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Covalently Bound Lipids in Reconstructed Human Epithelia

MARIA PONEC, ESTHER BOELSMA and ARIJ WEERHEIM

Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands

The composition of free and covalently bound lipids in reconstructed epithelia generated with normal human keratinocytes, HaCaT cells and squamous carcinoma cells was investigated and compared with native skin. Stratum corneum isolated from native human and reconstructed epidermis was subjected to extensive extraction with chloroform-methanol mixtures followed by alkaline hydrolysis to release covalently bound lipids. High-performance thin layer chromatography was used for analysis of solvent-extractable and non-extractable lipids and gas liquid chromatography was performed to assess the fatty acid profile in extractable lipids. In both native and reconstructed tissue covalently bound lipids consisted of ω-hydroxyceramides, ω-hydroxyacids and free fatty acids. Small amounts of ω-hydroxyacids could already be detected in solvent-extractable fractions. ω-Hydroxyceramides consisted of Ceramide A, Ceramide B and a small fraction of unknown ceramides with intermediate polarity. The relative proportions of individual ω-hydroxyceramides were similar in both native and reconstructed stratum corneum. In contrast, differences were found in profiles of both solvent-extractable and non-extractable lipids isolated from epithelia reconstructed with transformed cell lines (HaCaT, SCC-12F2 and SCC-13 cells). Compared with native or reconstructed epidermis, in epithelia reconstructed with transformed cell lines the ceramide content was low, the most polar ceramides were missing and the content of free fatty acids was low. The same holds true for covalently bound lipids that were virtually absent in these epithelia. Marked similarities were demonstrated in the overall lipid composition of free and bound stratum corneum lipids in native epidermis and in epidermis reconstructed with normal human keratinocytes. The observed imbalance in fatty acid profile may account for differences in phase behaviour of stratum corneum lipids. ***Key words: normal human keratinocytes; HaCaT cells; squamous carcinoma cells; lipid composition.***

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Maria Ponec, Department of Dermatology, Leiden University Medical Center, bld. 3, Sylvius Laboratory, PO Box 9503, NL-2300 RA Leiden, The Netherlands.
ponec@mail.medfac.leidenuniv.nl

It has long been recognized that the epidermis provides a barrier that protects the underlying tissue against desiccation and against a host of noxious environmental agents (1, 2). The permeability barrier resides in multiple lipid bilayers that fill the extracellular spaces between the keratinized cells (corneocytes) of the outermost skin layer, the stratum corneum. These extracellular bilayers consist mainly of cholesterol, ceramides and free fatty acids (3) and are organized in 2 lamellar phases with a periodicity of about 6 and 13 nm, respectively (4). The lipid lamellae are oriented approximately parallel to the corneocyte surface. Such a spatial organization is most probably achieved by the presence of a lipid monolayer (the lipid envelope) (5) covalently bound to the outer surface of a protein membrane, so-called cornified envelope (6). This cornified envelope, which consists of various proteins, is formed during final stages of keratinocyte maturation and is deposited as a thick band of protein on the inner surface of the plasma membrane. The lipid envelope consists of ω-hydroxyceramides, ω-hydroxyacids and fatty acids (5). The ω-hydroxyceramides have been demonstrated to consist of a long chain ω-hydroxyacid amide-linked to sphingosine (Ceramide A) (5) and 6-hydroxy-4-sphingenine (Ceramide B) (7). It has been proposed that ω-hydroxyceramides, the major component of bound lipids, are attached by their fatty acid hydroxyl group or by one of the sphingosine hydroxyl groups to the glutamate side of the protein cornified envelope (8).

Reconstructed epidermis generated by culturing human epidermal keratinocytes at the air-liquid interface provides an interesting model for studies to the processes regulating epidermal lipogenesis, since it closely mimics native epidermis both on morphological and biochemical level (9, 10). Electron microscopic studies revealed the presence of multilamellar lipid sheets throughout the stratum corneum as well as the presence of the protein cornified envelope both in human (9) and murine native and reconstructed epidermis (11). Analysis of epidermal lipids demonstrated the presence of all lipid classes necessary for the formation of a competent barrier (9). However, no information is presently available as to whether human keratinocytes *in vitro* are also capable of synthesizing covalently bound lipids, with the exception of a study of Kennedy et al. (12) who using electronmicroscopic approach could not detect the presence of covalently bound lipids in *in vitro* reconstructed epidermis.

