Cellular Fatty Acid Composition of Stratified Squamous Epithelia After Transplantation of Ex Vivo Produced Oral Mucosa Equivalent

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Purpose: We have fabricated, for clinical application, artificial oral mucosa that totally excludes both heterogenic protein interaction and xenotransplantation. The purpose of this study is to compare the fatty acid composition of cell membrane phospholipids related to post-transplantation epithelial regeneration.

Materials and Methods: Cultured keratinocytes, keratinocytes at 2, 3, 4, and 9 weeks after transplantation, and normal oral keratinocytes were compared by gas chromatography for the composition of 23 fatty acids. The relation between the composition of cell membrane fatty acids, and the glucose metabolism was immunohistochemically analyzed.

Results: 1. Even after transplantation, cultured keratinocytes retained the same ratio of palmitic acid as that of normal oral keratinocytes. 2. Essential fatty acids decreased markedly in cultured keratinocyte membranes to the same composition as that of normal oral mucosa 2 weeks after transplantation. 3. The percent composition of palmitoleic acid in cultured keratinocytes was significantly higher than that in post-transplanted keratinocytes; it decreased 2 weeks after artificial mucosa transplantation, but became similar to that in 3 weeks thereafter. 4. The entire population of stratified keratinocytes in EVPOME before transplantation expressed GLUT-1 protein.

Conclusion: Our findings suggest that post-artificial mucosa epithelialisation allows keratinocytes to proliferate while consuming palmitic acid, and then diet-provided essential fatty acids induce the keratinocytes to differentiate. Complete clinical epithelialisation of the transplant wound requires 4 weeks; however, within 3 weeks of transplantation, cultured cells of a specific metabolic mechanism change into or are replaced by keratinocytes of a normal metabolic mechanism similar to that of surrounding tissue.

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The first transplantation of cultured epidermal keratinocytes was carried out in 1980 (14); these cells engineered into tissue have since been widely used in the clinical treatment of various skin defects in Japan. Sheets of cultured oral keratinocytes developed by similar techniques have been used as stratified squamous epithelium in the oral mucosa (4, 15, 21). The sheets have been produced with the use of fetal calf serum (FCS) and a feeder layer (5, 16) and, not frequently, without a feeder layer (2, 3); in either case, FCS was used at some stage of production. To minimise the likelihood of infection from contact with heterogenic proteins (18), we use neither FCS nor self serum (7, 8), and have succeeded in producing a greater number of cells than with the use of FCS (19). Moreover, with acellular allogenic dermal matrix, AlloDerm® (LifeCell, Branchburg, NJ) as a scaffold, the world-first artificial oral mucosa composed of a complex of keratinocytes and dermis for clinical application has been produced and termed ex vivo produced oral mucosa equivalent (EVPOME) (7, 6, 8, 9, 19).

Comparative clinical studies on wound healing after the transplantation of EVPOME and of other artificial dermis without keratinocytes have confirmed the efficacy of EVPOME attributed to: 1) early epithelialisation, 2) a short period of time until complete healing, and 3) negligible scar contraction. Subsequent immunohistochemical, biochemical, and electron microscopic studies (6) have shown that fibroblasts are not capable of infiltrating EVPOME from surrounding areas soon after its transplantation because of the dense structure of AlloDerm® and that the keratinocyte-derived cytokine system plays a primary role in the wound healing mechanism. Unlike fibroblasts, vascular endothelial cells are capable of infiltrating AlloDerm® and promoting favourable vascularisation. In the next step of the wound healing process, keratinocytes on AlloDerm® were detached, but those in EVPOME acted on normal keratinocytes in the surrounding mucosa, promoting keratinocyte migration, which activated the wound healing neuromucosal system. Despite the degradation of AlloDerm® with time, the basic structure of the basement membrane that retains keratinocytes remained intact. These data indicate that the functions of both keratinocytes and AlloDerm® contribute to the clinical efficacy of EVPOME. The former is the keratinocyte-derived cytokine system of keratinocytes on AlloDerm® and those that have migrated from the surrounding area; the latter is the basement membrane structure of AlloDerm® that with its dense structure retains keratinocytes and physically prevents fibroblast invasion.

