Barrier Characteristics of Different Human Skin Types Investigated with X-Ray Diffraction, Lipid Analysis, and Electron Microscopy Imaging

Volker Schreiner, Gert S. Gooris,* Stephan Pfeiffer, Ghita Lanzendörfer, Horst Wenck, Walter Diembeck, Ehrhardt Proksch,† and Joke Bouwstra*

Paul-Gerson-Unna Skin Research Center, Beiersdorf AG, Hamburg, Germany; †Department of Dermatology, University of Kiel, Kiel, Germany; *Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

The stratum corneum requires ceramides, cholesterol, and fatty acids to provide the cutaneous permeability barrier. The lipids are organized in intercellular membranes exhibiting short- and long-periodicity lamellar phases. In recent years, the phase behavior of barrier lipid mixtures has been studied *in vitro*. The relationship of human stratum corneum lipid composition to membrane organization *in vivo*, however, has not been clearly established. Furthermore, the special function of the different ceramide species in the stratum corneum is largely unknown. We examined lipid organization and composition of stratum corneum sheets from different subtypes of healthy human skin (normal, dry, and aged skin). Lipid organization was investigated using X-ray diffraction and demonstrated that the 4.4 nm peak attributed to the long periodicity phase was frequently missing for skin with a low Cer(EOS)/Cer_{total} ratio, indicating an important part for Cer(EOS), which contains ω-hydroxy fatty acid (O) ester-linked to linoleic acid (E) and amide-linked to sphingosine (S). A deficiency in the 4.4 nm peak was predominantly observed in young dry skin. In one case of aged skin, however, and less often in young normal skin this peak was also missing. Furthermore, the ceramide composition of samples without the 4.4 nm peak showed a deficiency of Cer(EOH), which contains 6-hydroxy-4-sphingenine (H), and an increase in Cer(NS) and Cer(AS), which contain nonhydroxy (N) or α-hydroxy fatty acids (A). In addition, a 3.4 nm peak attributed to crystalline cholesterol occurred in most cases of aged and dry skin, but was not observed in young normal skin. Our results do not indicate a definite pattern of correlation between lipid organization and types of human skin. They demonstrate, however, that Cer(EOS) and Cer(EOH) are key elements for the molecular organization of the long periodicity lamellar phase in the human stratum corneum. ***Key words: ceramides/dry skin/lipids/skin permeability barrier/xerosis.***

J Invest Dermatol 114:654–660, 2000

The stratum corneum (SC) constitutes the main barrier to the diffusion of substances into the skin. It consists of corneocytes and intercellular lipids, mainly ceramides, sterols, and free fatty acids. Its integrity requires the organization of the lipids into intercellular membranes subsequent to the secretion of epidermal lamellar body contents at the stratum granulosum–SC interface (Elias and Menon, 1991).

Despite its great importance, the direct relationship of lipid composition, morphology of lipid organization, and barrier function *in vivo* has not been fully understood. Only a few studies using artificially altered lipid metabolism in skin have elucidated this interrelationship: a long-term inhibition of the 3-hydroxy-3-methylglutaryl coenzyme A reductase, the key enzyme of sterol biosynthesis, in murine skin results in an altered lipid composition, which coincides with structural alterations of the intercellular membranes and lamellar body secretory system (Feingold *et al*, 1991; Menon *et al*, 1992). In essential fatty acid deficiency (EFAD) Cer(EOS), previously called ceramide 1, has an altered molecular structure: oleic acid substitutes for linoleic acid, which normally is ester-linked (E) to an ω-hydroxy fatty acid (O), which in turn is amide-linked to sphingosine (S). This alteration results in an increased transepidermal water loss (TEWL), perturbations of the intercellular membranes and an altered epidermal homeostasis (Bowser *et al*, 1985; Wertz *et al*, 1987).

Several skin conditions with perturbed barrier function have been demonstrated to have abnormal lipid composition such as X-linked ichthyosis (Hamanaka *et al*, 1997). A disrupted lipid organization has also been demonstrated to result in a decreased barrier function in psoriasis and atopic dermatitis (Fartasch, 1997). In all skin conditions with a defect in barrier function was a paucity of Cer(EOS) (Imokawa *et al*, 1991; Motta *et al*, 1993; Di Nardo *et al*, 1998).

Barrier lipid organization can either be evaluated morphologically on electron micrographs or by physicochemical methods. Kitson *et al* (1994) have shown that aqueous dispersions of key

Manuscript received March 16, 1999; revised January 12, 2000; accepted for publication January 14, 2000.

Reprint requests to: Dr. Volker Schreiner, R&D Cosmed, Beiersdorf AG, Unnastrasse 48, 20245 Hamburg, Germany.

Abbreviations: A, α-hydroxy fatty acid; Cer_{total}, total quantity of ceramide; Cer(XX), ceramide-species (letters in brackets): E, ester-linked fatty acid; H, 6-hydroxy-4-sphingenine; LPP, long periodicity phase; N, nonhydroxy fatty acid; O, ω-hydroxy fatty acid; P, phytosphingosine; S, sphingosine; SAXD, small angle X-ray diffraction; SC, stratum corneum.

barrier lipid mixtures are mainly organized in a crystalline lattice. Small angle X-ray diffraction (SAXD) measurements on isolated SC sheets of healthy human skin revealed that the SC lipids are organized in lamellar phases with two periodicities of approximately 6 nm (short periodicity phase) and 13 nm (long periodicity phase, LPP) (White *et al*, 1988; Bouwstra *et al*, 1991). More recent *in vitro* studies revealed that mixtures prepared from ceramide species present in pig SC, cholesterol and long-chain free fatty acids ($C_{24/26}$) form two lamellar phases with repeat distances of 13.1 (LPP) and 5.3 nm (short periodicity phase). These values are similar to those present in intact SC (Bouwstra *et al*, 1996). If Cer(EOS) is omitted from the lipid mixture, there is no or either only a weak LPP is detectable, indicating a disturbance in the lipid organization.

