In Vitro Model of Essential Fatty Acid Deficiency

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The polyunsaturated fatty acids linoleic acid (18:2, n-6) and arachidonic acid (20:4, n-6) are essential for normal skin function and structure, both as eicosanoid precursors and as components of lipids forming cell membranes. Adult human keratinocytes grow optimally in serum-free medium (MCDB 153) that contains no fatty acids. These keratinocytes expand rapidly and produce normal epidermis upon *in vivo* grafting. Analysis of lipid extracts of epidermis and of cultured keratinocytes was done to determine the fatty acid composition of cells grown in essential fatty acid (EFA)–deficient medium. Gas chromatography and high-performance liquid chromatography analyses were done of the fatty acids in the entire cell and in a thin-layer chromatography separated fraction containing those lipids that form cellular membranes. Comparison of snap-frozen epidermis and epidermal basal cell suspensions to passage 1 to 4 cultures shows that the cells are in an extreme essential fatty acid–deficient state by the first passage. The amount of the saturated fatty acids 16:0, 18:0, and 14:0 is unchanged by culture. The polyunsaturated fatty acids are found to be significantly decreased, the cells balancing their lack with a significant increase in the relative abundance of the monounsaturated fatty acids, 18:1 and 16:1. Greater than 85–90% of the fatty acids was found in lipids associated with membranes and no unusual fatty acids were detected. Because the serum-free medium is fatty acid free and the cells cannot synthesize essential fatty acids, the rapid division of the cells results in the predominance of an extreme EFA-deficient cell type. The essential fatty acid–deficient keratinocyte is an excellent adult, normal epidermal cell model that can be used to study EFA deficiency and the effect of the eicosanoid and fatty acids on cell function and structure. *J Invest Dermatol 99:703–708, 1992*

The original, more classical systems for culturing primary epidermal basal cells, using 10–15% serum in standard medium formulations [1,2], have given way to a number of newer techniques [3–5]. One system developed by Boyce and Ham [6,7] employs serum-free, low-calcium MCDB 153. The keratinocyte strains grow rapidly, and can be induced to stratify and differentiate by restoring the cells to normal calcium and/or serum levels. The rapid growth of the primary human keratinocyte in serum-free medium characterizes this as one of the few cell strains to adapt successfully to the serum-free state.

In culture medium, serum is usually the only source of essential and non-essential fatty acids. Fatty acids are long hydrocarbon chains, with a carboxyl group at one end. They are usually esterified to the number 1 and 2 carbons of the glycerol backbone of phospholipids, to other amphipathic lipids (having polar and nonpolar ends), and to the carbons of glycerol, forming tri-, di-, and monoacylglycerol [8].

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Abbreviations:
BPE: bovine pituitary extract
EFA: essential fatty acid
EGF: epidermal growth factor
FA: fatty acid
FAMEs: fatty acid methyl esters
FBS: fetal bovine serum
GC: gas chromatography
HEPES: hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HPLC: high-performance liquid chromatography
P: passage
TLC: thin-layer chromatography

The human body can synthesize the saturated (thus, non-essential) fatty acids using cytosolic enzymes of the acetyl-CoA carboxylase and fatty acid synthase complexes [9], producing mainly 16:0 (palmitic acid) and minor amounts of 18:0 (stearic acid). Monounsaturated fatty acids are formed by direct oxidative desaturation (a removal of two hydrogen atoms) of a saturated fatty acid. A Δ^9 desaturase is the usual desaturation enzyme for production of monounsaturated fatty acids in human tissues (i.e., 16:1 and 18:1). Human tissue cannot introduce double bonds beyond the Δ^9 position, thus 18:2, $\Delta^{9,12}$, and 20:4 $\Delta^{5,8,11,14}$ cannot be synthesized by the epidermis and must be supplied by the diet, i.e., they are essential [10]. The intact epidermis also cannot convert 18:2 (linoleic acid) to 20:4 (arachidonic acid) although the induction by *in vitro* keratinocyte growth of the enzymes responsible for this conversion, a Δ^5 and Δ^6 desaturase, is reported [11].

Essential fatty acids (EFAs) are required for the normal function of mammalian tissues, and the lack of EFAs in the diet causes a human and animal disease known as essential fatty acid deficiency. In skin, it is manifested as a severe scaly lesion, accompanied by chronic epidermal hyperproliferation, abnormal differentiation, and abnormal cutaneous barrier function [12,13]; these symptoms are greatly lessened in humans by topical application of linoleic acid [14]. It is known that EFA deficiency causes, and that 18:2 alleviates, this disorder; however, the mechanism is unknown.

