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Lipid and ultrastructural characterization of reconstructed skin models

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Abstract

The study aimed at evaluating tissue architecture and quality of the permeability barrier in commercially available reconstructed human skin models; EpiDerm™, SkinEthic™ and Episkin™ in comparison to native tissue. For this purpose, tissue architecture was examined by electron microscopy and epidermal lipid composition was analyzed by HPTLC. Stratum corneum lipid organization was investigated by electron microscopy in combination with RuO_4 post-fixation and by SAXD. Ultrastructurally, the overall tissue architecture showed high similarities with native epidermis. In the stratum corneum extracellular space, lipid lamellae consisting of multiple alternating electron-dense and electron-lucent bands were present. This regular pattern was not seen throughout the whole stratum corneum probably due to the observed irregular lamellar body extrusion in some areas. Lipid analyses revealed the presence of all major epidermal lipid classes. Compared with native epidermis the content of polar ceramides 5 and 6 was lower, ceramide 7 was absent, and the content of free fatty acids was very low. These differences in lipid composition may account for differences observed in SAXD pattern of Episkin and EpiDerm penetration models. In the latter only the long-distance periodicity unit of about 12 nm was observed and the short periodicity unit was missing. In conclusion, all three skin models provide a promising means for studying the effects of topically applied chemicals, although the observed deviations in tissue homeostasis and barrier properties need to be optimized.

Keywords: Reconstructed epidermis; Skin barrier function; Epidermal lipids; Morphology

1. Introduction

There is an increasing pressure from the official authorities to encourage the development of in vitro test models as alternatives to animal testing.

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So far, reconstructed human epidermis is the first tissue generated in vitro that mimics its native counterpart to a high degree (reviewed in Ponec, 1992). During the past 10 years various skin equivalents have been generated and have been used in studies concerning skin permeability, pharmacology, toxicity and metabolism. Some of them, like TestSkin™, (Organogenesis, Canton, MA, USA), Skin^{2}™ (Advanced Tissue Sciences, La Jolla, USA), EpiDerm™ (MatTek Corporation, Ashland, USA), Episkin™ (Episkin SNC, Chaponost, France), SkinEthic™ (Laboratoire SkinEthic, Nice, France) appeared on the market. To facilitate the use of human skin equivalents in the future and to reduce animal testing, the European Community (DGXII) supported a project regarding testing and improvement of reconstructed skin kits. Part of this project aimed at evaluating the quality of three different skin models: Epiderm™, SkinEthic™ and Episkin™ on the basis of their ultrastructure and lipid composition and organization.

One of the most important criterions for evaluation of the performance of any in vitro skin culture system is the formation of a competent permeability barrier. The permeability of human skin resides in the uppermost layer of the epidermis, the stratum corneum, that consists of protein-enriched corneocytes embedded in a lipid-enriched, intercellular matrix (reviewed in Schürer and Elias, 1991; Wertz and Downing, 1991). The extracellular lipids are organized in multiple bilayers originating from small organelles, so-called lamellar bodies that are synthesized in the spinous and granular cells. These organelles contain a selected spectrum of lipids and hydrolytic enzymes and discharge their content into the intercellular space at the stratum granulosum-stratum corneum interface (Schürer and Elias, 1991; Ponec et al., 1997). Following secretion, the content of these organelles is reorganized into a system of broad lamellar bilayers consisting mainly of cholesterol, ceramides and free fatty acids (Gray and Yardley, 1975). These bilayers are organized in two lamellar phases with a periodicity of about 6 and 13 nm, respectively (Bouwstra et al., 1991). To establish whether a proper development of the permeability barrier takes place in vitro, the ultrastructure of various skin models and their lipid composition and organization has been examined extensively.

2. Materials and methods

2.1. Skin culture models

Three human epidermal constructs were used: EpiDerm™ (MatTek Corporation, Ashland, MA, USA) (Cannon et al., 1994; Klausner et al., 1996; Monteiro-Riviere et al., 1997), SkinEthic™ (Laboratoire SkinEthic, Nice France) (Rosdy and Clauss, 1990), and Episkin™ (Episkin SNC, Chaponost, France) (Tinois et al., 1991; Roguet et al., 1993, 1998). Two types of EpiDerm™ were evaluated: five batches of skin irritation kits (EPI-200-HCF) and three batches of penetration kits (EPI-606A); two types of Episkin™: three batches of skin irritation kits and three batches of penetration kits; and four batches of SkinEthic™ (skin irritation kit).

2.2. Electron microscopy

Cultures were cut into small blocks of 1 mm^3 and immersed immediately in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After fixation for 1 h at room temperature the specimens were rinsed twice in phosphate buffer and post-fixed with 1% osmium tetroxide for 1 h at 4°C. For optimal visualization of stratum corneum lipid bilayers, specimen blocks from each culture were subjected to an additional post-fixation with 0.5% ruthenium tetroxide for 30 min at 4°C, as described by van der Meulen et al., 1996. After rinsing, the specimens were dehydrated in 70% ethanol and embedded in an epoxy resin. Ultrathin sections were cut from at least four blocks (two of routine-fixed and two of ruthenium tetroxide-fixed material) of each culture batch, stained with uranyl acetate and lead hydroxide and examined in a transmission electron microscope (Philips 410 LS) at 80 kV.

For comparison of the ultrastructural morphology, photographs were taken according to a standard protocol, including an overview at

magnification $\times 2500$ and 11 details of the overview at magnification $\times 60\,000$ and two details at magnification $\times 88\,000$. Two investigators independently evaluated the electron micrographs.

Next to the examination of the morphological characteristics, the number of cell layers was counted and the thickness of the epidermal viable cell layers and of the stratum corneum was measured. Per batch, two samples were examined and each sample was evaluated in duplicate.

2.3. Lipid extraction and analysis

The reconstructed epidermis was separated mechanically from the dermal substrate with forceps. The harvested material was extracted according to Bligh and Dyer, 1959. The extracts were first dried under a stream of nitrogen then weighed and their lipid composition determined by analytical high performance thin layer chromatography (HPTLC), as described earlier (Ponec and Weerheim, 1990) with small modifications. The lipids were separated using the 'Total' and 'Ceramide' development systems.