This study is aimed to establish the relationship of SC lipid composition to membrane organization *in vivo* in different types of healthy human skin. Young normal and young dry skin were distinguished by clinical scoring and biophysical measurements. Additionally, a few cases of aged human skin were characterized accordingly. Lipid organization and composition were examined by electron microscopy, X-ray diffraction and high-performance thin layer chromatography. Our results clearly demonstrate that a balanced ratio of ceramides, with sufficient shares of Cer(EOS) and Cer(EOH) (H, 6-hydroxy-4-sphingenine), both containing long-chain (C_{30}–C_{34}) (ω-hydroxylated fatty acids with linoleic acid in ester-link, are necessary for a proper SC lipid organization *in vivo*.

MATERIALS AND METHODS

Chemicals and reagents Chloroform (LiChrosolv 102444), acetone (pro analysi/pa, 100014), methanol (LiChrosolv 106018), ethanol (pa, 100983), acetic acid (pa, 100062), n-hexane (pa, 104374), propionic acid (pa, 800605), diethyl ether (pa, 100921), and silica gel 60 plates (105641) were obtained from Merck (Darmstadt, Germany). Phosphoric acid (775899-8) was obtained from Aldrich Chemie (Steinheim, Germany). Trypsin/ethylenediamine tetraacetic acid solution (210242) for SC separation was from Boehringer Mannheim (Mannheim, Germany). Dulbecco's minimal Eagle's medium, phosphate-buffered saline, and fetal bovine serum were from Life Technologies (Eggenstein, Germany). Cupric sulfate pentahydrate/$CuSO_4$ (C-6283), Ninhydrin (N4876), L-ascorbic acid (A5960), propionic acid, sodium salt (P1880), ethylene glycol monomethylether (E5378), and standard lipids like ceramide IV (C-2512), ceramide type III (C-2137), palmitoleic acid (p-9417), and cholesterol (C-8667) were purchased from Sigma (Deisenhofen, Germany). *N*-stearoylphytosphingosine (ceramide 3) was a gift of Gist-Brocades (Delft, the Netherlands).

Skin types, clinical scoring, and season chosen for the investigation The skin of the lower legs of healthy Caucasian volunteers with no past or current history of atopic dermatitis was examined. The samples were divided into the following groups: young normal ($n = 10$, mean age ± (SD: 25.5 ± 2.5 y) young dry ($n = 5$, mean age ± SD: 30 ± 6 y) and aged skin ($n = 4$; mean age ± SD: 66 ± 3 y). The different skin types were scored on a clinical scale [visual score of scaliness: 1 (no scales) to 4 (very scaly); sensory score of suppleness: 1 (very smooth) to 7 (extremely rough)]. This study was largely conducted during the coldest months (October–March). Five samples of young normal skin, however, were taken in August. The volunteers were requested not to use any topical preparation 2 wk prior to the study.

SC hydration (corneometer readings) and TEWL All skin sites were characterized biophysically with respects to their degree of hydration by corneometer readings (Corneometer CM 820, Courage & Khazaka, Cologne, Staeb *et al*, 1997) and TEWL (Tewameter, Courage & Khazaka, Cologne; Pinnagoda *et al*, 1990).

Sample collection SC of shave biopsies taken from the previously examined skin sites was separated from the viable epidermis by trypsin digestion. The biopsies were incubated in a 1% trypsin solution overnight, followed by 37°C for 1 h. Trypsin digestion was stopped by adding 10% (vol/vol) in Dulbecco's minimal Eagle's medium, after which the SC was removed carefully from the viable epidermis. The SC was then dried and analyzed. In each SC sample lipid organization was examined using the small angle X-ray diffraction technique. Thereafter lipid composition was analyzed by high-performance thin layer chromatography in the same sample.

Small angle X-ray diffraction (SAXD) All measurements on isolated SC samples were carried out at the Synchroton Radiation Source at Daresbury (U.K.) using station 8.2. The samples were put into a specially designed sample holder with two mica windows as previously described (Bouwstra *et al*, 1991, 1995). The scattering intensities were measured as a function of the scattering angle (θ). From the scattering angle the scattering vector (Q) was calculated as $Q = 4\pi(\sin\theta)/\lambda$ where λ is the wavelength of 0.154 nm at the sample position.

In order to compare the lipid organization of SC of viable epidermis and of isolated SC a few measurements were performed at the synchrotron facilities in Grenoble (France) at station ID2A. At this station the beam cross-section at the sample position is 0.2 mm in height and 0.5 mm in width. The flux of the beam was very high being 2×10^{13} photons per second at a current of 200 mA. The wavelength was 0.0986 nm. The detector sample distance was set to 1.278 m. The small spot size, the high flux of the beam and a sample holder with two mica windows enabled us to focus the beam on the SC of a freshly excised skin biopsy (SC *in situ*) and to measure its X-ray scattering. During the measurements the epidermis and SC were fully hydrated. The diffraction pattern of the SC *in situ* could then be compared with that of the same SC after trypsinization. Owing to the small spot and the fully hydrated state of the tissue the signal noise ratio obtained with these measurements is less than of those measurements performed at the facilities in Daresbury.

Lipid analysis Lipids were extracted from the samples after measurement of the X-ray diffraction with chloroform/methanol (2:1, by vol.) according to a modified procedure of Bligh and Dyer (1959) using a sonicating water bath for 20 min. Samples were applied to silica gel 60 plates using a CAMAG Linomat IV. Lipids were analyzed with one-dimensional high-performance thin layer chromatography using a solvent system of Wertz *et al* (1985) (chloroform/methanol/glacial acetic acid, 190:9:1) and a modified solvent system of Lampe *et al* (1983) (hexane/diethylether/glacial acetic acid, 80:20:1.5). Quantitation was performed after charring of the chromatographed samples by using a photodensitometer with automatic peak integration (CAMAG TLC Scanner II). Lipid quantities were measured by densitometry and extrapolated from curves of authentic standards and then normalized to weight of protein (μg lipid per mg of total SC protein). $Cer(XX)/Cer_{total}$ ratios were calculated from of the total quantity of a particular ceramide species [Cer(XX)] and the total quantity of all ceramides (Cer_{total}) isolated from a single SC biopsy. Preparation of isolated SC samples by using trypsin does not influence the lipid composition as shown by Bowser and White (1985) and Lavrijsen *et al* (1994).

