Lipid composition of cultured human keratinocytes in relation to their differentiation

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Abstract The present study was undertaken to explore the possibility of the use of cultured human keratinocytes for the study of changes in lipid composition in relation to epidermal differentiation. In a submerged culture system, in which the stratification is incomplete, no significant differences have been found between the lipid composition of cells grown either at low calcium concentration (0.06 mM) (at which the keratinocyte differentiation is markedly retarded) or at normal calcium concentration (1.6 mM) (at which some differentiation takes place). Under these conditions the amount of phospholipids and sterols was high and that of ceramides was low. Furthermore, the acylglucosylceramides (AGC) and acylceramides (AC), the latter one known to be involved in water barrier function, were found to be absent. Contrary to this, both AGC and AC were found to be present in significant amounts in an air-exposed model using de-epidermized dermis (DED) as a substrate (in which, as judged from morphologic criteria, the extent of keratinocyte stratification is similar to that seen under the in vivo conditions). Fatty acid analysis revealed significantly lower content of 18:2 and higher content of 16:1 and 18:1 acids with all culture conditions used, as compared to the parent epidermis. This is probably a result of fatty acid levels and composition in fetal calf serum (which was used in the present study) that differ markedly from the in vivo situation. The 20:4 content was similar to that in the epidermis only in cells cultured under the submerged conditions, during which they have been found (Isseroff et al. 1987. *J. Lipid Res.* **28:** 1342–1349) to be able to convert 18:2 to 20:4. In DED cultures, however, the 20:4 content was markedly lower. Under all culture conditions used, the triglyceride content was higher as compared to the noncultured epidermis. The high content of triglycerides and the fatty acid composition of the various lipid fractions showed a resemblance with what is found in the epidermis in essential fatty acid-deficient animals. This resemblance was confirmed by electron micrographs which revealed the presence of some partially or completely empty lamellar bodies. The results of the present study suggest that the air-exposed culture model, in which the keratinocytes show a high extent of stratification, could be of great value in the study of epidermal lipid metabolism. However, further alterations in culture conditions are necessary to more closely approximate the lipid composition of noncultured epidermis.—**Ponec, M., A. Weerheim, J. Kempenaar, A-M. Mommaas, and D. H. Nugteren.** Lipid composition of cultured human keratinocytes in relation to their differentiation. *J. Lipid Res.* 1988. **29:** 949–961.

Supplementary key words air-exposed cultures • submerged cultures

Cultured human keratinocytes have become a popular model for the study of epidermal differentiation, since it has been demonstrated that the extent of keratinocyte differentiation can be modulated in vitro experimentally by changing the extracellular calcium concentration (1–4). Epidermal cells growing as a monolayer at low calcium concentration (< 0.1 mM) can be induced to differentiate to a certain extent by adding calcium to the level normally found in the culture medium (1–2 mM). To date, proteins, such as keratins (5), involucrin (4), extracellular matrix components (6, 7), desmosome precursors (8) and filaggrin (9) have primarily been used as markers of epidermal differentiation. In contrast, data about lipid composition in relation to differentiation of cultured keratinocytes are scarce.

It has been repeatedly shown (10) that under the above-mentioned conditions the maturation of keratinocytes does proceed to a lesser extent than under in vivo conditions. More nearly physiological in vitro conditions can be created by an attachment of the cells to a biological matrix and by exposing the cultured tissue to the ambient atmosphere. When keratinocytes are cultured on an air–liquid interface using de-epidermized dermis (DED) (to which the basement membrane remains associated) as the substrate, a multilayer of keratinocytes is formed that exhibits morphological features very similar to those seen in the epidermis under the in vivo conditions (11, 12). Therefore, in the present study, the lipid composition of keratinocytes grown on DED was compared with that in keratinocytes grown under submerged conditions.

The results show that no significant differences exist between the lipid composition of cells grown under the low and the normal Ca^{2+} concentrations in a submerged culture model. Under these conditions the cells contain high amounts of phospholipids and sterols and low amounts of

Abbreviations: FS, fetal calf serum; AC, acylceramides; AGC, acylglucosylceramides; EGF, epidermal growth factor; PBS, phosphate-buffered saline; DED, de-epidermized dermis; TLC, thin-layer chromatography.

ceramides. On the other hand, in cells cultured on DED, the lipid composition resembles to a great extent that found in the epidermis under in vivo conditions. The high triglyceride and ceramide contents and the low linoleic acid content show a similarity to the epidermal lipid composition of essential fatty acid-deficient animals.

MATERIALS AND METHODS

Cell culture

Submerged culture. Human keratinocytes (the first to fourth passage) isolated from juvenile foreskin were cultured in the presence of lethally irradiated (3000 R) 3T3 fibroblasts (13). The culture medium was a mixture of Dulbecco-Vogt and Ham's F12 (DV-H) medium (3:1) supplemented with 5% fetal calf serum (FS), 0.4 μg of hydrocortisone/ml, 10^{-6} M isoproterenol (14), and 10 ng of epidermal growth factor (EGF) (Sigma)/ml (15).

For the low-calcium cultures, a calcium-free Dulbecco-Vogt medium mixed with standard Ham's F12 medium (3:1) supplemented with 5% chelex-treated fetal calf serum was used (1). The final calcium concentration was 0.06 mM, as determined by flame photometry.

Air-exposed culture. The de-epidermized dermis (DED) for air-exposed cultures was prepared as described by Régnier, Pruniéras, and Woodley (11). Briefly, a piece of cadaver skin (stored at 4°C in 85% glycerol) was carefully washed with PBS and subsequently incubated for 3–5 days in PBS at 37°C. Next, the epidermis was scraped off and the remaining dermis was irradiated (3000 R) and washed several times with culture medium. The dermis was then placed on the stainless-steel grid and 0.5×10^6 epidermal cells (the second or third passage) were inoculated inside a stainless ring (diameter 1 cm) placed on the top of the dermis. After 24 hr the ring was removed. The level of culture medium in the culture dishes was adjusted so that it just reached the height of the grid. In this way the epidermal cells were exposed to air throughout the remaining period of culturing. The composition of culture medium was the same as that used for submerged cultures.

Electron microscopy

Small pieces of normal human skin (obtained from biopsies after intracutaneous administration of 1% lidocaine without epinephrine) and of keratinocytes cultured on DED were fixed in 2% paraformaldehyde with 2.5% glutaraldehyde in phosphate buffer for 1 hr at room temperature. They were postfixed in 1% osmium tetraoxide with 0.05 M potassium hexacyanoferrate in phosphate buffer for 1 hr at 4°C, dehydrated in a graded ethanol series up to 70%, and embedded in an epoxy resin. Ultrathin sections were stained with uranylacetate and lead hydroxide and viewed with a Philips EM 410 electron microscope.

