New Acylceramide in Native and Reconstructed Epidermis

Maria Ponec, Arij Weerheim, Peter Lankhorst,* and Phil Wertz†

Department of Dermatology, Leiden University Medical Center, Leiden, the Netherlands; *DSM Food Specialties, Delft, the Netherlands; †Dows Institute for Dental Research, Iowa, USA

Culturing of normal human keratinocytes at the air–liquid interface results in the formation of fully differentiated epidermis under *in vitro* conditions. Although the reconstructed epidermis shows a close resemblance to native tissue, there are still some differences in the stratum corneum lipid profile and intercellular lipid organization. As ceramides belong to one of the major stratum corneum lipid classes, the aim of this study was to characterize this fraction in more detail. For this purpose, individual ceramide fractions were isolated by column chromatography and characterized by a combination of nuclear magnetic resonance spectroscopy, high-performance thin-layer chromatography, and gas chromatography. The results of this study show that in both the native and reconstructed human epidermis the extractable ceramide fraction contains, in addition to the well known acylceramides (EOS, EOH), a new acylceramide in which the ω-O-acylhydroxyacid is amide-linked to phytosphingosine (EOP). The same three sphingoid base moieties (S, P, H) are also found in ceramides with amide-linked nonhydroxy and α-hydroxyacids. Whereas the same types of ceramides were present in both tissues, some differences in their fatty acid profiles have been found. In reconstructed epidermis the content of linoleic acid in all three acylceramides fraction was significantly lower; the ceramide (NS) fraction was enriched in short fatty acids and the ceramide(AS) fraction was enriched in long chain α-hydroxyacids. These differences together with a lower content of free fatty acids may explain the differences between native and reconstructed tissue in stratum corneum lipid organization observed earlier by X-ray diffraction. ***Key words: ceramides/fatty acids/keratinocyte/sphingoid base. J Invest Dermatol 120:581–588, 2003***

The uppermost layer of the epidermis—the stratum corneum (SC)—protects the human body against the loss of physiologically important components and against potentially damaging environmental insults. The SC consists of corneocytes embedded in a lipid-rich matrix. As the lipid-rich intercellular regions in the SC are the only continuous domains through the SC, they define the pathway through which molecules can diffuse across the SC and play a prominent role in skin barrier function. In the intercellular spaces of the SC ceramides (CER), cholesterol (CHOL), and free fatty acids dominate. Eight subclasses of ceramides have been identified in human SC (Wertz *et al*, 1985; Robson *et al*, 1994; Stewart and Downing, 1999). The ceramides include species in which a sphingoid base sphingosine (S), phytosphingosine (P) or 6-hydroxysphingosine (H) are amide-linked to a nonhydroxy fatty acid (N) or an α-hydroxy fatty acid (A). Two ceramides, ceramides 1 (EOS) and 4 (EOH), are exceptional in that they contain linoleic acid ester-linked to the ω-hydroxyl group of a very long chain amide-linked ω-hydroxyacid. For characterization of various ceramide classes, the extracted SC lipids are generally separated by thin-layer chromatography, and individual lipid fractions are isolated and further characterized. One-dimensional thin-layer chromatography usually allows separation of human ceramides into at least seven different fractions. It has been shown that the fifth ceramide fraction contains two different ceramides (ceramide 5 (AS) and ceramide 8 (NH)) that after acetylation can be separated by thin-layer chromatography (Stewart and Downing, 1999).

Reconstructed human epidermis can be generated by seeding human keratinocytes on an appropriate substrate and subsequent culturing at the air–liquid interface. Morphologic studies have shown that such reconstructed epidermis forms a multilayered epithelium composed of an organized stratum basale, stratum spinosum, stratum granulosum, and a SC. Furthermore, it displays characteristic epidermal ultrastructure (Ponec *et al*, 1988, 1997, 2001), and markers of epidermal differentiation are expressed (Ponec, 1991; Gibbs *et al*, 1997, 1998). Analysis of epidermal lipids by thin-layer chromatography revealed a high degree of similarity between native and reconstructed epidermis (Ponec *et al*, 1997, 2000, 2001). Detailed information on the molecular structures of the various ceramide classes, however, is still missing. The aim of this study was to characterize individual ceramide classes present in reconstructed epidermis and to compare these detailed structures with those present in the native tissue. A more detailed knowledge of the differences between the lipids from native SC and the culture model may suggest approaches for improving the barrier of the cultures so that they could be used, for example, in studies on transdermal drug absorption. For this purpose, extracted SC lipids were separated by column chromatography and individual fractions were analyzed. To determine the molecular structure of the sphingoid base in individual

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Reprint requests to: Dr M. Ponec, Department of Dermatology, Leiden University Medical Center, Sylvius Laboratory, Wassenaarseweg 72, 2333 AL Leiden, the Netherlands. Email: m.h.ponec-waelsch@lumc.nl

Abbreviations: CER, ceramides; EOH, ceramide 4; EOP, phytosphingosine; EOS, ceramide 1; FAME, fatty acid methylesters; NMR, nuclear magnetic resonance imaging; SC, stratum corneum.

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ceramides nuclear magnetic resonance imaging (NMR) was utilized. Gas chromatography was used to assess the fatty acid profiles in individual ceramide fractions.

MATERIALS AND METHODS

Cell culture Reconstructed human epidermis was generated on inert filter substrates, as previously described in detail (Ponec *et al*, 1997). In brief, adult human keratinocytes seeded on filters (Costar Corning BV, Schiphol-Rijk, The Netherlands) were cultured overnight in keratinocyte medium consisting of three parts Dulbecco–Vogt medium and one part Ham's F12 medium supplemented with 5% HyClone calf serum (Greiner, Nürtingen, Germany), 0.5 μM hydrocortisone, 1 μM isoproterenol, and 0.1 μM insulin. Subsequently, the cultures were fed with the same keratinocyte medium containing 1% serum, 1 mM L-carnitine, 10 mM L-serine, 1 μM DL-α-tocopherol-acetate, and a lipid supplement containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid, and 2.4 mM bovine serum albumin, and cultured under submerged conditions for an additional 5 d. Thereafter, the cultures were lifted to the air–liquid interface and cultured for an additional 3 wk in the same medium except that serum was omitted, the concentration of linoleic acid was increased to 30 μM and 1 ng epidermal growth factor per ml and 50 μg ascorbic acid per ml were added. All medium supplements were purchased from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands).