In the glucose metabolism, the primary role of glucose in cell energy metabolism is its incorporation into the cytoplasm by glucose transporters as membrane proteins; first produced in the pentose phosphate cycle is palmitic acid (16:0), followed by the production of a number of other fatty acids. Evaluation of the composition of palmitic acid in the cell membrane allows measurement of the capacity of energy metabolism, i.e., the glucose metabolising capacity of the cell, which is used as a parameter of proliferation (11, 12, 20). On the other hand, essential fatty acids are seen as being indispensable for keratinocyte differentiation and transformation such as apoptosis and immunocompetence (13). The evaluation of the essential fatty acid composition of the cell membrane of in vivo transplanted cells allows the determination of the degree of in vivo adaptation or differentiation of the cells, i.e., their assimilation with normal tissue. Here, we clarify the degree of keratinocyte maturity in the healing process after EVPOME transplantation, in terms of fatty acids constituting the cell membrane.

MATERIALS AND METHODS

Fabrication and transplantation of EVPOME to human oral mucosal defects

The concept and methods of culturing oral keratinocytes and fabrication of EVPOME were as described (6, 7, 8, 9, 19, 20). In short, the basic concept of our method of culturing oral keratinocytes is similar to that of Boyce and Ham expect that the 3T3 mouse feeder layer, regarded as a heterograft was excluded. In their method, although the medium is serum-free, FCS is necessary at certain steps of the procedure; we, on the other hand, use Epilife medium® (M-EPI-500-CA, Cascade Biologics Inc., Portland OR) that is composed of plant proteins and contains no animal proteins such as bovine pituitary extract. Informed consent was obtained from all patients on the form (Kobe Univ, No. 131) issued by the Ethical Committee of Kobe University. Tissue (about 2.5 mm2) was excised from normal buccal mucosa on the contralateral side of the lesion and reacted with enzymes; epithelial cells were then isolated, collected and cultured in serum-free medium (Epilife®) containing 0.06mM of Ca in a CO_2 incubator (Fig.1 (A)). An appropriate number of cultured cells was seeded onto AlloDerm® and after cell behaviour changed from proliferation to differentiation by increasing the Ca concentration to 1.2 mM, stratification was induced by the air-liquid interface method. A mean of 6.6 cm2 of EVPOME (cell count, 8.7×105) was obtained (Fig.1 (B)) after an average of 28.7 days. Since AlloDerm® was used as an artificial dermis, EVPOME could be manipulated with surgical instruments, in the same way as when conventional skin grafts are transplanted onto oral defects, allowing tie-over fixation (Fig.1(C)), a major advantage in clinical surgery.
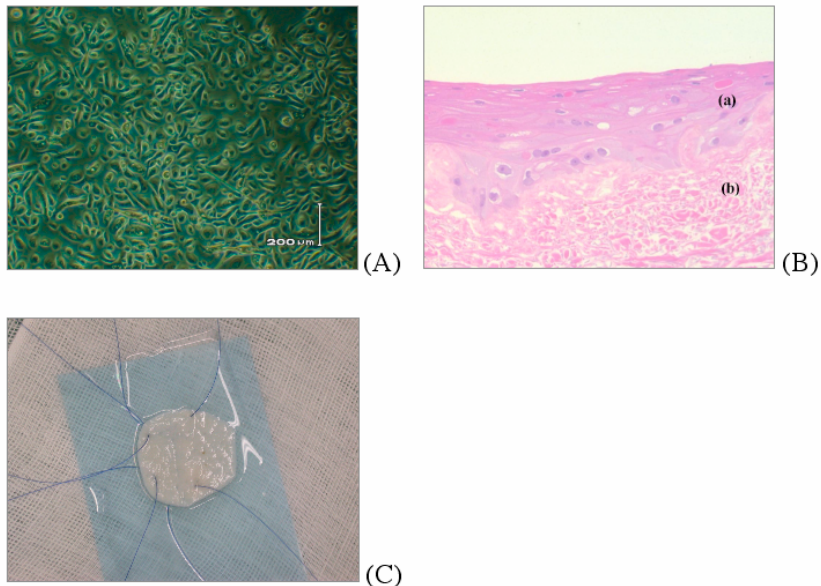

(A)

(B)

(C)

Figure 1. Fabrication of ex vivo produced oral mucosal equivalent (EVPOME)
    (A)Keratinocyte in vitro: After culturing for 14 days (confluent)
    (B)HE staining of EVPOME after culturing for 11 days ($\times 100$): Stratification of the cultured epithelium is observed, cells in the superficial layer are eosinophilic and show enhanced keratinisation [(a): cultured epithelium, (b): AlloDerm®]
    (C)Complete EVPOME (immediately before transplantation): Stratification of cultured keratinocyte on the acellular dermal matrix (AlloDerm®).