The aim of the present study was to examine whether cultured keratinocytes synthesize covalently bound lipids and, if so, whether their composition is comparable to that found in native epidermis. It has been shown that oral epithelia that differ in their differentiation pattern from the epidermis also differ in their lipid composition (13) and in the amount of covalently bound lipids (14). Therefore, the composition of free and covalently bound lipids in reconstructed epithelia generated with cells exerting a defect in their terminal differentiation program (HaCaT cells (15), and squamous carcinoma cells (SCC) SCC-12F2 and SCC-13 (16–18)) has also been examined and compared with that found in epidermis reconstructed with normal human keratinocytes.

MATERIAL AND METHODS

Cell culture

Normal human keratinocytes (NHK) were isolated from epidermis of female breast skin obtained after plastic surgery and cultured as described earlier (9, 19). The spontaneously transformed keratinocyte cell line HaCaT was a generous gift from Professor Dr N. Fusenig, Heidelberg (15) and SCC lines SCC-12F2 and SCC-13 were kindly

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provided by Dr J. Rheinwald (16). Normal human fibroblasts were obtained after outgrowth from skin explants (20).

Generation of reconstructed epithelia

Fibroblast-populated collagen matrices were prepared as described earlier (9, 21). Briefly, dermal equivalents were prepared by mixing a solution of rat collagen with a suspension of normal human dermal fibroblasts to achieve a final collagen concentration of 3.2 mg/ml and 2×10^5 fibroblasts/ml.

Epidermal equivalents were generated as described earlier (9, 18, 21). Briefly, suspensions of NHK, HaCaT, SCC-12F2 or SCC-13 cells (2×10^5 cells/cm^2) were seeded onto a dermal substrate and the cultures were first grown under submerged conditions for 1 day. A mixture of DMEM/Ham's F12 (3:1) media was used with the following supplements: 5% bovine serum (HyClone, Greiner, Germany), 0.5 μM hydrocortisone, 0.1 μM isoproterenol, 0.5 μg/ml insulin. One day after plating the cultures were lifted to the air-liquid interface, the serum concentration was reduced to 1% and the medium was further supplemented with 10 mM serine, 10 μM carnitine, 50 μg/ml ascorbic acid, 1 μM α-tocopherol, 8 μg linoleic acid coupled to β-dextrin/ml and 1.6 mg bovine serum albumin/ml. At day 3, serum was omitted whilst other supplements remained unchanged. All supplements were purchased by Sigma. Culture medium was renewed 3 times a week and cultures exposed to the air for 3 and 4 weeks were used for the experiments.

Tissue morphology

Samples were fixed in 4% paraformaldehyde and processed for embedding in paraffin. Vertical sections (5 μm) were cut and stained with haematoxylin and eosin for light microscopic examination.

Lipid extraction and analysis

The epithelia of HaCaT and SCC cultures were separated mechanically from the dermal substrate. For excised skin and reconstructed epidermis, the epidermis was separated from the underlying tissue by dispase (Grade II; Boehringer Mannheim, Germany) treatment (9) followed by incubation for 2 h in 0.1% trypsin (approximately 100 U/mg lyophilizate; Difco Laboratories, Detroit, MI, USA) in phosphate-buffered saline (PBS). Thereafter the tissue was washed with PBS and trypsinization was stopped by a short incubation in PBS containing soybean trypsin inhibitor (10 mg/ml). The epidermis was then washed several times with PBS and reincubated for 2 h in 0.01% proteinase-K (specific activity approximately 20 units/mg lyophilizate) in PBS (Boehringer Mannheim, Germany) (22). The harvested material was first extracted according to Bligh & Dyer (23) (followed by a series (3 – 6) of extractions with chloroform/methanol (1:2, 1:1 and 2:1). All extracts were first dried under a stream of nitrogen then weighted and their lipid composition was determined. The extracted and dried tissue was subjected to a mild saponification treatment, as described by Wertz et al. (5).