Collection of SC samples and lipid extraction The SC was isolated from the freshly excised human mammary skin or from reconstructed epidermis by treatment with dispase and trypsin followed by proteinase K, as described earlier (Ponec *et al*, 1997). Epidermis collected from two different skin donors and two independent keratinocyte cultures were used. The harvested material was first extracted according to Bligh and Dyer (1959) (followed by a series (3–6) of extractions with chloroform/methanol, 1:2, 1:1, and 2:1; Ponec *et al*, 2000). All extracts were first dried under a stream of nitrogen then weighted, and their lipid composition was determined. The extracted and dried tissue was subjected to a mild saponification and re-extracted to recover covalently bound lipids, as described by Wertz *et al* (1989a).

Isolation of human ceramides From 50 to 90 mg of solvent extractable lipids were separated by column chromatography (4 × 4 cm, 20 g of Silicagel LiChroprep Si 60, 15–25 μm; Merck, Darmstadt, Germany) by sequential elution with the following mixtures:

1. 100 ml hexane/chloroform/diethylether/acetic acid (70:10:20:1);
2. 100 ml hexane/chloroform/dioxane/2-propanol (55:40:4:1);
3. 200 ml hexane/chloroform/dioxane/ethyl acetate/methanol/2-propanol (51:40:2:6:1);
4. 200 ml hexane/chloroform/dioxane/ethyl acetate/methylethylketone/acetone/methanol (44:40:2:10:2:1:1);
5. 400 ml hexane/chloroform/dioxane/ethyl acetate/methylethylketone/acetone/methanol (38:40:2:12:4:2:2);
6. 200 ml hexane/chloroform/dioxane/ethyl acetate/methylethylketone/acetone/methanol (20:56:2:6:8:4:4);
7. 100 ml chloroform/methylethylketone/acetone/methanol (72:16:8:4);
8. 200 ml chloroform/acetone/methanol (76:16:8);
9. 100 ml chloroform/acetone/methanol (76:8:16);
10. 200 ml chloroform/methanol/ethanol/water/acetic acid (30:40:20:8:2);
11. 100 ml chloroform/methanol/ethanol/water/acetic acid (20:40:20:20:2).

All solvents were purchased from Merck. Fractions containing individual ceramides were pooled and if necessary further purified by preparative thin-layer chromatography.

High-performance thin-layer chromatography (HPTLC) Fifty microliters of lipid extracts were applied under a flow of nitrogen on the HPTLC plate (Merck) using a Linomat IV (CAMAG, Muttenz, Switzerland). The chromatograms were developed according to the following sequential development system:

1. dichloromethane/ethyl acetate/acetone (80:16:4);
2. chloroform/acetone/methanol (76:8:16);
3. hexane/chloroform/hexyl acetate/acetone/methanol (6:80:0.1:10:4).

Quantification was performed after spraying the thin-layer plate with 7.5% cupric acetate and 2.5% cupric sulfate in 8% phosphoric acid (w/w) and heating at 80°C, 120°C, and finally 160°C. A digitized image was captured after attaining each temperature. Quantification of lipid fractions was based on the known quantities of standards included on the same plate. For this purpose, cholesterol (Sigma), cholesterol sulfate (Sigma), free fatty acids (C18:1, C16:0, C24:0), and synthetic ceramide 3 (Cosmoferm, Delft, the Netherlands) were used.

Preparation and analysis of fatty acid methyl esters (FAME) Individual purified ceramide and free fatty acid fractions were dissolved in 50 μl of toluene and subsequently transmethylated in 1 ml of 10% boron trichloride in methanol using microwave irradiation, which was carried out at 85 W for 6 h. The reagents were evaporated at 30°C under a stream of nitrogen. Subsequently, the FAME were purified by column chromatography on a 60 × 5 mm column filled with Kieselgel 60 (0.040–0.063 mm, Merck 9385). The FAME were eluted with 4 × 1 ml hexane/diisopropyl ether (100:0.5) followed by 6 × 1 ml hexane/chloroform/diisopropyl ether (90:9:1); hydroxy FAME with 1 × 4 ml hexane/chloroform/ethyl acetate/acetone (40:40:16:4); and the remaining fractions with 2 × 1 ml chloroform/methanol (2:1) followed by 2 × 1 ml chloroform/methanol (1:2).

To examine the composition of hydroxy acids, the samples were applied on the HPTLC plate and separated using the following sequential development system:

1. hexane/chloroform/hexyl acetate/ethyl acetate/acetone/methanol (8:68:0.1:4:16:4),
2. hexane/chloroform/diethylether/ethyl acetate/dioxane (70:20:6:2:2).

This system allowed separation of the α- and ω-hydroxy FAME.

The FAME fractions containing either nonhydroxy fatty acids, α-hydroxy acids or ω-hydroxy acids were separated and analyzed with a Vega GC 6000 gas chromatograph (Carlo Erba Instruments, Milan, Italy) using a CP Wax 52 capillary column (Chrompack, Varian, Bergen Op Zoom, the Netherlands). An initial temperature of 80°C was increased to 160°C at a rate of 4°C per min followed by a 2°C per min increase to 250°C, which was maintained until all peaks were eluted. The peaks were identified by comparison with standards (non-hydroxy and α-hydroxy fatty acids, Alltech; Breda, The Netherlands and ω-hydroxy fatty acids, Cosmoferm and carnauba wax, Wertz *et al*, 1989a). Integration of peak asreas and calculation of weight percentages was performed by a Baseline 810 integrator. Heptadecanoic acid was used as the internal standard.