Quantitation of SC proteins The SC samples were completely hydrolyzed in 2 ml of 6 M NaOH for 5 h at 150°C following lipid extraction. After cooling, the hydrolysate was neutralized with 6 M HCl and 1 ml of an aqueous buffer containing 20.2% (wt/wt) propionic acid, sodium salt and 9.3% (vol/vol) of free propionic acid. Free amino acids were quantitated photospectrometrically after derivatization with ninhydrine. The aqueous ninhydrine reagent contained 20.2% (wt/wt) propionic acid sodium salt, 9.3% (vol/vol) free propionic acid, 50% (vol/vol) ethylene glycol monomethylether, and 2% (wt/wt) ninhydrine. Fifty microliters of the SC hydrolysate were diluted with 450 μl deionized water and then supplemented with 25 μl aqueous solution containing free ascorbic acid (0.4%, wt/wt) and 500 ml ninhydrine reagent. The mixture was shaken vigorously, heated to 100°C for about 20 min, cooled down to room temperature and supplemented with 50 ml of 50% ethanol (vol/vol). The extinction was measured at 570 nm.

Transmission electron microscopy Transmission electron micrographs were obtained from 2 mm punch biopsy samples of adjacent skin sites using the standard ruthenium and osmium tetroxide post fixation techniques as published earlier (Elias and Friend, 1975). Briefly, a part of each skin sample was prefixed overnight in modified Karnovsky's medium at 4°C, washed twice with 0.2 M sodium cacodylate buffer, each for 10 min, and postfixed either with 1% OsO_4 (wt/vol) in 0.133 M sodium cacodylate buffer containing 0.5% $K_4Fe(CN)_6$ (wt/vol) or with 0.5% RuO_4 (wt/vol) in 0.133 M sodium cacodylate buffer containing also 0.5% $K_4Fe(CN)_6$ (wt/vol) at 4°C for 45 min. Subsequently, the specimens were dehydrated in graded ethanol and embedded in Epon 812. The polymerization was carried out at 150°C overnight.

RESULTS

Visually assessed skin typing correlates with biophysical data For discrimination of different skin types clinical scorings and biophysical measurements were carried out. Furthermore, both

Table I. Clinical scoring and biophysical data of different skin types[a]

		Skin type		
Clinical scoring	Biophysical data	Young normal (n=10)	Young dry (n=5)	Aged (n=4)
Scaliness (score 1–4)		0.7 ± 0.5	3.1 ± 0.2**	2.5 ± 1.2*
Suppleness (score 1–7)		2.5 ± 0.8	6.0 ± 0.4**	4.5 ± 0.8*
	TEWL (g per $m^2 \times h$)	13.9 ± 3.9 (n=9)	16.2 ± 2.9	15.3 ± 4.3
	CR[b] (arbitrary)	76.3 ± 10.4	40.0 ± 1.6**	56.6 ± 8.9**

[a]Mean ± SD.
[b]CR=corneometer readings.
**$p < 0.01$ compared with corresponding value of young normal skin (Wilcoxon U test), *$p < 0.05$ compared with corresponding value of young normal skin (Wilcoxon U test).

kinds of evaluations were compared with each other. **Table I** shows the mean values (± SD) of scaliness, suppleness, hydration (corneometer readings), and TEWL values of the investigated volunteers. Young dry and aged skin were significantly different from young normal skin with respect to scaliness, suppleness, and hydration. There was no significant difference between young dry and aged skin by clinical scoring, TEWL, and hydration.

The different degrees of skin dryness (scaliness, visual score, and suppleness, sensory score) of the combined younger volunteers (mean age ± SD: 27 ± 4 y, n = 15) were best discriminated by corneometry. **Figure 1** demonstrates the linear relationship between visual scoring of scaliness and corneometer readings in young normal and young dry skin ($r^2 = 0.95$). In contrast, the visual score of aged skin (triangles) did not correlate with hydration. Furthermore, the TEWL of young normal skin did not significantly differ from young dry skin. Therefore, this parameter could not be correlated with clinical scoring.

In young skin scaliness clearly depends on skin hydration. Furthermore, in aged skin additional, age-dependent factors, which do not depend on hydration, seem to influence clinical scoring.

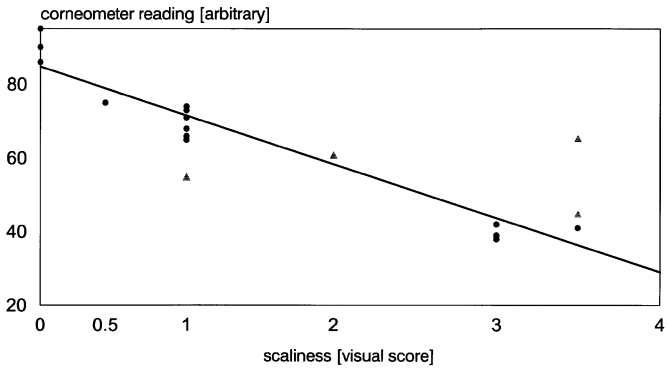

Figure 1. Relationship between visual scoring of scaliness and corneometer readings in young skin. $r^2 = 0.95$ for linear regression; data of aged skin (▲) were excluded from regression analysis.

The lipid compositions of different skin types do not differ significantly To evaluate whether the average barrier lipid composition or the lipid organization of the intercellular membranes differ between the normal and dry skin types X-ray diffraction measurements and lipid analysis were carried out on whole SC samples.

Mean values of overall lipid compositions are shown in **Table II.** Obviously, neither the proportions nor the amounts of the individual lipid classes differed between young dry skin and young normal. There was an alteration of the overall lipid composition in aged skin, however, which was not statistically relevant. In samples of aged skin there was an apparent increase of the percentage of free fatty acids and a compensatory decrease of ceramides. Therefore, there is little variation in the compositions of the key barrier lipids of the different types of healthy human skin. None of these alterations alone or in any combination could be related to changes in the X-ray-diffraction pattern.

The average ceramide compositions, expressed as Cer(XX)/Cer_{total} ratios, were similar in the different skin types (**Tables III–V)**, except for a significant increase of Cer(NS)/Cer_{total} in young dry skin in comparison with young normal skin and aged skin (**Table IV**). Ceramide profiles of individuals, however, varied a little within one group.

SAXD of isolated whole SC samples To evaluate the influence of the isolation procedure on X-ray diffraction characteristics SC of a whole fresh biopsy of human skin was measured before and after trypsinization. The congruence of both curves (**Fig 2**) clearly demonstrated that isolation of SC by trypsin treatment does not alter its X-ray diffraction pattern.