The lipid composition of all extracts was determined by analytical high-performance thin layer chromatography as described earlier (24) with small modifications using the following sequential development system: (i) hexane/chloroform/acetone (8:90:2), (ii) chloroform/acetone/methanol (76:8:16), (iii) hexane/chloroform/hexyl acetate/acetone/methanol (6:80:0.1:10:4), (iv) chloroform/acetone/methanol (76:4:20), (v) hexane/chloroform/hexyl acetate/ethyl acetate/methanol (8:80:0.1:6:6), (vi) hexane/diethylether/ethyl acetate (78:18:4). Serial dilutions of lipid standards (sphingomyelin, phospatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, cholesterol sulfate, cerebrosides type I and II, ceramide bovine III and IV, oleic acid, cholesterol, lanosterol, 1,2 diolein, 1.3-triolein, cholesterol oleate (Sigma) and lipid extracts obtained from normal human epidermis were run in parallel. Standards used for identification of covalently bound lipids (Ceramides A and B and ω-hydroxy fatty acids) were kindly provided by Professor Dr P. Wertz (5). Identification of individual lipid fractions was performed after charring (23).

Preparation and analysis of fatty acid methyl esters (FAMEs)

A 100 μg weight of total lipid extracts was dissolved in 100 μl 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. FAMEs dissolved in hexane and purified on a silica gel column were separated and analysed 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 4°C/min, followed by a 2°C/min increase to 250°C, which was maintained until all peaks had eluted. The peaks were identified by comparison with FAME standards (Sigma). Integration of peak areas and calculation of relative percentages were performed by Baseline 810 integrator. Heptadecanoic acid was used as an internal standard.

RESULTS

Terminal differentiation program is disturbed in HaCaT, SCC-12F2 and SCC-13 cells

Comparison of tissue architecture (Fig. 1) revealed that only NHK when grown at the air-liquid interface are capable of forming a fully differentiated epidermis *in vitro* consisting of stratum basale, stratum spinosum, stratum granulosum and stratum corneum. The morphology of epithelia reconstructed with HaCaT, SCC-12F2 and SCC-13 cells showed features of a defective terminal differentiation program, as judged from the absence of a stratum corneum and granulosum and a disorganized growth and differentiation pattern.

Transformed cells demonstrate an impaired capacity to synthesize stratum corneum barrier lipids

Harvested tissue was subjected to sequential solvent extraction to remove the extractable lipids. The majority of lipids (about 95%) appeared to be extracted already during the first extraction procedure. Since equivalent amounts of lipid from cultured and native tissue were applied onto silica plates, the distribution of the individual lipids could qualitatively be compared by their relative intensities (Fig. 2). The profiles in solvent-extractable stratum corneum lipid obtained from native tissue and from epidermis reconstructed with NHK showed high similarities (Fig. 2, lanes 1, 3). All major classes of stratum corneum lipids, including both polar (glucosphingolipids, ceramides and cholesterol sulfate) and non-polar subsets (cholesterol, free fatty acids and cholesterol esters) were present in comparable amounts. Only the amount of triglycerides in the native tissue was higher than in the reconstructed one. It should be noted that in native tissue the triglyceride content often varies. This is probably due to contamination of epidermal samples with small amounts of subcutanoeus lipids during the isolation procedure.

Due to the incomplete differentiation of HaCaT, SCC-12F2 and SCC-13 cells, the whole epithelium was subjected to the sequential extraction procedure. The profile in the first solvent-extractable lipid fraction obtained from all 3 cell lines differed from that seen in NHK (Fig. 2, lanes 5, 7 and 9), showing an extremely low content of free fatty acids and an incomplete profile of glucosphingolipids and ceramides.