In the 'Total' development system, the lipids were separated by sequential development in (1) hexane/chloroform/acetone (8:90:2), (2) chloroform/acetone/methanol (76:8:16), (3) hexane/chloroform/hexyl acetate/acetone/methanol (6:80:0.1:10:4), (4) chloroform/ethyl acetate/ethyl methylketone/2 - propanol/ethanol/methanol/water/hexyl acetate (36:6:6:6:16:28:2:1), (5) chloroform/ethyl acetate/ethyl methylketone/2-propanol/ethanol/methanol/water (48:6:6:6:16:24:4), (6) chloroform/acetone/methanol (76:12:8), (7) hexane/diethylether/ethyl acetate (78:18:4).

In the 'Ceramide' system the lipids were separated by sequential development in (1) hexane/chloroform/acetone (8:90:2), (2) chloroform/acetone/methanol (76:8:16), (3) hexane/chloroform/hexyl acetate/acetone/methanol (6:80:0.1:10:4), (4) chloroform/acetone/methanol (76:4:20), (5) hexane/chloroform/hexyl acetate/ethyl acetate/methanol (8:80:0.1:6:6), (6) hexane/diethylether/ethyl acetate (78:18:4). Serial dilutions of lipid standards (Sigma) and lipid extracts obtained from normal human epidermis were run in parallel. For each batch the analyses were performed in duplicate or triplicate.

2.4. Stratum corneum isolation

To isolate the stratum corneum, the reconstructed epidermis was washed several times with PBS and reincubated for 2 h in 0.01% proteinase-K in PBS (Bowser and White, 1985). After extensive washing with PBS and with distilled water, the stratum corneum was dried under vacuum and stored under nitrogen in the dark until use. Before use, the stratum corneum was rehydrated 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).

2.5. Small angle X-ray scattering

All measurements were carried out at the Synchrotron Radiation Source at Daresbury Laboratory (Daresbury, UK) 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, approximately 3 mg in weight, was put randomly in a special designed sample cell with two mica windows. The temperature of this sample cell could be adjusted between 25 and 120°C. All samples were measured for a period of 15 min. Checks for the appearance of radiation damage were performed but proved to be negative.

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 position 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 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.

3. Results

3.1. Electron microscopy

3.1.1. General structure

Detailed investigation of the individual cell layers, including their specific structures and the number of cell layers, both of the viable cell layers and of the stratum corneum, showed that the intra- and inter-batch variations for each model were very low, except for a single observation of the presence of melanocytes in a SkinEthic culture. It should be noted that during the preparation for electron microscopic examination the cultures were fragile and could easily be detached from the substrate.

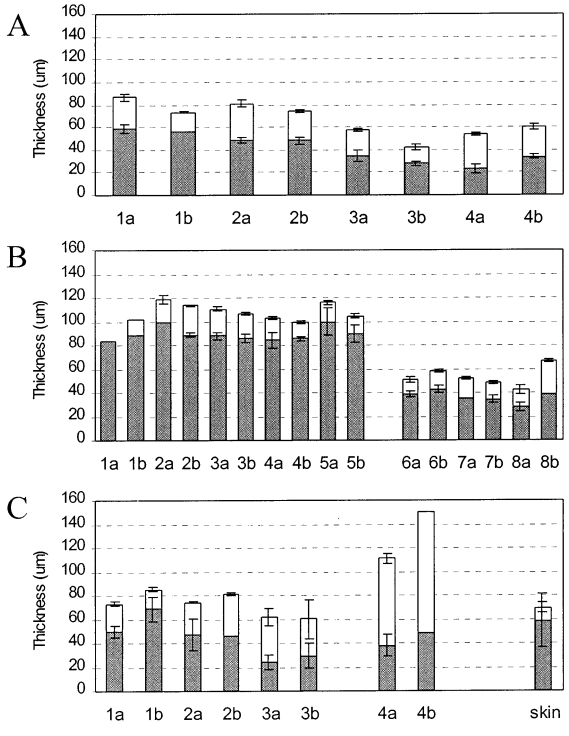

Fig. 1. Thickness of various skin models compared to native skin. Thickness (μm) of viable epidermal layers (grey bars) and stratum corneum (white bars) was evaluated at electron microscopic level in individual cultures of (A) four batches of SkinEthic models; (B) five batches of EpiDerm irritation models (1–5) and in three batches of EpiDerm penetration models (6–8); (C) three batches of Episkin irritation models (1–3), in one batch of an Episkin penetration model (4) and in native skin. In two batches of Episkin penetration models, the strata were too compact to discriminate between individual layers. Duplicate samples (a and b) of each batch were evaluated.

Epidermal and stratum corneum thickness was established in μm (Fig. 1) and in number of cell layers. EpiDerm cultures demonstrated an epidermal thickness consisting of about seven to 14 cell layers (83–100 μm) in skin irritation models and six to eight layers (28–43 μm) in penetration models. The stratum corneum was about 16–25 layers (12–28 μm) for both models. In SkinEthic cultures the epidermis consisted of five to nine cell layers (23–59 μm) and 14–24 layers (15–32 μm) in the stratum corneum. Episkin cultures consisted of seven to ten cell layers (24–69 μm) in irritation models and of seven cell layers (38–48 μm) in penetration skin models. The stratum corneum in Episkin irritation model consisted of 15–24 cell layers (17–37 μm). Remarkable was the presence of a thick stratum corneum of about 60–100 layers (73–102 μm) in the Episkin penetration model. In two batches of Episkin penetration models it was impossible to distinguish the different strata of the viable cell layers (basale, spinosum and granulosum), and therefore these samples were not included in Fig. 1.

3.1.2. Basement membrane

Models tested here were generated on different dermal substrates, e.g. on a thin layer of collagen on cell culture inserts (EpiDerm), on inert polycarbonate filters (SkinEthic), or on type I bovine collagen matrices surfaced with a film of type IV human collagen (Episkin). None of the cultures generated a normal, fully developed basement membrane. Occasionally, all three models exhibited a patchy distribution of lamina densa and lamina lucida. No anchoring fibrils were observed, but in basal cell layer hemidesmosomes could be demonstrated in all SkinEthic cultures, in about half of EpiDerm cultures and only in some Episkin cultures (data not shown).