Hypothetically, keratinocytes growing in serum-free medium can adapt to the EFA-deficient growth condition in a number of ways: 1) the cell could develop the capacity to desaturate beyond the Δ^9 position; 2) the keratinocyte could synthesize unusual fatty acids to compensate for the EFA-deficient state; and 3) the cell could stay in a classically defined EFA-deficient state. The first possibility invokes the expression of enzymes not found in the human epidermal cell. The last two possibilities have interesting ramifications because the fluidity of the cellular membranes, which control the function

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and communication of cells, depends on the nature of the fatty acids within these structures [15].

We have measured the fatty acid composition of a number of epidermal preparations and cultured keratinocyte strains to determine how this cell type adjusts to serum-free, rapid growth conditions.

MATERIALS AND METHODS

Materials Tissue-culture reagents were tissue-culture grade and all organics were high performance liquid chromatography (HPLC) grade. Gas chromatography and HPLC fatty acid standards and 17:0 were obtained from Nu-CHEK-PREP, Inc, Elysian, MN. Amino acids and medium reagents, fetal bovine serum (FBS), trypsin type IX, trypsin inhibitor type I-S, and gentamicin (10 mg/ml), were obtained from Sigma, St. Louis, MO. Plastics were from Laboratory Science Co, Corning, NY. Petroleum ether (30–60°C), benzene, HPLC water, and acetonitrile were purchased from Baker, Phillipsburg, NJ: methanol, and chloroform were obtained from B&J Baxter, McGraw Park, IL. Thin-layer silica gel 60 plates were obtained from Merck, Darmstadt, Germany. Frozen bovine pituitaries were from Pel-Freeze, Rogers, AR, and filters were obtained from Millipore, Corp, Bedford, MA.

Culture Chelexed (cation-free, BIO-RAD, Richmond, CA) fetal bovine serum (FBS) was prepared as previously described [16]. The basic medium, MCDB 153, was prepared as described by Boyce and Ham [6,7] and was supplemented with 0.6×10^{-6} M (0.218 μg/ml) hydrocortisone, 5 ng/ml epidermal growth factor (EGF), 5 μg/ml insulin, 6 mg % bovine pituitary extract (3 ml of extract, 10 mg protein/ml, in 500 ml of medium) [7], and 0.15 mM $CaCl_2$ to form complete growth medium. Discarded human skin from mastectomy and abdominoplasty cosmetic surgery was rinsed and soaked for 2 h in solution A (30 mM hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES] 10 mM glucose, 3 mM KCl, 130 mM NaCl, 1.0 mM Na_2HPO_4, pH 7.4) [7]. The skin was cut into 0.2 to 0.3 × 7 cm strips and digested overnight at room temperature with 0.18% trypsin in solution A, which separates the skin at the dermal-epidermal junction, leaving the basal cells on the dermal layer. The basal epidermal cells (keratinocytes) were gently scraped from the separated dermis into MCDB 153 plus 10% FBS. The filtered cell suspension (250 μ, mesh, PGC Scientific, Gaithersburg, MD) was plated at 20×10^6 cells in 15 ml MCDB 153 containing 2% chelexed FBS per T-75 flask and was grown at 37°C in 5% CO_2 in air gassing. The cultures were fed every 48 h with serum-free MCDB 153 and reached ≅ 70% confluence after 5–7 d. The monolayers were passaged every third to fifth day, yielding an expanding cell strain from each piece of surgical tissue [5–7].

Cell Growth Four keratinocyte strains were plated at 3.5×10^5 cells per 60-mm petri dish. Duplicate dishes were trypsinized and were counted by hemacytometer 2 and 4–5 d after plating. The cells were passaged at the 4–5 d time point until the sixth passage (P = 6).

Epidermal Biopsies and Cell Pellet Six epidermal biopsies were immediately snap-frozen in liquid nitrogen as previously described [17]. The biopsies were 0.1-mm-thick keratomed epidermal sheets containing trace amounts of dermal and blood elements, as previously shown by us [17]. The cell pellet was a centrifuged filtered cell suspension (see *Culture*) normally used to establish the primary cultures. The cell suspension contained >95% basal cells, some spinous cells, and small amounts of blood cells, melanocytes, and fibroblasts. The entire dermis, most of the spinous layer, and all of the granular and corneal layer remained as intact structures after basal cell removal. Similarly derived suspensions when used to establish keratinocyte cultures in serum containing medium showed a 75–95% viability and plating efficiency [1,2], indicating the high percent of basal layer derived cells.