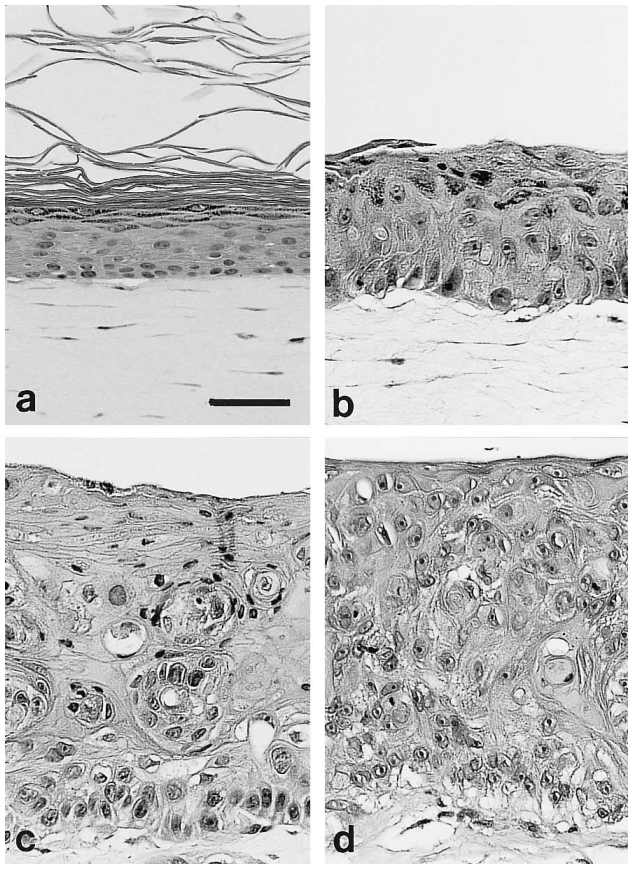

Fig. 1. Terminal differentiation program is disturbed in air-exposed cultures of HaCaT, SCC-12F2 and SCC-13 cells. Reconstructed epithelia were generated by seeding (a) normal human keratinocytes, (b) HaCaT, (c) squamous carcinoma cells SCC-12F2 and (d) SCC-13 cells on fibroblast-populated collagen matrices and culturing for 3 weeks at the air-liquid interface in serum-free medium followed by paraffin-embedding for hematoxylin and eosin staining, scale bar 10 μm.

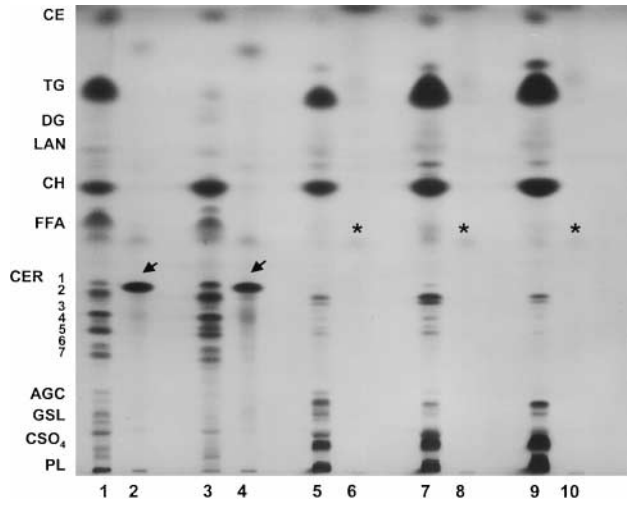

Fig. 2. Epidermis reconstructed with normal and transformed keratinocytes shows differences in composition of extractable lipids. Lipids were extracted from the stratum corneum isolated from the native (lanes 1, 2) and reconstructed (lane 3, 4) epidermis and from whole epithelia reconstructed with HaCaT (lanes 5, 6), squamous carcinoma cells SCC-12F2 (lanes 7, 8) and SCC-13 (lanes 9, 10) cells. The reconstructed epithelia were generated by seeding the cells on fibroblast-populated collagen matrices and culturing for 3 weeks at the air-liquid interface in serum-free medium. After harvesting, the stratum corneum was isolated from native and reconstructed epidermis, extracted and analysed by high-performance thin layer chromatography using the development system as described in Materials and Methods. From HaCaT, SCC-12F2 and SCC-13 cells the entire epithelium was extracted. Lanes 1, 3, 5, 7, 9 show the lipid profile in the 1st extract and lanes 2, 4, 6, 8, 10 in the 4th extract. Per lane 20 μg lipid of the 1st extract was applied and 10 μg lipid of the 4th extract. *Arrow*: ω-hydroxy acids (lanes 2,4), and *asterix*: free fatty acids (lanes 6, 8, 10). PL: phospholipids, CSO_4: cholesterol sulfate, GSL: glucosylceramides, AGC: acylglucosylceramides, CER: ceramides, FFA: free fatty acids, CH: cholesterol, LAN: lanosterol, DG: diglycerides, TG: triglycerides, CE: cholesterol esters.

Only in HaCaT cells the presence of acylglucosylceramide, the precursor of ceramide 1, could be detected. The profile of other glucosylceramides was incomplete as 2 fractions were present in detectable amounts but the most polar one was missing. In all cell lines small amounts of ceramides could be detected, the content of which was lowest in SCC-13 cells. In addition, the ceramide profile was not complete, since in SCC-13 cells only ceramide 2 could be detected, whilst in HaCaT and SCC-12F2 cells ceramides 1–5 were synthesized. However, their relative amount was much lower than in NHK and the most polar ceramides 6 and 7 were missing in spite of supplementation of media with vitamin C (9). As in NHK, also in the cell lines the ceramide fraction with slightly higher polarity than that of ceramide 2 was present. Modulation of culture conditions, like supplementation of media with serum and increasing the number of fibroblasts incorporated into the collagen matrix to 5×10^5/ml collagen did not lead to significant changes in lipid profiles (data not shown).