3.1.3. Stratum basale

Basal cells were columnar to round in shape in all SkinEthic cultures and in most EpiDerm cultures. In some EpiDerm cultures the basal cells were flatttened. In Episkin cultures the basal cell layer had a more irregular appearance. Striking

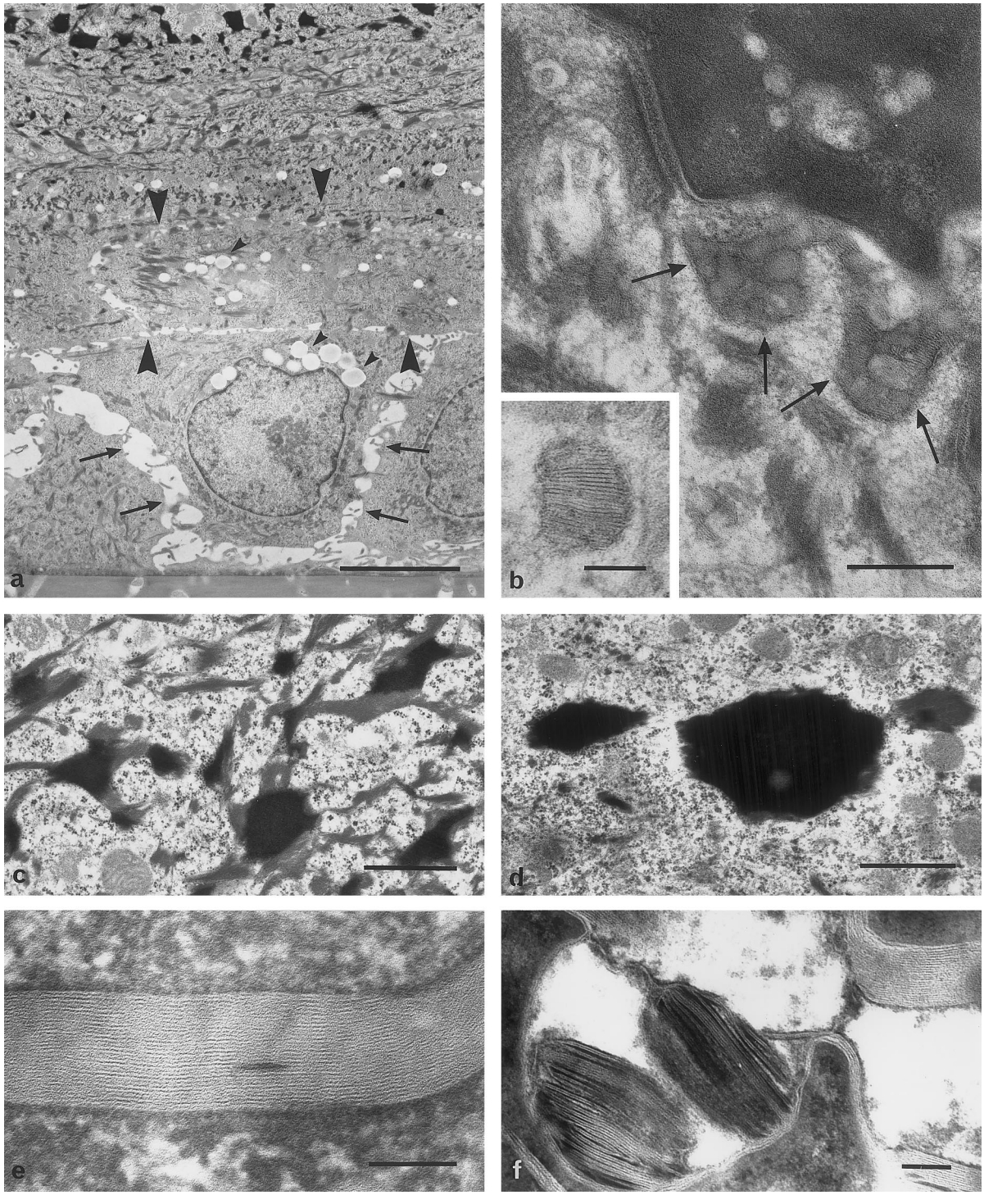

Fig. 2.

was the presence of lipid droplets in the cytoplasm of the basal cells in all EpiDerm and Episkin cultures, and in some SkinEthic cultures (Fig. 2a). Cells with morphological characteristics of apoptosis (condensed nucleus, vacuoles and cell fragments) were often observed in the basal layer of SkinEthic and EpiDerm cultures. In three batches of Episkin, sparse, short keratin filaments were observed, and in one batch the basal cells were completely disconnected from one another. Spongiosis was a frequently observed phenomenon in all culture models (Fig. 2a).

3.1.4. Stratum spinosum

In normal skin, keratinocytes in suprabasal layers loose their columnar shape and progressively flatten out as the apical surface of the tissue is reached. This phenomenon was readily found in all cultures of EpiDerm. However, the spinous cells were already flat in the cultures of SkinEthic and flat or irregular in shape in the cultures of Episkin (Fig. 2a). Only in Epiderm cultures the lamellar bodies were present in the upper stratum spinosum layer. Lipid droplets were found in the cytoplasm of the spinous cells in cultures of all three models, although not as abundantly as in the cells of the stratum basale.

3.1.5. Stratum granulosum

The stratum granulosum in native skin is characterized mainly by the presence of two cytoplasmic organelles: stellate shaped keratohyalin granules and lamellar bodies. As in normal skin, numerous lamellar bodies were ubiquitously present in all skin models at numbers similar to that seen in native tissue (Fig. 2b). Extrusion of lamellar bodies at the stratum granulosum/corneum interface was observed in all EpiDerm cultures but only in about half of the SkinEthic and Episkin samples (Fig. 2b). Keratohyalin granules were present in the granular cells of all cultures investigated. However, their shape varied from stellate and rounded/stellate in SkinEthic and EpiDerm cultures to rounded and enlarged in Episkin cultures (Fig. 2c and d).