Lipid extraction and separation

The cells grown under submerged conditions were harvested by trypsinization and washed several times with phosphate-buffered saline (PBS). The cells grown on DED and those from freshly isolated foreskin were harvested after the incubation of the cell culture or of the skin for 1 hr at 4°C in thermolysin solution (16) (0.5 μg/ml in PBS containing 1 mM $CaCl_2$) by which the epidermis is separated from the dermis as a whole sheet. The epidermal sheet was then washed several times with PBS. Lipids from the cells were extracted according to Bligh and Dyer (17) using 0.25 M KCl to ensure the complete extraction of all lipids. Organic phases were dried under a stream of nitrogen and the residues were weighed and then dissolved in a suitable volume of chloroform–methanol 2:1. Residue of the tissues after extraction and/or aliquots of cell suspension were taken for determination of protein (18).

The extracted lipids were separated by thin-layer chromatography using either a two- or one-dimensional system. In the two-dimensional TLC the separation of 1–2 mg of lipids was performed on Merck silica-gel plates using chloroform–methanol–acetic acid–water 90:70:1:0.7 (v/v) in the first direction and chloroform–methanol–25% NH_3 90:12:1.5 (v/v) in the second direction (19). For analytical one-dimensional TLC, three different developing systems were used. For the separation of total lipids by one-dimensional TLC, 5–50 μg of lipids was applied on HPTLC plates (10 × 20 cm) (Merck) and separated using chloroform–acetone–methanol 90:5:5 (up to 40 mm) as the first, chloroform–ethyl acetate–methanol–2-propanol–triethylamine–water 64:15:17:0.5:3:2 (first up to 65 mm, followed by up to 40 mm) as the second, and hexane–diethyl ether 90:10 (up to 90 mm) as the third developing system. For separation of ceramides, 5–50 μg of the total lipid extract was applied on an HPTLC plate (10 × 20 cm) (Merck) and separated using chloroform–acetone–methanol 76:4:20 (first up to 10 mm, followed by up to 25 mm) as the first, chloroform–acetone–methanol 80:10:10 (up to 75 mm) as the second, and chloroform–diethyl ether–ethyl acetate–methanol 72:6:20:2 (up to 90 mm) as the third developing system. Various sterol fractions were separated after application of 1–10 μg of total lipid extracts on HPTLC plates (Merck) using chloroform–methanol 90:10 (up to 10 mm) as the first and chloroform–diethyl ether–ethyl acetate 80:4:16 (up to 90 mm) as the second developing system.

For quantitative analytical TLC determination, increasing amounts of standards (0.2–5 μg) (sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, cholesterol sulfate,[1] cerebroside I and II, acylglucosylceramides,[2] acylceramides,[2] ceramides,

[1]Gift from Dr. M. Williams (Veterans Administration Hospital, San Francisco, CA).

[2]Isolated from pig epidermis.

oleic acid, cholesterol, lanosterol, lathosterol, triolein, 1,2-diglycerides, 1,3-diglycerides, and squalene) (Sigma) were applied. After lipid separation the plates were first heated for 5 min at 130°C, subsequently submerged for 20 sec in a mixture of acetic acid–sulfuric acid–water 5:0.1:95 containing 0.5% $CuSO_4$, and charred first at 150°C for 15 min followed by charring at 200°C for 15 min. The chromatograms were then cooled and scanned on a recording photodensitometer (Shimadzu) that provides peak integration and calculation of percentage composition.

Fatty acid analysis

One mg of total lipid extract was separated by two-dimensional TLC and individual lipid fractions were visualized under ultraviolet light after spraying the plates with 2% dichlorofluoresceine in 100% ethanol. Subsequently, the lipid fractions were scraped off the plates and the fatty acids were transmethylated for 3 hr at 70°C in 1–2 ml of a mixture of methanol–37% HCl 5:1 containing a known amount (5–10 μg) of internal standard heptadecanoic acid (17:0); the resultant fatty acid methyl esters (FAME) were extracted in diethyl ether. In order to extract sphingosine from the lipid fraction, the water phase was neutralized by adding 30% aqueous KOH to pH $\geq$ 8 and sphingosine was extracted in ethyl acetate. Both organic fractions were pooled and evaporated to dryness. The residue was dissolved in chloroform and the fractions were separated on a 20 × 20 cm silica-gel plate (Merck) using petroleum ether–diethyl ether 7:3 (up to 19 cm) as the first, petroleum ether–diethyl ether 45:55 (up to 15 cm) as the second, and chloroform–methanol–water 80:20:2 (up to 8 cm) as the third developing system. The FAME bands were scraped off the plate, extracted with chloroform–methanol 2:1, and analyzed on a Varian 3700 gas chromatograph, using a capillary column (Chrompack Sil 88, length 25 m, diameter 0.25 mm). The column temperature was programmed from 125°C to 200°C with a rate of 1°C/min, the prepressure being 1 atm and the split ratio 1:100. The FAMEs were identified by comparison of retention times to those of known standards (Nu-Chek-Prep., Copenhagen) and the relative percentages were calculated by integration of the area under each peak using the Hewlett Packard 33908 integrator.

[^{14}C]Acetate incorporation

The keratinocytes cultured on DED were labeled for various time intervals with [^{14}C]acetate (0.5 mM or 5 mM, 59 mCi/mmol, Radiochemical Centre, Amersham). Harvesting of the cells, lipid extraction, and separation by one-dimensional TLC were performed as described above. After development, Fuji X-ray film was placed over the plates for 1–2 weeks and the film was then processed with a commercial Kodak X-ray developer.

RESULTS

Cell morphology

Submerged cultures. Striking modulation of epidermal differentiation in relation to variations in calcium concentration in the culture medium has been reported for both cultured mouse (1–3) and human (4) keratinocytes. When the keratinocytes were maintained in low-calcium medium (< 0.1 mM) they did not stratify at all. Under these conditions there was little cell contact and the cells did not form desmosomes (1–4, 8) (**Fig. 1a**). Raising the level of calcium up to 1–2 mM, which is the standard concentration for most commercial culture media, induced keratinocyte monolayers to stratify in three to six cell layers (Fig. 1b, c). However, the extent of keratinocyte maturation under these culture conditions remained much lower than that seen under the in vivo conditions.

Air-exposed cultures. The situation was completely different when the keratinocytes were grown in an air-exposed culture using de-epidermized dermis as the substrate. Here, the cells were forming a multilayer already after 2–3 days of culture (**Fig. 2a**), with a number of cell layers increasing with the prolongation of the cell culture to 8–10 days (Fig. 2b, c). Further prolongation of the culture up to 15 days resulted in a relative thinning of layers containing living cells (Fig. 2d). By morphological criteria, the keratinocytes cultured for 8 to 15 days on DED closely resembled the epidermal structure in vivo. The studies on both the light microscopic and the electron microscopic level revealed the presence of stratum basale, spinosum, granulosum, and corneum as well as of the basement membrane, numerous desmosomes, lamellar bodies and keratohyalin granules, and a high degree of stratification. Many of the lamellar bodies had the same features as those that are found in biopsies of normal skin (**Fig. 3a**). Some of them, however, appeared partially or completely empty (Fig. 3b).