Acetylation of ceramides Purified ceramide fractions were dissolved in a 1:1 mixture of pyridine and acetic anhydride and allowed to react for 2 h at 60°C. The reagents were then removed by evaporation under a stream of nitrogen, and the acetylated ceramides were subjected to HPTLC. A small fraction of the sample was applied to an HPTLC plate in 250 μl chloroform/methanol (2:1). The development system used was:

1. hexane/chloroform/hexyl acetate/ethyl acetate/acetone/methanol (6:68:0.1:4:16:4);
2. hexane/chloroform/diethyl ether/ethyl acetate (10:70:16:4).

This procedure separated the fifth ceramide fraction into two distinct subfractions, as described previously (Stewart and Downing, 1999). Acetylated ceramides have been used for NMR analysis in our study.

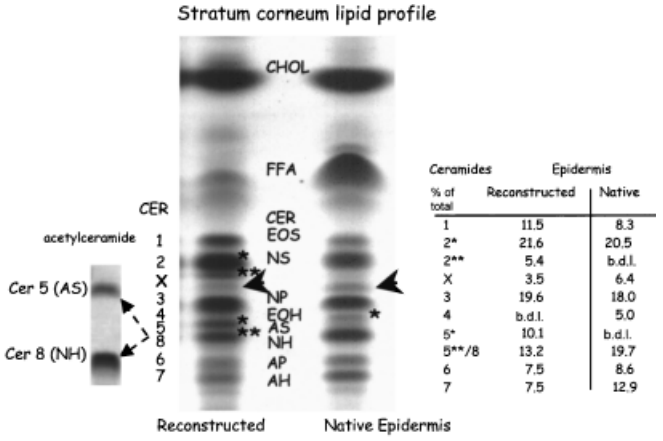
NMR The purified ceramide acetates (50–100 μg) were dissolved in 800 μl of deuterated chloroform (Isotec Campro, Scientific Veenedaal, The Netherlands) and were kept at room temperature overnight. Then, the mixtures were filtered (Gelman acrodisc CR PTFF, 0.2 μm) and transferred to NMR tubes. One-dimensional proton NMR spectra were obtained at 500 MHz using a Varian NMR spectrometer or with a Bruker DRX-600 operating at 600 MHz. Assignments of the NMR spectra of several ceramides (ceramides 3, 4, 5, 8, 9, A, B) were made using a combination of the following two-dimensional NMR techniques: COSY (correlation spectroscopy) (Hurd, 1990), TOCSY (total coherence spectroscopy) (Griesinger *et al*, 1988), HSQC (heteronuclear single quantum coherence) (Kay *et al*, 1992), and HMBC (heteronuclear multiple bond correlation) (Summers *et al*, 1986). All two-dimensional NMR spectra were recorded using the 600 MHz spectrometer equipped with a 5 mm inverse triple resonance probe with self-shielded gradients. The temperature of the probe was set at 300 K. Chemical shifts are referenced against tetramethylsilane.

RESULTS

The ceramide profile in reconstructed epidermis deviated from that of native tissue Analysis of SC lipids extracted from reconstructed epidermis revealed the presence of all major lipid fractions, including cholesterol, free fatty acid and ceramides (**Fig 1**). With the lipids from reconstructed epidermis the second ceramide fraction split into a prominent fraction (*) and a slightly more polar minor fraction (**). In addition, the fifth ceramide fraction appeared to split into two subfractions

(* and **, respectively) and ceramide 4 was difficult to detect as its band was obscured by the ceramide 5* band. The relative amounts of individual ceramide fractions in reconstructed epidermis showed some differences from that found in native tissue (table in **Fig 1**). In both tissues an unidentified fraction X with polarity between ceramide 2 and ceramide 3 was clearly visible on the HPTLC chromatogram.

It remained to be established whether ceramide 4 is indeed present in reconstructed epidermis and whether in native epidermis ceramides 2** and 5* could also be detected. As the isolation of individual ceramides by HPTLC is not satisfactory, lipids extracted from both the native and the reconstructed epidermis have been subjected to column chromatography. More than 100 fractions were collected, and the lipid composition of each individual fraction was determined by HPTLC. Cholesterol esters, triglycerides, cholesterol, and free fatty acids were eluted in the first 40 fractions. Ceramides were generally detected in later fractions. As can be seen from **Fig 2**, several fractions contained more than one ceramide; therefore, additional fractionation was required. This was achieved either by column chromatography using small columns or by HPTLC followed by scraping and elution of individual fractions.



Ceramides	Epidermis	
% of total	Reconstructed	Native
1	11.5	8.3
2*	21.6	20.5
2**	5.4	b.d.l.
X	3.5	6.4
3	19.6	18.0
4	b.d.l.	5.0
5*	10.1	b.d.l.
5**/8	13.2	19.7
6	7.5	8.6
7	7.5	12.9

Figure 1. Differences in ceramide profile in reconstructed and native epidermis. Lipids from SC collected from native and reconstructed epidermis were extracted and subjected to HPTLC. The content of individual ceramides was established after charring using synthetic ceramide 3, as the standard. Data are presented in the inserted table. Note the presence of double bands in the ceramide 2 and 5 positions. HPTLC separation of isolated ceramide 5 subjected to acetylation showed the presence of two bands (ceramide 5 and ceramide 8, respectively).