3.1.6. Stratum corneum

For visualization of intercorneocyte lipid bilayers, ruthenium tetroxide was used as an additional fixative. All culture models showed the unique organization of the characteristic alternating electron-dense and electron-lucent lipid lamellae (Fig. 2e). In addition, in some Episkin cultures the processing of lamellar bodies into lipid lamellar sheets was locally disturbed and the presence of not fully transformed lamellar bodies was noticed in the upper stratum corneum layers (Fig. 2f).

In nearly all EpiDerm cultures the stratum corneum depicted a basket-wave pattern like in normal skin (Fig. 3b), whereas in some SkinEthic samples and all Episkin samples the stratum corneum appeared to be very compact (Fig. 3a and c). The intercellular space in these cultures was not continuously filled with lipid lamellae, and in some locations only two lamellae corresponding to the lipid envelope of two adjacent corneocytes in close apposition were seen. In the Episkin penetration model the stratum corneum consisted of a remarkable high number of corneocyte layers (Fig. 3a). All three-culture models were characterized by the presence of intracellularly located lipid droplets in corneocytes, although not to the same extent. In EpiDerm they were found only occasionally, but in SkinEthic

Fig. 2. Survey of the ultrastructural characteristics of EpiDerm, SkinEthic and Episkin models. (a) Electron micrograph showing an overview with columnar to round basal cells containing multiple lipid droplets (small arrowheads) and an abrupt flattening of the cells in the stratum spinosum (large arrowheads) often seen in Episkin and SkinEthic cultures. Spongiosis was often observed (arrows); scale bar, 5 μm. (b) The presence of numerous lamellar bodies (insert; scale bar, 0.1 μm) and their extrusion at the stratum granulosum–stratum corneum interface (arrows; scale bar, 0.5 μm). (c) Keratohyalin granules were either stellate, rounded/stellate in EpiDerm and SkinEthic cultures (scale bar, 1 μm) or (d) rounded and enlarged in most Episkin cultures (scale bar, 1 μm). (e) Lipid lamellae showing a characteristic electron-dense and electron-lucent pattern were observed in all culture models (scale bar, 0.1 μm), but (f) in some Episkin cultures lamellar bodies were found in the upper stratum corneum layers indicating indicating disturbance in the processing of the lamellar bodies into lipid lamellar sheets; scale bar, 0.1 μm. (a–d) osmium-postfixation; (e and f) RuO_4 staining.

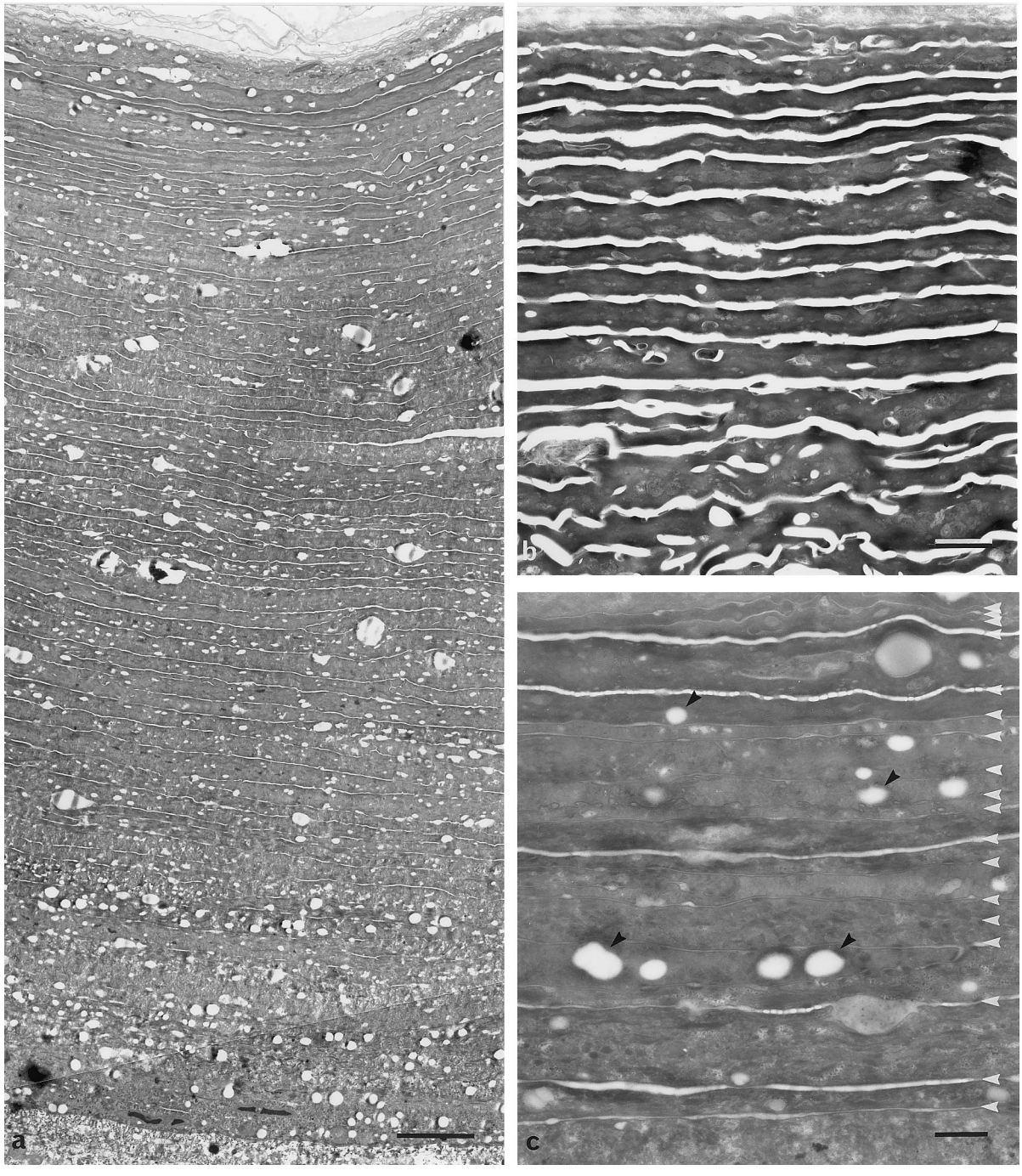

Fig. 3. Ultrastructural appearance of the stratum corneum in EpiDerm, SkinEthic and Episkin models. Osmium-postfixation. (a) Electron micrographs showing stratum corneum of Episkin consisting of a remarkably high number of densely packed cell layers; scale bar 5 μm. (b) Normal basket-wave pattern of the stratum corneum as observed in most EpiDerm cultures; scale bar, 1 μm. (c) Very compact stratum corneum seen in some SkinEthic and in most Episkin samples; scale bar, 1 μm. Cell borders are depicted with white arrowheads. Note the presence of multiple lipid droplets in the corneocytes (black arrowheads).

and Episkin regularly, and especially in Episkin abundant lipid droplets were seen in all samples (Fig. 3a and c).