Lipid composition

The use of the two-dimensional thin-layer chromatography system (19) that allows a good separation of ceramides, the presence of which is associated with a high degree of keratinocyte differentiation (20), enabled us to make a proper comparison of lipid composition of cells grown under various culture conditions. Inspection of two-dimensional chromatograms revealed that the ceramide content was low in cells grown under submerged conditions in both the low and the normal calcium cultures. On the other hand, the presence of ceramides, especially of acylceramides (AC) and acylglucosylceramides (AGC), was observed in cells grown on DED, whereby the AGC and AC content increased with prolongation of the cell culture.

In order to make a quantitative comparison of the lipid composition in keratinocytes cultured under various condi-

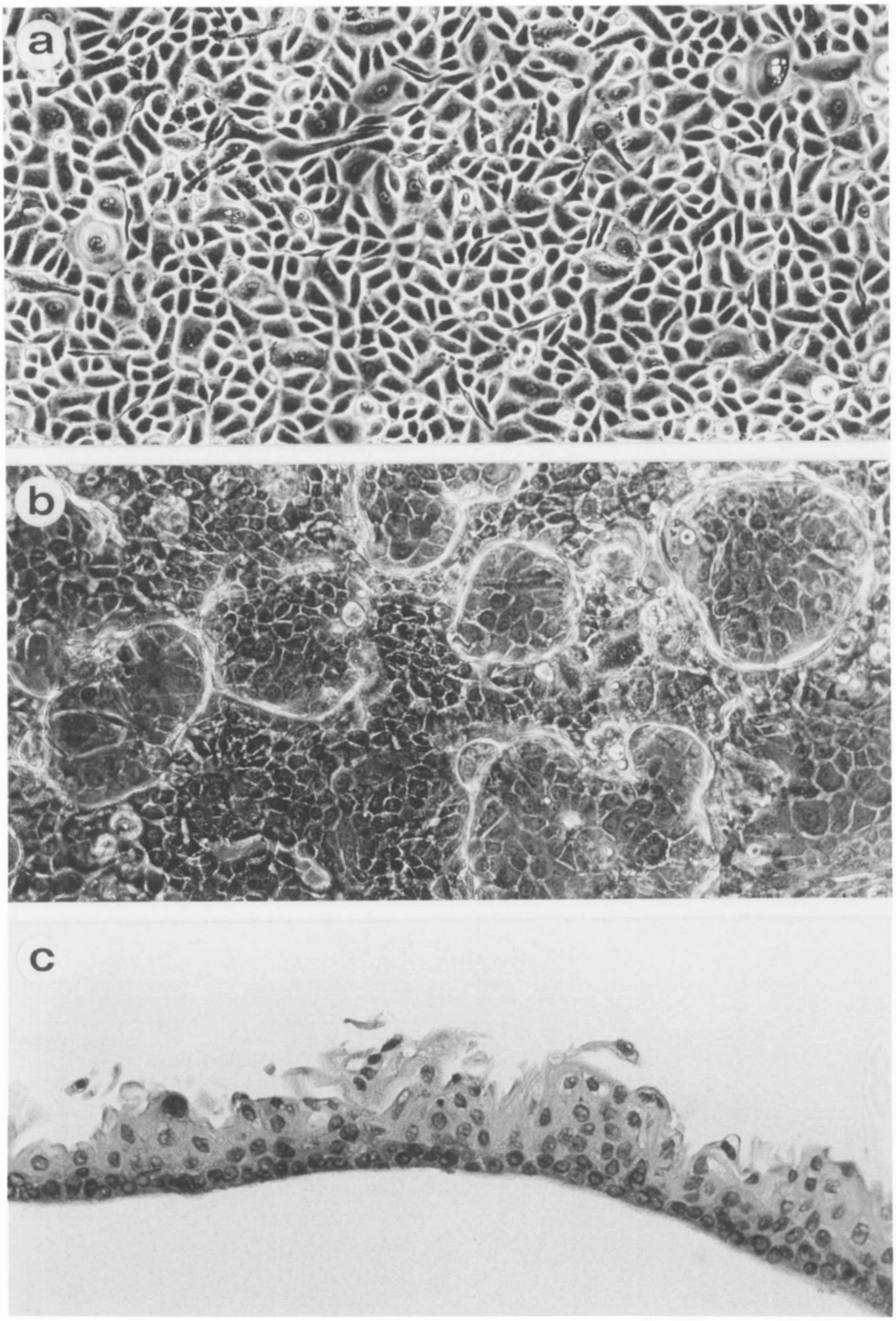

Fig. 1. Phase-contrast photomicrograph of human keratinocytes grown to confluency in submerged culture system either under a) low Ca^{2+} or b) normal Ca^{2+} concentrations; c) hematoxylin and eosin staining of keratinocytes grown to confluence in submerged culture at normal Ca^{2+} concentration.