Sphingoid bases in individual ceramide fractions The NMR analysis of acetylated ceramides has been performed to identify the sphingoid base in individual ceramides (Freeman, 1988; Jie and Mustafa, 1997). The chemical shifts of the acetylated ceramides are extremely useful for their quick identification. It can be seen from **Table I**, that a triplet at 4.00 ($\pm$0.05) ppm shows that the fraction must contain a ceramide with an ω-hydroxyacid (ceramide 1, 4, 9, A, and B). This signal is attributed to the terminal methylene protons of the ω-hydroxyacid adjacent to the ester linkage. Ceramides containing an α-hydroxyacid have a doublet of doublets at 5.00–5.05 ppm (ceramides 5, 6, and 7). This is attributed to the proton on the α-carbon of the fatty acid coupled to each of the nonequivalent protons on the β-carbon. It is shifted down field by the O-acetyl group. Ceramides containing phytosphingosine as the base have the H5 signal in the high field region of the spectrum at approximately 1.5 ppm (ceramides 3, 6, and 9). Ceramides containing 6-hydroxysphingosine can be recognized easily because H6 appears as a pseudoquartet between 5.10 and 5.20 ppm (ceramides 4, 7, 8, and B). The ceramides containing sphingosine, finally, have a characteristic doublet of doublets attributed to H4 at 5.30–5.35 ppm and a pseudoquartet (actually a doublet of triplets with the two central peaks superimposed) attributed to H5 at 5.70–5.75 ppm (ceramides 1, 2, 5, and A). Sphingosine was present in both ceramide 5 (ceramides 5* and 5**) fractions. As shown in **Fig 1**, acetylation of ceramide 5** fraction isolated from reconstructed epidermis resulted in identification of two different ceramides. These ceramides contained sphingosine and 6-hydroxysphingosine, respectively. Phytosphingosine was identified in ceramides 3 and 6. In addition, from the NMR spectrum it could be deduced that phytosphingosine is the base moiety of the still unidentified

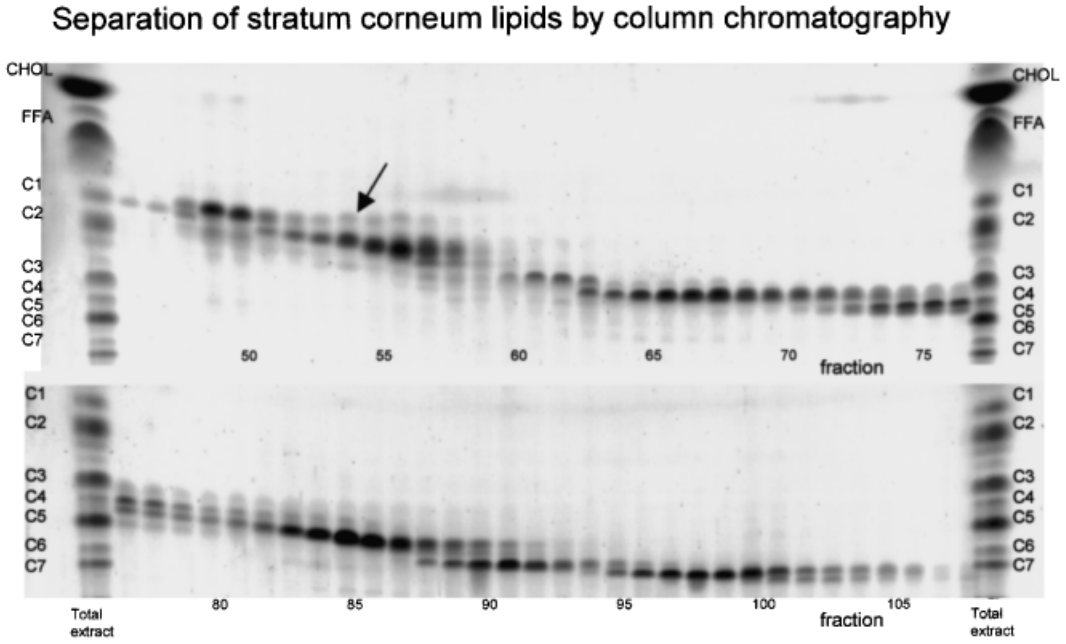


Figure 2. Separation of SC lipids by column chromatography. Lipids from SC collected from native epidermis were extracted and subjected to column chromatography. Shown are lipid profiles of total SC and of ceramide containing fractions 45–107. *Arrow*: fraction containing Cer(EOP) and used for further purification and analysis.

Table I. Sphingoid bases in ceramides of the human SC. NMR chemical shifts, δ, of their fully acetylated derivatives in $CDCl_3$ solution (ppm, $\delta_{TMS} = 0$)

	NMR chemical shifts at 300 K (ppm)									
Ceramide	H1	H1′	H2	H3	H4	H5	H6	Hα	Hω	Base
Free ceramides										
Cer1	4.23	3.96	4.39	5.20	5.30	5.71	2.05	2.10	3.99	Sphingosine
Cer2	4.23	3.96	4.38	5.20	5.32	5.72	1.97	2.08	–	Sphingosine
Cer3	4.22	3.92	4.41	5.03	4.85	1.55	n.o.	2.13	–	Phytosphingosine
Cer4	4.22	4.00	4.48	5.35	5.62	5.73	5.18	2.18	4.05	6-OH-sphingosine
Cer5*	4.33[a]	3.90[a]	4.35[a]	5.23	5.33	5.75	1.95	5.05	–	Sphingosine
Cer5**	4.31[a]	3.93[a]	4.34[a]	5.21	5.32	5.73	1.95	5.03	–	Sphingosine
Cer6	4.28	3.95	4.39	5.04	4.88	n.o.	n.o.	5.02	–	Phytosphingosine
Cer7	4.27	3.91	4.37	5.33	5.57	5.67	5.13	5.05	–	6-OH-sphingosine
Cer8	4.17	3.94	4.42	5.28	5.57	5.65	5.12	2.10	–	6-OH-sphingosine
Cer9	4.30	4.00	4.50	5.12	4.94	1.62	n.o.	2.20	4.04	Phytosphingosine
Protein-bound ceramides										
CerA	4.24	3.97	4.39	5.22	5.33	5.73	2.03	2.10	4.00	Sphingosine
CerB	4.18	3.94	4.40	5.27	5.56	5.65	5.10	2.10	3.97	6-OH-sphingosine