The amount of lipids collected by subsequent extraction procedures with a chloroform/methanol mixture (1:2, 1:1 and 2:1) was low (2nd extract: 2.7%, 3rd extract: 1.5%, 4th extract: 0.8%). It should be noted that the 3rd and 4th extracts of the stratum corneum isolated from both the native and the reconstructed epidermis contained significant amounts of ω-hydroxyacids (Fig. 2, lanes 2, 4). Analysis of the 4th extract of the cell lines revealed the presence of minute amounts of free fatty acids (Fig. 2, lanes 6, 8, 10).

NHK but not transformed cells generate a complete spectrum of covalently bound lipids

The amount of lipids released by saponification of previously extracted stratum corneum isolated from reconstructed and native epidermis was about 5% of total lipids. These lipids consisted of 4 major fractions with mobilities like Ceramides A and B, ω-hydroxyacids and fatty acids, and a few fractions of still unknown lipids with thin layer chromatographic (TLC) mobilities between Ceramide A and B (Fig. 3).

In transformed cell lines the fraction of non-extractable lipids was about 0.2% of totally recovered lipids. As shown in Fig. 3, only free fatty acids could be detected in these extracts. No change in the amount and the composition of non-extractable lipids has been observed upon prolongation of the culture time at the air-liquid interface up to 4 weeks. Also, modulations of culture conditions, like the use of other dermal supports (de-epidermized dermis or inert filters), the

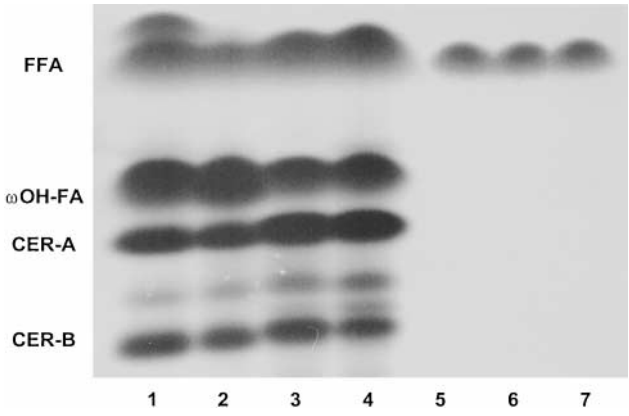

Fig. 3. Normal but not transformed keratinocytes are capable to synthesize covalently bound lipids *in vitro*. Composition of lipids released by saponification of lipids extracted from the stratum corneum isolated from the native epidermis (lane 1, 2) and from reconstructed epidermis (lane 3, 4), and from whole epithelia reconstructed with HaCaT (lane 5), squamous carcinoma cells SCC-12F2 (lane 6) and SCC-13 (lane 7) cells. Reconstructed epithelia were generated by seeding the normal human keratinocytes, HaCaT, SCC-12F2 and SCC-13 cells on fibroblast-populated collagen matrices and culturing for 3 weeks at the air-liquid interface in serum-free medium. After harvesting, the lipids were extracted from the stratum corneum isolated from native and reconstructed epidermis and from whole epithelia reconstructed with HaCaT, SCC-12F2 and SCC-13 cells. The residues were subjected to a mild saponification treatment. The released lipids were analysed by high-performance thin layer chromatography using the development system, as described in Material and Methods. CER: ceramides, ω-OH-FA: ω-hydroxy fatty acids, FFA: free fatty acids.

increase of the number of fibroblasts incorporated into the collagen matrix to 5×10^5/ml collagen and supplementation of media with serum did not lead to changes in composition of bound lipids (data not shown).

Low content of linoleic acid in stratum corneum lipids extracted from epidermis reconstructed with NHK

FAMEs were identified and quantified by gas liquid chromatography in extractable lipid fraction isolated from the stratum corneum of the native and reconstructed epidermis after their transmethylation. Data presented as weight percent in Fig. 4 show that the relative amounts of linoleic acid (C18:2) in reconstructed epidermis is much lower than in native tissue, and the amounts of C20:0 and C22:0 were slightly higher. The relative amounts of other fatty acids were similar.

