Stratum Corneum Lipid Composition and Structure in Cultured Skin Substitutes is Restored to Normal after Grafting onto Athymic Mice

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Restoration of an epidermal barrier is a definitive requirement for wound closure. Cultured skin substitutes grafted onto athymic nude mice were used as a model for a long-term study of stratum corneum barrier lipid metabolism and organization. Samples of stratum corneum collected after 12 and 21 d *in vitro* and 6, 11, and 24 mo postgrafting were examined for their lipid and fatty acid composition, and their lipid organization and structure using electron microscopy and small angle X-ray diffraction, respectively. All of these methods confirm the impaired barrier function of cultured skin substitutes *in vitro*, as judged from the deviations in lipid composition and from poor organization of the stratum corneum lipids that show no lamellar structure. At 6 mo postgrafting, the total stratum corneum lipid profiles of the epidermal grafts is close to that of the human stratum corneum with the exception of the presence of mouse specific lipids. The increase of ceramides 4–7 in cultured skin substitutes after grafting indicates restored activity of processes involved in the hydroxylation of fatty acids and sphingoid bases. Conversely, the ceramide profile still reveals some abnormalities (elevated content of ceramide 2 and slightly lower content of ceramide 3) and the content of long-chain fatty acids remains below its physiologic level at 6 mo postgrafting, but normalizes by 2 y postgrafting. The ultramicroscopic observations revealed the formation of lamellar extracellular lipid domains by 4 mo postgrafting. Despite these findings, the X-ray diffraction showed differences in the diffraction pattern at 2 y after grafting, suggesting that the organization of stratum corneum lipids in all epidermal grafts differs from that of the native skin. ***Key words: barrier function/transplantation/X-ray diffraction. Journal of Investigative Dermatology Symposium Proceedings 3:114–120, 1998***

The primary function of the skin is the formation of a highly impermeable protective barrier, the stratum corneum (SC). For the SC to function as a permeability barrier, generation of lipid-enriched intercorneocyte lamellae is required. The SC lipids consist mainly of ceramides and neutral lipids such as free cholesterol, cholesterol esters, and free fatty acids (reviewed in Schürer and Elias, 1991; Wertz and Downing, 1991). Small amounts of glucosphingolipids, cholesterol sulfate, triglycerides, squalene, and n-alkanes were also found in the SC (the latter originates from exogenous sources) (Lampe *et al*, 1983). The quantitatively dominant SC lipids, ceramides, cholesterol, and free fatty acids were shown to be present in an approximately equimolar ratio (Man *et al*, 1993). The ceramides differ in their molecular structures and are composed of various types of long-chain hydrophobic sphingosine bases and mainly long-chain acids (Wertz and Downing, 1983; Wertz *et al*, 1985; Robson *et al*, 1994). Seven ceramide classes were shown to be separated by thin-layer chromatography in pig (Wertz and Downing, 1983) and human (Wertz *et al*, 1985; Robson *et al*, 1994) SC. The ceramide classes differ in their proportions and their profiles are a critical determinant for the SC barrier. Abnormal ceramide patterns are often associated with defective barrier function, e.g., oleate-linoleate substitution in sphingolipids occurs in association with defective barrier in essential fatty acid deficiency (Wertz *et al*, 1983).

In recent years, attention has been focused on the development of skin substitutes to provide biologic materials for the treatment of acute and chronic wounds, and to provide models permitting toxicologic and pharmacologic studies *in vitro*. Although most of these cultured substitutes exhibit morphologic and biochemical features similar to native skin, barrier function of these analogs is significantly deficient in direct comparisons (Ponec *et al*, 1990; Régnier *et al*, 1990; Mak *et al*, 1991; Simonetti *et al*, 1995), and the epidermal lipids show abnormalities in composition (reviewed in Ponec, 1994). The organization of intercorneocyte lipid structures in cultured epidermis also deviates in a number of features from the native tissue. It displays abnormalities in the lamellar body delivery system, transformation of lamellar bodies into lamellar lipid bilayers, and an impaired structural organization and distribution of SC lipids in the intercellular space (Fartasch and Ponec, 1994; Bouwstra *et al*, 1995).

Human skin grafted onto athymic mice has long been considered an attractive *in vivo* model for understanding processes related to epidermal proliferation and differentiation to allow preparation *in vitro* of a more physiologic and stable skin substitute (Demarchez *et al*, 1986, 1987; Boyce *et al*, 1991; Higounenc *et al*, 1994a). It was shown that cultured keratinocytes grafted onto athymic mice retain the capability of generating a well-differentiated epidermis exhibiting epidermal lipid profile and structure comparable with those of normal human skin and competent SC barrier (Higounenc *et al*, 1994b; Boyce *et al*, 1996).

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Abbreviations: CSS, cultured skin substitute; FAME, fatty acid methyl ester; HPTLC, high performance thin layer chromatography; SAXD, small angle X-ray diffraction; SC, stratum corneum.

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Some deviations in ceramide profile and fatty acid composition of epidermal lipids, however, were also seen at 4 mo after grafting (Vičanová *et al*, 1997). In this study, cultured skin substitutes (CSS) were examined between 6 mo and 2 y after grafting to athymic mice for their SC lipid composition and organization of intercellular lipid lamellar domains. We show that although the SC lipid composition and ultrastructural organization closely resembles that seen in the native human SC, the X-ray diffraction studies suggest abnormalities in the SC lipid organization.

