Improved Barrier Structure Formation in Air-Exposed Human Keratinocyte Culture Systems

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The epidermis (including stratum corneum) of human keratinocytes cultured at the air-liquid interface attached to an appropriate substrate shows a morphology closely mimicking that of its *in vivo* counterpart. In spite of the histologic similarities, the barrier function seems to be impaired. The aim of the present study was to characterize development and structure of the epidermal permeability barrier in two human skin recombinants using electron microscopy (including ruthenium tetroxide-post fixation technique) and analysis of lipid composition. The epidermis was reconstructed by growing human keratinocytes either on de-epidermized dermis or on a bovine collagen-containing matrix with active fibroblasts (Living Skin Equivalent). Ultrastructurally both culture systems showed a) an abnormal lamellar body delivery system, b) disturbance of transformation into lamellar lipid bilayers, c) an impaired structural organization and distribution of the epidermal lipids in the intercellular spaces. In either of the systems used, prolongation of the culture period did not induce any significant improvement in the stratum corneum lipid organization. Whereas the Living Skin Equivalent showed only sparse lamellar bodies, the number of lamellar bodies in the human keratinocyte culture on de-epidermized dermis grown in regular medium seemed to be comparable to native skin. Contrary to the Living Skin Equivalent, the keratinocyte culture on de-epidermized dermis contained a higher number of intracorneocytic lipid droplets correlating with a higher triglyceride content in the lipid analyses. By reconstructing the keratinocyte culture on de-epidermized dermis with the same medium as used for the Living Skin Equivalent, both lipid composition (lower triglyceride, higher ceramide contents) and structural organization were improved, and regular lamellar lipid bilayers comparable to those of native skin appeared. *Key words: epidermal lipids/ultrastructure/triglyceride/culture media. J Invest Dermatol 102:366–374, 1994*

Air-exposed (A/E) human and murine keratinocyte cultures have been demonstrated to possess a multilayered stratum corneum (SC) [1–5] that exerts a substantial diffusion barrier. However, recent studies show that in spite of comparable architecture of the epidermis and of a histologically normal-looking SC, the A/E keratinocyte cultures show a reduced permeability barrier to water and other substances [4,6–9] when compared with intact human skin.

It is generally accepted that the epidermal lipids (ELs) contribute substantially to the regulation of the water permeability barrier of the SC [10,11]. Recent studies propose the structural organization of the SC lipids may be as important in barrier function as the unique lipid composition [11–14].

In contrast to conventional, submerged keratinocyte cultures, the A/E cultures grown on various dermal substrates (such as de-epidermized human dermis [1,2], collagen-coated nylon membranes [15] or collagen gels with or without metabolic active fibroblasts [9,16]) display the capacity to synthesize all lipid species that are present in native tissue; some lipids are synthesized and/or metabolized at rates different from those *in vivo*. This leads to the difference in bulk lipid composition between the A/E keratinocyte cultures and the native tissue [2,3,15,17]. Air-exposed human keratinocyte cultures were found to contain a significantly higher content of triglycerides and a lower content of essential fatty acids [2]. But important precursors of barrier lipids (acylglucosylceramides) [18,19] and ceramides [20], the lipid class most indicative of completed differentiation, are present there in significant amounts [2,21].

Furthermore, A/E cultures are capable of producing lamellar bodies [2,3,5,15], which are the putative source of the SC lipids [22,23]. Similar to *in vivo*, lamellar bodies in A/E murine keratinocyte cultures were found to extrude their contents into the intercellular spaces of the SC [5], and there form multilayered lipid lamellae lacking the regular pattern observed in human skin [14,24,25].

In A/E human keratinocyte cultures, freeze-fracture electron microscopy has shown local anomalies in the lamellar structure of the intercellular lipids in the cornified layers [3], which may be responsible for the impaired barrier function of these cultures. However, this method bypasses the more polar domains because of the preferential deviation of the fracture plain to the most hydrophobic surfaces [14]. The aim of the present study was to obtain further insight into the intercellular lipid organization of *in vitro*-reconstructed epidermis using the ruthenium tetroxide (RuO_4) post-fixation technique. In addition to biochemical analyses of epidermal lipids, the SC lipid structures in various human skin recombinants (constructed by growing keratinocytes on de-epidermized dermis [RE-DED] or on fibroblast-populated collagen matrices [LSE]) were ex-

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Abbreviations: A/E, air-exposed; EL, epidermal lipids; GM, Genesis Living Skin Construct Media; ICS, intercellular space; LB, lamellar body; LSE, Living Skin Equivalent; M, regular culture medium; RE-DED, air-exposed human keratinocyte culture on de-epidermized dermis; SC, stratum corneum; SG, stratum granulosum

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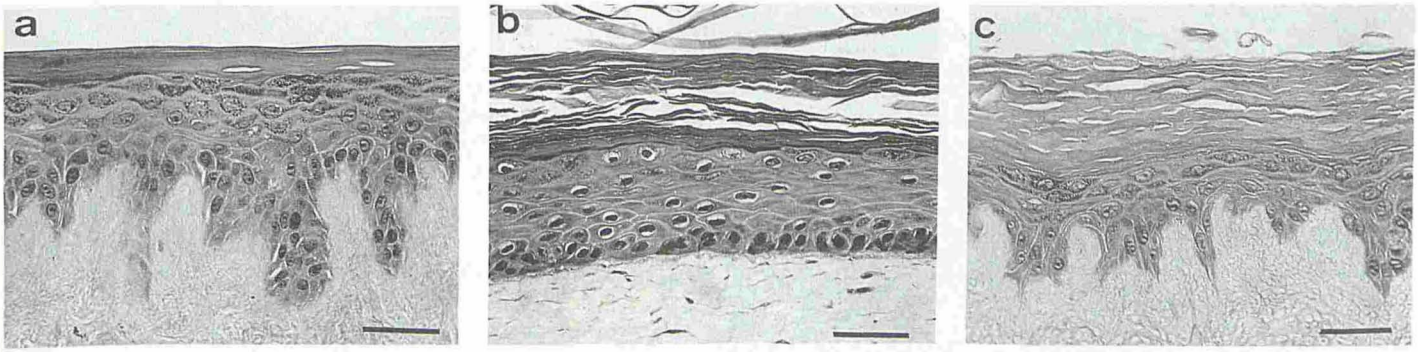

Figure 1. Light microscopy of the A/E cultures. *a*) RE/DED/M grown in regular medium displaying a distinct, prominent stratum granulosum and a convoluted dermal-epidermal junction with a better organized basal layer than in LSE. The stratum corneum layer seemed to be more compact (×486). *b*) LSE showing no rete projection and a more "basket-weave" arrangement of SC than RE-DED/M (magnification ×486). *c*) RE-DED grown in GM. On the light microscopical level the SC structure resembles *b*. (H&E; *bar*, 20 μm.)

amined and compared with those of the native epidermis. Furthermore, improvement of the quality of the RE-DED system by a modification of the media composition could be demonstrated not only by modulations of the EL profile, but also by the ultrastructural assessment of the lamellar body (LB) secretory system and the lipid structure organization of the SC.