3.2. Lipid composition

Total amount of lipids extracted from the collected tissue showed little intra- and inter-batch variations. However, differences in lipid content between the models were observed. The total lipid content was lowest in SkinEthic models (0.290 ± 0.06 mg/cm^2), higher in Episkin irritation models (0.438 ± 0.081 mg/cm^2), and was highest in EpiDerm irritation models (0.526 ± 0.170 mg/cm^2). In both penetration models the total lipid content was higher as compared to the irritation ones, being for Episkin 0.556 ± 0.040 mg/cm^2 and for EpiDerm 0.997 ± 0.06 mg/cm^2.

Epidermal lipid profile of different skin models is shown in Figs. 4 and 5. The lipid extracts derived from all skin models tested contained major epidermal lipid classes, including phospholipids, cholesterol sulfate, glucososphingolipids, ceramides, free fatty acids, cholesterol, triglycerides and cholesterol esters. Compared with native epidermis some differences were noticed in the content and profile of some lipid classes. These differences comprised:

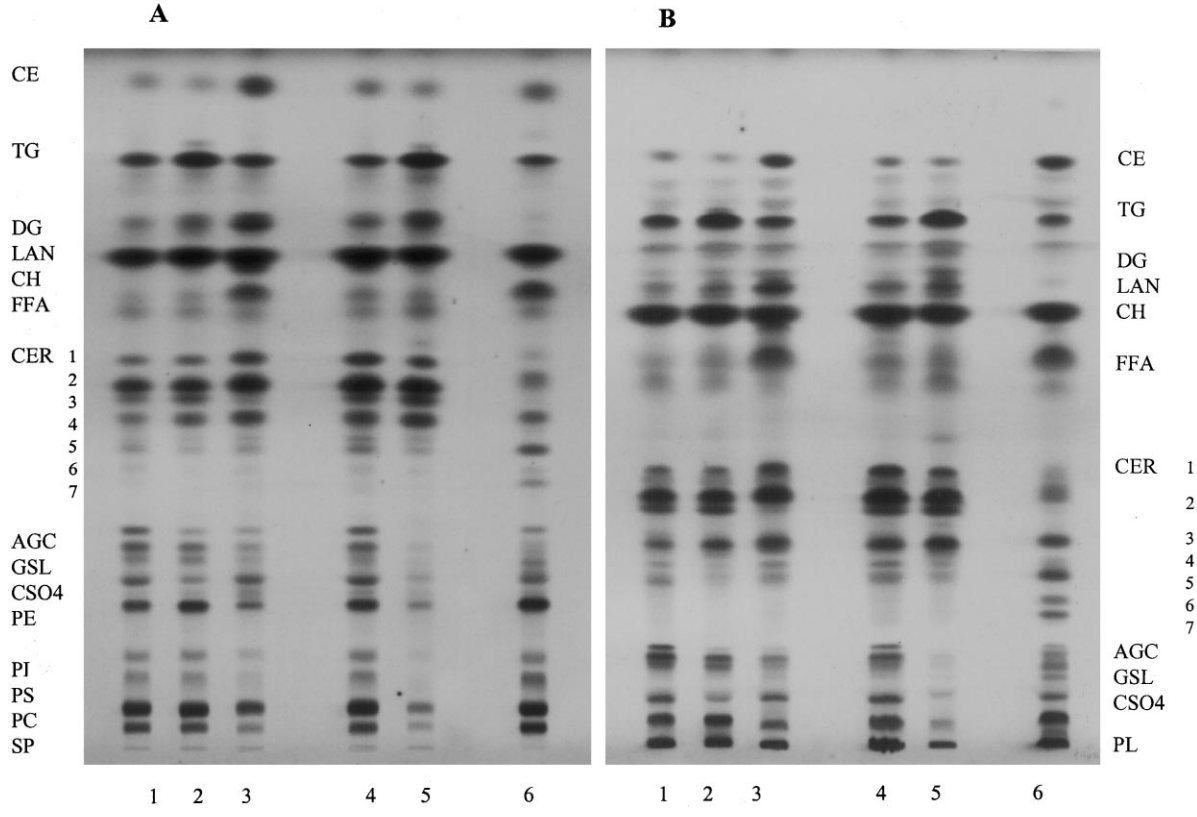

Fig. 4. Thin-layer chromatographic separation of lipids extracted from various skin models and native skin. Total epidermal lipids were extracted and subjected to separation by HPTLC using the (A) 'total' and (B) 'ceramide' development systems. Representative profiles are shown. PL, phospholipids; CSO_4, cholesterol sulfate; GSL, glucosphingolipids; CER, ceramides (1–7); FFA, free fatty acids; CHOL, cholesterol; LAN, lanosterol; DG/TG, di/triglycerides; CE, cholesterol esters. Skin irritation models: EpiDerm (lane 1), Episkin (lane 2), SkinEthic (lane 3). Skin penetration models: EpiDerm (lane 4), Episkin (lane 5). For comparison, the lipid profile is shown obtained form native human epidermis (lane 6). Per lane, 10 μg (A) and 20μg (B) of total lipids were applied.