MATERIALS AND METHODS

Keratinocyte cultures and grafting technique CSS were prepared as described in previous studies (Boyce *et al*, 1988, 1995; Boyce and Williams, 1993). Secondary cultures of human keratinocytes (isolated from surgical discard of split-thickness skin from a pediatric donor) were used to prepare CSS by inoculation onto collagen-glycoaminoglycan substrates populated with human fibroblasts. CSS were incubated for 3 d submerged in supplemented MCDB 153 medium, lifted to the air–liquid interface, and incubated for another 12 or 21 d. Grafting to athymic mice was performed at day 14 of incubation.

Animal studies were approved by the University of Cincinnati, Institutional Animal Care and Use Committee. Grafting to athymic mice was performed as described previously (Boyce *et al*, 1991, 1996). Tissue samples were collected after 12 and 21 d *in vitro* and at 6, 11, and 24 mo postgrafting [samples from 1 mo for small angle X-ray diffraction (SAXD) analysis and from 4 mo postgrafting for transmission electron microscopy were also included].

Isolation of SC The human SC was obtained from healthy young donors undergoing surgical corrections (most often breast corrections). To separate epidermis from the underlying tissue, the pieces of human skin and cultured skin substitutes were first incubated for 2 h in 0.1% trypsin in phosphate-buffered saline (PBS). Thereafter the tissue was washed with PBS and the trypsinization was stopped by a short incubation in PBS containing soybean trypsin inhibitor (10 mg per ml). Subsequently, the epidermis was washed several times with PBS and reincubated for 2 h in 0.01% proteinase K in PBS (Bowser and White, 1985). The isolated SC was extensively washed with PBS and distilled water.

Lipid extraction and separation The extraction and separation was performed as previously described by Ponec and Weerheim (1990). SC lipids were extracted using the method of Bligh and Dyer (1959), with the addition of 0.25 M KCl to extract polar lipids, and stored in chloroform:methanol (2:1, vol/vol) under nitrogen at −20°C until use. The extracted lipids were separated by one-dimensional high performance thin layer chromatography (HPTLC) on 20 × 10 cm glass plates coated with silica gel (Kieselgel 60, Merck, Darmstadt, Germany) using the "ceramide development system."

Serial dilutions of appropriate lipid standards were used for quantitation. The quantitation was performed after staining (copper acetate and copper sulfate in phosphoric acid) and charring, using a photodensitometer (Shimadzu CS-9000) with automatic peak integration (Shimadzu FDU-3). Data were expressed as a mean of three different samples ± SD (MicroCal Origin 3.0).

Preparation and analysis of fatty acid methyl esters (FAME) One hundred micrograms of the total lipid extract were dissolved in 100 μl of toluene and transmethylated in 1 ml BCl_3/methanol (10%) using microwave irradiation, which was carried out at the lowest power setting (85 W) for 4 h. FAME were dissolved in hexane and purified on a silica gel column. FAME and α-hydroxy-FAME fractions were separated and analyzed on a Vega GC 6000 gas chromatograph (Carlo Erba Instruments, Italy) using capillary column CP Wax 52 (Chrompack, The Netherlands). An initial temperature of 80°C was increased to 160°C at a rate of 40°C per min followed by a 2°C per min increase to 250°C, which was maintained until all peaks had eluted. The peaks were identified by comparison with FAME and α-hydroxy-FAME standards (Sigma). Integration of peak areas and calculation of relative percentage were performed by Baseline 810 integrator. Heptadecanoic acid was used as the internal standard. To determine fatty acid composition of mouse specific lipids, fractions of these lipids after separation were scraped off the HPTLC plate, extracted, and transmethylated, as described above. Data were expressed as a mean of two different samples ± SEM (MicroCal Origin 3.0).

SAXD The isolated SC was dried under vacuum and stored under nitrogen gas in the dark until analyzed. Before analysis, the SC was hydrated for 24 h over 27% NaBr solution, which resulted in an approximate hydration level of 20% [(weight hydrated SC − weight dry SC)/(weight hydrated SC)]. All measurements were carried out at the Synchrotron Radiation Source at Daresbury Laboratory using station 8.2. This station has been built as part of a NWO/SERC agreement. The small angle camera was connected with a position sensitive multiwire quadrant detector. The sample to detector distance was set to 1.40 m. A more detailed description of the experimental set-up has been given elsewhere (Bouwstra *et al*, 1991). The SC, ≈3 mg in weight, was put randomly in a specially designed sample cell with two mica windows. The measurements were performed at room temperature. Two to three samples were measured for each time point.

The scattering intensities have been plotted as a function of the scattering vector Q defined as $Q = 4\Pi\sin\theta/\lambda$ in which λ and θ are the wavelength and scattering angle, respectively. The positions of the diffraction peaks are directly related to the repeat distance of the molecular structure, as described by Braggs law $2d\sin\theta = n\lambda$, in which n is the order of the diffraction peak. In the case of a lamellar structure the various peaks are located at equal interpeak distances, $Q_n = 2n\Pi/d$, Q_n being the position of the nth order peak.

Transmission electron microscopy Ultrastructure of the intercorneocyte lipid lamellae was examined after preparation of samples for transmission electron microscopy. Skin was fixed immediately after excision in 2% glutaraldehyde/2% formaldehyde in 0.1 M cacodylate buffer. Post-fixation was performed with 1% (wt/vol) osmium tetroxide followed by (wt/vol) ruthenium tetroxide (0.25%) (Swartzendruber *et al*, 1989) followed by embedment in MedCast-Araldite resin and ultramicrotomy. Microscopy and photography were performed with a JEOL 100 CX transmission electron microscope.