SAXD curves are characterized by the 6.2 nm peak being assigned to LPP and short periodicity phase, the 4.4 nm peak being attributed to LPP, and occasionally a 3.3 nm peak assigned to crystalline cholesterol. Three representative curves are shown here for each skin type (**Fig 3**, young normal skin; **Fig 4**, young dry skin, and **Fig 5**, aged skin). In the groups of the various skin types the X-ray diffraction curves varied in two aspects: the presence of a peak attributed to phase-separated cholesterol and the presence of the 4.4 nm peak.

In this study no systematic changes were found between the diffraction curves of SC samples isolated from aged, young dry, and young normal skin, respectively. But, it was noticed that the 4.4 nm peak was frequently absent in the diffraction curve. In total two of 10 curves (20%) of young normal skin were lacking the 4.4 nm peak (**Fig 3**; **Table III).** One of these two cases clearly showed a 3.3 nm peak (curve not shown here). In total three of five curves (60%) of young dry skin were lacking the 4.4 nm peak (**Fig 4**; **Table IV**). Four of five samples of young dry skin showed additional cholesterol reflections. Finally, one diffraction curve of aged skin (**Fig 5**; **Table V**) was lacking the 4.4 nm peak (volunteer 15). The two other curves of aged skin showed cholesterol reflections, of which one was very strong (volunteer 5).

Ceramide composition (CerX/Cer_{total}) and relationship to 4.4 nm peak As no significant differences were found between the diffraction curves of the different skin types, and the only pronounced variation in the diffraction peaks attributed to the lamellar phases was the presence of the 4.4 nm phase, it was decided to subdivide the SC diffraction patterns and the corresponding lipid profiles of all investigated volunteers (young normal skin + young dry skin + aged skin) into two groups: one group with, and the other group without a 4.4 nm peak in the X-ray diffraction curve.

The mean ceramide profiles of the two groups with or without a 4.4 nm peak in the X-ray diffraction curve are shown in **Fig 6**. Each bar represents a mean Cer(XX)/Cer_{total} ratio ± SD. A lack of the 4.4 nm peak coincided with a significantly reduced Cer(EOS)/Cer_{total} ratio. Furthermore, the deficiency of Cer(EOS) frequently

Table II. Percentage and quantity (μg lipid per mg SC protein) of different barrier lipid classes in different skin types[a]

Skin type	Cer_{total}		Free sterols		Free fatty acids	
	Percentage	Quantity	Percentage	Quantity	Percentage	Quantity
Young normal (n=9)	38 ± 2	21 ± 4	31 ± 2	17 ± 4	31 ± 2	17 ± 3
Young dry (n=4)	39 ± 3	20 ± 2	31 ± 4	15 ± 1	31 ± 6	18 ± 5.5
Aged (n=3)	28 ± 3	26 ± 11	28 ± 4	23 ± 6	43 ± 5	38 ± 12

[a]Mean ± SEM.

Table III. Individual and mean[a] ceramide distribution[b] in young normal skin and corresponding diffraction characteristics

Volunteer	Cer(EOS)/ Cer_{tot}	Cer(NS)/ Cer_{tot}	Cer(NP)/ Cer_{tot}	Cer(EOH)/ Cer_{tot}	Cer(AS)/ Cer_{tot}	Cer(AP)/ Cer_{tot}	Cer(AH)/ Cer_{tot}	SAXD: 4.4 nm peak	SAXD: cholesterol reflections
11	0.10	0.19	0.15	0.07	0.21	0.09	0.19	+	–
12	0.05	0.20	0.24	0.04	0.23	0.10	0.14	–	+
16	0.12	0.16	0.18	0.08	0.20	0.09	0.16	+	–
20	0.12	0.18	0.16	0.10	0.20	0.08	0.16	+	–
22	0.07	0.16	0.18	0.08	0.19	0.13	0.19	+	–
23	0.10	0.12	0.15	0.10	0.23	0.08	0.22	+	–
24	0.06	0.16	0.13	0.11	0.24	0.09	0.23	–	–
25	0.06	0.15	0.26	0.07	0.16	0.15	0.16	+	–
26	0.08	0.12	0.20	0.08	0.22	0.12	0.19	+	–
Mean ± SD	0.08 ± 0.03	0.16 ± 0.03	0.18 ± 0.04	0.08 ± 0.02	0.21 ± 0.02	0.10 ± 0.02	0.18 ± 0.03		

[a]Mean ± SD.
[b]Quantity of Cer(XX)/quantity of Cer_{total}.

Table IV. Individual ceramide distribution[a] in young dry skin and corresponding diffraction characteristics

Volunteer	Cer(EOS)/ Cer_{tot}	Cer(NS)/ Cer_{tot}	Cer(NP)/ Cer_{tot}	Cer(EOH)/ Cer_{tot}	Cer(AS)/ Cer_{tot}	Cer(AP)/ Cer_{tot}	Cer(AH)/ Cer_{tot}	SAXD: 4.4 nm peak	SAXD: cholesterol reflections
13	0.15	0.20	0.14	0.10	0.19	0.07	0.16	+	+
14	0.13	0.19	0.13	0.09	0.18	0.08	0.19	+	+
19	0.07	0.21	0.16	0.09	0.24	0.05	0.17	–	–
18	0.04	0.21	0.18	0.03	0.27	0.08	0.20	–	+
17	0.04	0.23	0.17	0.02	0.26	0.09	0.20	–	+
Mean ± SD	0.09 ± 0.05	0.21** ± 0.01	0.16 ± 0.02	0.7 ± 0.04	0.23 ± 0.04	0.07 ± 0.02	0.18 ± 0.02		

[a]Quantity of Cer(XX)/quantity of Cer_{total}.
**$p < 0.01$ compared with young normal skin (Wilcoxon U test).