Lipid Analysis Passage 1 to 4 keratinocyte cultures were rinsed twice with calcium-free, phosphate-buffered saline and were scraped into ice-cold methanol. The pelleted cell suspension and snap-frozen biopsies were ground while frozen under liquid nitrogen and homogenized in methanol. The preparations were extracted with a 1:2:1.5 ratio of methanol:chloroform:0.1 M KCl in 50% methanol, and the aqueous phase was re-extracted with 2.5× volume of 0.1 M KCl in 50% methanol [18]. The precipitated protein and DNA were processed for Lowry protein and Burton DNA as described [17]. The organic phase, containing the polar phospholipids, neutral lipids, complex polar lipids, such as the sphingosine-based lipids, cholesterol and cholesterol esters [19], and free fatty acids, was split into two aliquots and evaporated under a nitrogen stream. One aliquot was transmethylated to form fatty acid methyl esters (FAMEs) using 6% methanolic-HCL for 2 h at 80°C [20] with 50 μg of 17:0 added as a gas chromatography (GC) and high-performance liquid chromatography (HPLC) reference and quantitative standard. The second aliquot was suspended in 50 μl of 1:1 chloroform:methanol, applied to a thin-layer chromatography (TLC) plate, and chromatographed in one direction using $CHCl_3$:MeOH:acetic acid (90:8:1). Neutral lipids, including cholesterol and its esters, and free fatty acids were separated from the phospholipids and other amphipathic lipids [21] (*i.e.*, sphingolipids that are less than 10% of all lipids in cultured human keratinocytes [22]). The phospholipid fraction was scraped and the material was eluted from the silica during transmethylation with 6% methanolic-HCl + 50 μg 17:0. The FAMEs from the whole cell and TLC-separated aliquots were extracted into petroleum ether, evaporated to dryness, and stored frozen under benzene [21]. The FAMEs were resuspended in 200 μl of benzene, filtered using a 0.45 μm filter, evaporated, and resuspended in either 12–30 μl filtered chloroform (GC) or 25 μl methanol (HPLC) for analysis. One microliter of chloroform or 20 μl of methanol was injected for analysis.

Analysis of FAMEs FAMEs were analyzed using a Hewlett Packard gas chromatograph (GC) model 5710A equipped with a J and W Scientific (Folsom, CA) fused silica Megabore DB225, 0.53 μ diameter column. The FAMEs were eluted with scrubbed helium at a flow rate of 2.79 ml/min at 215°C for 8 min, at a gradient of 4°C/min until 230°C, and then isothermic until 25 min. The flame ionization detector output of the gas chromatograph was digitized by an IBM-PC computer interface (model AN-146, Alpha Products, Darien, CT). Both the recording and data evaluation software were written by us in BASIC or FORTRAN. The GC area evaluation routines use sophisticated curve fitting procedures to minimize error, which will be described in detail in a future publication. FAMEs were also analyzed by C-18 reverse-phase chromatography with a Hewlett Packard 1090M HPLC equipped with diode array spectrometer. The initial solvent was 70% acetonitrile and 30% water adjusted to pH 3.6 with phosphoric acid. At 2 min, a continuous gradient of increasing percent acetonitrile was initiated, reaching 90% at 48 min, followed by a gradient to 95% acetonitrile at 49 min, and a third gradient to 100% acetonitrile at 60 min. All materials and equipment exposed to human tisssue or keratinocytes were soaked in a 30% bleach solution prior to disposal or washing. The human use protocol was approved by the University of Michigan Internal Review board (IRB #87-224). The tissue source included women and minorities.

RESULTS

Keratinocyte Growth in Essential Fatty Acid–Deficient Medium The cells grow rapidly in monolayers. The addition of serum and/or calcium (1.2 to 2.0 mM) causes the cessation of proliferation, and induces differentiation and stratification of the cultures (data not shown). The doubling time is approximately 2 d for the first five passages, after which time the cells begin to lose the ability to divide (data not shown, n = 4). By passage six, there is an expansion from 1 to over 10,000 cells, during a 30-d period (15 doublings).