RESULTS

The composition of overall SC lipids in CSS normalizes after transplantation onto athymic mice SC lipids extracted from CSS after 12 and 21 d in culture, 6, 11, and 24 mo after grafting, and from human and murine skin were separated corresponding to their polarity by HPTLC using the "ceramide development system" (Ponec and Weerheim, 1990) (**Fig 1**). Amounts of individual fractions, determined according to appropriate calibration curves and expressed as a weight percentage of all selected lipids, are summarized in **Table I**. In the human SC, ceramides and free fatty acids represent more than 20% of total lipids each, cholesterol and cholesterol ester are present in slightly lower quantities (about 18%). A different lipid profile was obtained for CSS *in vitro*. CSS incubated in culture for both 12 and 21 d contained higher levels of triglycerides and lower levels of cholesterol esters and free fatty acids. Prolongation of culture time did not modulate lipid synthesis. The results show normalization of the SC lipid profile by 6 mo after grafting. Samples collected after 11 and 24 mo revealed great similarity with lipid profile of the native human SC, although an elevated level of triglycerides was detected at 11 mo postgrafting. Compared with human SC, the murine SC contained a higher level of cholesterol esters (30%). Mouse specific fraction represented about 30% of SC lipids in murine SC and about 20% in CSS grafts.

Normalization of ceramide profile in grafted CSS requires longer than 6 mo after transplantation onto athymic mice The chromatographic separation of the ceramides is shown in **Fig 1**, where the ceramides are separated into major fractions and identified according to their mobility, comparable with that of standards obtained from pig epidermis (kindly provided by P. Wertz, College of Dentistry, University of Iowa, USA). With this approach seven different ceramide fractions detected in native human SC, but only four major fractions in the murine SC. In the human SC, ceramides 2, 3, and 5 accounted for about 20%, ceramide 7 for 14%, and ceramides 1, 4, and 6 for about 6%–8% (**Table II**). Analysis of lipids extracted from SC of CSS incubated *in vitro* for 12 or 21 d revealed the absence of ceramides 6 and 7 and a low level of ceramide 5, whereas ceramide 2 was the major ceramide present (58%). Six months postgrafting, both ceramide 6 and ceramide 7 reached normal levels, but the content of ceramide 2 was still above the normal value, and it normalized at later time points. Unlike the human skin, murine SC contained very high levels of ceramide 2 (67%), 11% of ceramide 4, and 14% of ceramide 5. Other ceramide classes were present in trace amounts. Slightly different mobility of some murine ceramides as compared with the standards suggests differences in molecular structure of individual ceramides, which remains to be established. Similar ceramide profiles were reported for neonatal BALB mouse (Madison *et al*, 1989), athymic mouse (Higounenc *et al*, 1994a), and hairless mouse (Lavrijsen, 1997).

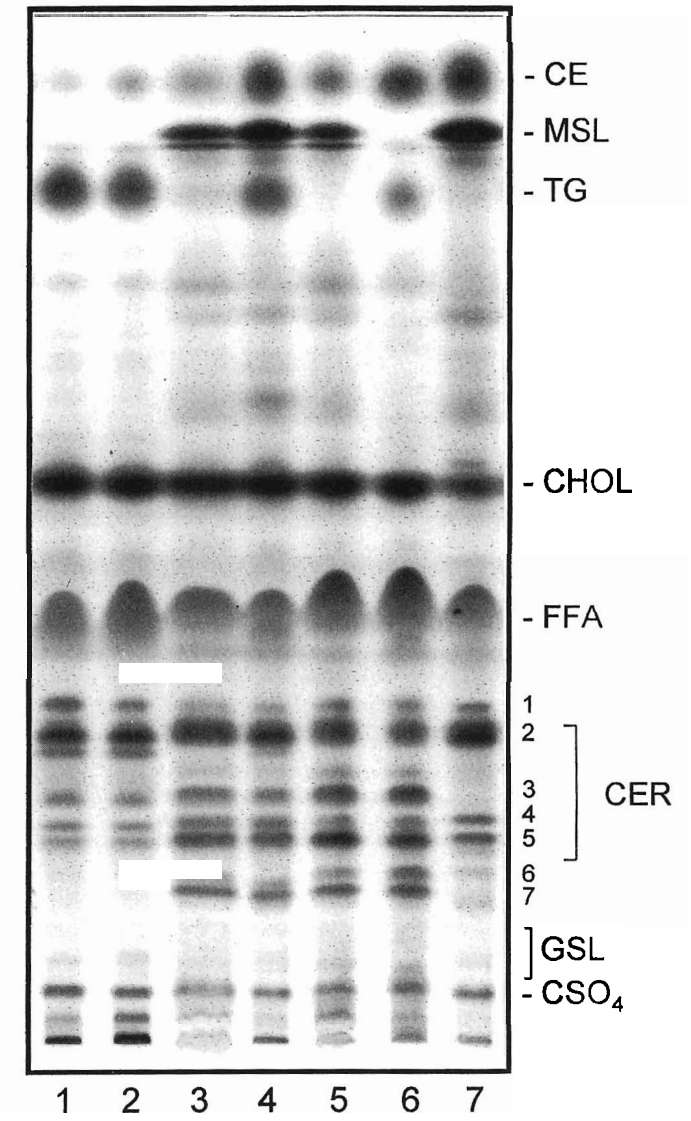

Figure 1. SC lipid profile separated by thin layer chromatography. SC lipids extracted from CSS after 12 (*lane 1*) and 21 (*lane 2*) d *in vitro*, 6 (*lane 3*), 11 (*lane 4*), and 24 (*lane 5*) mo after grafting, and from human (*lane 6*) and murine (*lane 7*) skin were separated corresponding to their polarity by HPTLC. CSO_4, cholesterol sulfate; GSL, glucosphingolipids; CER, ceramides (1–7); FFA, free fatty acids; CHOL, cholesterol; TG, triglycerides; MSL, mouse specific lipids; CE, cholesterol esters.