n.o. Signal overlaps with bulk of CH_2 resonances and/or impurities in the high field region, and could therefore not be observed.
[a]Chemical shifts are approximate, due to strong coupling of H1 and H2.
The N-H signal can be observed in all spectra. Because the chemical shift strongly depends on solvent conditions, the chemical shift is not given in this table. In the spectra of ceramides 1, 4, and 9 also the characteristic signals of the double bonds of linoleic acid (5.27 ppm) and of the CH_2 α to the ester function (2.2–2.3 ppm) are observed.

fraction X, which has HPTLC mobility between ceramides 2 and 3 (**Fig 1**). The sphingoid bases in covalently bound ceramides from reconstructed epidermis are the same as that found in native tissue (Wertz *et al*, 1989a). Ceramide A contains sphingosine and ceramide B contains 6-hydroxysphingosine. Owing to its low content, the third minor component (probably containing phytosphingosine[1]) could not be analyzed in this study. As the nomenclature introduced by Motta *et al* (1993) clearly indicates the molecular structure of individual ceramides, it will be used in the following paragraphs.

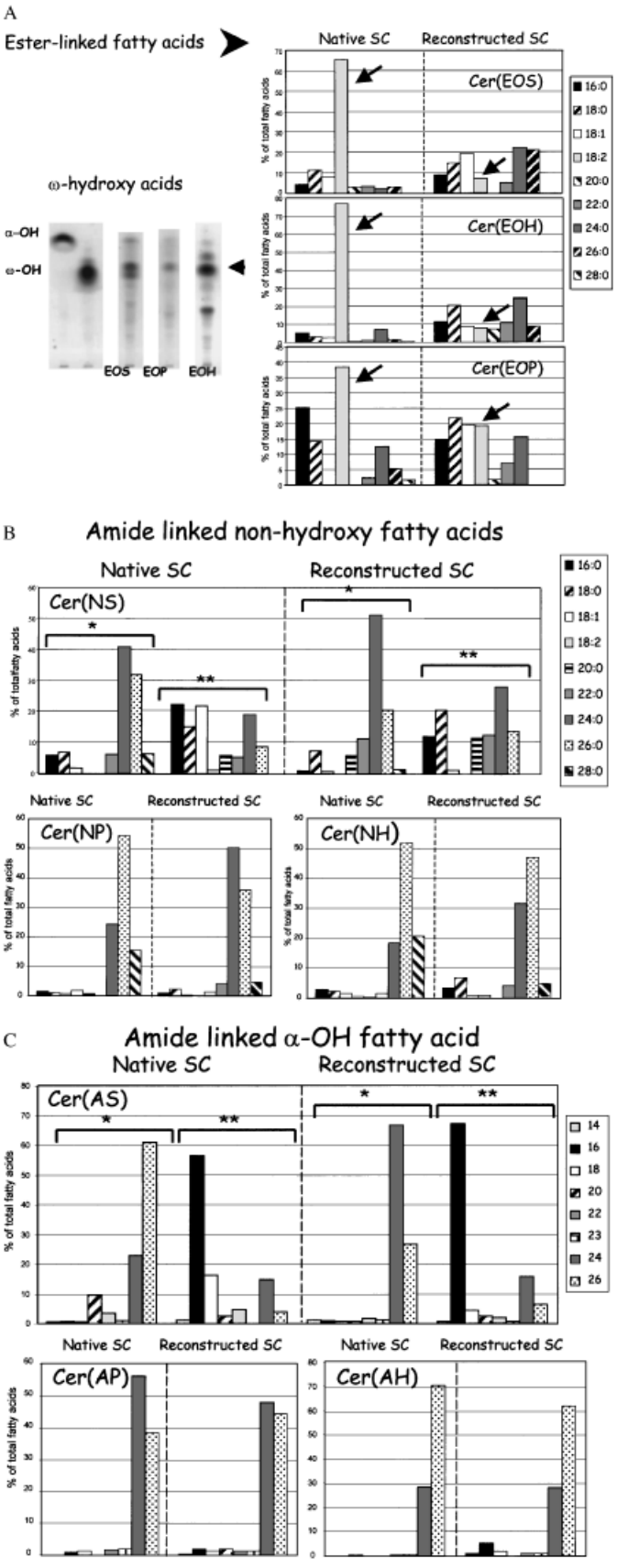
Identification of acetyl derivative of new ceramide The identity of the acetyl derivative of the new ceramide was established by means of 600 MHz NMR. NMR is an extremely useful tool for structural elucidation of organic compounds. With the advent of modern high-field instruments operating at 500, or 600 MHz or higher frequency, sensitivity has increased tremendously. Moreover, the use of two-dimensional techniques has enormously facilitated the assignment of NMR spectra, and hence, the identification of unknown compounds. The most often used two-dimensional NMR experiments have been reviewed extensively by, e.g., Croasmun and Carlson (1994) and Braun *et al* (1998).