Fatty Acid Content of Bovine Pituitary Extract (BPE) and Growth Medium Five microliters of BPE was extracted using a modification of the methanol/chloroform/salt method used to ex-

Table I. Fatty Acid Content, Percent Total Lipids

Fatty Acid	Bio	Pellet	P1	P2	P3	P4
14:0	1.1 ± 0.2	0.9 ± 0.2[b]	3.0 ± 0.5[c]	3.5 ± 0.5[c]	2.4 ± 0.3[c]	4.9 ± 1.3[b]
14:1	0.5 ± 0.1	0.2 ± 0.1[b]	0.4 ± 0.2[b]	0.2 ± 0.2[b]	0.6 ± 0.3[b]	0.5 ± 0.3[b]
16:0	16.1 ± 0.7	18.8 ± 0.3[c]	19.4 ± 3.7[b]	21.1 ± 1.6[c]	17.1 ± 1.4[b]	20.6 ± 5.2[b]
16:1,n-7[a]	0.2 ± 0.2	2.5 ± 1.0[b]	12.5 ± 0.9[d]	12.6 ± 1.6[c]	14.1 ± 1.3[d]	14.5 ± 0.1[d]
18:0	12.7 ± 0.3	14.2 ± 1.7[b]	14.4 ± 1.3[b]	16.6 ± 1.2[d]	12.7 ± 0.6[b]	12.2 ± 1.7[b]
18:1,n-9	16.0 ± 0.4	25.1 ± 3.1[b]	42.1 ± 3.6[d]	32.5 ± 3.0[c]	41.3 ± 3.2[f]	41.2 ± 7.6[b]
18:2,n-6	24.8 ± 0.8	23.8 ± 1.4[b]	1.6 ± 0.2[c]	4.0 ± 1.0[c]	3.2 ± 1.7[d]	1.5 ± 0.8[c]
18:3,n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:0	1.2 ± 0.0	0.8 ± 0.1[b]	0.0 ± 0.0	0.2 ± 0.2[c]	0.0 ± 0.0	0.0 ± 0.0
20:1,n-9	0.4 ± 0.1	0.1 ± 0.1[b]	0.6 ± 0.4[b]	0.5 ± 0.2[b]	0.7 ± 0.4[b]	0.2 ± 0.2[b]
20:2,n-6	0.2 ± 0.1	0.4 ± 0.3[b]	1.3 ± 0.6[b]	0.9 ± 0.2[d]	1.3 ± 0.7[b]	0.0 ± 0.0
20:3,n-6	2.1 ± 0.2	1.5 ± 0.2[b]	0.2 ± 0.2[c]	1.1 ± 0.3[c]	1.0 ± 0.4[b]	0.1 ± 0.1[c]
20:4,n-6	8.1 ± 0.5	5.8 ± 0.3[d]	0.8 ± 0.5[c]	2.0 ± 0.4[c]	2.9 ± 1.1[c]	0.8 ± 0.4[c]
20:3,n-9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:0	3.7 ± 0.2	1.8 ± 0.2[d]	0.7 ± 0.3[c]	1.2 ± 0.3[c]	0.4 ± 0.2[c]	0.3 ± 0.3[c]
22:1,n-9	0.2 ± 0.2	0.3 ± 0.2[b]	0.2 ± 0.1[b]	0.5 ± 0.3[b]	0.3 ± 0.1[b]	0.3 ± 0.3[b]
22:4,n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:6,n-3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:0	12.3 ± 0.9	2.8 ± 0.5[c]	1.4 ± .4[c]	1.6 ± 0.2[c]	0.9 ± 0.5[c]	0.9 ± 0.5[c]
24:1,n-9	0.3 ± 0.3	0.9 ± 0.2[b]	1.4 ± 0.5[b]	1.4 ± 0.4[f]	0.9 ± 0.5[b]	2.0 ± 0.3[d]
26:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
n	6	4	4	12	3	3

[a] The (n-) nomenclature indicates the double bond closest to the methyl end, whereas the Δ nomenclature indicates the distance of the double bond from the carboxyl end.
[b] Not significant.
[c] $0.05 > p > 0.02$.
[d] $0.01 > p > 0.001$.
[e] $p < 0.001$.
[f] $p \cong 0.05$.

tract the cell samples. After methylation, fatty acid analysis by HPLC showed that no fatty acids were present in this amount of BPE, which is sufficient to make 850 ml of complete growth medium. This was expected because BPE is a 0.15 M NaCl (aqueous) homogenate of frozen-powdered pituitaries that was clarified by ultracentrifugation twice at 200,000 × *g*, and sterile filtered through 0.8-, 0.45-, and 0.22-μ filters, processes that would minimize the presence of lipids in the BPE. Similar extraction of complete growth medium showed that the medium was completely fatty acid free.