MATERIALS AND METHODS

Keratinocyte Cell Culture

Air-exposed Keratinocyte Culture Grown on De-epidermized Dermis (RE-DED): Secondary cultures of adult human keratinocytes (obtained from healthy patients undergoing surgical corrections) cultured using the Rheinwald-Green method [26] were seeded on de-epidermized dermis and cultured under air-exposed conditions [1] in our regular culture medium (M) (Dulbecco-Vogt and Ham's F12 [3 : 1] medium supplemented with 5% HyClone calf serum [Greiner], 0.4 μg/ml hydrocortisone, 1 μM isoproterenol, 5 μg insulin/ml, and 10 ng/ml epidermal growth factor) [2] or in Genesis Living Skin Construct Media (GM): 3 d under submerged conditions in Genesis Epidermal Growth Medium (GM1), 1 week after lifting to the air-liquid interface in Genesis Cornification Medium (GM2), and subsequently for various time intervals in Genesis Maintenance Medium (GM3), according to the instructions of the manufacturer. The base medium of GM1 is a 3 : 1 mixture of Ham's F-12 medium and calcium-free Dulbecco's modified Eagle's medium. This medium is supplemented with 1.88 mM $CaCl_2$, 0.3% chelated newborn calf serum, and 4 mM L-glutamine. The medium has a final concentration of each of the following components: not in excess of 50 μg/ml gentamicin sulfate, adenine, and ethanolamine/o-phosphorylethanolamine; and not in excess of 10 μg/ml selenium, insulin, transferrin, triiodothyronine, progesterone, and hydrocortisone. GM2 is a 1 : 1 mixture of calcium free Dulbecco's modified Eagle's medium and Ham's F-12. It is additionally supplemented to 1.95 mM $CaCl_2$, 2% newborn calf serum, and 4 mM L-glutamine (other components like in GM1). GM3 is a 1 : 1 mixture of calcium free Dulbecco's Modified Eagle's Medium and Ham's F-12. It is additionally supplemented with 1.95 mM $CaCl_2$, 1% newborn calf serum, and 4 mM L-glutamine (other components like above).

Living Skin Equivalent (LSE) (Organogenesis, Cambridge, MA) was, after arrival, kept for various time intervals in GM3.

Normal Human Skin To compare the architecture of the culture system with human skin, morphologic studies of normal skin from seven control subjects without skin disease undergoing excision of tattoos (from arms) were performed.

Morphologic Studies To assess the morphology that accompanies the age of the cultures, DED cultures were harvested on d 10, 14, and 25 after the air exposure (n = 5) and the LSE cultures were harvested 15 d after lifting the cultures to the air (1 d after the arrival of the cultures), and on d 25 and 43 post air-lifting (n = 6), respectively. (According to the obtained information from the manufacturer, the LSE were shipped 14 d after lifting to the air). At each time point, the cultures were split for morphologic studies and for lipid analyses.

Light Microscopy: For routine histology, parts of the cultures were fixed in 4% buffered formaldehyde and processed for embedding in paraffin. Vertical sections were stained with hematoxylin and eosin. For immunohistochemistry, embedment was performed in OCT compound and stored at −70°C. Samples were then sectioned to 4–6 μm at −20°C, air-dried, and immunolabeled using the streptavidin-biotin method and the three-stepped peroxidase method. Monoclonal antibodies used were anti-filaggrin (B.T.I. Stoughton, MA) (1 : 150 dilution), anti-membrane-bound transglutaminase (1 : 1000) [27], anti-keratin 1, AF 87 (1 : 500) [28], anti-keratin 6, AF 124 (1 : 1500) [29], and anti-keratin 10, RKSE60 (1 : 5) [30], and the rabbit antiserum directed against involucrin (1 : 2000) [31].

Enzyme reactivity was visualized using 3-amino-9-ethyl-carbazole as their chromogenic substrates. For control the primary antibodies were replaced with 1% bovine serum albumin in phosphate-buffered saline. All the controls revealed negative staining.

Electron Microscopy: After rinsing in buffer, cultures were divided and fixed in cacodylate-buffered 2.5% glutaraldehyde and then post-fixed with 0.5% ruthenium tetroxide (RuO_4) and 0.25% aqueous potassium ferrocyanide in darkness at 4°C [25,32]. The other half was processed routinely (fixation with 2.5% glutaraldehyde, post-fixation with 1% osmium tetroxide [OsO_4]). Tissue sections were then dehydrated in graded ethanol and embedded in Spurr's resin (RuO_4-fixed samples) or Epon 812 (OsO_4-fixed samples).

Thin sections were examined before and after double staining with ethanolic uranyl acetate plus lead citrate, *via* Jeol 100 CX electron microscope. Serial semithin sections of the OsO_4-fixed samples were stained with 1% methylene blue. The relative volume of LB of the uppermost subcorneal cell layers of the epidermis (first layer of stratum granulosum [SG]) was measured by applying standard ultrastructural stereologic methods [32–35]. Details of these methods are published in Fartasch *et al* [32]. In brief, two blocks (osmium-fixed and Epon-embedded) from cultures and controls (three RE-DEDs reconstructed with M, two LSEs and two RE-DEDs reconstructed with GM, and three samples of native epidermis) were sectioned perpendicular to the skin surface. At randomly selected areas at least five cells of the SG cell layer were photographed consecutively in slightly overlapping fields, at a primary magnification of × 6600. The negatives were printed to give a final magnification of × 66000. By fitting the slightly overlapping electron micrographs together a complete picture of the SG layer was obtained. The boundary of the five cells of SG were determined on the electron micrographs and outlined by a marker. LBs were identified by their typical lamellar internal structure and their cell membrane-like coating membrane. Using commercial computer software (Videoplan, Kontron, Bildanalyse GmBH, Munich, Germany— the volume of LB (in percent) from sections of at least five SG cells were measured. The following stereologic parameters were measured.