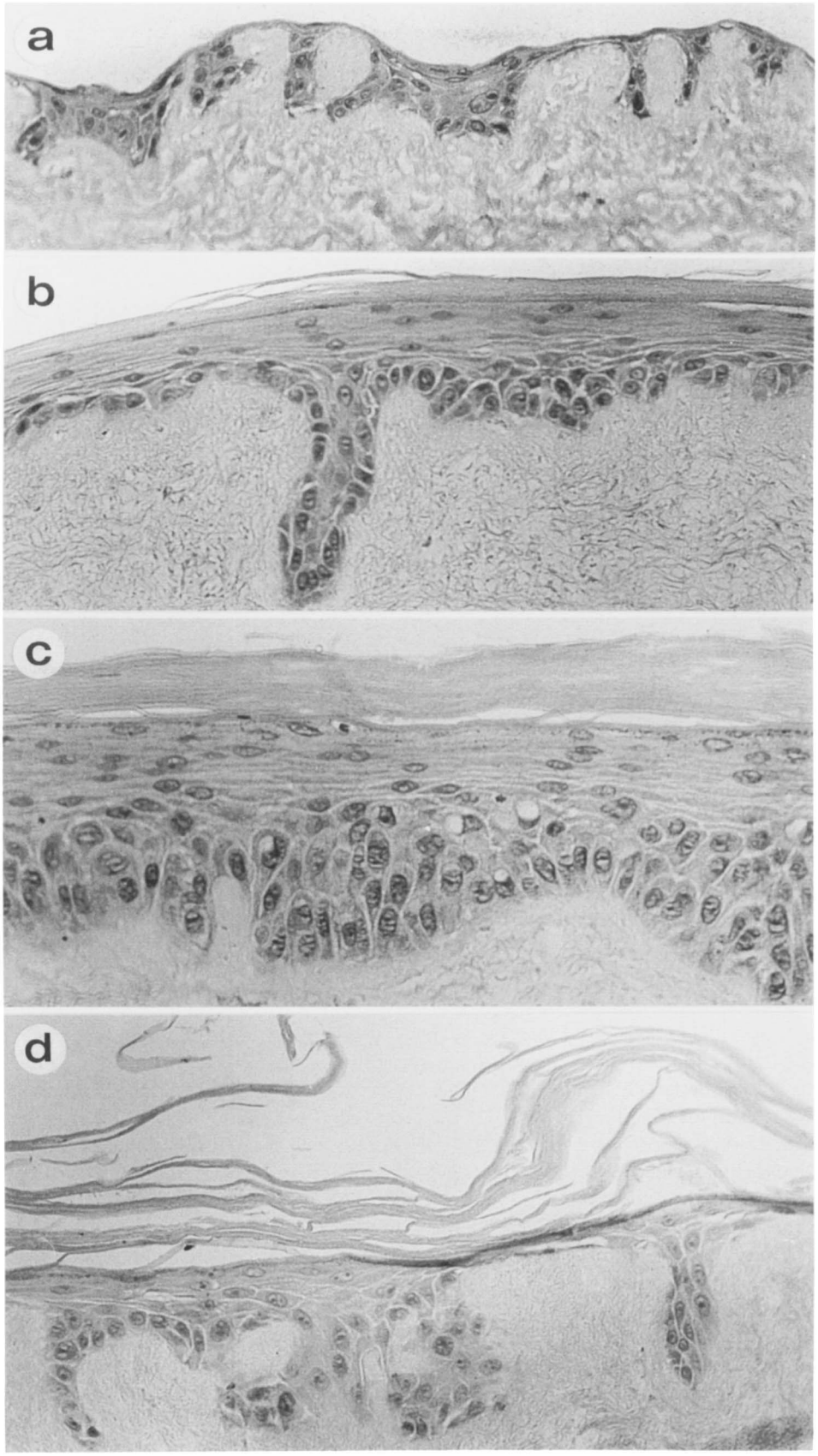

Fig. 2. Vertical paraffin sections stained with hematoxylin and eosin of air-exposed cultures of human keratinocytes grown for a) 3, b) 5, c) 8, and d) 14 days on de-epidermized dermis.

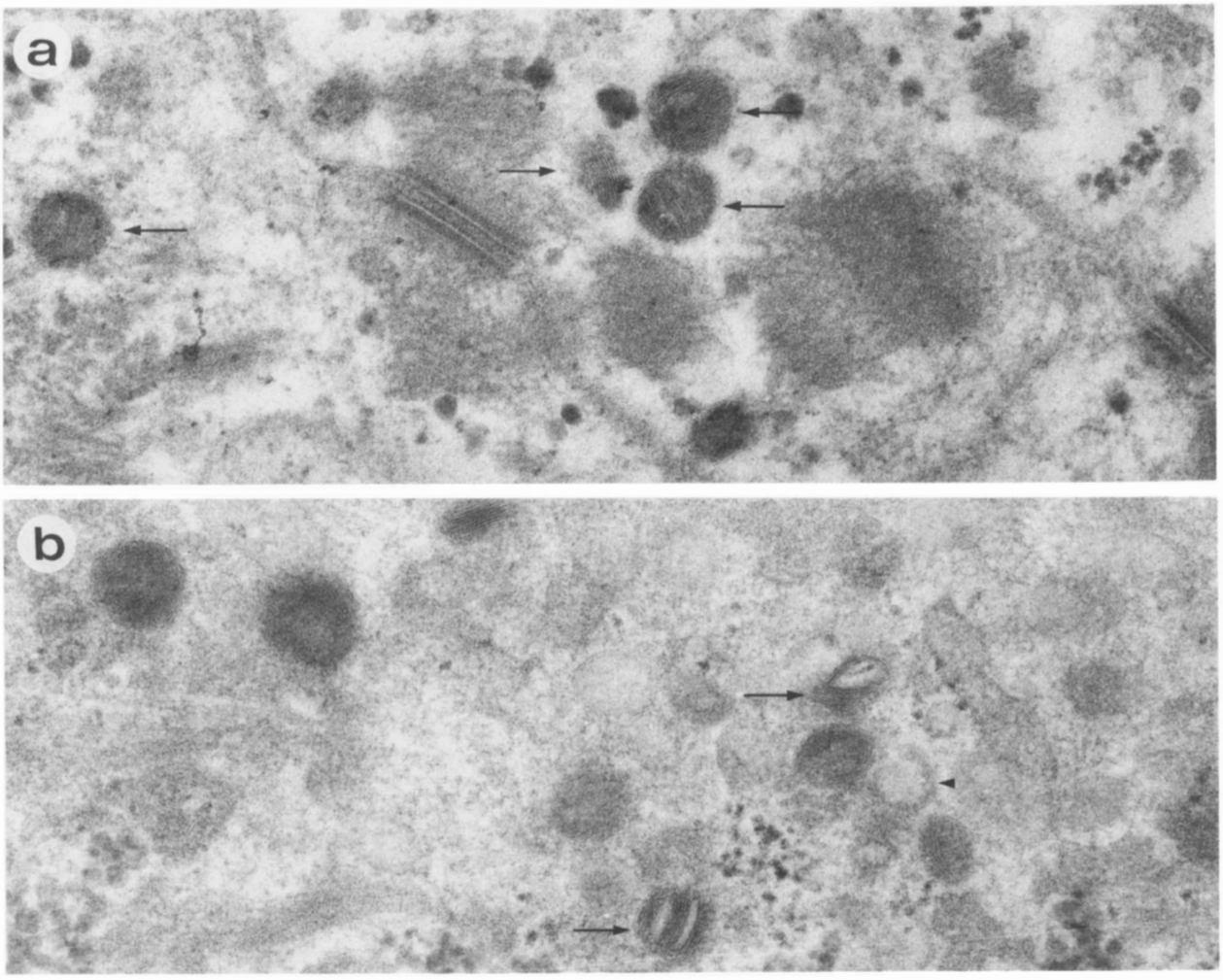

Fig. 3. Detail of keratinocytes from the upper spinous layer cultured for 6 days in air-exposed culture on de-epidermized dermis. a) Most of the lamellar bodies have normal fine lamellar structure (arrow). b) Some of the lamellar bodies have partially (arrow) or completely (arrow-head) empty appearance (note also the trilamellar structure of the desmosomes). Magnification 77,000 ×.

tions, several one-dimensional TLC systems were developed (**Fig. 4, Fig. 5, and Fig. 6**) and the lipid composition was determined by photodensitometry. This enabled the analysis of small quantities of lipids (5–50 μg of total lipids). A summary of lipid analyses, presented in **Table 1,** showed a high phospholipid and cholesterol content and a low ceramide content in cells cultured under submerged culture conditions at both the low and the normal calcium concentrations. However, in cells grown on DED, the phospholipid content was lower and it progressively decreased with the prolongation of the culture period. Furthermore, the triglyceride and ceramide contents were higher and increased further with the prolongation of the culture time. The presence of AGC and AC could be detected in the DED cultures only. While the AC content increased upon the prolongation of the culture period, the AGC content reached a maximum after approximately 8 days, followed by a drop in the 14-day-old cultures. In contrast to submerged cultures, in the DED cultures the presence of other lipids such as lanosterol and lathosterol was also observed. Lathosterol, which has a TLC mobility very close to that of cholesterol (Fig. 6), has been tentatively identified by the brownish color produced after charring the plates at 100°C. Under all culture conditions used, cholesterol sulfate could be detected; the relative amount was lower in cells grown under submerged conditions as compared with that seen in cells grown on DED and/or under the in vivo conditions.