Change in Total Fatty Acid Content from Biopsy and Pellet to Keratinocyte Cultures In Table I the fatty acid compositions of snap-frozen biopsies, pelleted cell suspensions, and P1 to P4 keratinocyte strains are compared. The biopsy material contains basal cells and differentiating epidermal cell layers; the cell pellet consists of isolated basal cells that were used to initiate the cultures, plus small amounts of other skin cell types (see *Materials and Methods, Epidermal Biopsies and Cell Pellet*). The P1 to P4 cultures are pure basal epidermal cells because only these cells grow well in complete serum-free MCDB 153 (data not shown). The data are expressed as micrograms of a fatty acid per milligram of protein or microgram of DNA, with the total protein and DNA extracted from the samples being used as indicators of cell number in the tissue, cell pellet, or cultures. In the cell cultures, 3×10^6 cells contains 1 mg of protein and approximately 150 ± 28 μg fatty acids (SEM, N = 5). The values were also normalized as percent of the total fatty acid in the sample (μg fatty acid/100 μg of total fatty acid present).

Twenty-one biologically occurring fatty acids plus the 17:0 standard are easily identified and quantified by gas chromatography at the 1 μg/injection range. The snap-frozen biopsy values were compared to the pellet and P1 to P4 values using a two-tailed Student t test. The biopsy values in Table I are equivalent to those analyzed and reported by Chapkin et al in a previous study with us [20], indicating consistency in obtaining isolated epidermis by our keratome/freeze method and in the lipid extraction and analysis procedures. The fatty acid values for biopsy epidermis reflect the levels for the whole tissue, not just the basal cell layer, which is the source of the cell type that grows in culture. The cell pellet fatty acid levels closely resemble the *in vivo* basal cell values because the suspension consists almost entirely of epidermal basal cells. Even after overnight trypsinization at room temperature (no serum) the isolated cell suspension (pellet) shows a fatty acid profile similar to the snap-frozen biopsy material (Table I). A decrease in 24:0 and 22:0 when the pellet is compared to the biopsy is seen, suggesting that these long-chained fatty acids may be present in greater quantities in the differentiating epidermal component. Summation of the data presented in Table I shows that the ratio of saturated to unsaturated fatty acids changes from 47:53 in the biopsy to about 39:61 in the cell cultures. The saturated fatty acids 14:0, 16:0, 18:0, and 20:0 show no or little significant change between the keratinocyte cultures and the snap-frozen biopsy values. The amounts of 22:0 and 24:0 also decrease to approximately one-tenth the biopsy value in P3 and P4 cultures. This suggests lessened synthesis or increased turnover of these two long-chained fatty acids by the cultured keratinocytes. Although the percent of total unsaturated fatty acid is somewhat constant among the various epidermal preparations and cell passages, large shifts in the ratio of monounsaturated to polyunsaturated fatty acids occur. The ratio of the polyunsaturated, essential fatty acids (18:2, 20:2, 20:3, and 20:4) to the monounsaturated fatty acids (14:1, 16:1, 18:1, 20:1, and 24:1) is 35:18 in the biopsy, 32:29 in the pellet, 4:57 in P1 cells, and down to 2:59 in the P4 cultures. The decrease from the 35% biopsy value to the 2% P4 value for the total polyunsaturated fatty acids is balanced by a relative increase in the abundance of 16:1 and 18:1 in the cultured cells. The percent palmitoleic acid (16:1) increases from a biopsy value of 0.2 ± 0.2 to a P4 value of 14.5 ± 0.1 (mean ± SEM, Table I). Oleic acid (18:1) comprises 16.0 ± 0.4% of the total fatty acids in the biopsy material and is 41.2 ± 7.6% of the fatty acid content of P4 cells (Table I). Analysis of similar samples using HPLC verifies the extreme shift in keratinocyte culture fatty acid composition (data not presented).