$$\%\ \text{volume of LB in SG layer} = \frac{\text{area of LB in layer}}{\text{total area of cell layer}} \times 100.$$

To quantify the lipid droplets in the corneocytes of the different culture systems (three RE-DEDs reconstructed with M, two LSEs and two RE-DEDs reconstructed with GM, and three samples of native epidermis), their total volume from sections of at least two adjacent corneocytes from the first SC layer and two superposed corneocytes from the second SC layer (total volume of four corneocytes) was assessed. From each culture (n = 7) two RuO_4-fixed blocks were sectioned. Three ultrathin sections were mounted on every Formvar-coated copper grid. Randomly selected areas were photo-

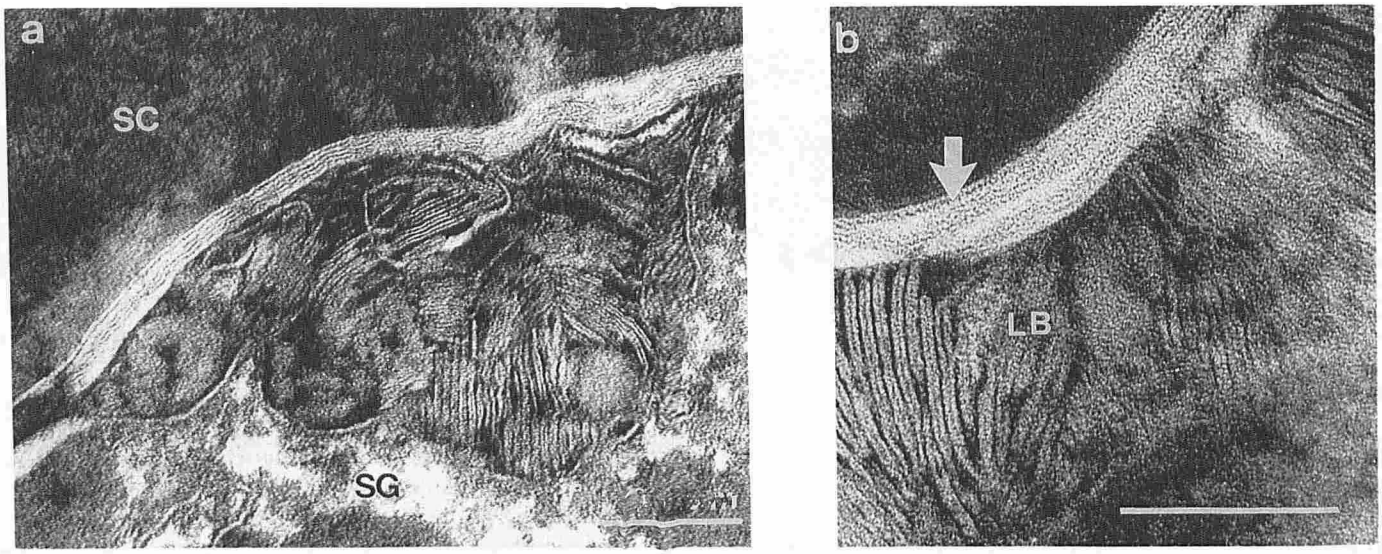

Figure 2. Prelaminar LB sheets in normal human skin. In saccular extracellular domains predominantly coiled lipid sheets are found. In the upper region of the dilatated ICS laminar lipid bilayers and a lipid envelope (→) have already appeared (*bar*, 0.1 μm).

graphed and the corneocytes were outlined. Lipid droplets were identified by their round or oval non-membrane-bound form and their fairly opaque interior laying within the corneocytes.

Comparisons between the different cultures and the controls were made by the Wilcoxon-Mann-Whitney test. All reported p values are two tailed.

Number of Stratum Corneum Cell Layers and Viable Epidermis of the Cultures All cell numbers were counted from the semithin sections (Epon-embedded) of the epidermis and were controlled by electron microscopy. Three Epon blocks (from random selection) were used per time point. Six counts were made per specimen (three over rete projections and three above dermal papillae in RE-DEDs) and the total mean was calculated for each time point.

Lipid Extraction and Separation Lipid analyses were performed parallel to the morphologic studies at different time points of cell culture. Reconstructed epidermis was harvested by heating the tissue for 1 min at 60°C. Lipid extraction, separation, and quantification were performed as described earlier [2].

RESULTS

Comparison of Keratinocyte Cultures Grown on De-epidermized Dermis (RE-DED/M) with Living Skin Equivalent (LSE)

Light and Immunohistochemistry: The evaluation of tissue architecture of RE-DED/M and LSE cultures (Fig 1*a* vs *b*) was performed at comparable time points (RE-DED/M after 10–12 d and LSE 15 d after air exposure). In contrast to LSE, the RE-DED displayed prominent rete projections. The keratohyalin granules in RE-DED/M (Fig 1*a*) appeared to be more prominent as compared to LSE (Fig 1*b*); additionally, the stratum corneum layer of RE-DED/M appeared to be more compact whereas the LSE depicted a more basket-weave pattern. RE-DED/M shows a more organized basal layer with perpendicular oriented basal cells and five to eight living cell layers. No differences between the cultures could be detected in the immunohistochemical distribution pattern of the epidermal maturation and differentiations markers, i.e., of keratins 1, 10, involucrin, transglutaminase, and filaggrin. Both culture systems showed keratin 6 expression (data not shown).

The evaluation of culture morphology at different time intervals after the air exposure (in RE-DED/M: 15 d, 25 d; in LSE: 25, 43 d) revealed that the viable parts of the epidermis had rapidly thinned with only one or two viable cell layers left, whereas the thickness of the SC had increased (data not shown).

Electron Microscopy

Lamellar Body Structure and Delivery System at the SG/SC Interface: The cells of the SG in both culture systems revealed pleomorphic keratohyalin granules that were not interconnected with tonofilament, as was the case in freshly excised skin. Comparing the relative total volumes of LBs there were no statistically significant differences between RE-DED/M and the native epidermis (RE-DED/M, volume of LBs in percent of total volume of cell layer; V_L: median 3.42% [range 2.2%–3.6%]; native epidermis; V_L: median 3.87% [range 2.73%–4.92%]). However, in LSE the relative total volume of LBs were significantly decreased compared with RE-DEDs and native epidermis ($p < 0.01$). In RE-DED/M and LSE the internal contents of LBs seemed to be normal both by OsO_4 and RuO_4 fixation, consisting of lamellar arranged disc-like structures, showing major electron-dense lamellae, which were separated by a electron-lucent material divided centrally by a minor, electron-dense band [36,37].