When the lipid composition in the DED cultures was compared with that of the epidermis from which the cells were isolated for culture, the following differences were observed. In the DED cultures, the phospholipid, glycosphingolipid, and the AGC contents were lower and that of triglycerides higher. Since some glycosphingolipids (showing a TLC mobility in the 'cerebroside' region) could not

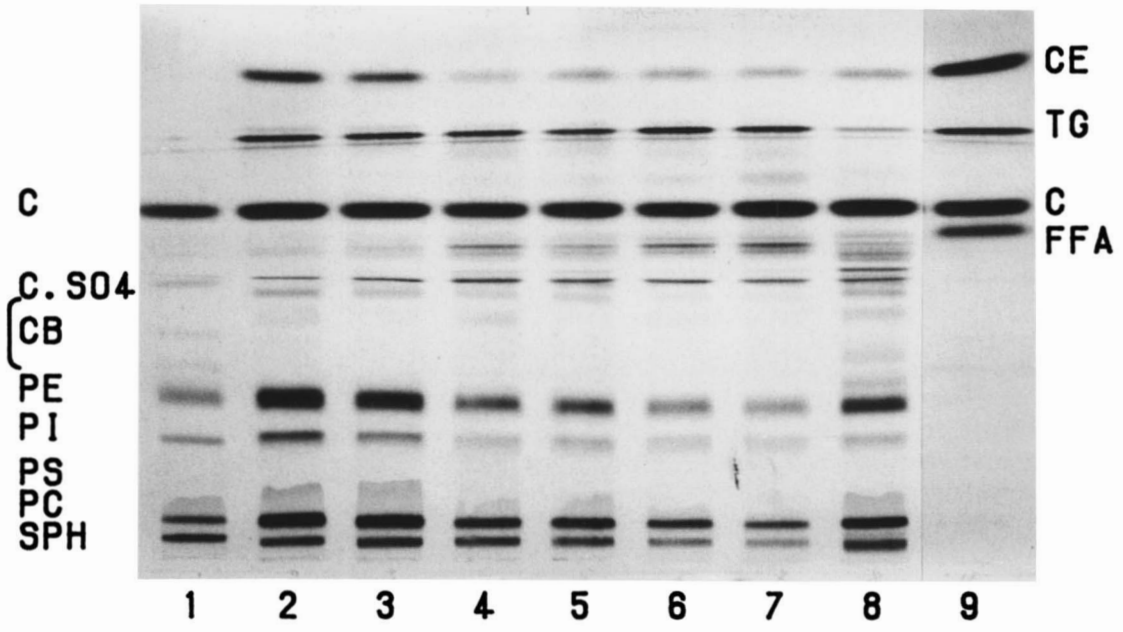

Fig. 4. One-dimensional TLC separation of total lipid extract in 'total lipid solvent system' of a mixture of standards (lane 1) containing sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), cerebrosides (CB), cholesterol sulfate (CSO_4), cholesterol (C), and keratinocytes grown under low Ca^{2+} (lane 2), normal Ca^{2+} (lane 3), on DED for 3 days (lane 4), for 5 days (lane 5), for 8 days (lane 6), for 14 days (lane 7), or isolated directly from the epidermis (lane 8), and a mixture of standards free fatty acids (FFA), cholesterol (C), triglycerides (TG), cholesteryl esters (CE) (lane 9). Five to 50 μg of total lipid extract was applied on HPTLC plates and separated using chloroform–acetone–methanol 90:5:5 as the first, chloroform–ethyl acetate–methanol–2-propanol–triethylamine–water 64:15:17:0.5:3:2 as the second, and hexane–diethyl ether 90:10 as the third developing system. The lipid fractions were visualized after charring as described in Materials and Methods.

be detected in the DED cultures, the question arose as to whether the keratinocytes were able to synthesize these lipids under the in vitro conditions used. In order to test this, the cells were labeled with [^{14}C]acetate for various time intervals (1–4 days) using two different acetate concentrations (0.5 and 5.0 mM) at two different times (on days 5 and 9), and the synthesized lipids were extracted and separated by one-dimensional TLC (Fig. 5b). In these experiments, the composition of synthesized lipids was found to be similar and glycosphingolipids could be detected. As an example, the labeling pattern of cultures labeled for 4 days is shown; the label was added on day 5 (Fig. 5b). The results clearly show that the difference found between the lipid composition of the keratinocytes cultured on DED and that of the epidermis in vivo is only quantitative.

The total lipid content when expressed per mg of cellular protein was similar under all experimental conditions used (Table 1).

Fatty acid composition

The procedure for the determination of the fatty acid composition of the various lipid classes (see Materials and Methods) also allowed the detection of the long chain 32:1-ω-hydroxy fatty acids and α-hydroxy fatty acids, both being present only in the cells cultured on DED and in the epidermis. The 32:1-ω-hydroxy fatty acids were found to be present in the triglyceride, AC, A-acid, and polar ceramide (AGC) fractions. Marked differences in the distribution of fatty acids in various lipid classes were observed between cells grown under submerged and air-exposed conditions (**Table 2**). Based on the fatty acid analysis, the relative amount of phospholipids was high in submerged cultures and in the epidermis and low in DED cultures, confirming the results obtained by photodensitometry (Table 1). The relative amount of apolar lipids, ceramides, and fatty acids was much lower in submerged cultures as compared with the air-exposed ones; the content of apolar lipids was, however, lowest in the noncultured epidermis. The fatty acid analysis revealed a high content of oleic (18:1), lignoceric (24:0), and cerotic (26:0) acids, and a very low content of linoleic acid (18:2) under all in vitro conditions as compared with the content of these fatty acids in the epidermis in vivo.

DISCUSSION

Epidermal differentiation has been shown to be accompanied by marked changes of lipid composition. A progressive depletion of phospholipids coupled with an increase