Comparison of Whole Cell and Phospholipids Fatty Acid Content The membranes of mammalian cells contain the majority of the phospholipid and other amphipathic, lipid molecules in cells [8]. It is possible that the fatty acid profiles of the phospholipids (the membranes) of keratinocytes grown serum free would differ

Table II. Comparison of Whole Cell and Phospholipid Fatty Acid Content

Fatty Acid	Whole Cell Mean ± SEM (μg/mg protein)	Whole Cell (%) Ttl Lipid	Phospholipid[a] Mean ± SEM (μg/mg protein)	Phospholipid[a] (%) Ttl Lipid
14:0	3.0 ± 0.3	2.85	2.3 ± 0.3	2.51
14:1	0.5 ± .07	0.45	0.1 ± .02	0.15
16:0	13.7 ± 0.7	13.05	11.9 ± 0.8	13.21
16:1,n-7	14.6 ± 0.7	13.89	12.7 ± 0.7	14.15
18:0	11.5 ± 1.0	10.95	10.1 ± 0.6	11.25
18:1,n-9	49.6 ± 2.4	47.30	43.0 ± 2.5	47.88
18:2,n-6	3.6 ± 0.5	3.46	3.4 ± 0.3	3.78
18:3,n-6	0.0 ± 0.0	0.00	0.0 ± 0.0	0.00
20:0	0.2 ± .01	0.16	0.1 ± .03	0.16
20:1,n-9	0.6 ± 0.1	0.54	0.4 ± .05	0.48
20:2,n-9	1.4 ± 0.9	1.28	1.3 ± 0.4	1.48
20:3,n-6	0.5 ± .05	0.47	0.4 ± .07	0.48
20:4,n-6	1.0 ± .07	0.95	0.9 ± .05	1.05
22:0	0.5 ± .02	0.50	0.4 ± .02	0.47
22:1,n-9	0.3 ± .09	0.29	0.2 ± .05	0.24
22:4,n-6	0.0 ± 0.0	0.00	0.0 ± 0.0	0.00
22:6,n-3	0.0 ± 0.0	0.00	0.0 ± 0.0	0.00
24:0	1.8 ± 0.4	1.74	1.4 ± 0.3	1.59
24:1,n-9	2.1 ± 0.5	2.12	1.0 ± 0.3	1.12
26:0	0.0 ± 0.0	0.00	0.0 ± 0.0	0.00

[a] TLC separates the polar phospholipid and other amphipathic lipids from the neutral, and other fatty acid containing lipids. Addition of vitamin E (1 μM, Aldrich Chemical Co.) to the medium did not alter the values (n = 2, data not presented).

from that of the whole cell lipid or that the cells contain greater than normal amounts of free fatty acids and acylglycerols. Table II presents a comparison of whole-cell extract and TLC-separated phospholipid and other amphipathic molecules from P3 cell strains (n = 6). The mean micrograms of fatty acid per millogram of protein (normalized values) of the TLC fraction is approximately 85–90% of the whole cell value and the percent phospholipid fatty acids is almost identical to the whole cell value. The composition of the fatty acids in the phospholipids obtained by TLC of the biopsy lipid extract is presented in Fig 1. Comparison of the data from the whole cell extract (Table I) and to the TLC-separated phospholipid values (Fig 1) shows that greater than 90% of the fatty acids extracted from the whole biopsy is found in the TLC-separated phospholipid fractions of the tissue; the absolute and normalized values are almost identical in the two fractions. Thus, in both intact epidermis and keratinocytes, approximately 90% of fatty acids is found in the phospholipid component of the cell.

Percent Composition of Major Fatty Acids in the Phospholipid Fraction from Biopsies, Cell Pellet, and Keratinocyte Cultures The percent composition of major fatty acids in the phospholipid and other amphipathic molecules extracted from the tissue and cells is presented in Fig 1. The data in *a* demonstrate that the cells maintain approximately the *in vivo* (biopsy) amounts of the saturated fatty acids. In the cells, the relative abundance of 18:1 and 16:1 is increased *(b)*, whereas the percentages of the two major polyunsaturated fatty acids, 18:2 and 20:4, are greatly decreased *(c)* when compared to the biopsy and cell pellet values. Thus, the extreme fatty acid deficiency of these cells is reflected in the phospholipids that form the membrane structures of these keratinocytes.