Comparison of both RE-DED/M and LSE cultures with freshly excised skin showed that the secretion of LB lipids was disturbed. In normal human skin, exocytosis of LB lipids into the intercellular space (ICS) was completed at the stratum granulosum (SG)/stratum corneum (SC) interface [32]. In the secreted stage they form unfurling LB sheets (the electron-lucent material still being divided by a striated electron-dense band) within hemispherical saccular dilatation of the ICS at the SG/SC interface [25,38] (Fig 2*a*,*b*). The LB-derived lipid sheets precede the lamellar intercellular lipid bilayers. In the cultures in some areas regular but short stacks of LB contents were formed in very small dilatations of the underlying SG cell (Fig 3*a*,*c*). In the area in which the LB already had fused with the SG cell membrane, tangles of lipid membranes were found, which could have originated from the disturbed secretion of LB lipids (Fig 3*b*,*c*). Additionally, individual corneocytes in the lower portion of the SC displayed profiles of unsecreted LBs (not shown).

Inclusions of the Stratum Corneum Cells: The low-power magnification of both air-exposed cultures (RE-DED/M and LSE) shows the appearance of numerous intracellular non-membrane-bound lipid droplets (Fig 4*a*). In some areas additionally crystalline inclusions (postulated to be cholesterol clefts) were seen (not shown). No such inclusions were found in the native epidermis. The relative total volume of lipid droplets in LSE was lower than seen in RE-DED/M ($p < 0.01$, data not shown). Prolongation of culture time did not lead to the reduction of the number of lipid droplets. In addition, in

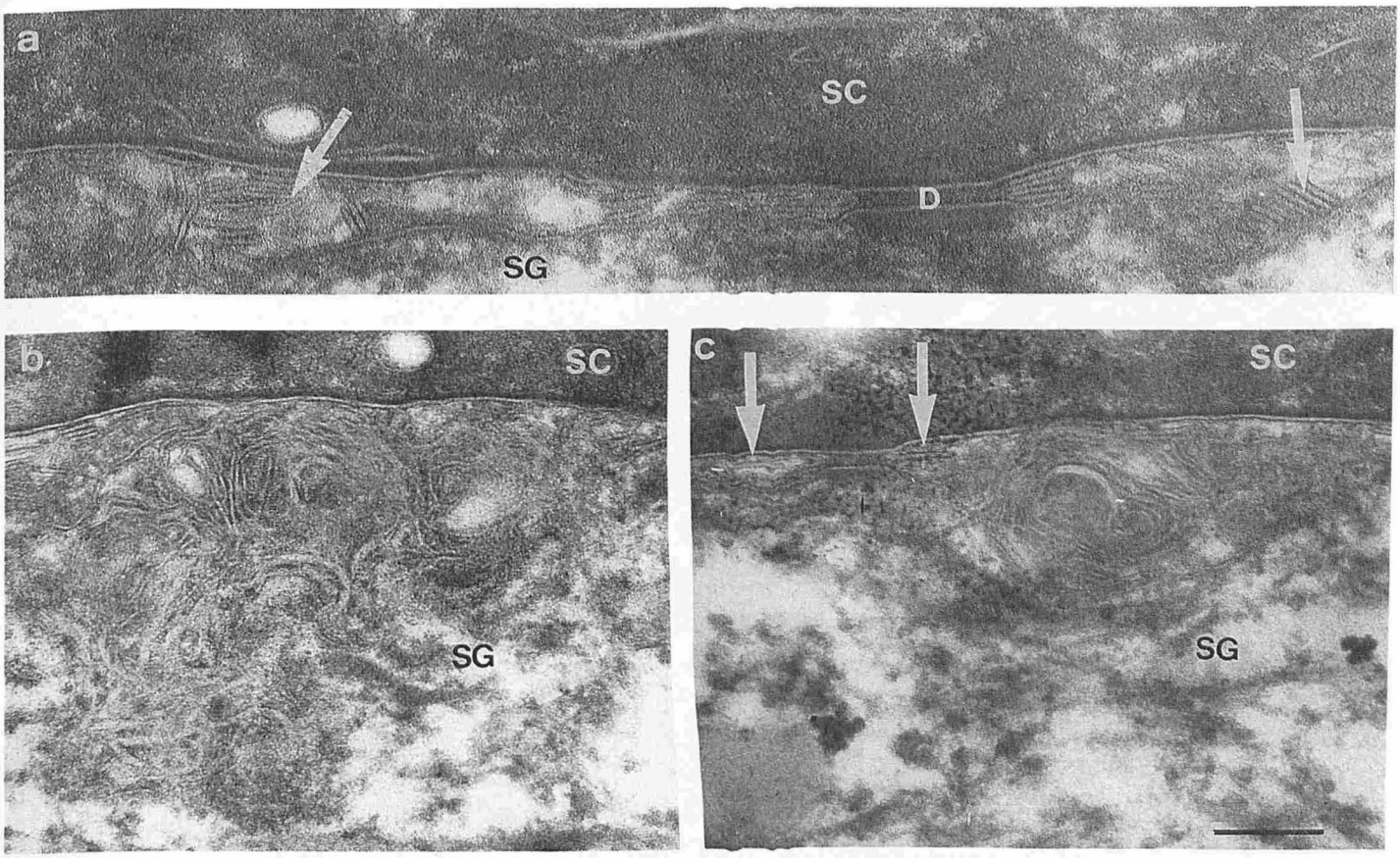

Figure 3. In the A/E cultures only shortened LB sheets were extruded at the SG/SC interface (*a*). No prominent indenting of the underlying SG and transitional cell is found (D = desmosome). Tangles of lipid membranes (*b*, *c*) and extruded short LB sheets (*arrow* in *c*) are seen simultaneously (*bar*, 0.1 μm).

the lower parts of the SC incompletely degraded organelle membranes were found.

Intercellular Arrangement of Epidermal Lipids: In normal human skin the ICS appears to be continuously filled with lipid lamellae forming a repetitive pattern [14,24,25]. The lipid envelope, found both in human epidermis [39] and in A/E murine cultures [40], is complexed to the cornified envelope, followed by an electron-dense lamellae [14,25]. Then two electron-lucent lamellae, separated by a narrow-appearing interrupted electron-dense lamellae, are depicted.