Experimental design

EVPOME was transplanted onto oral mucosal defects in 15 patients, and informed consent was obtained from 10 of the 15. Samples were then collected 2, 3, 4 and 9 weeks after transplantation from 2, 4, 2 and 2 patients, respectively. Details of the patients are shown in Table I.

Table I. *Patients*

Case No	Age	Sex	Diagnosis	Treatment	Grafting (cm2)	Biopsy period after transplantation (week)
1	74	M	Alveolar ridge hypoplasia	Alveoplasty	5.7	2
2	51	M	Leukoplakia (tongue)	Laser resection	7.6	2
3	53	F	Alveolar ridge hypoplasia	Alveoplasty	7.6	3
4	64	M	Leukoplakia (tongue)	Laser resection	7.6	3
5	66	M	Leukoplakia (tongue, buccal mucosa)	Laser resection	7.6	3 (buccal mucosa)
6	61	M	Leukoplakia (maxillary gingival)	Laser resection	5.7	3
7	65	M	Leukoplakia (maxillary gingival)	Laser resection	7.6	4
8	71	F	Leukoplakia (multiple oral mucosa)	Laser resection	9.5	4 (buccal mucosa)
9	64	F	Leukoplakia (tongue)	Laser resection	7.6	9
10	74	F	Alveolar ridge hypoplasia	Alveoplasty	3.8	9

Lipid analysis

Biopsy samples were rinsed twice with calcium-free phosphate buffered saline (PBS), scraped into methanol and extracted at a ratio of methanol: chloroform: 0.1 M of KCl of 1:2:1.5 in 50% methanol; the organic phase was re-extracted with 2.5 times the volume of 0.1 M of KCl in 50% methanol (11). Each of the protein precipitates was measured with the modified Lowry protein assay (1). The extracted fraction was then suspended in 75 μl chloroform: methanol (1:1) after evaporation under a nitrogen stream, applied to a thin-layer chromatography (TLC) plate, and chromatographed in one direction using a mixture of chloroform, methanol and glacial acetic acid (90:8:1). After TLC of the cell lipids, the area of the plate containing the phospholipids was scraped and the material thus obtained was eluted from the silica during transmethylation with 6% methanolic-HCl. A total of 50 μg 17:0 was then added (internal standard) and the sample was heated for 3.5 h at 80°C to form fatty acid methyl esters (FAMEs). The FAMEs were resuspended in 200 μl benzene, filtered through a 0.45 μm filter, evaporated, and resuspended in 50-150 μl (as determined by the quantity of protein) filtered chloroform for analysis. A total of 0.5 μl chloroform was injected for analysis.

Analysis of fatty acid methyl esters (FAMEs)

The FAMEs were analysed with the use of a Shimazu gas chromatograph (GC) model GC-14A (Shimazu, Kyoto, Japan) equipped with a J and W Scientific (Folsom, CA) fused silica Megabore DB225 and a 0.53 μm diameter column, and eluted with scrubbed helium at a flow rate of 2.79 ml/min at 210°C for 16 min, heated at a gradient of 4°C/min until 220°C, and kept isothermic for 18.5 min. The flame ionization detector output of the gas chromatograph was digitised and evaluated with a C-R8A Shimadzu Chromatopac (Shimazu, Kyoto, Japan).

Immunohistological studies

Before transplantation, EVPOME was fixed with 10% formalin and embedded in paraffin blocks. Immunohistochemical staining of the section for GLUT-1 was carried out by

standard procedures. Sections (5 μ m thick) were incubated at 37°C overnight, deparafinised with xylene and rehydrated, endogeneous peroxydase activity was blocked for 15 min in 80% methanol and 0.6% hydrogen peroxide. Antigen was retrieved from all sections before immunostaining with 0.01 μ mol/L citrate buffer (pH 6.0) in a microwave oven at 98°C for 15 min. After cooling, the sections were washed with TBST, incubated with the primary polyclonal antibody at a dilution of 1:1000 for GLUT-1 (AB1340, Chemicon International Inc., Temecula, CA), and washed with TBST; signals were then detected with DAB. Finally, the specimens were counterstained with haematoxylin-eosine (HE), dehydrated, and mounted.