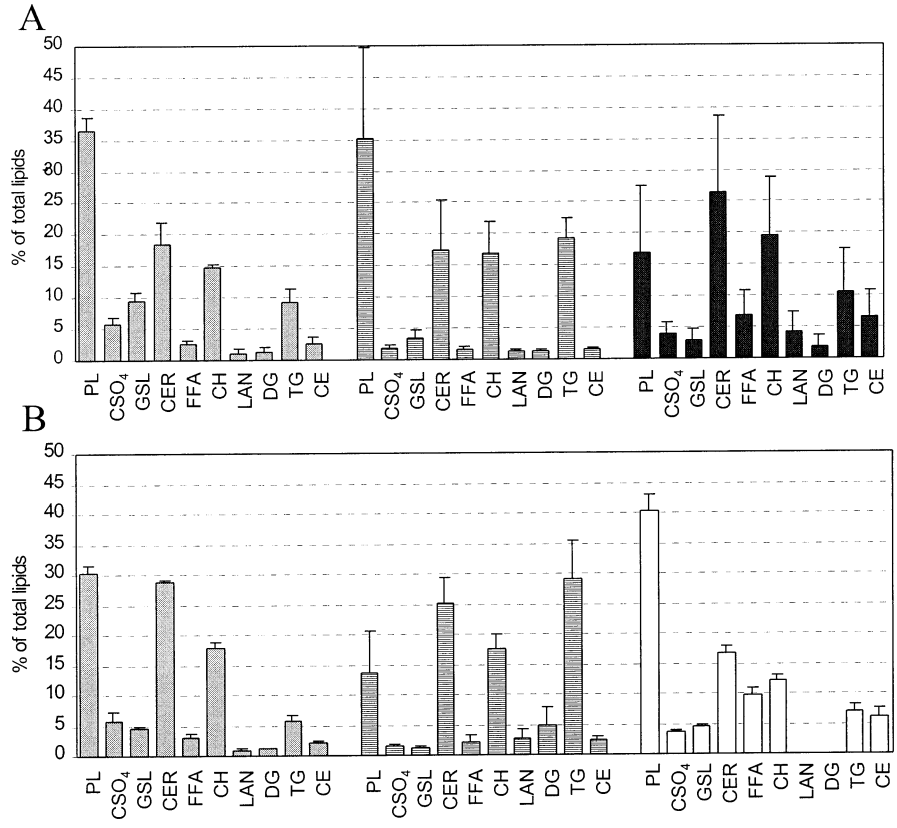

Fig. 5. Relative amounts of epidermal lipids in various skin models and in native skin. Total epidermal lipids were extracted and subjected to separation by HPTLC as described in Section 2. Data are presented as weight percent of total lipids in EpiDerm irritation models ($n = 5$) (grey bars), Episkin irritation models ($n = 3$) (striped bars), SkinEthic models ($n = 4$) (black bars), (B) EpiDerm penetration models ($n = 3$) (grey bars), Episkin penetration models ($n = 3$) (striped bars), and native epidermis (white bars). Note the high interbatch variation in Episkin and SkinEthic cultures. PL, phospholipids; CSO_4, cholesterol sulfate; GSL, glucosphingolipids; CER, ceramides (1–7); FFA, free fatty acids; CHOL, cholesterol; LAN, lanosterol; DG/TG, di/triglycerides; CE, cholesterol esters.

3.2.1. Phospholipids

Phospholipids are constituents of membrane bilayers in cellular organelles of viable cell layers and their content gradually decreases during keratinocyte differentiation (Gray and Yardley, 1975). The analysis of lipid profiles revealed that the overall phospholipid content differed between various skin irritation models. In EpiDerm and Episkin the values were closest to those found in native epidermis (35 vs. 41%). In Episkin and SkinEthic samples the inter-batch variations were high probably reflecting batch-to-batch differences in the thickness of viable epidermal cell layers. Comparison of epidermal lipid profiles between irritation and penetration kits revealed no significant differences for Epiderm but marked differences for Episkin. In the latter the relative amount of phospholipids decreased from about 35% in irritation models to about 15% in penetration models.

The relative amounts of individual phospholipid classes were similar in all skin models, being approximately for sphingomyeline 16%, phosphatidylcholine 42%, phosphatidylserine 8%, phosphatidylinositol 10%, phosphatidylethanolamine 25%, and close to values found in

the native tissue (sphingomyeline 12%, phosphatidylcholine 39%, phosphatidylserine 14%, phosphatidylinositol 10%, phosphatidylethanolamine 26%).

3.2.2. Glucosphingolipids and ceramides

Using the HPTLC separation approach, as shown in Fig. 4, four different glucosphingolipid fractions differing in their polarity can be distinguished in lipid extracts of the native epidermis. These lipids are precursors of ceramides that can be separated by HPTLC in at least seven different fractions (reviewed in 3). The separation of GSL into a lower number of fractions most probably results from smaller differences in polarity between various GSL fractions caused by the presence of a glucose moiety. The overall GSL content in Episkin and SkinEthic models was close to that seen in native skin. In EpiDerm the relative amount was higher. In all skin models tested the least polar GSL - acylglucosylceramide (precursor of ceramide 1) was present. The second least polar GSL was mostly present in relatively higher amounts than in native epidermis. The other two GSL fractions could not be detected in most of the samples tested.

The ceramides were abundantly present in all skin models. In SkinEthic cultures the content was high reaching levels of 25% of total lipids. In both EpiDerm and Episkin penetration models the ceramide content was higher (between 25 and 30%) compared with the irritation models (about 18%). Inspection of the ceramide profiles revealed differences from that seen in native tissue (Table 1): in all skin models ceramide 2 was the most abundant ceramide fraction amounting for about 50% compared with 25% in native epidermis. In addition, an additional ceramide fraction with a slightly higher polarity than that of ceramide 2 (ceramide 2a) could be detected in high amounts in EpiDerm and Episkin cultures. The level of ceramide 2a was lowest in Skinethic cultures, reaching the levels found in native epidermis. In addition, the levels of more polar ceramides also differed from those seen in native tissue, as demonstrated by low relative amounts of ceramides 5 and 6 and the absence of ceramide 7.

3.2.3. Free fatty acids

The content of one of the major stratum corneum lipid fractions, i.e. free fatty acids, was very low in EpiDerm and Episkin cultures (about 2%) and moderate in SkinEthic cultures (about 6%) as compared with native epidermis (10%). This was observed in both the irritation and penetration skin models.