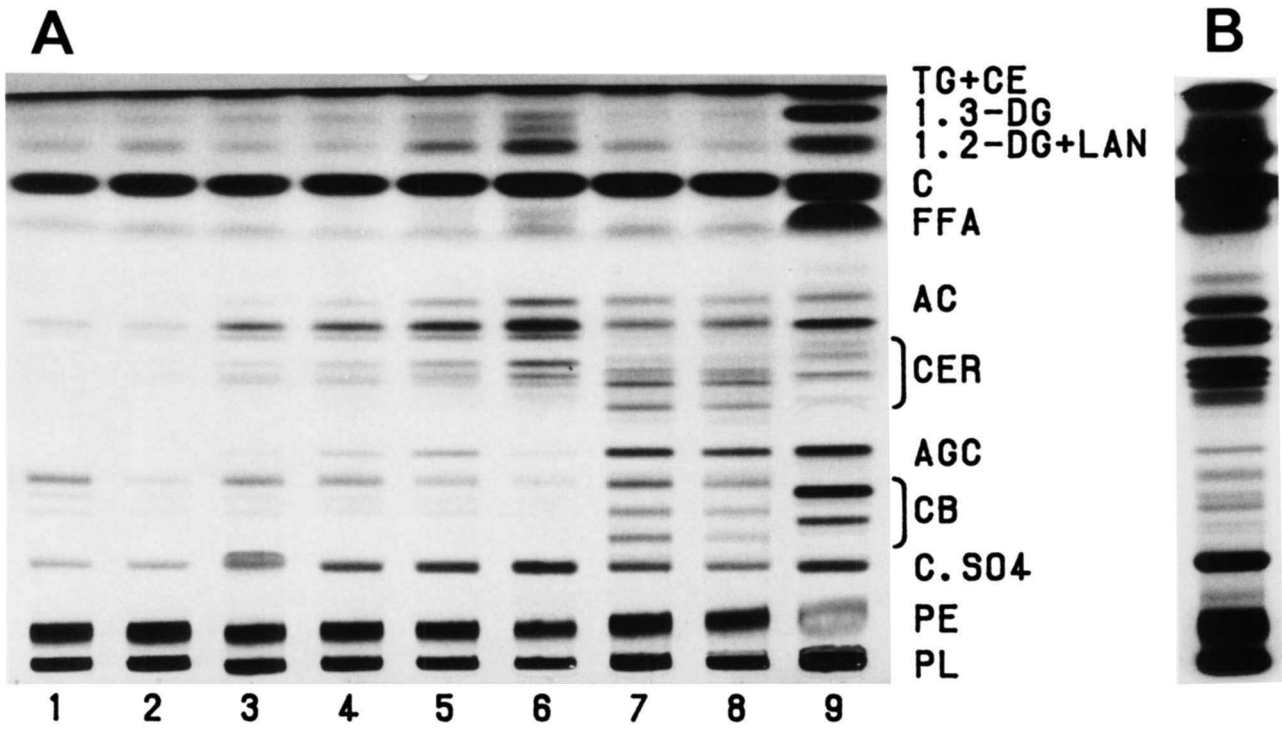

Fig. 5. One-dimensional TLC separation of total lipid extract in 'ceramide lipid solvent system'. A) Keratinocytes grown under low Ca^{2+} (lane 1), normal Ca^{2+} (lane 2), on DED for 3 days (lane 3), for 5 days (lane 4), for 8 days (lane 5), for 14 days (lane 6), or isolated directly from the epidermis (lanes 7 and 8). The mixture of standards (lane 9) contained phospholipids (PL), (sphingomyelin (SPH), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI)), phosphatidylethanolamine (PE), cholesterol sulfate (CSO_4), cerebrosides I and II (CB), acylglucosylceramide (AGC), ceramides (CER), acylceramide (AC), free fatty acids (FFA), cholesterol (C), lanosterol (LAN), 1,2-diglycerides (1,2-DG) and 1,3-diglycerides (1,3-DG), cholesteryl esters (CE), triglycerides (TG). Five to 50 μg of total lipid extract was applied on HPTLC plates and separated using chloroform–acetone–methanol 76:4:20 as the first, chloroform–acetone–methanol 80:10:10 as the second, and chloroform–diethyl ether–ethyl acetate–methanol 76:6:20:2 as the third developing system. The lipid fractions were visualized after charring as described in Materials and Methods. B) Autoradiogram of keratinocytes grown for 5 days on DED and subsequently labeled for 4 days with [^{14}C]acetate. The lipid extraction and separation were performed as described under (A).

of sterols and certain classes of sphingolipids was found to occur during differentiation in both human and animal epidermis (20–30). Similar phenomena related to differentiation are also observed in this study.

By morphologic criteria, the keratinocyte stratification in submerged cultures does not proceed further than what is observed in the lower layers of the epidermis, namely mainly the basal and suprabasal layers. On the other hand, the air-exposed keratinocyte culture looks almost identical to the epidermis in vivo (11, 12, Fig. 2). Although the lipid content (expressed as mg of recovered lipid/mg of protein) was similar whatever culture conditions were used, the lipid composition showed marked differences (Table 1). In submerged cultures, the keratinocytes grown under the low and/or normal calcium conditions showed a different degree of differentiation (1–5, Fig. 1) but not a significant difference in lipid composition (Table 1). Obviously, the differences occurring in the stage of incomplete differentiation did not result in measurable differences in lipid composition. The keratinocyte lipid composition in submerged cultures was, however, found to differ to some extent from that reported for basal and suprabasal human (22) and nonhuman (20, 24–26) epidermal cells. A comparison with data presented in this report reveals that keratinocytes cultured under submerged conditions distinguish themselves by the following features: *a*) the virtual absence of AGC and AC, *b*) a change in fatty acid composition, and *c*) an increase in triglyceride content. In apparent contradiction to our results are those reported by Madison et al. (27) who established the presence of AGC and AC in primary mouse keratinocyte cultures. This discrepancy might be due either to the differences between human and mouse keratinocytes or to the fact that we performed our studies with secondary and tertiary cultures. In any case, air-exposed cultures showed the presence of much higher amounts of AGC and AC than any submerged cultures do. It should be noted that the air-exposed cultures grow on a dermal substrate. At present we cannot exclude the possible influence of dermis on lipid biosynthesis.

The next point to discuss is the fatty acid composition. Here the submerged and air-exposed keratinocyte cultures are rather similar and they both differ from the noncul-