Fatty acid profiles of individual ceramide fractions To compare the fatty acid profiles in reconstructed and native epidermis, the individual ceramide fractions were subjected to transmethylation with boron trichloride in methanol followed by purification of the FAME by column chromatography. In this way fractions containing methyl esters of either nonhydroxy or hydroxy fatty acids have been collected. These fractions were subsequently analyzed by HPTLC and gas chromatography. As shown in **Fig 3**(***A***), three different fractions contained ω-hydroxy fatty acids and ester-linked fatty acids (EO). These fractions contained sphingosine (S), phytosphingosine (P), or 6-hydroxysphingosine (H) as the base component. Whereas in native epidermis these acylceramide fractions contained high amounts of linoleic acid [up to 70–80% in Cer(EOS) and Cer(EOH) and about 40% in Cer(EOP)], in reconstructed epidermis the linoleic acid content was much lower (10–20%) in all acylceramide fractions, and the content of $C_{18:0,18:1}$ and of long chain $C_{24,26}$ fatty acids was increased. As established by gas chromatography, the majority of ω-hydroxy fatty acids in all three acylceramide fractions had a chain length of C_{30} (about 75%) next to a smaller fraction with C_{32} (about 15%). The amide linked nonhydroxy fatty acids (N) were found in three ceramides Cer(NS), Cer(NP), and Cer(NH). As shown in **Fig 3**(***B***), in these ceramide fractions a high degree of similarity has been observed in fatty acid profiles between native and reconstructed epidermis. Fatty acids with chain lengths between C_{24} and C_{28} were the most prominent ones. In both native and reconstructed epidermis two different Cer(NS) fractions [Cer(NS*) and Cer(NS**), respectively] were isolated and characterized. Whereas the major Cer(NS*) fraction contained predominantly long chain fatty acids (about 80% of $C_{24,26}$), the minor fraction Cer(NS**) was substantially enriched with $C_{16,18}$ fatty acids (about 40–55%). Also the profile of amide-linked α-hydroxy fatty acids (A) was similar in both the native and reconstructed tissue (**Fig 3*C***). The short chain α-hydroxypalmitic acid, C_{16}, was the major component in the Cer(AS**) fraction. The Cer(AS*) fraction, which was present only in small amounts in native epidermis, was present in substantial amounts in the reconstructed tissue (about 9% of

Figure 3. Fatty acid profiled in different ceramide fractions. Fatty acid profile of isolated fractions of acylceramides Cer(EOS), Cer(EOP), and Cer(EOH) was established after conversion to methyl esters (FAME) and subsequent purification by column chromatography. The collected fractions were dried under the stream of nitrogen. The fractions containing FAME were analyzed by gas chromatography and the presence of ω-hydroxy acids was established by means of HPTLC. Shown are percentages of total fatty acids in individual ceramide fractions. *Arrow*: linoleic acid. (*A*) Acylceramides in reconstructed epidermis contain long chain ω-hydroxy acids (left panel) and low linoleic content (right panel). (*B*) Less abundant Cer(NS) fraction in reconstructed epidermis is enriched with short chain fatty acids. Note the great similarity in fatty acid profile in Cer(NP) and Cer(NH) between reconstructed and native epidermis. (*C*) Less abundant Cer(AS) fraction in reconstructed epidermis is enriched with long chain fatty acids. Note the great similarity in fatty acid profile in Cer(NP) and Cer(NH) between reconstructed and native epidermis.

[1]Chopart, M, Castiel-Higounenc I, Arbey E, Schmidt R: A new type of covalently bound ceramide in human epithelium. Poster, SC III meeting, Basel, 2001.

total ceramides), and contained predominantly $C_{24,26}$. These long chain α-hydroxyacids also prevailed in the Cer(AP) and Cer(AH) fractions. The profile of free fatty acids showed great similarity between native and reconstructed epidermis (**Fig 4**), in spite of the lower content in the latter.



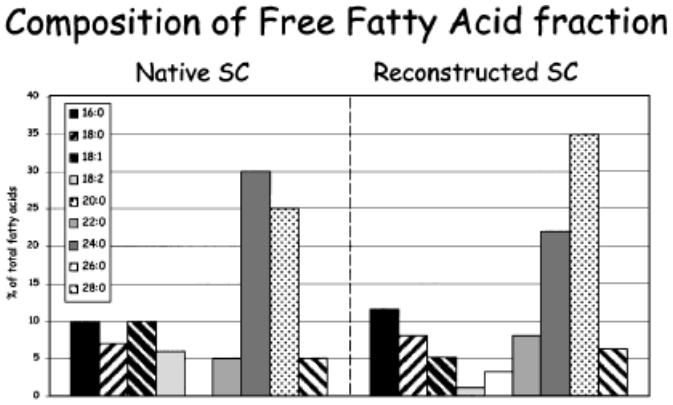


Figure 4. Similarity of profiles of free fatty acids in native and reconstructed epidermis. Fractions containing free fatty acids were pooled and subsequently FAME prepared analyzed by means of gas chromatography. Shown are percentages of total fatty acids.

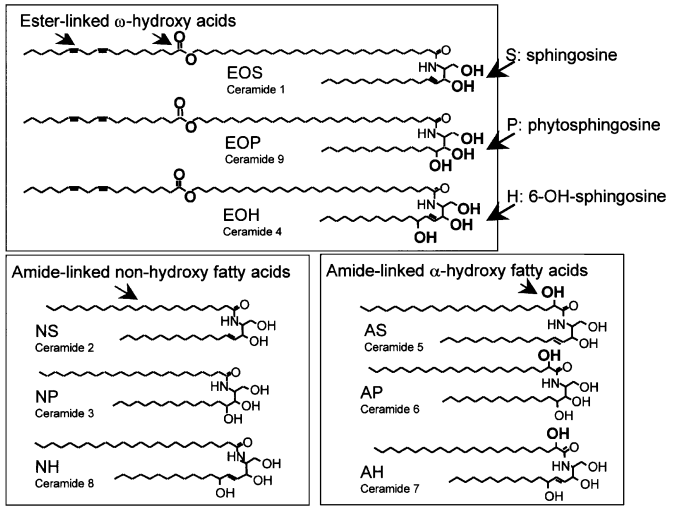


Figure 5. Sphingosine, phytosphingosine, and 6-hydroxysphingosine serve as a base in ceramides containing ester-linked, nonhydroxy and α-hydroxy acids. Molecular structure of individual ceramides is depicted.