3.2.4. Lanosterol

A precursor of cholesterol, was present in detectable amounts (1–5%) in most of the cultures. The absence of this lipid in native tissue indicates anomalies in cholesterol biosynthetic pathway in vitro.

Table 1
Ceramide profile in different skin models

Ceramides (% of total)	Irritation models			Penetration models		Native epidermis ($n=6$)
	EpiDerm ($n=5$)	SkinEthic ($n=4$)	Episkin ($n=3$)	EpiDerm ($n=3$)	Episkin ($n=3$)	
1	10.5 ± 3.6	15.4 ± 3.2	9.8 ± 2.9	13.7 ± 2.9	7.6 ± 1.6	9.2 ± 1.9
2	45.9 ± 2.8	53.7 ± 5.5	42.6 ± 3.6	43.9 ± 2.1	40.4 ± 1.9	24.7 ± 5.7
2a	12.6 ± 3.9	2.4 ± 2.4	16.7 ± 4.2	10.2 ± 2.3	18.1 ± 1.6	2.7 ± 2.3
3	15.1 ± 2.6	18.8 ± 3.7	23.8 ± 2.6	18.8 ± 0.4	26.4 ± 0.9	21.7 ± 0.5
4	4.5 ± 0.6	3.5 ± 1.6	3.0 ± 1.4	4.4 ± 0.3	2.8 ± 0.4	4.8 ± 1.1
5	8.9 ± 1.8	4.7 ± 1.3	3.4 ± 1.8	6.6 ± 0.7	3.5 ± 0.2	20.9 ± 0.3
6	2.4 ± 0.6	1.4 ± 0.5	0.6 ± 0.4	2.3 ± 1.5	1.2 ± 0.3	5.7 ± 0.5
7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	10.3 ± 0.8

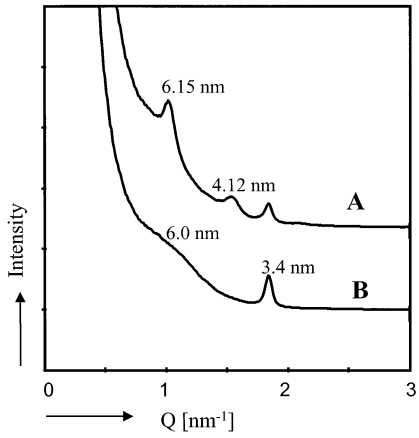

Fig. 6. SAXD pattern of stratum corneum isolated from skin penetration models (A) EpiDerm, (B) Episkin. The 6.15- and 4.12-nm spacings correspond to the second and third order of the 12-nm lamellar phase. The peak with 3.4-nm spacing corresponds to crystalline cholesterol that is phase separated.

3.2.5. Di-/triglycerides

Small amounts of diglycerides have been noticed in most of the batches tested. This fraction is absent in the native tissue. The triglyceride contents in EpiDerm and SkinEthic were comparable with those found in native tissue (about 10%). In contrast, triglyceride content was high in Episkin (about 20%). In EpiDerm penetration models the triglyceride content was only 6% compared to about 30% in Episkin penetration models.

3.2.6. Cholesterol esters

In all analyzed samples the content of cholesterol esters was much lower than the amounts found in native tissue.

3.3. Small angle X-ray diffraction (SAXD)

Only two skin penetration models — EpiDerm and Episkin — were included in the SAXD experiments. SAXD profile of SC isolated from EpiDerm (Fig. 6, curve A) revealed the presence of two peaks at 6.15 and 4.1 nm that can be attributed to a long periodicity phase of about 12.3 nm. In addition, a peak at 3.4 nm indicate the presence of the crystalline cholesterol. The same cholesterol peak was detected in SAXD pattern (Fig. 6, curve B) of Episkin. In contrast to EpiDerm only a weak broad peak was seen at 6 nm, indicating poor lipid organization in the stratum corneum isolated from Episkin.

4. Discussion

In the three skin models evaluated in the present study the epidermis was reconstructed by culturing normal human keratinocytes at the air–liquid interface. Both the substrate and the medium composition differed between models (Rosdy and Clauss, 1990; Tinois et al., 1991; Cannon et al., 1994; Klausner et al., 1996; Monteiro-Riviere et al., 1997).

In general, the overall ultrastructural appearance of the epidermis in the skin models was highly similar to that of native tissue. All major epidermal strata including stratum basale, stratum spinosum, stratum granulosum and stratum corneum were present. However, some differences were noticed compared with native epidermis. In human skin the presence of columnar-shaped basal cells resting on the basement membrane is important for mechanical association with underlying dermis. In cultures examined here, the epidermis could easily be detached from the underlying substrate probably due to the observed absence of a fully developed basement membrane. This may have implications for the experimental design for irritation and penetration studies, in which the firm attachment of the cultured tissue to the support is frequently required.

In native skin basal cells are bordered on the apical site by spinous cells. The proper shape and polarity of individual epidermal cell layers probably plays an important regulatory role in terminal differentiation program. In all models the cells flatten out as the apical surface of the tissue is approached. However, this transition from cubical basal cells to flat granular cells seemed to occur relatively abrupt in SkinEthic and Episkin cultures, indicating disturbances in the regulation of the differentiation process. Only in EpiDerm cultures the lamellar bodies were present in the upper stratum spinosum layers as in native epidermis. In addition, basal and spinous cells in Episkin cultures showed an irregular and flat ap-

pearance and presence of short keratin filaments. The global shape of keratohyalin granules together with less formed keratin filament network and reduced expression of differentiation-specific keratins (keratin 1 and 10) (Boelsma et al., 2000) often seen in differentiation disorders (Holbrook et al., 1987), is indicative for disturbances in the terminal diiferentiation process observed in a number of samples tested.