In both culture systems (RE-DED/M and LSE) a great variability in intercellular lamellar organization was found. In some sites, there were no electron-lucent and electron-dense lamellae present. One lamellae corresponding to the lipid envelope then remained at the cell surface and the lipid envelopes of adjacent corneocytes were brought into close apposition. In some areas (found at all levels of the SC) fusiform interdesmosomal dilatations of ICS were detected. The interior of the dilated interdesmosomal regions contained a mixture of an amorphous electron-dense substance and lamellar material that resembles extruded elongated LB-derived sheets, with the same substructure and dimension as initially secreted LB sheets [25,28]. They have apparently failed to reorganize into lamellar lipid bilayers. These accumulations of membranous material, revealed by the RuO_4, staining showed either a more string-like arrangement of the intercellular lipids (A_1) (Fig 4*b–d*), or, with an increase of the lipid amount, a whorl-like arrangement (A_2) of the lipid layers, displaying the same pattern (electron-lucent material separated by a minor electron dense band) as was apparent in the LB sheets (Fig 4*e*). Intermediate or transitional forms between these two different types of lipid accumulation were also found. Only in some areas four to six multilamellar lipid structures were found in the ICS but lacked the periodicity seen in normal skin (Fig 4*f*). There was no improvement of the EL organization with prolongation of the culture time up to 30 d.

Lipid Composition: The results of the analysis of the epidermal lipid composition of RE-DED reconstructed in regular medium (Fig 5, *lane 3*; Table I, *column 2*) and LSE cultures (Fig 5, *lane 1*; Table I, *column 1*) revealed that the keratinocytes synthesize all lipids that are found in normal human epidermis. In both types of cultures the relative amounts of most of the lipid classes were similar, the only exception being the content of triglycerides, which was much higher in RE-DED/M cultures as compared with the LSE and the amount of ceramides in LSE, which seemed to be slightly higher than in RE-DED/M.

Prolongation of the culture time of both LSE and RE-DED led to a marked thickening of the stratum corneum and to the decrease of the number of viable cell layers (data not shown). This resulted in a gradual decrease in total phospholipid content and in an increase in the ceramide content. During the whole culture period the triglyceride content in RE-DED grown in regular culture medium M was higher as compared with LSE (data not shown).

Effect of Genesis Medium (GM) on Keratinocytes Grown on De-Epidermized Dermis (RE-DED/GM) On the light microscopical level the stratum corneum of LSE compared with the RE-DED/GM cultures showed no differences (Fig 1*b,c*). The patterns of expression of immunohistologic markers tested were also the same (data not shown).

Comparing the relative volume of LBs of the RE-DED/GM (V_L, median 4.27% [range 3.26%–4.81%]) with RE-DED/M and the native epidermis there were no significant differences. However,

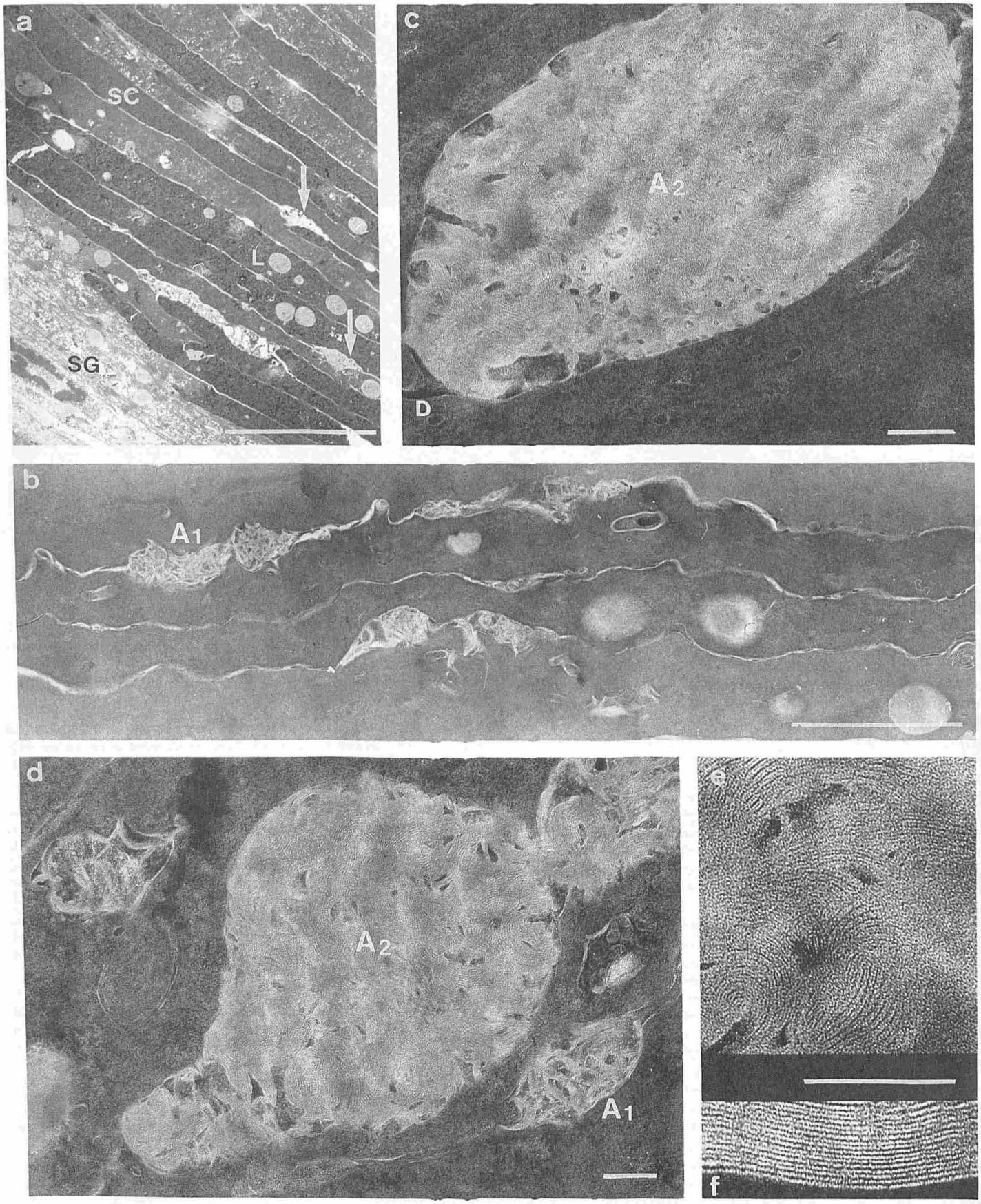

Figure 4. Survey of the RE-DED/M culture (*a*) shows multiple lipid droplets (L) in the corneocytes. Additional irregular dilatations of the ICS (→) (×4780; *bar* = 20 μm). Higher magnifications of the dilated ICS showing them filled with accumulations of lipid membranes (*b,c,d*) forming string-like (A1) or whorl-like (A2) structures. (*b*, magnification ×33200, *bar*, 1 μm; *c* × 125,400; *bar*, 0.1 μm; *d*, ×100,000; *bar*, 0.1 μm). Comparison of the membrane structures of the lipid accumulations of the cultures (*e*) with the regular lamellar lipid pattern of human skin (*f*) showed structural similarities with the broad LB sheets in Fig 2 (*bar*, 0.1 μm).