RESULTS

Percent composition of fatty acid in keratinocyte cell membrane of EVPOME
Palmitic acid (16:0)

As a parameter of cell proliferation and of the metabolism of the overlying keratinocytes in EVPOME, the percent composition of palmitic acid (16:0) in cell membranes of normal oral mucosal, cultured oral and post-transplanted keratinocytes was analysed. The percent composition in the cultured oral keratinocytes (23.10±0.30%) was already similar to that in normal oral mucosal keratinocytes (buccal mucosa: 27.18±3.74%, gingival: 23.00±1.40%) and did not change even after transplantation (Fig.2).

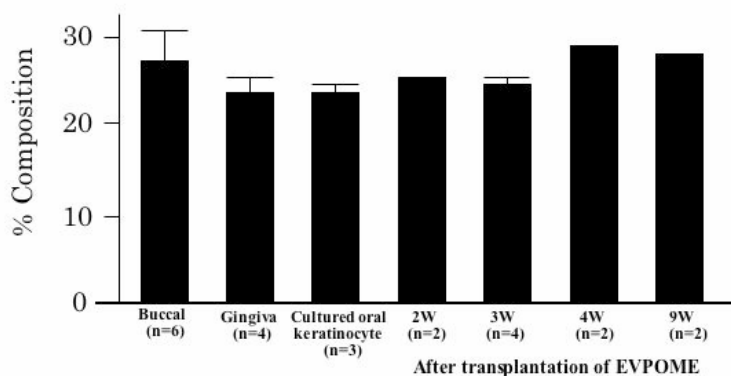**Figure 2.** Percent composition of palmitic acid (16:0) in keratinocyte cell membrane of EVPOME

Linoleic acid (18:2) and arachidonic acid (20:4)

As a parameter of cell differentiation and of the metabolism of the overlying keratinocytes in EVPOME, the percent composition of total linoleic acid (18:2) and arachidonic acid (20:4), essential fatty acids in cell membranes of the cultured oral keratinocytes, was only 5.3 ± 0.10 %; however, it changed to 27.6 % (n=2, average) after a little over 2 weeks, to 24.8 ± 1.21 % (n=4) after 3 weeks, to 21.9% (n=2, average) after 4 weeks and eventually reached 21.7% (n=2, average), which was similar to the percent composition in normal gingival keratinocytes (23.8±4.0%) and in normal buccal mucosal keratinocytes (23.12±3.47%) (Fig.3).
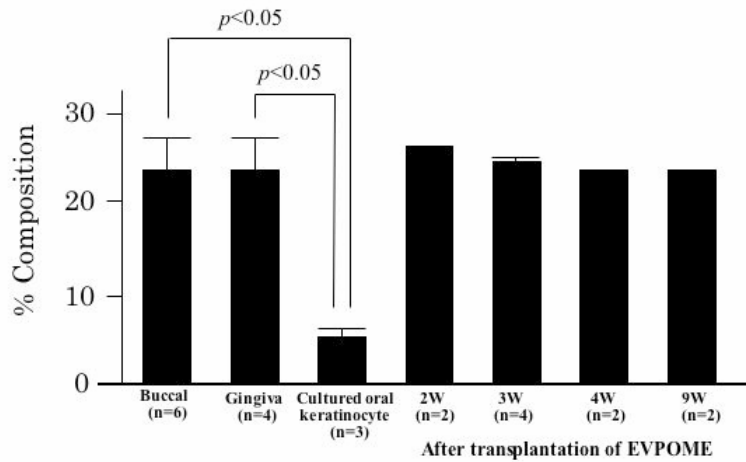
Figure 3. Percent composition of linoleic acid (18:2) and arachidonic acid (20:4) in keratinocyte cell membrane of EVPOME

Palmitoleic acid (16:1)