Gas Chromatograms of Biopsy and Keratinocyte Fatty Acid Content Figure 2 presents two gas chromatograms of the fatty acid profiles of the TLC-separated fractions obtained from a biopsy *(a)* and a P3 keratinocyte culture *(b)*. The whole cell extract chromatograms are identical (data not presented). The chromatograms, depicting the raw, non-normalized data, demonstrate that the keratinocytes show greatly increased 18:1 (1.5 min) and 16:1 (−0.5 min) peaks *(b)*, whereas the biopsy material *(a)* has a smaller 18:1 and no detectable 16:1 peaks. Additionally, the large 18:2 (2.5 min) and 20:4 (7.0 min) polyunsaturated fatty acid peaks seen in the frozen epidermal samples *(a)* are seen to be greatly reduced in the keratinocyte cultures *(b)*. The amounts of 18:2 and 20:4 are often barely detectable (1 μg range) in keratinocyte samples that contained large amounts of the saturated and monosaturated fatty acids. Note that the 16:0 (−1.0 min) and 18:0 (1.5 min) peaks are approximately equivalent in both chromatograms. All the fatty acids are identified using lipid standards. No fatty acid breakdown is seen because no large peaks with retention times similar to 26:0 and

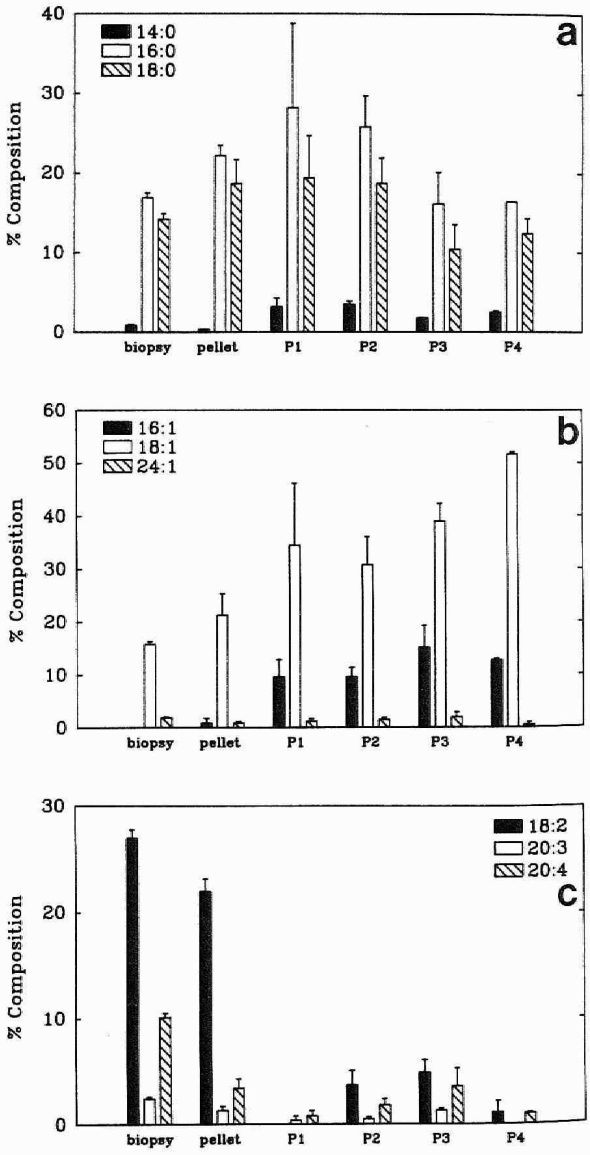

Figure 1. Percent composition of major fatty acids in the TLC fractions. The fatty acids are extracted, TLC separated, methylated, and prepared for GC analysis as described in the *Materials and Methods* section. The p values are as presented in Table I for the whole cell fatty acid values. *Error bar,* SEM.