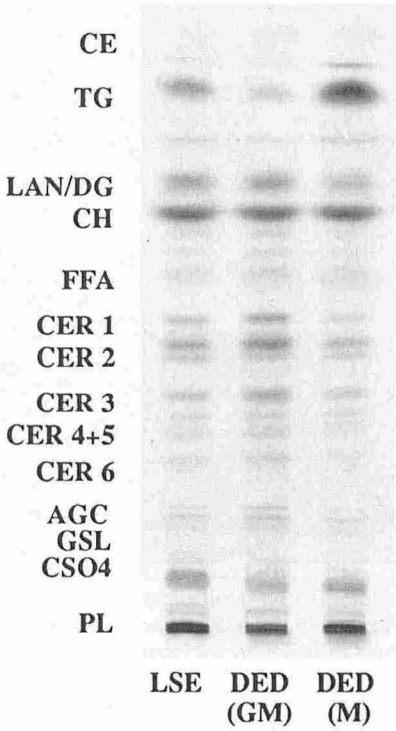

Figure 5. Thin-layer chromatography separation of lipids extracted from LSE, RE-DED grown in Genesis medium (GM) or regular (high) glucose (M) medium. PL, phospholipids; CSO4, cholesterol sulfate; GSL, glucosphingolipids; AGC, acylglucosylceramides; CER, ceramides; FFA, free fatty acids; CH, cholesterol; LAN/DG, lanosterol/diglycerides; TG, triglycerides; CE, cholesterol esters.

Table I. Lipid Composition of Reconstructed Epidermis[a]

Skin Recombinant	LSE (n = 4)		RE-DED (M) (n = 3)		RE-DED (GM) (n = 3)	
Percentage of total lipids[b]						
Phospholipids	29.6	(1.2)	24.6	(3.5)	28.0	(8.7)
Sphingomyelin	5.0	(0.7)	3.8	(0.5)	4.1	(1.1)
Phosphatidylcholine	10.7	(0.4)	9.1	(1.7)	10.1	(2.7)
Phosphatidylserine	2.2	(0.5)	1.7	(0.3)	2.5	(1.0)
Phosphatidylinositol	4.6	(1.5)	4.6	(0.5)	4.0	(1.7)
Phosphatidylethanolamine	7.1	(1.8)	5.4	(2.2)	7.3	(2.6)
Cholesterol sulfate	3.9	(0.4)	2.2	(0.7)	3.0	(0.2)
Glycosphingolipids	2.0	(1.0)	1.0	(0.6)	2.7	(0.3)
Acylglucosylceramide	0.7	(0.2)	0.2	(0.1)	1.5	(0.2)
Ceramides	10.6	(1.0)	8.8	(0.8)	14.1	(2.3)
Free fatty acids	2.2	(0.7)	3.1	(1.6)	39	(2.5)
Cholesterol	35.7	(1.6)	31.0	(5.4)	32.3	(5.6)
Lanosterol/diacylglycerols	5.9	(1.0)	2.8	(1.1)	5.0	(1.0)
Triacylglycerols	8.6	(2.2)	25.1	(2.9)	6.6	(1.2)
Cholesterol esters	0.7	(0.2)	1.2	(1.0)	2.9	(2.4)
Individual ceramides, percentage of total ceramides[c]						
Ceramide 1[d]	15.4	(1.0)	13.2	(1.1)	16.4	(2.1)
Ceramide 2	43.0	(2.1)	40.7	(3.2)	41.2	(2.2)
Ceramide 3	18.2	(0.6)	19.0	(1.1)	21.6	(0.3)
Ceramide 4 + 5	14.0	(2.0)	17.3	(0.9)	11.9	(1.1)
Ceramide 6	9.4	(1.1)	9.8	(0.6)	8.8	(0.7)

[a] DED cultures grown in standard or Genesis medium were harvested on d 10 after the air exposure and the LSE cultures were harvested 1 d after the arrival of the cultures, i.e, on d 15 post air-lifting. Reconstructed epidermis was harvested by heating tissue for 1 min at 60°C and the lipid extracted and separated by means of HPTLC.

[b] Values are given as average percentage of total lipids ± SD (in parentheses).

[c] Various detected ceramide fractions were grouped together into six fractions that correspond closely in terms of mobility (same Rf value) on TLC to the pig epidermal ceramide fractions (1,2,3 4 + 5, and 6) kindly provided by P. Wertz [21,57].

[d] Ceramide 1 = acylceramide.

the relative total volume of intracellular lipid droplets in the corneocytes of RE-DED/GM decreased significantly as compared with that of RE-DED/M and LSE ($p < 0.001$, data not shown). Only a few droplets were found in the whole sections. Whereas in RE-DED grown in regular medium and in LSE the regular pattern of epidermal lipid (EL) lamellar arrangement was not observed and mainly accumulations of intercellular lipids were seen, in the modified culture (RE-DED/GM) the regular pattern of EL lamellar arrangement appeared (Fig 6*a*); only a few accumulations of the A_1 type intermediate between the regular lamellar arranged lipids and the A_1 type were depicted (Fig 6*b*). In general the intercellular spaces of the RE-DED/GM were more regular (Fig 7*a*), but still regions with high numbers of lamellar lipid membranes were found throughout the middle and upper part of the SC (Fig 7*b*).

The lipid analyses of the reconstructed epidermis on de-epidermized dermis with the medium of the same composition (GM) as used for the preparation of LSE resulted in a marked decrease in triglyceride content and increase in ceramide and acylglucosylceramide contents (Fig 5, *lane 2*; Table I, *column 3*).

A summary of the morphologic and biochemical characteristics of the culture systems is found in Table II.