Table V. Individual ceramide distribution[a] in aged skin and corresponding diffraction characteristics

Volunteer	Cer(EOS)/ Cer_{tot}	Cer(NS)/ Cer_{tot}	Cer(NP)/ Cer_{tot}	Cer(EOH)/ Cer_{tot}	Cer(AS)/ Cer_{tot}	Cer(AP)/ Cer_{tot}	Cer(AH)/ Cer_{tot}	SAXD: 4.4 nm peak	SAXD: cholesterol reflections
4	0.14	0.14	0.16	0.06	0.24	0.10	0.18	+	+
5	0.12	0.16	0.13	0.07	0.21	0.11	0.18	+	++
15	0.04	0.20	0.20	0.01	0.23	0.12	0.20	–	–
Mean ± SD	0.10 0.05	0.17 ± 0.03	0.16 ± 0.04	0.05 ± 0.03	0.23 ± 0.02	0.11 ± 0.01	0.19 ± 0.01		

[a]Quantity of Cer(XX)/quantity of Cer_{total}.

coincided with a significant Cer(EOH) deficiency. These deficiencies are compensated by a significant increase in Cer(NS) and Cer(AS).

Transmission electron micrographs Electron microscopy was conducted in order to visualize perturbances of lipid organization indicated by the lack of the 4.4 nm peak in X-ray diffraction curves.

Transmission electron micrographs did not reveal any differences between young dry skin and young normal skin, neither with OsO_4, nor the RuO_4 postfixation protocol. Alterations could only be observed in micrographs of AS. **Figure 7** shows the stratum granulosum–SC interface of an OsO_4 postfixated sample of aged skin. The morphology of lamellar bodies was changed, with many small organelles visible, filled with pleated sheets of pro-barrier membranes, which were not as obvious as in young normal skin (not shown). Moreover, sometimes amorphous depositions were detectable after RuO_4 postfixation in the intercellular domain in SC samples of aged skin (**Fig 8**). Intercellular membranes, however, were clearly visible and obviously not altered.

DISCUSSION

In this study young normal, young dry, and aged skin were characterized (i) *in vivo* according to appearance (clinical scoring),

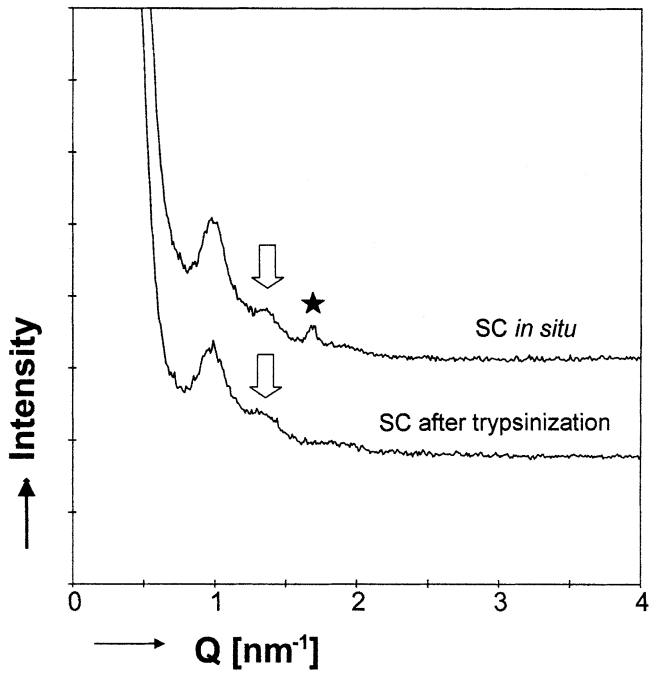

Figure 2. SAXD of stratum corneum *in situ* and after trypsinization. A fresh biopsy of human skin was placed in the X-ray beam in such a way that only the SC was irradiated (SC *in situ*). Thereafter, the SC was isolated by trypsinization and measured again (SC after typsinization). The *asterisk* marks a small peak, which is caused by a membrane used to support the whole biopsy. The *arrows* indicate the 4.4 nm peaks, which are present in both spectra.

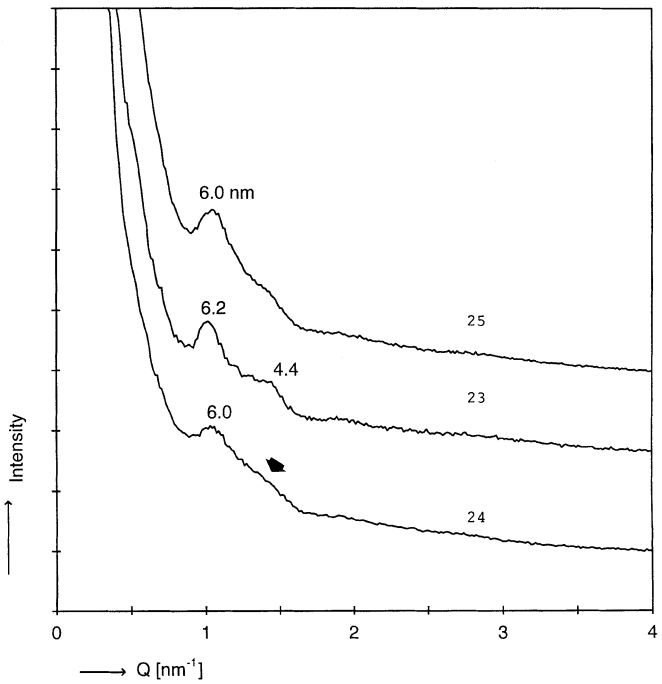

Figure 3. Three representative SAXD curves of young normal skin. The numbers of the curves match the numbers of the volunteer in **Table III**. The 4.4 nm peak assigned to LPP is missing in curve 24 (*arrow*).