Fatty acid synthesis in grafted CSS recovers human profiles between 1 and 2 y after transplantation onto athymic mice Twenty-six FAME were identified and quantitated by gas–liquid chromatography in different SC samples after their transmethylation. Data presented as weight percentages are given in **Figs 2** and **3**. Changes in C18 profile of CSS *in vitro* and after its grafting onto athymic mice are shown in **Fig 2**. The relative amount of oleic acid (18:1) (originated from triglycerides) significantly decreased from 30% to 40% *in vitro* to 6.1 ± 1.3% by 6 mo and to 3.5% by 24 mo after grafting. The level of oleic acid is 4.7 ± 0.9% for human SC and 4.4% for murine SC. A decrease in stearic acid (18:0) after grafting was also seen, it comprised 9.3 ± 0.8% of the total fatty acids in CSS cultured for 12 d and 3.3 ± 0.4% by 6 mo postgrafting, compared with 2.3 ± 0.3% in human and 2.3% in murine SC. The level of linoleic acid (18:2) increased from 1.3 ± 0.6% *in vitro* to 5.4 ± 1.3% by 11 mo after grafting, the value not significantly differing from that detected in human SC (4.6 ± 1.2%).

Changes in levels of saturated and unsaturated FAME containing more than 20 carbon atoms that are summarized according to their carbon number are shown in **Fig 3**. Levels of C20 and C22 fatty acids after grafting reached higher values than those detected in native human SC (1 ± 0.3% for C20:0 and 3.5 ± 0.5% for C22:0). The relative amount of C24 remained unchanged at all time points, being about 25% of the total fatty acids, which is slightly lower than the 30% present in the human SC. On the other hand, the relative amount of long-chain fatty acids C25–30 increased after grafting and approximated 50% by 24 mo postgrafting, reaching values comparable with those in the human SC (53.0 ± 2.4%). Murine skin, however, contains only 19.4 ± 1.3% of C25–30.

Because ceramides 4–7 contain α-hydroxy acids (Wertz and Downing, 1983), α-hydroxy acid composition of CSS *in vitro*, after grafting, and in native human and murine SC was examined. In the human SC, α-hydroxy 24:0 and α-hydroxy 26:0 account for the majority of 39.7 ± 1.3% and 37.2 ± 0.6%, respectively, of the total α-hydroxy acids. In the murine SC only low levels of α-hydroxy 16:0 and α-hydroxy 23:0 were detected. A very low content of total α-hydroxy acids was present in CSS *in vitro*. Upon transplantation of the CSS the total α-hydroxy acid content increased (**Fig 4**) in parallel with the normalization of ceramide 4–7 synthesis (**Table II**). The profile of α-hydroxy 24:0 and 26:0 normalized in about 4 mo postgrafting, accounting for 35.6 ± 1.5% and 36.6 ± 2.4%, respectively, of total α-hydroxy acids. These values remained unchanged at later time points (24 mo after grafting), being 33.3 ± 0.8% and 41.1 ± 2.6%, respectively, and were similar to those found in the human SC.

The mouse specific lipids contain mainly saturated and monounsaturated medium–long chain fatty acids (18:1, 20:0, 20:1, 22:0, and 22:1) (Table III) The presence of these fatty acids in mouse specific lipids may explain their elevated levels in samples containing mouse specific lipids (murine SC and CSS grafted to mice) as compared with the levels found in native human SC (**Fig 3**, C20 and C22).

Intercorneocyte lipid lamellae form after CSS are grafted onto athymic mice Intercellular spaces of SC in CSS before grafting contained lamellar bodies, but intercorneocyte lamellae were infrequent and incomplete, as described in Boyce and Williams (1993). Transmission electron microscopy of the intercellular space of the SC of CSS 4 mo postgrafting revealed multilayered lamellar lipid structures that showed the characteristic alternating electron dense and electron lucent pattern. The repeating pattern of broad-narrow-broad electron-dense bands in healed CSS (**Fig 5*A***) was consistent with the pattern of native human skin (**Fig 5*B***).

Well-ordered SC lipid lamellar phase is formed during the second year after grafting of CSS onto athymic mouse The SAXD curves obtained with SC isolated from CSS incubated *in vitro* and after grafting onto athymic mice and with SC of human and murine epidermis are depicted in **Fig 6**.

The diffraction profiles of SC isolated from native human epidermis (**Fig 6*A***) showed a typical profile as reported by Bouwstra *et al* (1991). Two lamellar structures were found with periodicity of ≈6.4 nm (a short periodicity lamellar phase) and 13.4 nm (a long periodicity lamellar phase), respectively. On the diffraction curve, the third reflection of the long periodicity phase showed a peak corresponding to 4.6 nm spacing. The diffraction pattern of murine SC, however, slightly deviated from that observed in hairless mice (Bouwstra *et al*, 1994). In hairless mice, the lipid lamellar structure was characterized by a repeat distance of 13.4 nm and only occasionally a second lamellar phase was found with a repeat distance of 6.1 nm. In this study, four peaks corresponding to spacings of 12.0, 6.4, 4.6, and 3.2 nm were detected in the athymic mouse SC (**Fig 6*A***), indicating the presence of a long lamellar phase with a periodicity of ≈12.8 nm. Because the experimental peak positions slightly deviated from those expected for a 12.8 nm lamellar phase, the presence of a second phase in the murine SC cannot be excluded.