Remarkable was the presence of lipid droplets in basal cells in all models and in suprabasal cells in some of them. The presence of lipid droplets has been associated with a hyperproliferative status of the reconstructed epidermis (Boyce and Williams, 1993; Nolte et al., 1993). A disturbance in the differentiation process in the skin models tested is supported by recent observations showing deviations in the expression of a variety of differentiation specific protein markers in these models, like keratin 6, involucrin, small proline rich proteins (SPRRs), skin-derived antileukoproteinase (SKALP), transglutaminase (Boelsma et al., 2000). In untreated (control) cultures levels of SKALP (Boelsma et al., 1998), integrins (von den Driesch et al., 1995), and cytokines (Nickoloff and Naidu, 1994; S. Gibbs, personal communication) are often already elevated compared to levels found in normal human skin. It should be noticed that a basal presence of these 'irritation markers' may interfere with their induction upon topical application of irritating agents since differences in the expression of above mentioned markers between treated and untreated cultures may be too small to detect. This may limit the use of skin equivalents for testing of mild irritants.

For the application of reconstructed human skin equivalents in studies concerning pharmacology, toxicity, metabolism and permeability, the formation of a competent stratum corneum barrier is of crucial importance, since topically applied compounds first have to pass the stratum corneum before they can affect cellular functions of the viable cells. The extent of the effect will depend on the actual concentration of the test agent in the viable cell layers and thus on the quality of the barrier. Electron microscopic evaluation of the stratum corneum structures revealed the presence of characteristic intercellular lipid lamellae consisting of multiple alternating electron-dense and electron-lucent bands in the extracellular space in all three models. It should be noticed, however, that this pattern did not exist throughout the whole intercellular space. This was especially the case in SkinEthic and Episkin samples in which the stratum corneum revealed a very compact appearance. In these cultures, the non-homogeneous distribution of lipid lamellar structures within the stratum corneum might most probably be attributed to disturbances in the lamellar body extrusion at the stratum granulosum/corneum interface that was observed in about half of the samples. In some Episkin cultures also areas with not fully transformed lamellar bodies were noticed in the upper stratum corneum layers, which is indicative for disturbed processing of the secreted lamellar bodies. Disturbances in the fusion of adjacent lamellar structures and/or in the formation of unit membranes may be due to a lack in proper extracellular processing by e.g. phospholipase A_2 (reviewed in Elias and Menon, 1991) or β-glucocerebrosidase (Holleran et al., 1992). The number of intracellularly located lipid droplets was low in the stratum corneum of EpiDerm and SkinEthic cultures but high in Episkin cultures. In general, the presence of serum in culture medium used for generation of air-exposed cultures may explain the abundant presence of lipid droplets in the stratum corneum as well as the high triglyceride content (Fartasch and Ponec, 1994; Ponec et al., 1997).

While in skin irritation models the thickness of the stratum corneum was more or less similar, marked differences have been observed between EpiDerm and Episkin penetration models. In the latter the stratum corneum was often composed of a remarkably high number of cell layers, which may lead to a misinterpretation of penetration data. Namely, the diffusion of compounds through the stratum corneum is a passive diffusion process governed by Fick's law (Scheuplein and Blank, 1971). This implies that the penetration rate is inversely dependent on the thickness of the stratum corneum. Penetration studies performed so far revealed that the rate of percutaneous absorption in reconstructed human epidermis is generally higher than in native skin

(Bell et al., 1991; Mak et al., 1991; Slivka et al., 1993; Roguet et al., 1994; Ponec, 1996; Doucet et al., 1998; Rosdy et al., 1998). However, this has been shown to occur even when the thickness of the stratum corneum exceeds several times that of the native skin (Lotte et al., 1998a,b).

This apparent contrast may be explained by the absence of continuous lipid lamellar domains throughout the whole extracellular space, by observed differences in lipid composition and in lipid lamellar organization. It has been shown that in stratum corneum cholesterol, ceramides and free fatty acids are present in approximately equimolar ratios (Man et al., 1993). In addition, the ceramide fraction consists of at least seven different ceramides (Wertz and Downing, 1991). Individual ceramides differ in their head-group architecture and the chain length distribution and in addition, ceramides 1 and 4 contain linoleic acid chemically bound to a long chain ω-hydroxyacid (Robson et al., 1996). Recent studies demonstrated that the presence of most of the ceramides is required for the proper formation of lipid lamellae (Bouwstra et al., 1996a,b, 1998). Lipid analyses revealed that the major barrier lipids are synthesized in vitro but not in the same proportions as found in native skin, as was demonstrated by the low content of free fatty acids and the incomplete profiles of glucosphingolipids and of ceramides. The content of ceramides 5 and 6 was markedly reduced and the most polar ceramide 7 was missing. This may indicate deficiencies in hydroxylation of long chain fatty acids and sphingoid bases in vitro (Wertz and Downing, 1983; Robson et al., 1996). Therefore, stimulation of hydroxylation, e.g. by ascorbic acid (Ponec et al., 1997) may facilitate synthesis of ceramides 6 and 7. Furthermore, we have observed that an additional ceramide (here assigned as ceramide 2a) was abundantly present in EpiDerm and Episkin cultures. The deviation in lipid profile may also explain the differences in SAXD pattern seen in EpiDerm and Episkin cultures in which only the long-range lipid lamellar phase could be detected. A recent study in which the barrier properties of human epidermis reconstructed on de-epidermized dermis (Ponec et al., 1997) have been examined disclosed that even upon normalization of ceramide profile and increase in the fatty acid content still deviations in lamellar lipid organization persisted. In these cultures the intercellular space of the entire stratum corneum was abundantly filled with multiple broad lipid lamellar structures, as observed by electron microscopy. The presence of well-ordered lipid lamellar phases was confirmed by small angle X-ray diffraction. However, in this reconstructed epidermis only the long-range lipid lamellar phase was present and the short lamellar phase, usually present in native skin, was missing. Similar observations were made with EpiDerm cultures. It seems likely that reduced free fatty acid content (especially the long-chain ones) may be the cause of differences in lipid organization (Fartasch and Ponec, 1994). A similar situation was observed in human skin in which treatment with sodium lauryl sulfate induced disturbances in barrier function (Fulmer and Kramer, 1986).

Although scientific and ethical needs to develop alternative models have been established, intra- and inter-laboratory evaluations of quality and reproducibility form a vital and mandatory part in the development process. Satisfactory performance in this phase, which often includes culture modifications and optimization, provides the basis for the application of skin models in toxicity screening, permeability studies, and pharmacological risk assessments. In conclusion, the skin models described here represent a promising tool for both fundamental and applied research although still attention has to be paid to the observed deviations form native skin.

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