absence of free ω-OH GlcCer in epidermis, support the hypothesis that acylGlcCer but not free ω-OH GlcCer is a precursor of CLE-Cer. A recent study indicated that ω-OH Cers in the CLE are decreased in a rat deficient in essential fatty acids (Meguro *et al*, 2000), suggesting that the linoleate moiety of acylGlcCers may play a critical role in forming CLE structures. The enzyme transglutaminase is known to form isopeptide bonds. Steinert and coworkers discovered a novel function of transglutaminase, i.e., transglutaminase 1, but not transglutaminase 2 and 3, forms the ester linkage between involucrin and a synthetic pseudo-ω-OH Cer, 16-(16hydroxy-hexadecyl)oxypalmitoyl sphingenine, which displays similar solubility and chromatographic properties to natural ω-OH Cers (Nemes *et al*, 1999). They hypothesized that the thioester bond between transglutaminase 1 and a Gln residue of involucrin is replaced by the ω-OH group of ω-OH Cers, forming a Glu-O-Cer linkage. Based on this hypothesis, acylGlcCers may be hydrolyzed to ω-OH GlcCer followed by a nucleophilic ω-OH attack on the thioester bond. As Doering *et al* (1999b) suggested, it is also possible that transesterification occurs between acylGlcCers and the carboxy group of CE proteins, resulting in the formation of CE-ω-O-Cers and the subsequent release of linoleic acid, mediated by an as-yet unknown enzyme(s) or by nonenzymatic reaction.

In addition to ω-OH GlcCers, it is known that the sphingosine base and ester-linked FA of acylGlcCers are different between the epidermis of humans and other mammals. We reported that there are two types of human acylGlcCers characterized by their unique sphingosine bases, sphingenine and phytosphingosine with one double bond. Animal epidermis contains only one acylGlcCer group with sphingenine, however. Furthermore, linoleic acid is the ester-linked FA in 95% of human epidermal acylGlcCers (Hamanaka *et al*, 1989), but this figure is only 75%–77% in pig (Gray *et al*, 1978; Wertz and Downing, 1983a), 32% in rat (Wertz *et al*, 1984), 45% in mouse (Wertz and Downing, 1985), and 84% in guinea pig (Uchida *et al*, 1988). As linoleic acid is essential in epidermal barrier function (Elias *et al*, 1980; Hansen, 1986), acylGlcCers carrying linoleic acid must play a key role in forming this barrier. In this respect, human epidermis may possess a selective mechanism for the production of functional acylGlcCers. These linoleic-acid-carrying acylGlcCers were named epidermosides to stress their role in barrier function (Hamanaka *et al*, 2001). Moreover, epidermosides may be important in the formation of the CLE, as described above.

Alterations of the content and composition of SC Cers in atopic dermatitis patients have been reported by several investigators (Melnik *et al*, 1988; Imokawa *et al*, 1991; Yamamoto *et al*, 1991; Di Nardo *et al*, 1998; Bleck *et al*, 1999). In particular, Cer 1 is markedly decreased in skin from such patients. Their β-GlcCer'ase and sphingomyelinase activity were normal, however (Jin *et al*, 1994), and no accumulation of GlcCers and SM were reported in their SC. Imokawa and coworkers found that glucosylceramide sphingomyelin deacylase (or sphingomyelin deacylase) but not sphingomyelinase or ceramidase activity was increased in atopic dermatitis lesions (Hara *et al*, 2000; Higuchi *et al*, 2000). They proposed that the reduction in SC Cers is due to increased glucosylceramide sphingomyelin deacylase activity. The structural preferences of glucosylceramide sphingomyelin deacylase have not been defined, however, to explain why acylGlcCer-derived, but not SM-derived, Cer 1 is specifically decreased. Interestingly, Bleck *et al* (1999) reported that short chain α-OH acyl (C16 and C18) sphingenine or hydroxysphingenine is increased in unaffected skin of atopic dermatitis patients, whereas normal control subjects contain long chain α-OH acyl (C22 to C26) sphingenine or hydroxysphingenine. Our previous results (Uchida *et al*, 2000) and these results suggest that Cer containing short chain α-OH FAs (primarily C16) with sphingenine derives from SMs (SM-2), whereas Cer with long chain α-OH FAs is derived from GlcCers. Thus, alterations in GlcCer or SM synthesis as well as increasing glucosylceramide sphingomyelin deacylase activity may occur in atopic dermatitis. A recent study revealed that increases in Cer 2 and Cer 5 are evident in the human SC with dry skin symptom, whereas Cer 4 decreases (Schreiner *et al*, 2000). Changes in Cer proportion derived from GlcCer and SM may often occur in this skin type; however, the mechanism responsible for the metabolic changes has not yet been defined.

Our previous studies (Uchida *et al*, 2000) and this study of epidermal SM and GlcCer structures further define the synthetic pathway for SC Cer formation. These results not only elucidate the metabolic source for specific epidermal Cer species, but also help to define potential metabolic steps that are modified in/by skin disease(s).

[2]Uchida Y *et al*: Formation of the lipid-bound envelope (LBE): insights from glucocerebrosidase-deficient Gaucher mouse epidermis. *J Invest Dermatol* 112:543, 1999 (abstr.)

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