In diffraction curves obtained with SC isolated from the CSS grown for 12 or 21 d *in vitro*, only a weak shoulder at $Q = 0.98$ nm^{-1} corresponding to a spacing of 6.4 nm was observed (**Fig 6*B***). Four weeks after grafting, only a weak peak at $Q = 1.87$ nm^{-1} was detected,

Table I. Composition of overall SC lipids in CSS after grafting onto nude mice is similar to that of native human skin

Lipid fraction[a]	*In vitro* (d)		Post-grafting (mo)			Human SC	Murine SC
	12	21	6	11	24[b]		
CSO_4	6.8 ± 0.2	6.0 ± 0.0	6.6 ± 0.4	4.1 ± 0.3	7.1	7.6 ± 0.7	4.5 ± 0.6
CER	24.8 ± 0.2	24.0 ± 3.6	35.4 ± 0.1	23.0 ± 1.6	31.6	25.2 ± 1.2	22.8 ± 2.0
FFA	13.5 ± 2.0	16.0 ± 4.2	18.3 ± 0.8	14.9 ± 2.1	24.1	23.8 ± 0.8	20.3 ± 0.8
CH	22.5 ± 1.1	21.7 ± 2.8	21.8 ± 1.4	20.0 ± 0.6	20.6	17.3 ± 3.0	12.7 ± 1.3
TG	27.7 ± 1.1	26.2 ± 0.4	6.6 ± 0.6	13.7 ± 4.5	4.1	7.7 ± 3.1	8.8 ± 0.4
CE	4.7 ± 0.6	6.1 ± 1.8	11.3 ± 1.0	21.3 ± 0.5	12.6	18.4 ± 1.1	30.9 ± 4.5

[a]CSO_4, cholesterol sulfate; CER, ceramides; FFA, free fatty acids; CH, cholesterol; TG, triglycerides; CE, cholesterol esters.
[b]Only one sample was available.

Table II. Grafting of CSS onto nude mouse results in normalization of ceramide profile[a]

Ceramide	*In vitro* (d)		Post-grafting (mo)			Human SC	Murine SC
	12	21	6	11	24[b]		
	15.8 ± 2.6	13.1 ± 0.9	6.6 ± 0.3	7.4 ± 0.1	7.5	6.6 ± 0.6	7.9 ± 0.8
	57.9 ± 1.7	59.7 ± 0.2	32.0 ± 1.2	27.0 ± 1.2	25.8	22.7 ± 0.2	67.1 ± 1.5
	13.8 ± 0.3	14.1 ± 0.5	13.0 ± 0.6	15.0 ± 0.3	16.6	**20.2 ± 1.6**	**tr.**
	6.2 ± 0.2	6.2 ± 0.5	7.8 ± 0.1	8.7 ± 0.2	7.1	6.0 ± 0.8	11.2 ± 2.2
	6.3 ± 0.4	6.9 ± 0.3	18.3 ± 0.2	21.5 ± 0.2	24.5	21.5 ± 1.9	13.8 ± 0.1
	tr.	tr.	7.0 ± 0.7	6.0 ± 0.9	6.3	7.5 ± 1.5	tr.
	0	0	15.3 ± 0.5	13.9 ± 0.8	12.3	14.7 ± 0.8	tr.

[a]The data are expressed as weight percentages of total ceramides; mean ± SD (n = 3). tr = traces.
[b]Only one sample was available.

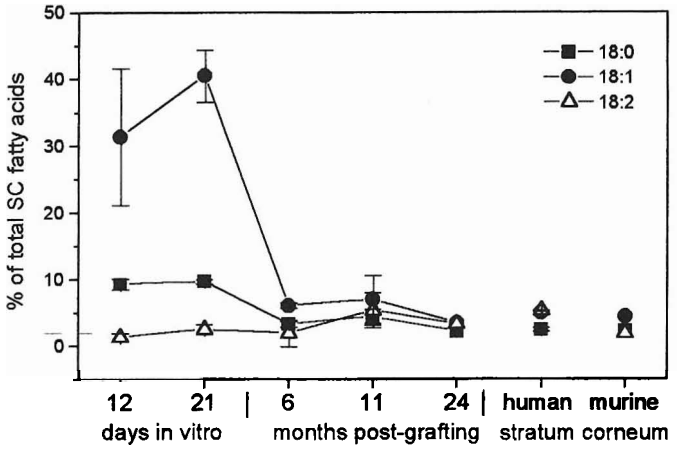

Figure 2. Grafting of CSS induces decrease of oleic acid content in SC lipids. Relative amounts of stearic (18:0), oleic (18:1), and linoleic (18:2) acid in SC isolated from CSS *in vitro* (12 and 21 d), after grafting (6, 11, 24 mo) and in native human and murine SC are present as a percentage of total fatty acid. The fatty acid methyl esters obtained by transmethylation of SC lipids were subjected to gas–liquid chromatography and the amounts of individual fatty acids calculated using C17 fatty acid as an internal standard. *Error bars*, SEM (n = 2).

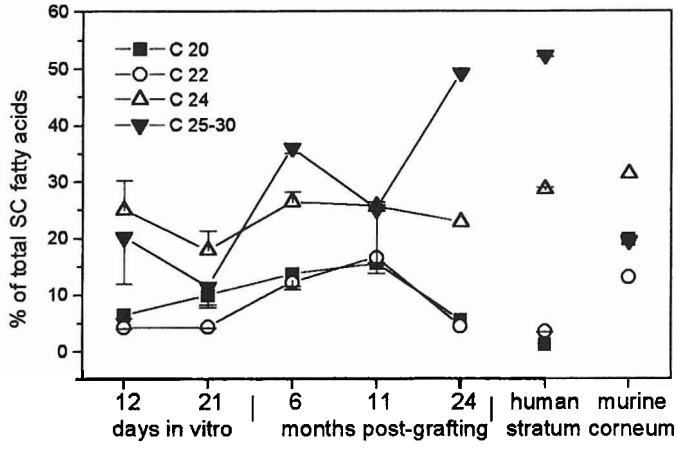

Figure 3. Content of long-chain fatty acids in CSS normalizes within 2 y of grafting onto athymic mice. The SC lipids isolated from CSS *in vitro* (12 and 21 d), after grafting (6, 11, 24 mo), and from native human and murine SC were transmethylated, subjected to gas–liquid chromatography, and the amounts of individual fatty acids were calculated using C17 fatty acid as an internal standard. Fatty acids were grouped according to the number of carbon atoms (C20, C22, C24, C25–30) and their relative amounts summarized. Data are presented as percentage of total fatty acid. *Error bars*, SEM (n = 2).