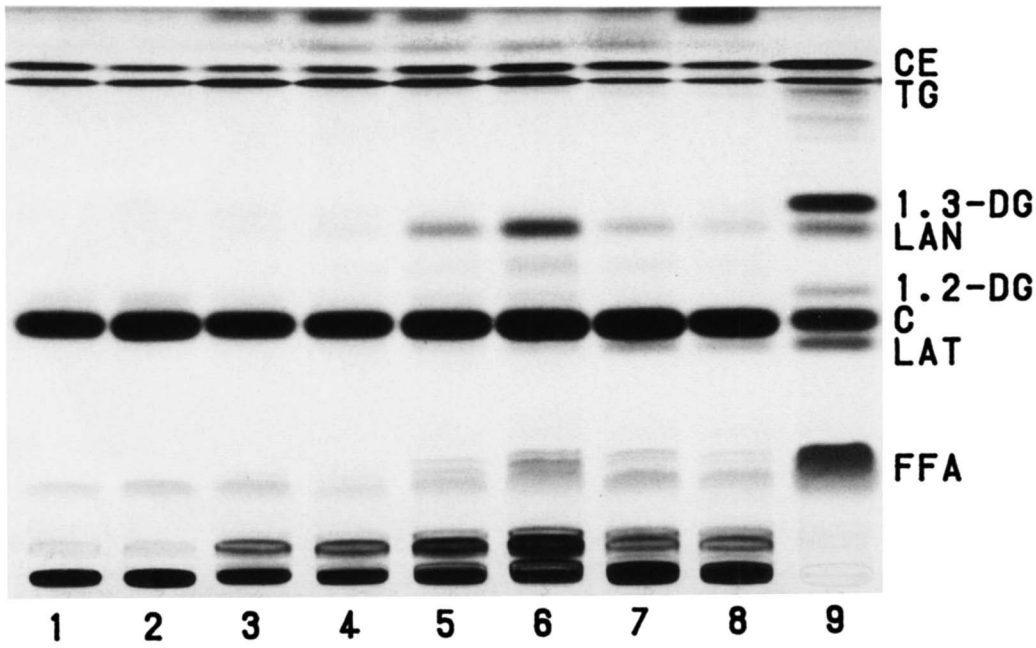

Fig. 6. One-dimensional TLC separation of total lipid extract in 'lanosterol lathosterol lipid solvent system' of keratinocytes grown under low Ca^{2+} (lane 1), normal Ca^{2+} (lane 2), on DED for 3 days (lane 3), for 5 days (lane 4), for 8 days (lane 5), for 14 days (lane 6), or isolated directly from the epidermis (lanes 7 and 8). The mixture of standards (lane 9) contained free fatty acids (FFA), lathosterol (LAT), cholesterol (C), lanosterol (LAN), 1,2-diglycerides (1,2-DG), 1,3-diglycerides (1,3-DG), triglycerides (TG), and cholesteryl esters (CE). Five to 50 μg of total lipid extract was applied on HPTLC plates and separated using chloroform–methanol 90:10 as the first, and chloroform–diethyl ether–ethyl acetate 80:4:16 as the second developing system. The lipid fractions were visualized after charring as described in Materials and Methods.

TABLE 1. Variation of lipid composition in relation to human keratinocyte differentiation

Fraction	Submerged Culture		Period of Cultivation on De-epidermized Dermis in Air-exposed Model				Freshly Isolated Epidermis
	Low Ca^{2+}	Normal Ca^{2+}	3 Days	5 Days	8 Days	14 Days	
Phospholipids	68.8 ± 5.0[a]	66.5 ± 5.2	58.8 ± 3.9	56.5 ± 1.5	49.4 ± 3.6	33.5 ± 2.6	65.5 ± 4.5
Sphingomyelin	5.5 ± 0.2	7.2 ± 0.7	6.8 ± 0.6	6.7 ± 0.2	6.4 ± 0.2	4.1 ± 1.2	9.3 ± 1.2
Phosphatidylcholine / Phosphatidylserine	32.0 ± 4.7	30.2 ± 5.6	30.4 ± 5.1	28.0 ± 0.5	22.0 ± 2.3	14.4 ± 2.5	31.9 ± 3.1
Phosphatidylinositol	8.2 ± 2.2	7.3 ± 0.7	5.7 ± 1.0	5.9 ± 0.9	6.6 ± 0.7	4.4 ± 0.9	6.5 ± 0.5
Phosphatidylethanolamine	23.1 ± 2.5	21.8 ± 3.0	15.9 ± 0.3	15.9 ± 0.3	14.4 ± 0.4	10.6 ± 2.2	17.8 ± 0.9
Cholesterol sulfate	0.6 ± 0.2	0.7 ± 0.1	1.5 ± 0.1	1.7 ± 0	1.9 ± 0	2.5 ± 0.2	1.4 ± 0.1
Glycosphingolipids	1.2 ± 0.5	0.7 ± 0.1	1.4 ± 0.3	0.9 ± 0.2	0.9 ± 0.1	0.5 ± 0.2	3.4 ± 0.9
Acylglucosylceramides	0	0	0.1 ± 0	0.2 ± 0	0.4 ± 0.1	0.1 ± 0	1.8 ± 0.1
Acylceramide	0	0	0.2 ± 0.1	0.3 ± 0	0.8 ± 0.1	1.2 ± 0.1	0.7 ± 0.1
Other ceramides	0.8 ± 0.3	0.8 ± 0.2	2.8 ± 0.4	2.5 ± 0.5	5.0 ± 0.6	7.4 ± 0.1	4.3 ± 1.5
Neutral lipids	28.5 ± 2.9	31.2 ± 3.2	35.1 ± 2.7	37.9 ± 1.8	41.6 ± 2.1	54.8 ± 1.8	22.9 ± 1.1
Cholesterol	12.9 ± 4.2	15.6 ± 4.3	9.0 ± 0.5	11.4 ± 1.0	13.9 ± 1.5	20.6 ± 1.2	14.7 ± 1.8
Lanosterol	0	0	0	0.3 ± 0.1	1.7 ± 0.3	4.6 ± 0.6	1.1 ± 0.2
Free fatty acids	0.5 ± 0.2	0.4 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.9 ± 0.2	2.9 ± 0.7	1.1 ± 0.4
Triglycerides	10.9 ± 1.6	12.4 ± 3.0	24.7 ± 2.8	24.6 ± 1.5	23.3 ± 2.5	24.3 ± 1.3	3.9 ± 1.2
Cholesteryl esters	4.2 ± 1.1	2.8 ± 1.2	0.8 ± 0.1	1.1 ± 0.2	1.8 ± 0.1	2.4 ± 0.4	2.1 ± 0.5
mg lipid/mg protein	0.25 ± 0.01	0.24 ± 0.05	0.30 ± 0.05	0.29 ± 0.07	0.21 ± 0.03	0.26 ± 0.05	0.24 ± 0.02

The lipid compositions of keratinocytes, grown either in submerged or air-exposed cultures, and of freshly isolated foreskin epidermis were determined after lipid extraction, one-dimensional TLC, and photodensitometry, as described in Materials and Methods. The results are given as an average percentage of total lipids ± SEM (n = 3).

TABLE 2. Fatty acid composition of major lipid fractions of

Fatty Acid	Total Lipid Extract				Phospholipids			
	Low Ca^{2+}	Normal Ca^{2+}	DED	Epidermis	Low Ca^{2+}	Normal Ca^{2+}	DED	Epidermis
	% of total fatty acids				*% of total fatty acids*			
16:0	17.7	16.7	22.2	15.8	16.1	14.2	23.2	15.1
16:1	10.4	11.8	13.1	1.8	9.6	8.7	15.7	1.2
18:0	9.1	9.3	10.8	14.8	9.7	10.7	10.5	15.3
18:1	52.0	52.0	45.8	16.6	53.2	57.1	45.9	20.1
18:2	1.1	1.2	1.1	23.3	1.4	1.2	1.0	28.7
20:4	2.4	2.0	0.7	2.1	3.4	2.6	0.7	2.6
24:0	0.8	1.6	3.6	10.2	1.0	1.1	1.6	7.5
25:0	0	0	0.1	2.7	0	0	0	1.6
26:0	0.3	1.1	1.9	5.3	0.4	0.7	0.5	2.7
28:0	0	0	0	2.7	0	0	0	0.8
% of total lipids	100.0	100.0	100.0	100.0	75.4	67.0	32.7	68.6

[a]Excluding lipids containing esterified hydroxy acids.