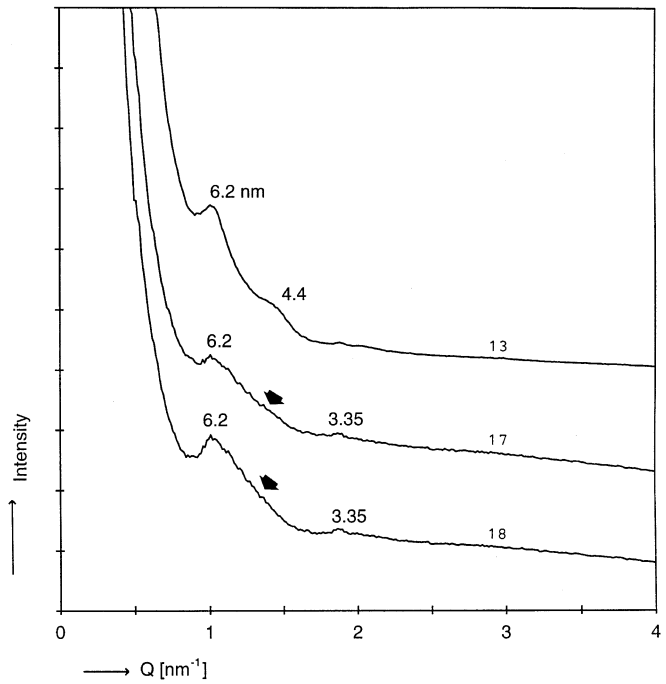

Figure 4. Three representative SAXD curves of young dry skin. The numbers of the curves match the numbers of the volunteer in **Table IV.** Curves 17 and 18 lack of the 4.4 nm peak (*arrow*) but show a reflection at 3.35 nm representing crystalline cholesterol.

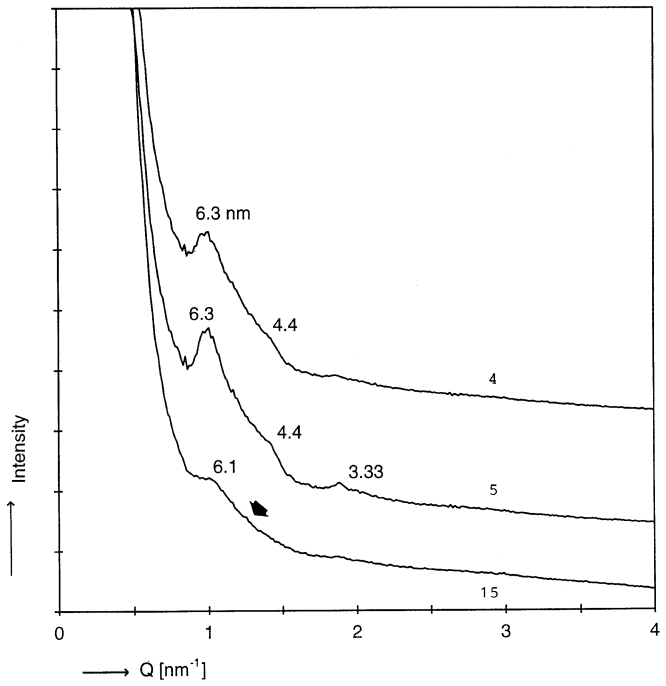

Figure 5. SAXD curves of aged skin. The numbers of the curves match the numbers of the volunteer in **Table V.** The 4.4 nm peak is lacking in curve 15 only (*arrow*). Curve 5 shows a strong reflection of crystalline cholesterol.

hydration (corneometry), skin barrier function (TEWL), and (ii) *ex vivo* according to skin lipid organization (by small angle X-ray diffraction) and lipid analysis (by high-performance thin layer chromatography). Information on the ultrastructure of the epidermis was also obtained by transmission electron microscopy. This multimethodologic approach was chosen to comprehensively understand the characteristics of the barrier and the interrelationship between these properties and dry and normal skin.

There was an excellent correlation between the clinical score and hydration of young skin. In the case of aged skin additional age-dependent factors influenced the skin's clinical appearance. In accordance with previous studies, this study also confirms that dry

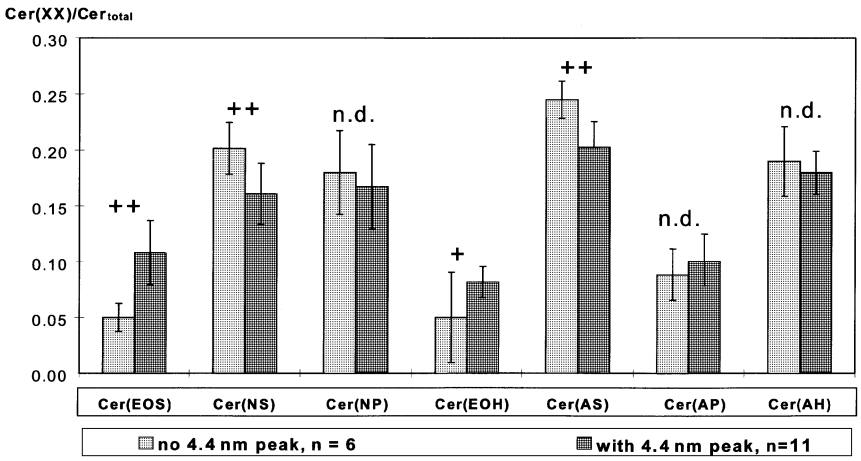

Figure 6. Means of ceramide ratios. Means of ceramide ratios of all samples from healthy skin (young normal, young dry, and aged skin) belonging to group A with no 4.4 nm peak ($n = 6$) or to group B with 4.4 nm peak ($n = 11$). Data presented in the graph represent mean values, error bars: ± SD. Values of group A significantly deviating from group B are marked as follows: ++ $p < 0.01$; + $p < 0.05$; n.d., no difference, Wilcoxon U-test.

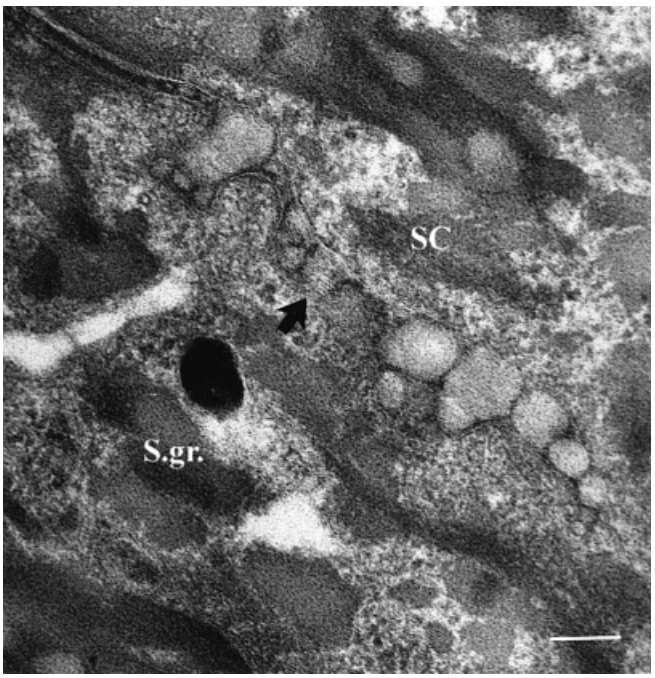

Figure 7. Ultrastructure of stratum granulosum-SC interface of aged skin. OsO_4-postfixated sample: many small lamellar bodies are visible (*arrow*). Stacks of pro-barrier membranes inside these organelles are not as obvious as in young normal skin. SC, stratum corneum; S.gr., stratum granulosum. *Scale bar*: 0.5 μm.