DISCUSSION

The results of this study show that in both the native and reconstructed human epidermis the extractable ceramide fraction includes three acylceramides species, in which the ω-hydroxyacid is amide-linked to sphingosine, phytosphingosine, and 6-hydroxysphingosine, respectively. The same sphingoid moieties serve as bases in ceramides with amide-linked nonhydroxy and α-hydroxyacids (**Fig 5**). Also, lipids covalently bound to the cornified envelope contained these sphingoid bases (this study; Wertz *et al*, 1989a; Chopart *et al*, 2001[1]). These findings suggest that common pathways are involved in the biosynthesis of epidermal ceramides in native epidermis and in culture.

In contrast to the most polar ceramides, the synthesis of Cer (EOP) *in vitro* was not modulated by vitamin C and by serum, as this ceramide fraction was clearly detectable in lipids extracted from cultures grown in earlier studies in vitamin C-deficient and serum containing media (data not shown; Gibbs *et al*, 1997; Ponec *et al*, 1997). Re-inspection of lipid profiles recently analyzed by HPTLC of tape strips collected from healthy human volunteers (Weerheim and Ponec, 2001) revealed the presence of Cer(EOP) in most of the harvested samples. As for other ceramide species, no significant change of the relative Cer(EOP) content

throughout the whole SC has been noticed. Similar observations have been made earlier by Lavrijsen *et al* (1994) in lipid samples collected by sequential scraping of SC in human volunteers. In that study the presence of Cer(EOP) was further detected in lipids collected by topical extraction of SC in human volunteers and in lipids extracted from isolated human SC with a chloroform/methanol mixture. The presence of Cer(EOP) can be deduced from thin-layer chromatograms of lipids extracted from the SC that was stripped by cyanoacrylate resin (Imokawa *et al*, 1991). In addition, reduction in Cer(EOP) content has been noticed by Lavrijsen *et al* (1995) in patients with lamellar ichthyosis. The presence of acylceramide containing phytosphingosine has been suggested by Vietzke *et al* (2001) and very recently by Chopart *et al*.[2]

Presumably, all three acylceramides are derived from glycosylated precursors. Glucosyl-EOS is well known, and a second acylglucosylceramide has been reported to exist in human epidermis (Hamanaka *et al*, 1989). Although the structure of this second human acylglucosylceramide was not completely deduced in the original report, it is apparent from the published NMR spectrum that it is Cer(EOH). Evidence for glycosyl-EOP is yet to be produced.

Glucosyl-EOS has been shown to be present in relatively high proportions in isolated lamellar granules (Madison *et al*, 1998), and evidence has been presented indicating that this molecule may be involved in assembly of the lamellar contents of the granule (Landmann, 1988). It has also been proposed that a high proportion of the lamellar granule-associated glucosyl-EOS is actually in the bounding membrane of the granule. This pool of glucosyl-EOS is presumed to serve as the source of the ω-hydroxyceramide that becomes covalently attached to the outer surface of the cornified envelope. Subtle differences among the fatty acid compositions and patterns of hydroxylation among Cer(EOS), Cer(EOH), and Cer(EOP) may determine which molecular species partition into the bounding membrane and which associate with the internal lamellae. Consistent with this view, the reported ratio of covalently bound OS/OH in human SC is 2:1 (Wertz *et al*, 1989a); however, the ratio of Cer(EOS) to Cer(EOH) in this study appears to be much greater.

Using electron microscopy it has been established that the SC lipids are organized in lamellar sheets (Madison *et al*, 1987) oriented approximately parallel to the corneocyte surface. These lipid lamellae have an unusual repeating pattern consisting of a broad–narrow–broad sequence of electron lucent bands. The exceptional lipid composition results in the predominant formation of a crystalline sublattice in the lamellar sheets as established by X-ray diffraction studies (White *et al*, 1988; Bouwstra *et al*, 1991). It has been demonstrated that in human SC two lipid lamellar phases with periodicities of approximately 13 nm and 6 nm are present (Bouwstra *et al*, 1991). As the 13 nm phase has been present in all species examined and is thought to reflect an unusual lipid arrangement (Bouwstra *et al*, 2000), this phase has been considered to be very important for the permeability barrier of the skin.