Fatty acid compositions of keratinocytes, grown to confluency in submerged culture either under low or normal Ca^{2+} conditions or for 14 days on DED, and of freshly isolated foreskin epidermis were determined after lipid extraction, two-dimensional TLC separation, fatty acid methylation, and gas-liquid chromatography, as described in Materials and Methods. Only the major fatty acids are shown in the table. Small quantities of 14:0, 22:0, 22:5, 22:6, and 24:1 were found in most of the samples, but these are not included in the table.

tured counterparts. This difference might be caused by culture conditions used, since Iserroff et al. (31, 32) recently demonstrated that the fatty acid content of fetal calf serum plays an important role in the determination of lipid and especially of fatty acid composition. These authors (31, 32) demonstrated that there is a gradual marked decrease in linoleic (18:2) acid content, a small decrease in linolenic (18:3) and arachidonic (20:4) acid contents, and an increase in the palmitoleic (16:1) acid content over a relatively short period (5 days) of primary culture of mouse keratinocytes as compared with the noncultured mouse keratinocytes. Since cultured cells incorporate fatty acids that are present in the serum-containing culture medium, the low levels of 18:2 and 18:3, and the high levels of 16:1 and 18:1 found in the fetal calf serum (32, 33) might account for the observed modulations of the fatty acid composition in cultured keratinocytes as compared with noncultured ones (Table 2). The higher amount of 18:2 acid found in the primary mouse keratinocyte cultures (27, 30, 31) as compared with the secondary and tertiary cultures of human keratinocytes used in the present study may be explained by the differences in the length of the cell culture period.

Contrary to the case for linoleic acid, the content of arachidonic acid in keratinocytes cultured under submerged conditions did not significantly differ from that seen in noncultured cells (Table 2). A similar observation has recently been reported by Iserroff et al. (32) for both murine and human keratinocytes. These authors showed that, contrary to the in vivo situation, the conversion of linoleic acid into arachidonic acid takes place in cultured keratinocytes. The process might, however, be switched off again when the keratinocytes are cultured on DED, since the arachidonic acid content in these cultures was found to be significantly lower than that seen in the submerged cultures (Table 1). This hypothesis might be supported by a recent finding from our laboratory that showed that the culture conditions play an important role in the determination of biosynthesis of some cellular components. Namely, keratinocytes cultured under submerged conditions in a medium containing a normal calcium concentration do express cytokeratins 4, 13, and 19, which were found to be absent not only in the noncultured foreskin epidermis (5) but also in keratinocytes cultured on DED (Ponec, M. et al., unpublished results).

The third point concerns the triglyceride content. All our results show rather dramatic and systematic differences between keratinocyte cultures (both the submerged and the air-exposed ones) and keratinocytes in vivo (Tables 1 and 2). At present we cannot offer a definitive explanation for this particular phenomenon. We can speculate that the higher level of triglycerides in cultured keratinocytes is a consequence of one of the two following situations: *1*) the essential fatty acid deficiency leads to a relatively higher triglyceride content (as observed in rat epidermis by D. H. Nugteren, unpublished results) or *2*) modulation of the activity of triacylglycerol hydrolase, an enzyme involved in triglyceride catabolism (34). This enzyme has been found to be present in the cytosol of living epidermal cells, in the lamellar bodies of cells of the granular layer, and located intercellularly in the stratum corneum. This enzyme is probably activated after the extrusion of lamellar bodies (35), a process that might be modified under the in vitro conditions used.

human keratinocyte lipid extracts

Free Fatty Acids				Ceramides[a]				Neutral Lipids			
Low Ca^{2+}	Normal Ca^{2+}	DED	Epidermis	Low Ca^{2+}	Normal Ca^{2+}	DED	Epidermis	Low Ca^{2+}	Normal Ca^{2+}	DED	Epidermis
% of total fatty acids				*% of total fatty acids*				*% of total fatty acids*			
24.8	16.5	21.0	12.4	35.4	36.3	27.6	9.4	21.2	19.8	21.0	32.4
8.5	6.4	3.1	0.2	4.2	2.4	1.6	0.4	13.2	16.0	14.5	11.7
26.0	17.5	28.4	17.2	44.5	26.7	11.6	8.6	5.7	5.7	9.1	13.6
31.4	32.9	24.6	1.8	9.3	5.9	6.0	1.4	54.6	47.0	53.4	17.8
1.6	1.3	3.7	0.5	0.5	0.6	1.1	0.5	0.5	1.3	0.8	11.4
0.6	1.3	3.7	0	0.5	0.4	1.0	0.1	0.2	0.9	0.3	0.8
1.4	9.9	10.4	29.4	0.5	13.2	29.1	26.2	0.1	0.9	0.5	3.0
0	0	0	7.4	0	0	0	10.6	0	0	0	0.7
0.4	9.9	3.9	14.2	0	9.5	18.4	25.2	0.1	0.8	0.2	1.4
0	0	0	7.0	0	0	0	11.5	0	0	0	0
1.3	0.6	4.2	5.6	1.4	2.0	3.2	8.0	21.2	28.0	44.8	5.2

In air-exposed cultures, as compared with submerged cultures, changes in the fatty acid composition as well as changes in the total lipid composition were observed. At day 8 of the culture, the lipid composition was similar to that found in the epidermis (Table 1), with the exception that the content of triglycerides was found to be much higher and that of phospholipids, glycosphingolipids and AGC to be lower than in noncultured cells. The linoleic content of AGC in keratinocytes cultured on DED was considerably lower than that of noncultured epidermis (**Table 3**) and to a certain extent similar to that seen in the epidermis of fatty acid-deficient rats (36, 37, and Nugteren, D. H., unpublished observations). The abnormalities of some of the lamellar body structures confirm this biochemical evidence of essential fatty acid deficiency. Similar observations were made by Elias and Brown (38) on experimental animals.

As reported for mouse keratinocytes (39), the lipid analyses of both cultured and noncultured human keratinocytes revealed the presence of lanosterol and lathosterol, intermediates in biosynthetic pathways for cholesterol (40). In human keratinocytes the presence of both lanosterol and lathosterol was also related to the stage of differentiation of the keratinocytes. Namely, these sterols were found to be absent in cells grown under submerged culture conditions and during a short period of culture on DED (up to 5 days) (Table 1). As soon as the extent of stratification was comparable to that seen under the in vivo conditions, these sterols appeared. The situation in human keratinocytes was found to be, however, slightly different from that seen in mouse keratinocytes. In the latter case, lathosterol was already present in the submerged culture system in cells induced to differentiate by incubation in medium containing normal calcium concentration (39).

TABLE 3. Fatty acid composition of acylglucosylceramides in human keratinocytes

Fatty Acid	Cultured for 14 Days on DED	From Freshly Isolated Epidermis
	% of total fatty acids	
16:0	28.4	16.2
16:1	0	2.6
18:0	17.9	13.0
18:1	20.5	6.5
18:2	2.6	58.4
24:0	20.5	0
26:0	10.7	0
% of total lipids	1.1	1.8

Fatty acid compositions of acylglucosylceramides in keratinocytes cultured for 14 days on DED and in freshly isolated epidermis were determined as described in Table 2.

In conclusion, the air-exposed culture system, using de-epidermized dermis as a substrate, offers a very attractive model for studying the differentiation-related changes in lipid composition of the keratinocyte. Minor differences in lipid composition between cultured keratinocytes and their in vivo counterparts were still observed. This might be due to differences in available nutrients. Further studies are needed to see whether changes in culture conditions, such as the supplementation of culture media with essential fatty acids, a decrease in relative humidity, or the inclusion of dermal fibroblasts, will enable in vitro conditions to approximate the in vivo situation.

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