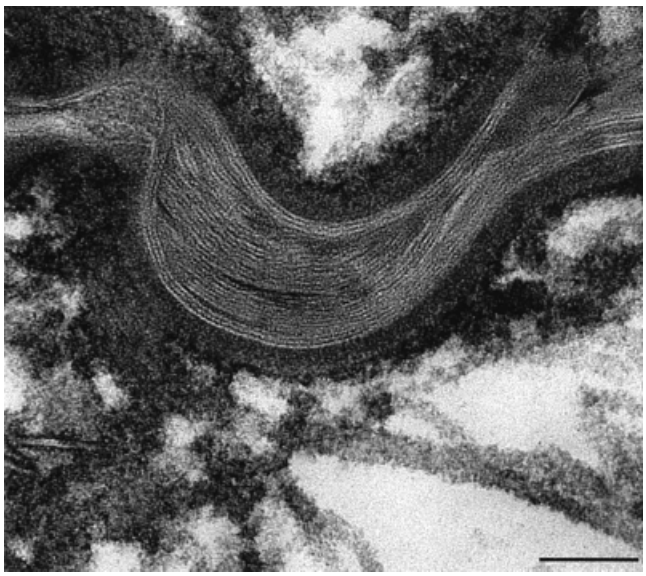

Figure 8. Ultrastructure of stratum corneum of aged skin. RuO_4-postfixated sample. Some amorphous depositions are detectable in the intercellular domain (*arrow*), which is otherwise unchanged if compared with young normal skin. *Scale bar*: 0.1 μm.

skin types display only a slight, statistically nonsignificant increase in TEWL (Engelke *et al*, 1997; Gfesser *et al*, 1997).

Despite clear differences in the clinical appearance of the skin, there were only slight and nonsystematic differences in the compositions of the key barrier lipids, which could not be related to changes in the X-ray diffraction pattern. Our results, however, show a tendency towards phase separation of crystalline cholesterol in young dry and aged skin. Because of the unchanged free cholesterol content in the whole SC samples of these skin types it would be of interest to study their cholesterol sulfate content, which plays an important part in the solubilization of cholesterol (Bouwstra *et al*, 1998).

As clinical appearance could not clearly be related to pronounced differences in lipid phase behavior or lipid composition, it was decided to look at whether there was any association of the X-ray diffraction characteristics of the whole set of SC samples and lipid composition. Samples were divided as to whether they possessed a 4.4 nm peak attributed to the LPP or not. The ceramide profiles of these two groups are significantly different. The most pronounced difference was a decrease of the Cer(EOS) fraction in samples lacking the 4.4 nm peak. The changes in the 4.4 nm peak may be either due to a strong decrease in the quantity of lipids that form the LPP or a change in the molecular organization in the LPP. The latter deduction is based on the fact that a change in the molecular organization within a repeat distance is indicated by a change of the relative intensity of the corresponding peak.

The importance of Cer(EOS) in the formation of the LPP has previously been demonstrated using lipid mixtures containing pig SC ceramide (Bouwstra *et al*, 1998). The role of Cer(NS) in the lipid phase behavior is less pronounced as also shown in this study. A significant increase in Cer(NS) was observed in young dry skin samples with no change in the diffraction pattern. It has been suggested that the distinguished role of Cer(EOS), which is similar in pig and humans, in the formation of the LPP is due to its exceptional molecular structure. Cer(EOS) has a very long chain ω-hydroxy fatty acid with linoleic acid linked to an ω-hydroxy group which forms a scaffold for the organization of the lipids. As in the skin lipid mixtures the absence of Cer(EOS) results in a disappearance of the LPP phase, the most likely explanation for the absence of the 4.4 nm peak in the diffraction pattern of the human skin samples is a reduction in the fraction of lipids that form the

LPP phase, rather than a change in the molecular organization within the LPP.

Comparative analysis of transmission electron micrographs does not give any hints for ultrastructural changes in the SC of dry or aged skin. The ultrastructural alterations seen in the underlying tissue of aged skin were found in all investigated samples and might indicate perturbances of lamellar body formation. This in turn could have resulted in not yet discovered alterations of the barrier lipid composition. These possible alterations, however, have not changed the morphology of the intercellular membranes but might be causative for the amorphous depositions demonstrated in **Fig 8**. The lack of ultrastructural changes in dry skin is in agreement with a comparative study on dry, noneczematous skin of atopic dermatitis with a reduced content of Cer(EOS) and healthy skin (Fartasch *et al*, 1991; Imokawa *et al*, 1991). Other authors, however, have shown morphologic alterations of the intercellular membranes in aged human skin (Ghadially *et al*, 1995) or in the essential fatty acid deficiency syndrome (Wertz *et al*, 1987). A possible explanation for the discrepancies to our results could be that the volunteers investigated by Ghadially *et al* were very old (> 80 y) and that in essential fatty acid deficiency syndrome the molecular structure of Cer(EOS) was completely changed, whereas in our cases it may be assumed that there still was Cer(EOS) in the samples lacking the 4.4 nm peak in the X-ray diffraction curves. Furthermore, disrupted lamellae, especially in the outer SC have been demonstrated in dry skin of young individuals as visualized by transmission electron micrographs of tape-stripped SC.[1] This may possibly be partly owing to environmental insult or stress on intercellular membranes, which leads to reduced lipid ordering.