Based on studies with mixtures of isolated SC lipids a model recently has been proposed for the molecular organization of the long periodicity phase (Bouwstra *et al*, 2000). In this model the lipids are organized in three layers: a narrow layer that is located in the center with broad layers on both sides. Cer(EOS) links this tri-layer unit together and consequently dictates the broad–narrow–broad sequence in the tri-layer unit (Bouwstra *et al*, 1998b). The two broad layers are formed by ceramides with the long chain fatty acids (predominantly C_{24} to C_{26}) and by cholesterol, whereas the narrow low-electron density region is formed by the short chain ceramides (predominantly C_{16}). In the central layer most probably the unsaturated linoleate present on Cer(EOS) and cholesterol together with Cer(AS) containing predominantly C_{16} acyl chain are located, whereas the ceramides with long saturated hydrocarbon chains are present in the adjacent layers. The finding that in native epidermis Cer(AS) is predominantly a short chain ceramide differs from earlier published data (Wertz *et al*, 1985, 1987). This may be explained by the use in earlier studies of a mixture of at least two ceramides for fatty acid analysis. The presence of a significant amount of Cer(AS) with long hydrocarbon chains [Cer(AS*)] in reconstructed epidermis may also account for the differences in lamellar lipid organization between the native and reconstructed tissue. Furthermore, the content of linoleic acid in all three acylceramides—Cer(EOS), Cer(EOH), and Cer(EOP)—is much lower in the reconstructed epidermis than in native tissue. At present it is unclear why the incorporation of linoleic acid into ceramides *in vitro* is less efficient than *in vivo*, in spite of supplementation of culture media with this essential fatty acid (Ponec *et al*, 1997; Vicanova *et al*, 1999). The differences in linoleic acid content and enrichment of the Cer(NS) fraction with short chain fatty acids [fraction Cer(NS**)], lower content of free fatty acids, and the presence of significant amounts of long chain Cer(NS**) may explain the differences in SC lipid organization between native and reconstructed epidermis. Namely, using small angle X-ray diffraction, it has been established that in reconstructed SC only the long lamellar phase of about 12 nm is formed and the short lamellar phase is missing (Ponec *et al*, 1997). Furthermore, using wide angle and electron diffraction techniques it has been established that the lateral packing in native SC lipids is predominantly orthorhombic, whereas in reconstructed SC hexagonal packing prevails (Ponec *et al*, 2000). The latter could be ascribed to differences in fatty acid profile and to the reduced free fatty acid content in the reconstructed tissue. These differences may have implications for the observed impaired barrier function of reconstructed epidermis. The important role of fatty acids on SC barrier function and lipid organization has been demonstrated in various *in vivo* and *in vitro* studies. The reduction of free fatty acid content in patients with lamellar ichthyosis resulted in changes in both lamellar and lateral SC lipid organization (Lavrijsen *et al*, 1995; Pilgram *et al*, 2001). From studies performed with isolated SC lipid mixtures it became clear that their phase behavior was dependent on the fatty acid chain length, as only in the presence of long chain fatty acids appropriate lipid lamellar and lateral organization has been noticed. (Bouwstra *et al*, 1996, 1998a). Also *in vivo*, deviations in lipid composition in patients with various skin disorders are accompanied by impairment in SC barrier function. Yamamoto *et al* (1991) found in patients with atopic dermatitis a significant decrease in the proportion of Cer(EOS) in which levels of oleic acid esterified to Cer (EOS) increased. Similar observations have been made by Rogers *et al* (1996) in aged skin during the winter months. A general decrease in the total ceramides in nonlesional skin of atopic eczema patients accompanied by a significant reduction of Cer(EOS) and Cer(NP) has been demonstrated in several studies (Imokawa *et al*, 1991; Yamamoto *et al*, 1991; Di Nardo *et al*, 1998; Bleck *et al*, 1999). Deficiencies in Cer(EOS) and Cer(NP) have been also detected in psoriatic scale (Motta *et al*, 1993). The reduction in relative contents of Cer(NS), Cer(EOP), and Cer(EOH) and increase in Cer(AS) was reported in patients with lamellar ichthyosis (Lavrijsen *et al*, 1995). Fulmer and Kramer (1996) reported an increase in Cer(NS) and Cer(EOH) and a decrease in Cer(NP) and free fatty acid contents in surfactant-induced dry scaly skin. Furthermore, age- and sex-dependent changes in ceramide profile have been observed by Denda *et al* (1993). The presence of two distinct Cer(AS) subfractions containing either ($C_{16,18}$) or C($_{22,24,26}$) α-hydroxyacids has also been noticed in nonlesional skin of atopic eczema (Bleck *et al*, 1999). The double peak in Cer(AS) subfractions has also been noticed earlier by Wertz *et al* (1985).

Structural details of the different acylceramide species as well as the ω-hydroxyceramide may be related to their functions. In this regard, it has been suggested that the covalently bound lipid may serve to provide cohesion between the ends of adjacent corneocytes (Wertz *et al*, 1989b) as well as providing a template upon which the broad lamellae of the SC intercellular spaces

[2]Chopart M, Castiel-Higounenc I, Arbey E, Schmidt R: The normal human SC: A new ceramide profile. In: Brain KR, Walters KA (eds) *Prediction of Percutaneous Penetration*, Vol.8a, 2002, p 35.

form (Wertz and van den Bergh, 1998). The acylceramide species in the intercellular spaces of the SC are most likely involved in determining the state of organization of the lipids and the barrier function of the skin. It has been shown that Cer(EOS) promotes formation of a 13 nm trilaminar structure as judged by either X-ray diffraction (Bouwstra *et al*, 1998b) or by transmission electron microscopy (Kuempel *et al*, 1998). The observation that removal of EOS did not result in a complete disappearance of the 13 nm phase (Bouwstra *et al*, 1998b, 2002) indicates that EOH or EOP or both may also serve in organizing the intercellular lipids into the trilaminar repeat units. That these units are important for barrier function is supported by several observations: In some skin disorders in which barrier function is impaired, the 13 nm trilaminar units are not observed (Fartasch, 1997). In addition, in keratinized oral epithelium, the proportion of acylceramide is much lowered, and the trilaminar structures are not observed (Law *et al*, 1995). Keratinized oral tissue is an order of magnitude more permeable than the skin (Law *et al*, 1995). Adding an extra hydroxyl group in going from EOS to EOP or adding both a hydroxyl group plus a double bond in going to EOH effectively makes the polar head group region of the molecule larger. This will require greater motion of the aliphatic chains beneath the polar head group, which would make the membrane domain more fluid and, thereby, more permeable. Whereas highly ordered membrane domains will be favorable for impermeability, some degree of fluidity is also necessary for flexibility. The three acylceramides may be necessary to balance the requirement for a relatively impermeable skin with the need for flexibility.

In conclusion, the use of column chromatography to isolate individual SC lipids has enabled identification of minor ceramide fractions, the presence of which is often obscured by more prominent ceramide fractions when HPTLC is used. This approach has made it possible to isolate from both native and reconstructed epidermis the new acylceramide Cer(EOP) (referred also as ceramide 9) and both Cer(NS) and Cer(AS) subfractions. In addition, the isolation of Cer(EOH) enabled the establishment of its presence in reconstructed epidermis, identification of which was difficult with the HPTLC method due to a prominent Cer(AS) subfraction with an almost identical polarity.

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