indicating the presence of polycrystalline cholesterol. The absence of other strong peaks indicates very poor lipid lamellar ordering at this time point. Six months after grafting the SAXD curves exhibited a broad peak at $Q = 1.16\ nm^{-1}$, corresponding to a spacing of 5.4 nm (**Fig 6C**). No higher order reflections of the lipid phase were detected, making it impossible to attribute this peak to a particular lipid phase. A second peak at $Q = 1.87\ nm^{-1}$ (3.36 nm spacing) indicated the presence of polycrystalline cholesterol. Eleven months after grafting the broad peak at $Q = 1.19\ nm^{-1}$ (5.5 nm spacing) remained the only detected peak, suggesting no significant improvement in lamellar arrangement between 5 and 11 mo postgrafting (**Fig 6C**). In contrast, the SAXD pattern of CSS grafted for 24 mo exhibited four strong reflections at $Q = 0.50$, 1.0, 1.43, and $1.99\ nm^{-1}$ corresponding to spacings of 12.6, 6.3, 4.4, and 3.15 nm (**Fig 6C**). These reflections are most probably the first, second, third, and fourth reflections of a lamellar phase with a periodicity of ≈12.6 nm. In addition, a weak peak at $Q = 1.86\ nm^{-1}$ was found with SC isolated 24 mo postgrafting, indicating the presence of phase separated cholesterol.

DISCUSSION

Synthesis of barrier lipids and their arrangement in the extracellular lamellar domains is a very complex and highly regulated process. Similarly to previous studies (Ponec *et al*, 1988; Boyce and Williams, 1993; Fartasch and Ponec, 1994) we show disturbance of this process *in vitro* as the analysis of the SC lipid and fatty acid composition revealed abnormal profiles and the organization of the SC lipids was impaired. Major aberrations of carbohydrate-lipid metabolism are represented by excessive accumulation of triglycerides in a form of

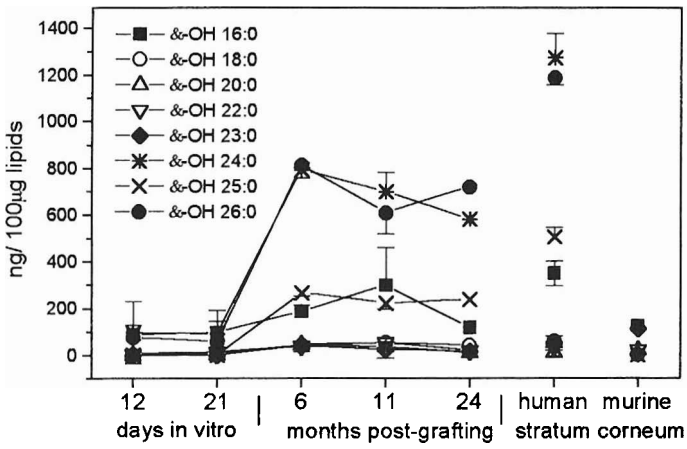

Figure 4. Synthesis of α-hydroxyacids in CSS normalizes after grafting onto nude mouse. The α-hydroxy-FAME fractions were isolated from the total fatty acid methyl ester mixture obtained by transmethylation of SC lipids from CSS *in vitro* (12 and 21 d), after grafting (6, 11, 24 mo), and from native human and murine SC. The data are expressed as ng per 100 µg of total lipids. *Error bars*, SEM (n = 2).

Table III. Fatty acid composition of the mouse specific lipid fraction[a]

Fatty acid	Weight percentage
16:0	4.1 ± 0.8
16:1	tr.
18:0	4.4 ± 0.1
18:1	20.2 ± 0.2
18:2	2.4 ± 0.2
20:0	15.7 ± 0.2
20:1	12.6 ± 0.6
20:2	0.4 ± 0.0
20:4	0.6 ± 0.1
22:0	11.7 ± 0.1
22:1	15.9 ± 0.9
22:2	2.1 ± 1.8
24:0	2.8 ± 1.3
24:1	2.2 ± 0.1
25:0	1.8 ± 0.7
26:0	0.7 ± 0.5
27:0	0.5 ± 0.3
28:0	0.3 ± 0.2
29:0	0.6 ± 0.1
30:0	0.3 ± 0.2

[a]The data are expressed as a percentage of total fatty acids present in the mouse specific fraction and presented, mean ± SEM (n = 2).

lipid droplets within the SC, and an imbalance in synthesis of individual ceramide classes. The abundant presence of triglycerides correlates with an excessive synthesis of oleic acid *in vitro*, which is the major fatty acid present in triglycerides (Vičanová *et al*, 1997). The high level of oleic acid and a low content of linoleic acid and of long-chain fatty acids in CSS *in vitro* indicates disturbances in fatty acid metabolism. Insufficient synthesis of long-chain fatty acids *in vitro* results in an abnormal profile of free fatty acids and ceramides. The ceramide profile of the CSS *in vitro* reveals great disproportions: the synthesis of ceramides 4–7 is inhibited and ceramides 6 and 7 are virtually absent. Ceramides 6 and 7 contain α-hydroxyacids linked to hydroxylated derivative of sphingosine base (Long *et al*, 1994; Robson *et al*, 1994), indicating possible defects in the hydroxylation process of fatty acids and sphingoid bases. In contrast, ceramide 2, which has been shown to consist of nonhydroxylated fatty acids and sphingosine (Wertz and Downing, 1983), is the major ceramide present *in vitro*. We can speculate that a low rate of synthesis of polar ceramides is balanced by an increased synthesis of ceramide 2, similarly to the shortage of linoleic acid enhancing synthesis of oleic acid (Wertz *et al*, 1983; Marcelo and Dunham, 1993).