Our *ex vivo* data show that the ω-hydroxy acid containing Cer(EOS) and Cer(EOH) seem to be of importance for the ordering of barrier lipids in healthy human skin. A deficiency in Cer(EOS) and Cer(EOH), which coincides with a lack of the 4.4 nm peak attributed to the LPP, occurs predominantly in young dry skin. It could also be observed in a few cases of aged and young normal skin. Accordingly, our data only partly support a relationship between lipid organization and dry skin. This picture can best be explained by the fact that ''dry skin'' is still not clearly defined and certainly not monocausal. In conclusion, Cer(EOS) and Cer(EOH) containing ω-hydroxy fatty acids are key elements for the molecular organization of SC lamellar phase. A deficiency in these ceramides is frequently correlated to dry skin conditions.

REFERENCES

Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917, 1959

Bouwstra JA, Gooris GS, van der Spek JA, Bras W: Structural investigations of human stratum corneum by small angle x-ray scattering. *J Invest Dermatol* 97:1004–1012, 1991

Bouwstra JA, Gooris GS, Bras W, Downing DT: Lipid organization in pig stratum corneum. *J Lipid Res* 36:685–695, 1995

Bouwstra JA, Cheng K, Gooris GS, Weerheim A, Ponec M: The role of ceramides 1 and 2 in the stratum corneum lipid organization. *Biochim Biophys Acta* 1300:177–186, 1996

Bouwstra JA, Gooris GS, Dubbelaar FER, Weerheim AMI, Jzerman AP, Ponec M: Role of ceramide 1 in molecular organization of the stratum corneum lipids. *J Lipid Res* 39:186–196, 1998

Bowser PA, White RJ: Isolation, barrier properties and lipid analysis of stratum compactum, a discrete region of the stratum corneum. *Br J Dermatol* 112:1–14, 1985

Bowser PA, Nugteren DH, White RJ, Houtsmuller UMT, Pottrey C: Identification, isolation and characterization of epidermal lipids containing linoleic acid. *Biochim Biophys Acta* 834:419–428, 1985

Di Nardo A, Wertz PW, Gianetti A, Seidenari S: Ceramide and cholesterol composition of skin of patients with atopic dermatitis. *Acta Derm Venereol* 78:27–30, 1998

Elias PM, Friend DS: The permeability barrier in mammalian epidermis. *J Cell Biol* 65:180–191, 1975

Elias PM, Menon GK: Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv Lipid Res* 24:1–26, 1991

Engelke M, Jensen JM, Ekanayake-Mudiyanselage S, Proksch E: Effects of xerosis and ageing on epidermal proliferation and differentiation. *Br J Dermatol* 137:219–225, 1997

Fartasch M: Epidermal barrier in disorders of the skin. *Microsc Res Tech* 38:361–372, 1997

Fartasch M, Diepgen TL, Bassukas ID: Ultrastrukturelle Untersuchungen zur Funktion der Hornschichtbarriere an der nicht-ekzematösen Haut von Atopikern. *Allergologie* 14:315–321, 1991

Feingold KR, Man MQ, Proksch E, Menon GK, Brown BE, Elias PM: The lovastatin-treated rodent: a new model of barrier disruption and epidermal hyperplasia. *J Invest Dermatol* 96:201–209, 1991

Gfesser M, Abeck D, Rügemer J, Schreiner V, Stäb F, Disch R, Ring J: The early phase of epidermal regeneration is faster in patients with atopic eczema. *Dermatology* 195:332–336, 1997

Ghadially R, Brown BE, Sequeira-Martin SM, Feingold KR, Elias PM: The aged epidermal permeability barrier. Structural, functional, and lipid biochemical abnormalities in humans and a senescent murine model. *J Clin Invest* 95:2281–2290, 1995

Hamanaka S, Ujihara M, Serizawa S, Nakazawa S, Otsuka F: A case of recessive X-linked ichthyosis: scale-specific abnormalities of lipid composition may explain the pathogenesis of the skin manifestation. *J Dermatol* 24:156–160, 1997

Imokawa G, Abe A, Jin K, Kawashima M, Hidano A: Decreased level of ceramides in stratum corneum of atopic dermatitis: an etiologic factor in atopic dry skin? *J Invest Dermatol* 96:523–526, 1991

Kitson N, Thewalt J, Lafleur M, Bloom M: A model membrane approach to the epidermal permeability barrier. *Biochemistry* 33:6707–6715, 1994

Lampe MA, Burlingame AL, Whitney JA, Williams ML, Brown BE, Roitman E, Elias PMJ: Human stratum corneum lipids: characterization and regional variations. *J Lipid Res* 24:120–130, 1983

Lavrijsen AP, Higounenc IM, Weerheim A, Oestmann E, Tuinenburg EE, Bodde HE, Ponec M: Validation of an in vivo extraction method for human stratum corneum ceramides. *Arch Dermatol Res* 286:495–503, 1994

Menon GK, Feingold KR, Mao-Qiang M, Schaude M, Elias PM: Structural basis for the barrier abnormality following inhibition of HMG CoA reductase in murine epidermis. *J Invest Dermatol* 98:209–219, 1992

Motta S, Monti M, Sesana S, Caputo R, Carelli S, Ghidoni R: Ceramide composition of the psoriatic scale. *Biochim Biophys Acta* 1182:147–151, 1993

Pinnagoda J, Tupker RA, Agner T, Serup J: Guidelines for transepidermal water loss (TEWL) measurement. *Contact Dermatitis* 22:164–178, 1990

Staeb F, Sauermann G, Hoppe U. Evaluation of moisturizers. In Wilhelm KP, Elsner E, Beradesca E, Maibach HI (eds). *Bioengineering and Skin: Skin Surface Imaging and Analysis*. Boca Raton, FL: CRC Press, 1997, pp 315–329

Wertz PW, Miethke MC, Long SA, Strauss JS, Downing DT: The composition of ceramides from human stratum corneum and from comedones. *J Invest Dermatol* 84:410–412, 1985

Wertz PW, Swartzendruber DC, Abraham W, Madison KC, Downing DT: Essential fatty acids and epidermal integrity. *Arch Dermatol* 123:1381–1384, 1987

White SH, Mirejovsky D, King GI: Structure of lamellar lipid domains and corneocyte envelopes of murine stratum corneum. An x-ray diffraction study. *Biochem* 27:3725–3732, 1988

[1]Warner RR, Boissy YL, Lang SA: Microscopy of lipid structure in the outer stratum corneum: The effect of age, skin grade, and frequent soap use. *J Invest Dermatol* 110:585, 1998 (abstr.)