DISCUSSION

The present study demonstrates the presence of covalently bound lipids in the stratum corneum of human reconstructed epidermis in amounts which were comparable with those found in native tissue. The major components comprise ω-hydroxyceramides (Ceramides A and B), ω-hydroxyacids and free fatty acids, as judged from the comparison of TLC mobilities of these lipids with standards. Similarly as in native epidermis, in reconstructed epidermis the relative amount of Ceramide A was higher than the amount of Ceramide B. Next to these major components a few still unknown components

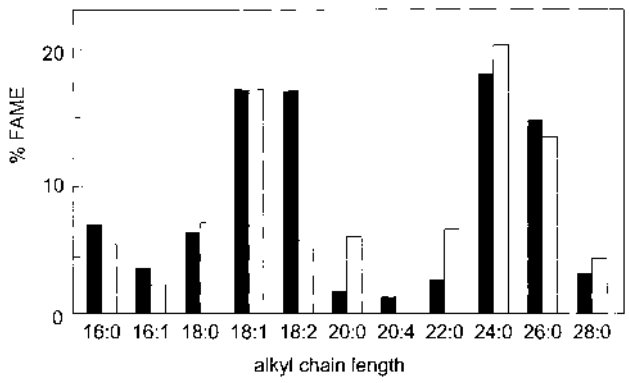

Fig. 4. Low content of linoleic acid in stratum corneum lipids extracted from epidermis reconstructed with normal human keratinocytes. Fatty acid methyl esters (FAME) were prepared from solvent-extractable lipid fraction from the native (*black bars*) and reconstructed stratum corneum (*white bars*), as described in detail in Material and Methods. Data are presented as weight percent and show the mean of 2 independent experiments.

with TLC mobilities between Ceramides A and B were detected. At present it is not known whether these components are also ceramides that may differ in the polarity of the long chain base moiety or in the chain length of the fatty acid moiety.

The nature of the linkage of the other non-extractable lipids (ω-hydroxyacids and free fatty acids) to the protein envelope has not yet been elucidated. Compared with ω-hydroxyceramides and free fatty acids, the binding of ω-hydroxyacids seemed to be less strong, since this fraction could already be partially extracted in later phases of the extraction procedure of the stratum corneum. However, it should be noted that this fraction represented less than 1% of the extractable lipids.

From our findings it can be concluded that no major differences exist in the composition of covalently bound lipids between native and reconstructed epidermis. The same holds true for solvent-extractable lipids, except for some small differences. These include the presence of an additional ceramide fraction that is slightly more polar than ceramide 2, a relatively lower content of free fatty acids, and a slightly altered fatty acid profile (25). The imbalance in the relative amounts of C18 fatty acids in ceramides and possible differences in molecular structure of the sphingoid bases (such as acyl chain length) may account for differences observed in the phase behaviour of stratum corneum lipids in native and reconstructed SC. Namely, in native tissue two lamellar phases with repeat distances of 6 and 13 nm, respectively, have been demonstrated (4), whilst in reconstructed epidermis only a long periodicity phase of 12 nm has been found (9). In addition, in native stratum corneum the lamellar packing of lipids is orthorhombic but in reconstructed tissue the hexagonal lamellar packing is prevailing (26, G. Pilgram, unpublished observation).

In contrast to the high degree of resemblance between native and reconstructed epidermis, epithelia reconstructed with transformed cell lines showed abnormal differentiation patterns as judged from the absence of clear granular and cornified layers, the expression of a broader spectrum of keratins, and disturbed expression of the precursors of cornified envelope proteins (18, 20). The low contents of glucosylceramides, ceramides and free fatty acids showed

remarkable similarities to immature and "wet" epithelia (27) and to non-keratinized oral epithelia (14). The observed low amount of bound lipids (about 0.2% of totally recovered lipids), has also been demonstrated in keratinizing and non-keratinizing epithelia (13). However, the fraction of covalently bound lipids in the HaCaT and SCC lines consisted only of free fatty acids.

In conclusion, the absence of a clear stratum corneum, of the full spectrum of stratum corneum barrier lipids together with the absence of covalently bound ω-hydroxyceramides in transformed epithelial cell lines is indicative for a defective terminal differentiation program in these cells. As it has previously been shown (28) that differentiation processes normalized after transplantation of HaCaT cells onto nude mice, additional factors present *in vivo* may contribute to a normalization of tissue homeostasis. It remains to be established whether under such *in vivo* conditions the synthesis of free and bound lipids can also take place.

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