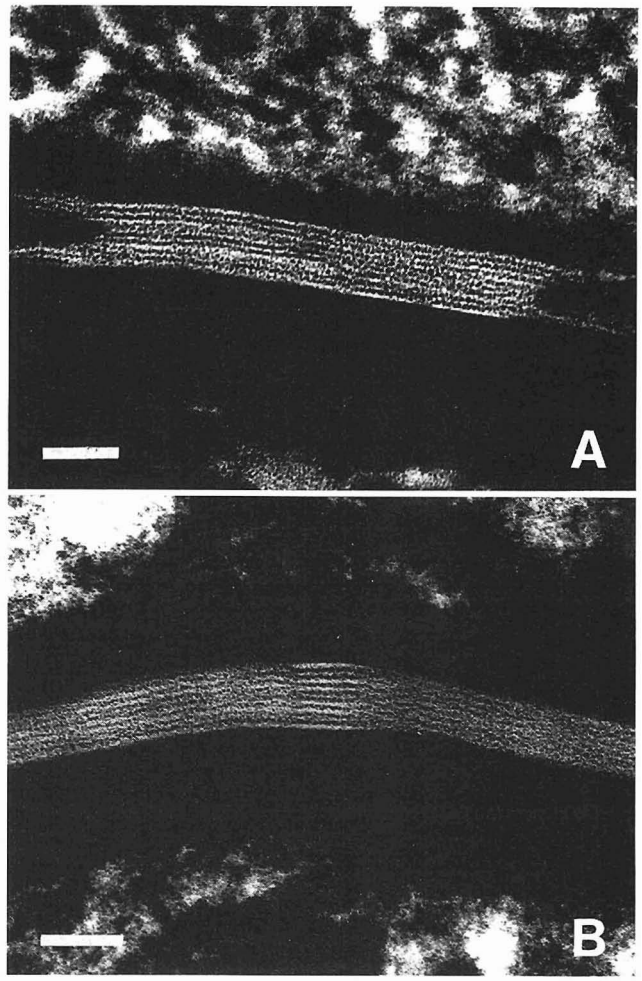

Figure 5. Intercorneocyte lipid lamellae in CSS at 4 mo after grafting onto athymic mice (*A*) ultrastructurally resemble those of native human skin (*B*). Both multilayered lamellar lipid structures show the characteristic alternating electron dense and electron lucent repeating pattern, as visualized by transmission electron micrography with ruthenium tetroxide staining. *Scale bar*, 25 nm.

The observed metabolic errors in CSS *in vitro* can be attributed to the culture environment, because overall synthesis of SC lipids is restored when CSS is grafted onto a physiologic host. The total SC lipid profile of the epidermal grafts is similar to that of the human SC at 6 mo postgrafting with the exception of the presence of mouse specific lipids, which, however, have been demonstrated not to be a constituent of the human graft (Higounenc *et al*, 1994b). The presence of these lipids is probably caused by lipids spreading over the surface of the collected skin sample, as it was not detected when the human graft was covered by a nonocclusive dressing or when they were removed from the skin surface by treatment with acetone wipe. Normalization of α-hydroxyacid profile and the increase of ceramides 4–7 content in CSS after grafting indicates restored activity of processes involved in the hydroxylation. Surprisingly, the level of ceramide 2 remains slightly elevated until 2 y postgrafting. As the proper composition of ceramides is required for the formation of competent epidermal barrier (Bouwstra *et al*, 1996a, b), greater attention should be paid to optimization of these processes *in vitro*. In a recent study (Ponec *et al*, 1997) we demonstrated that supplementation of culture media with vitamin C results in an increased synthesis of ceramides 4–7 and normalization of the lipid profile, suggesting that vitamin C as a cofactor and/or an antioxidant may play a crucial role in the processes involved in hydroxylation of fatty acids and sphingoid bases.

The SC lipid composition and fatty acid profile in CSS improves within 6 mo and is very similar to that of native human SC 2 y postgrafting, indicating that the host (mouse) does not significantly