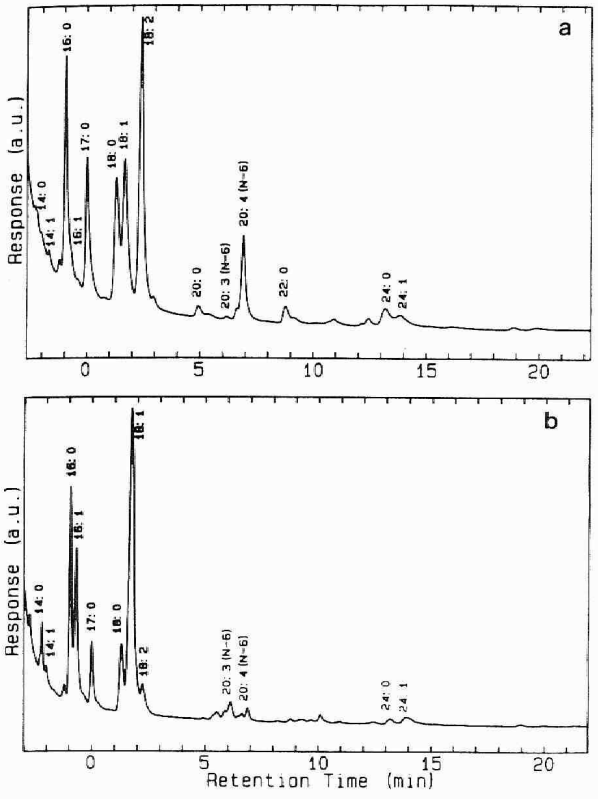

Figure 2. Gas chromatograms of fatty acid analysis. Gas chromatograms of the fatty acids from the TLC-separated fraction of *(a)* a snap-frozen biopsy and *(b)* a P3 keratinocyte strain. The data are not normalized and represent material in 1 μl of sample. The large 18:2 (2.5 min) and 20:4 (7.0 min) essential fatty acid peaks seen in the frozen epidermis *(a)* are greatly reduced in the keratinocyte cultures *(b)*, which show greatly increased 18:1 (1.5 min) and 16:1 (−1.0 min) peaks. All peaks are identified, indicating no production by the cells of unusual fatty acids and no breakdown of fatty acids during tissue and keratinocyte processing.

longer saturated fatty acids are observed, which are characteristic of sample oxidation.

DISCUSSION

Minor alterations in the fatty acid composition of cells grown *in vitro* have been reported by numerous investigators. Because most culture systems contain serum, reported decreases in the amount of 18:2 (N = 6) were thought to reflect an amount of 18:2 in the serum lower than tissue level [21,23–25]. Ponec et al [23] reported very low amounts of 18:2, normal levels of 20:4, and increased amounts of other fatty acids in neonatal keratinocyte cultures; these cells were grown in serum and on feeder layers. The authors suggested that the very low amounts of 18:2 seen in these cells resulted from the activation of the Δ^5 and Δ^6 desaturases in culture, converting all the 18:2 to 20:4, and thus maintaining 20:4 at the *in vivo* tissue levels [11,23].

Growth of cells in serum-free medium or medium containing delipidized serum decreases the proliferation of most cells in culture [26], although another skin-derived cell type, the melanocyte, has been reported to grow reasonably well in both serum-free [27] and serum-containing medium [28]. In contrast, adult human keratinocytes grown in serum-free MCDB 153 medium show optimal growth and expansion of epidermal cell monolayers. The plating efficiency using this culturing technique is low, approximately 5–8% when compared to other primary keratinocyte culture methods that support 70–80% plating of cells [2,3].

The primary cultures begin to proliferate rapidly after the first medium change, when the 2% chelexed serum used in the plating medium is removed. At first passage, quite rapid growth is seen, which allows the keratinocyte cell strains to expand greatly their numbers, the cells being subcultured numerous times without the loss of viability [29,30]. The serum- and EFA-free medium ensures the EFA deficiency of the rapidly dividing cells because there is no source of essential fatty acids available to the keratinocytes. Although the cell selectivity of this growth system is unknown, i.e., it may select for cells already EFA deficient *in vivo* or for a subpopulation of basal cells that can successfully adjust to EFA deficient *in vitro* growth conditions, the rapid proliferation of EFA-deficient cell type allows it to dominate the culture population by passage one.

The proliferation of confluent keratinocyte monolayers is markedly decreased, and the cultures stratify and differentiate when calcium and/or serum is added to the medium [7]. This manipulation creates an epidermal tissuelike structure that has been used successfully in grafting burn patients with cultured epidermal cells [31]. The keratinocyte culture grafts formed a viable epidermis, 10–12 cells thick with structures characteristic of *in vivo* epidermis [32]. Thus, the transplant studies indicate that the EFA-deficient cells have the capacity to revert to normal *in vivo*. Other studies by us will report how the fatty acid composition of the EFA-deficient keratinocytes can be altered to biopsy/pellet values by *in vitro* addition of fatty acids to serum-free medium, a manipulation that decreases the expansion and the capacity for successful passage of the cultures.* Thus, it is possible that the EFA-deficient cells are exhibiting, under serum-free culture conditions, the hyperproliferation characteristic of diseased EFA deficient epidermis [14].

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