DISCUSSION

Our ultrastructural studies have shown that even though both culture systems (RE-DED and LSE) expressed signs of hyperproliferation as judged from the expression of involucrin, transglutaminase, keratin 6 [17], and cell cycle time [41], the processes involved in the formation of intercorneocyte lipid structures seem not to be totally comparable with those observed in other hyperproliferative disorders. Namely, in hyperproliferative diseases, such as psoriasis [42], ichthyosis congenita [43], irritated, and then latex-occluded, murine epidermis [38], congenital ichthyosiform erythroderma [44], and atopic dry skin [32,45], the LB-derived membrane structures persist to higher layers within the SC interstices. In contrast, in recon-

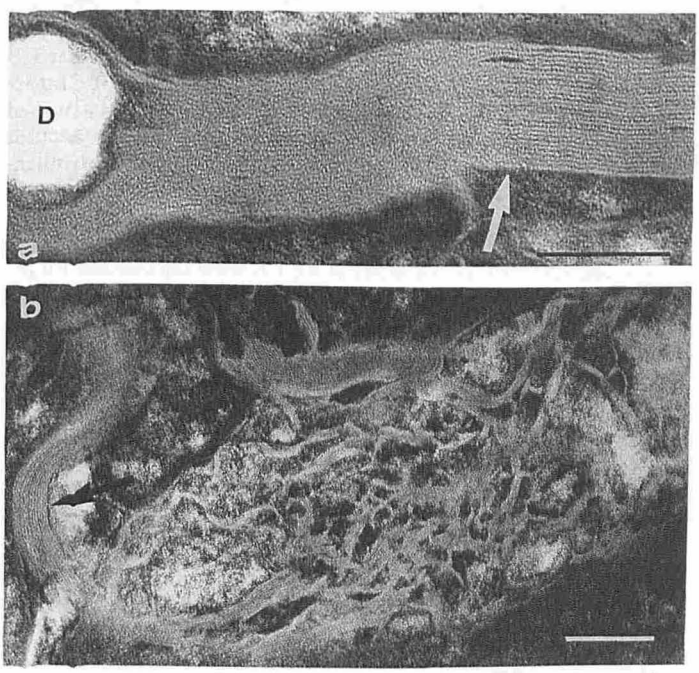

Figure 6. *a*) High numbers of lipid lamellae were seen, focally showing the regular pattern (→) comparable to normal human skin (magnification × 220,000; *bar*, 0.1 μm). *b*) In the RE-DED culture grown in GM medium only accumulations of the A1 type were found, showing simultaneously regular lamellar lipid layers in some regions (*bar*, 0.1 μm).

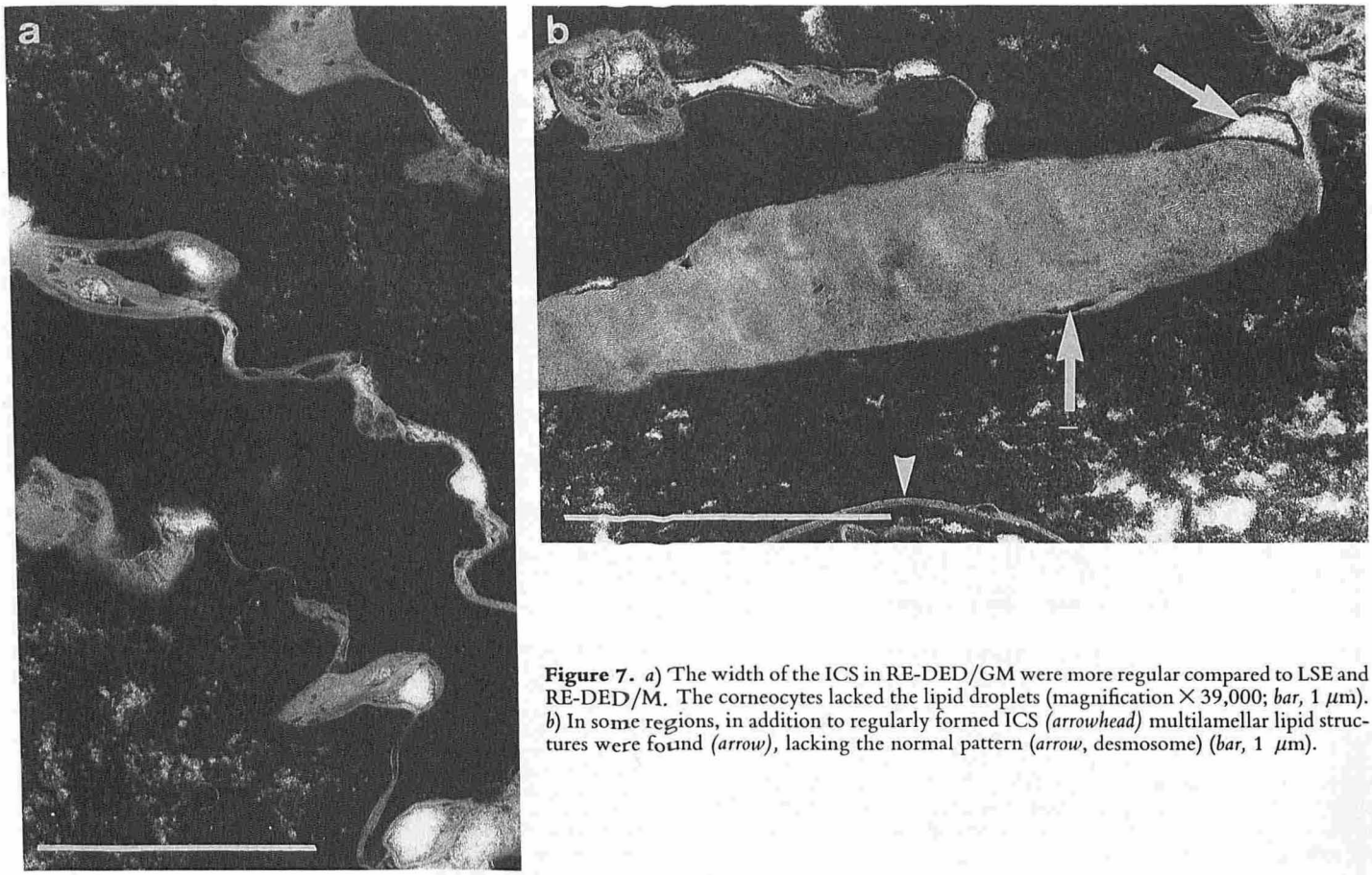

Figure 7. *a*) The width of the ICS in RE-DED/GM were more regular compared to LSE and RE-DED/M. The corneocytes lacked the lipid droplets (magnification × 39,000; *bar*, 1 μm). *b*) In some regions, in addition to regularly formed ICS *(arrowhead)* multilamellar lipid structures were found *(arrow)*, lacking the normal pattern (*arrow*, desmosome) (*bar*, 1 μm).

structed epidermis there seems to be an alteration of the LB delivery system because LB lipids were not fully extruded from the cells, resulting in partial retention of LBs in SG cells and in corneocytes.