Among components other than palmitic acid (16:0) and essential fatty acids, palmitoleic acid (16:1) also showed changes. In cultured keratinocytes it was $9.20 \pm 0.20\%$, which was significantly higher than in normal oral keratinocytes (buccal: $3.42 \pm 1.15\%$, gingival: $4.30 \pm 0.85\%$), decreased markedly to 1.34% (n=2, average) 2 weeks after the intraoral transplantation of oral keratinocytes in the form of EVPOME, but turned similar to that in normal oral keratinocytes 3 weeks after transplantation (Fig.4).
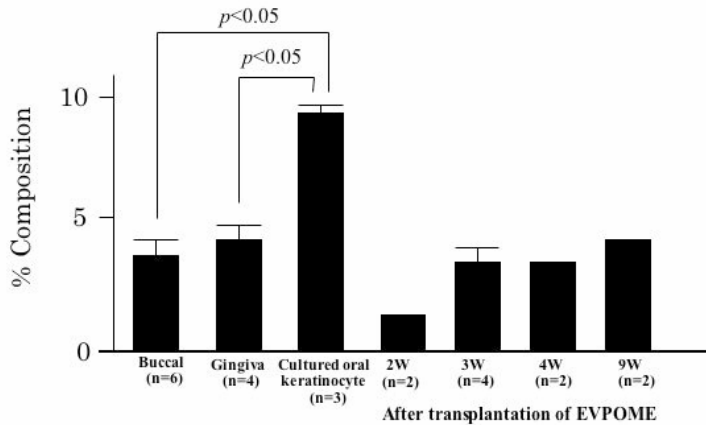

Figure 4. Percent composition of palmitoleic acid (16:1) in keratinocyte cell membrane of EVPOME

Immunohistological glucose transporter 1 (GLUT-1) staining of EVPOME

The entire population of stratified keratinocytes in EVPOME before transplantation showed marked expression of GLUT-1 protein in spite of an environment that tended to induce a shift from proliferation to differentiation (stratification) with high calcium concentration (1.2mM). The stratification of keratinocytes in EVPOME differed from that of the differentiation gradient and from that of the cell metabolism gradient in the normal oral mucosal epithelium (Fig.5).
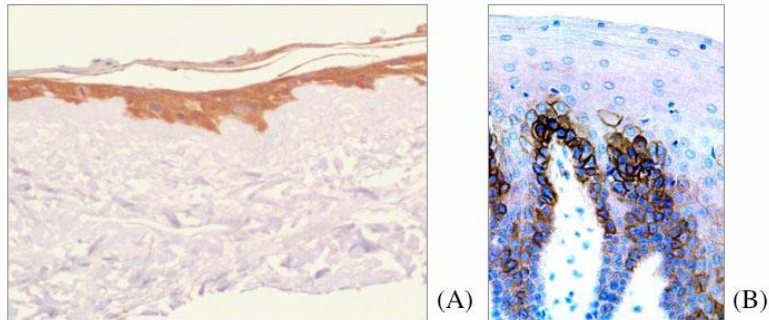

Figure 5. Immunohistological glucose transporter 1 (GLUT-1) staining of EVPOME

The entire population of stratified keratinocytes in EVPOME before transplantation showed marked expression of GLUT-1 protein in spite of an environment that tended to induce a shift from proliferation to differentiation (stratification) with high calcium concentration (A). The stratification of keratinocytes in the EVPOME differed from that the of the differentiation gradient and from that of the cell metabolism gradient in the normal oral mucosal epithelium (B).
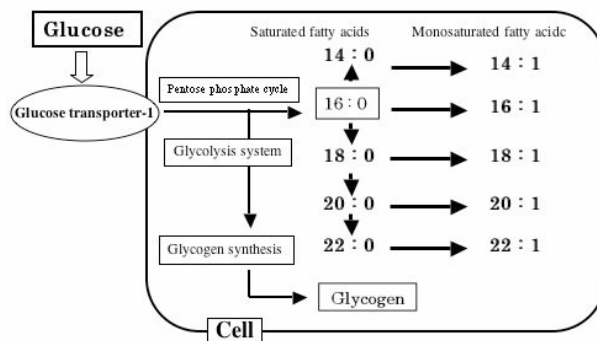

Figure 6. Relationship between GLUT-1 and palmitic acid (16:0)

During glucose metabolism, the core stage of epithelial cell energy metabolism, glucose is, by glucose transporter 1 (GLUT-1), incorporated into the cytoplasm as a membrane protein, while palmitic acid (16:0) is first during the pentose phosphate cycle, then by the production of many fatty acids.

14:0, myristic acid; 14:1, myristoleic acid; 16:0, palmitic acid; 16:1, palmotoleic acid; 18:0, stearic acid; 18:1, oleic acid; 20:0, alachidic acid; 20:1, gadoleic acid, 22:0, behenic acid; 22:1, ericic acid.