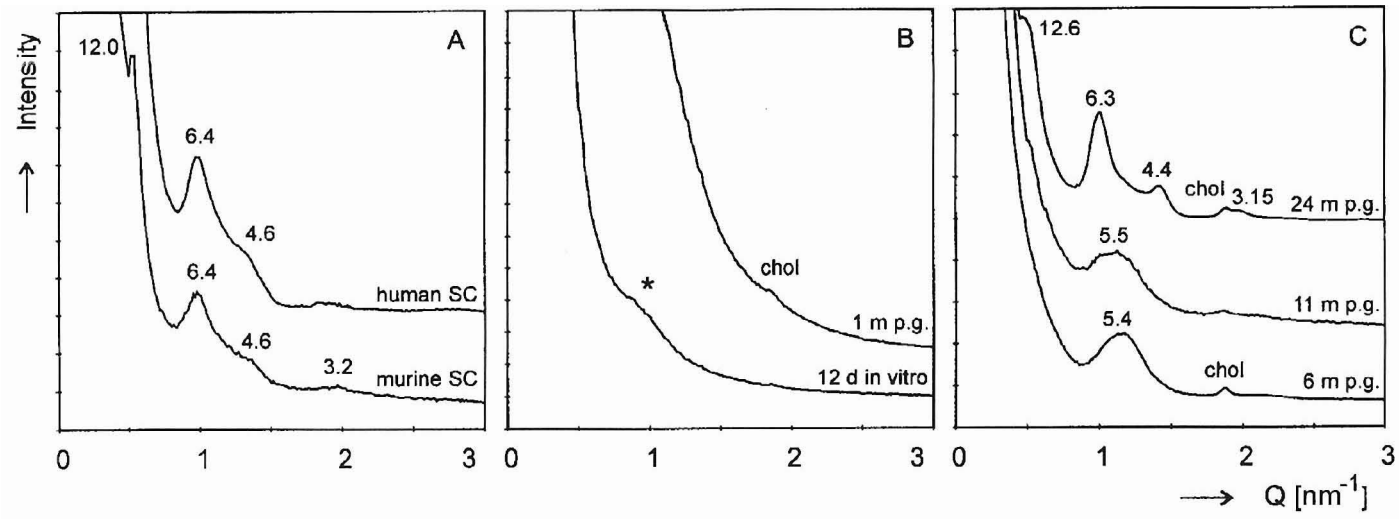

Figure 6. Organization of SC lipids in CSS changes between 6 mo and 2 y after transplantation. SAXD profiles were measured with SC isolated from native human and murine SC (*A*), with CSS *in vitro* (12 d), and after grafting (1, 5, 11, and 24 mo). The values corresponding to peaks and shoulders on diffraction curves denote the spacings of the various orders of the lamellar phases. (*B*) Only a weak shoulder at $Q = 0.98$ nm^{-1} was observed (*) in diffraction curves obtained with SC isolated from the CSS incubated for 12 d *in vitro*, indicating poor lamellar ordering. (*C*) A single broad peak corresponding to spacing 5.4 (5.5, respectively) nm obtained with SC 6 and 11 mo postgrafting is impossible to attribute to any lamellar phase. Four strong reflections on the SAXD curve at 24 mo postgrafting indicate the presence of ordered lamellar phase. chol, diffraction peak of cholesterol. Spacings are given in nm.

affect the restoration of lipogenesis. In addition, ultramicroscopic observations revealing the formation of extracellular lipid lamellae in grafted CSS are suggestive for a complete restoration of the barrier function after grafting. A restoration of barrier was also seen in a recent study (Boyce *et al*, 1996) in which the surface electrical capacitance was measured. Despite these observations, the SAXD analysis indicates differences in the organization of SC lipids in epidermal grafts as compared with the native skin. Evaluation of SAXD patterns of epidermal grafts 5 and 11 mo postgrafting demonstrates similar diffraction curves showing the presence of a broad peak corresponding to a 5.4 nm (5.5 nm, respectively) spacing. This single reflection, however, cannot be attributed to any particular lipid phase indicating poor lipid lamellar ordering. A different SAXD profile was obtained with SC isolated from epidermal grafts 24 mo postgrafting, indicating significant changes in the organization of SC lipids during the second year and a presence of well ordered lipid lamellar phase. Three strong and one weak reflection on the diffraction curve represent a long lamellar phase with the periodicity of 12.6 nm. The second, short periodicity lamellar phase normally present in native epidermis, however, seems to be absent in epidermal grafts. Similarly, different diffraction patterns were demonstrated with recrystallized human SC lipids (Bouwstra *et al*, 1991), in hairless mice (Bouwstra *et al*, 1994), and with SC isolated from reconstructed epidermis generated in vitamin C-supplemented media (Ponec *et al*, 1997). It remains to be established whether the differences in SAXD pattern between grafted CSS and native skin can be attributed to: (i) different pH, cholesterol sulfate, or calcium gradients; (ii) the differences in composition of covalently bound lipids; (iii) minor changes in ceramide and fatty acid profiles in grafted samples; (iv) differences in structure of individual ceramide classes or: (v) insufficient desquamation. It is unlikely that the mouse specific surface lipids are responsible for the observed differences in the diffraction patterns as these patterns clearly change at different time points after grafting, whereas the amounts of mouse specific lipids in epidermal grafts were similar at all time points after grafting.

Studies with model systems of isolated mixtures of ceramides, cholesterol, and free fatty acids (Bouwstra *et al*, 1996a, b) demonstrate the importance of properly balanced composition of the lipid mixtures and of the fatty acid chain length for lamellar phase formation. The enrichment with long-chain fatty acids 2 y postgrafting might explain differences in diffraction patterns of SC isolated 5 mo and 1 y postgrafting, as compared with that obtained 2 y postgrafting. pH and ion gradients may also account for the abnormalities of the SC lipid structure as delivery, transformation, and arrangement of barrier lipids is a highly regulated process involving enzymes and cofactors, and these activities are dependent on the microenvironmental conditions. These may also change after the grafting of the CSS onto the murine host. It seems unlikely, however, that differences in pH could cause deviations in SAXD pattern, as data obtained with a surface contact pH electrode showed no statistical differences between human or murine skin *in situ* and CSS at 1 mo postgrafting (pH = 5, data not shown). In addition, physiologic conditions such as increased humidity during wound healing or insufficient desquamation of the horny layer might be indirectly involved in the regulation of SC lipid organization and ordering.

In conclusion, SC lipid and fatty acid profiles of cultured skin substitutes grafted onto the athymic mouse exhibit the normal human pattern. It remains to be established why the molecular organization of the SC lipids in human skin substitutes after grafting to athymic mice seems to differ from that of the native human skin.

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