In normal human skin the formation of lamellar arranged bilayers (lipid layers) is preceded by the formation of long prelaminar LB sheets situated extracellularly in saccular compartments of the intercellular spaces, the SC/SG interface [25]. The observed failure of extrusion in reconstituted epidermis and the failure to form saccular invagination of the upper SG cell membrane may be due to simultaneously occurring alterations of the cytoskeleton (microfilaments, microtubules, and keratin intermediate filaments). There is evidence that the individual components of the cytoskeleton are structurally associated with each other as well as with the cellular membrane. The forces for the extrusion of secretory products are probably provided by the action of the actin-myosin contractile systems and studies of Zamansky [46,47] have shown that actin appears to be intimately associated with keratin. Immunohistochemical studies have convincingly shown that the cultures show a pathologic expression of keratin [17,48]. It is tempting to speculate that these alterations may have an influence on the cytoskeleton and may 1) inhibit the formation of a special saccular compartment that might offer a unique micro-environment to facilitate the transformation process of the LB sheets into bilayers [25] and 2) alter the secretion and intracellular movement of the LBs, which, assuming a lysosomal origin of the LBs, depends probably on the functional integrity of both actin filaments and microtubules [49,50].

The situation in reconstructed epidermis is also different from that found in mice treated topically with inhibitors of HMGCoA reductase [51] in which a mixture of LB sheets and mature lamellar bilayers was observed in the lower and middle part of SC. The finally formed stratum corneum intercellular lamellar bilayers were indistinguishable from those seen in the control animals.

On the contrary, the *in vitro* reconstructed epidermis demonstrated great variability in intercellular lipid organization. There was not only a deficiency in the quantity of EL but also a defect in the distribution of abnormal structured EL, with considerable variations in ICS width throughout the whole stratum corneum. The abnormalities in lamellar substructures did not improve with the age of the culture, as our timecourse series on the cultures have shown. Within dilated interdesmosomal regions the cultures displayed lamellar material that resembled extruded LB sheets that have apparently failed to undergo rearrangement into lamellae bilayers, instead forming typical whorl-like accumulations. Similar atypical and incomplete processed lamellar lipid structures have been shown to occur in the ICS after inhibition of epidermal β-glucocerebrosidase [52] and in β-glucosylcerebrosidase–deficient transgenic mice,†

Table II. Biochemical and Ultrastructural Characteristics of A/E Cultures[a]

				Intercellular Epidermal Lipids			
	TG[a]	Lipid droplets	LB	A_1	A_2	Lamellar	Reg Lam
LSE	+	+	D	+	−	+	−
RE-DED/M	++	++	N	+	+	+	−
RE-DED/GM	(+)	(+)	N	+	−	++	[+]

[a] TG, triglycerides; reg lam, regular lamellar pattern comparable to normal human skin; A_1, lipid sheets with string-like arrangement; A_2, lipid sheets with onion-like arrangement; N, normal; D, diminished; −, absent; [+], ultrastructurally only focally present; (+), biochemically slightly increased compared to normal skin, ultrastructurally low amounts.

†Holleran WH, Menon GK, Elias PM, Ginns EI, Sidransky E: β-Glucocerebrosidase-deficient transgenic mice have abnormal stratum corneum lamellar bilayers. *J Invest Dermatol* 100:513A, 1993.

suggesting that the extracellular transformation of the lipids in the culture systems may be disturbed due to the alteration of enzyme activities. The enzyme activity may be disturbed possibly due to a high buffer capacity of the culture medium (preventing the formation of appropriate pH gradient for enzyme activity), the failure of LB (and their proton pumps [53]) to fuse with the apical cell membrane and induce an optimum extracellular pH necessary for the activation of enzymes, and/or a decreased secretion of appropriate hydrolases in the ICS.

An important question that deserves further studies is why the LSE culture system forms fewer LBs than the RE-DED. One can speculate that the presence of a basal membrane (in the RE-DED) plays an important role for the epidermal morphogenesis and, among other factors, may affect the LB formation. On the other hand our studies have shown that the presence of fibroblasts seemed not to be crucial for LB formation, as lower amounts of LBs were found in LSE.

In spite of the differences in the composition of culture medium and of the dermal substrate, the irregularities in EL formation (organization and distribution) were similar. To a certain degree, this was also the case with the occurrence of neutral lipid droplets in the corneocytes. Inspection of OsO_4- and RuO_4-fixed material revealed the presence of numerous intracellular non-membrane-bound lipid droplets that have been shown to contain neutral lipids [44,54], especially within the corneocytes of RE-DED grown in regular medium. The appearance of lipid droplets seems to be a common finding in various air-exposed culture systems with different dermal models [7,15]. The biochemical lipid analyses showed the highest triglyceride contents of the RE-DED grown in regular medium (M) and this correlated with the presence of lipid droplets in the corneocytes. *In vivo,* lipid droplets are found in different types of ichthyosis, psoriasis, and essential fatty acid deficiency, all these diseases showing signs of hyperproliferative abnormalities. It has been suggested that the occurrence of these neutral lipids in the corneocytes is due to an effect of cellular hypoxia, which could probably be induced by a hyperproliferative state of the air-exposed cultures. Under these conditions it would be easier for the cells to metabolize carbohydrate than fat, so that there would be a tendency for intracellular fat to accumulate [55]. The hypothesis of an anaerobic condition of the cultures is also corroborated by a high lactate production (taken as a measure of anaerobicity) and its dependence on glucose levels in culture medium [56]. By lowering the glucose level both lactate production and triglyceride contents in RE-DED cultures were markedly decreased.

In summary, by applying the RuO_4 staining techniques, the barrier structures of human skin recombinants could be analyzed in detail, revealing consistent abnormalities in membrane structure that have not previously been possible to evaluate, due to the lack of methods for the visualization of the contents of the SC intercellular spaces. The data suggest that improvements of barrier structures and barrier-associated epidermal lipids in the A/E cultures can probably be induced by the regulation of keratinocyte metabolism. Modification of culture medium composition showed that the keratinocytes in de-epidermized dermis culture system have the potential and the ability to form mature barrier structures.

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