DISCUSSION

Phospholipids of the cellular membrane are essential for cellular function and morphological maintenance, while fatty acids (a major component of these phospholipids) are key factors in the regulation of cell proliferation and differentiation (11, 12). Among the fatty acids that make up the cell membrane, palmitic acid (16:0) is a basic unit present in the membranes of all human cells. During glucose metabolism, the core stage of epithelial cell energy metabolism, glucose is, by glucose transporter 1 (GLUT-1), incorporated into the cytoplasm as a membrane protein, while palmitic acid (16:0) is first during the pentose phosphate cycle, then by the production of many fatty acids (Fig.6). For the maintenance and proliferation of human cells, the storage of palmitic acid (16:0) as an energy source is therefore indispensable. Determination of the percent composition of palmitic acid (16:0) in the membranes of keratinocytes allows the measurement of energy metabolism capacity, i.e., the glucose metabolism capability of cells, which is a parameter of proliferative activity. The percent composition of palmitic acid (16:0) in cultured oral keratinocytes was similar to that in vivo, and did not change after intraoral transplantation, implying that the glucose metabolism capability, as the most predominant metabolic capability of keratinocytes, had already been acquired in vitro. This inference was supported by the marked expression of GLUT-1, which is a membrane protein used as a parameter of glucose metabolism (10, 17, 22), in stratified keratinocytes observed by immunohistologic analysis. In the fabrication of EVPOME, keratinocyte proliferation was transformed to differentiation by increasing the calcium concentration from 0.06 to 1.2 mM. Since GLUT-1 was strongly expressed in the keratinocytes in the high-concentration calcium culture, EVPOME was the special stratified epithelium containing keratinocytes (with their character transformed to differentiation by a change in the calcium concentration) that maintained their proliferation potential. Izumi et al. (8) have confirmed these characteristics by immunostaining with PCNA and Ki-67 antibodies. Our study confirmed and supported these characteristics of palmitic acid(16:0) in the keratinocyte cell membrane and glucose metabolism capacity evaluated with the GLUT-1 antibody. The stratification of keratinocytes in EVPOME differed from that of the differentiation gradient and from that of the cell metabolism gradient in the normal oral mucosal epithelium.

Essential fatty acids cannot be produced by cells themselves, but need to be obtained from external dietary nutrition. To induce cell proliferative activity, cells are cultured with a decrease in essential fatty acids (13). The percent fatty acid composition is very low (18:2+20:4=5.5%; normal gingiva: 24%, normal buccal mucosa: 23%). Moreover, essential fatty acids are indispensable for keratinocyte differentiation; also, the glucose metabolism shifts to a fatty acid metabolism with the progression of differentiation. Therefore, the extent of cell adaptation or differentiation, i.e., assimilation with normal tissue, can be determined by evaluating the percent composition of essential fatty acids transplanted into the body. The percent composition of total linoleic acid (18:2) and arachidonic acid (20:4) 2 weeks after EVPOME transplantation was already comparable to that of normal oral mucosa, suggesting that keratinocytes on EVPOME had acquired a similar cell metabolic capability.

These findings suggest that epithelialisation, i.e., mucosal wound healing, after EVPOME transplantation is a phenomenon whereby keratinocytes proliferate while consuming palmitic acid (16:0) and subsequently differentiate after being provided with essential fatty acids from diet.

The percent composition of palmitoleic acid (16:1) in cultured oral keratinocytes was initially significantly higher than that in oral keratinocytes in vivo; it decreased markedly 2 weeks after the intraoral transplantation of cultured oral keratinocytes in the form of

EVPOME, but after 3 weeks turned similar to that in oral keratinocytes in vivo. The significance of palmitoleic acid (16:1) for the cell metabolism remains unclear. We speculate, however, that oral keratinocytes in culture as a specific environment express their cell metabolism in a manner that differs from that of the normal cell metabolism in vivo. Changes in the percent composition of palmitoleic acid (16:1) may thus represent the process by which keratinocytes in EVPOME change to or are replaced by keratinocytes with normal metabolic function after intraoral transplantation.

The results of our comprehensive evaluation of the percent composition of essential fatty acids (linoleic acid, 18:2; arachidonic acid, 20:4) and palmitoleic acid (16:1) in the keratinocyte membrane after intraoral transplantation suggest that in the clinical setting 4 weeks (mean, 28.7 days) are requisite for the complete epithelialisation of the wound. Nonetheless, cultured cells in EVPOME with a specific metabolic mechanism change to or are replaced by keratinocytes with a normal metabolic mechanism similar to that in the surrounding tissue within 3 weeks after transplantation.

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