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Epithelialization in Oral Mucous Wound Healing in Terms of Energy Metabolism

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Key Words: wound healing, oral mucosa, energy metabolism, GLUT-1, palmitic acid, essential fatty acids.

Purpose: The purpose of this study was to clarify the "healing capacity" of wounds of the oral mucosa in comparison to those of the skin, and to evaluate the wound healing mechanism of oral mucosa using a cytobiological approach from the aspect of energy metabolism in oral keratinocytes.

Materials and methods: Samples of epidermal and oral keratinocytes collected at surgery and of cultured oral keratinocytes were used to analyze (1) by gas chromatography the composition of fatty acids (16:0, 18:2, 20:4) in the cell membranes of keratinocytes, (2) by immunohistochemical staining of GLUT-1 antibody and specific PAS staining the localization of glucose metabolism, and (3) by RT-PCR and Western blotting the expression of GLUT-1 mRNA and of protein in the keratinocytes of the basal and parabasal layers of each epithelial tissue.

Results: 1. The % composition of palmitic acid (16:0) was significantly higher in buccal mucosal keratinocytes (27.18±3.74%) and in the gingiva (23.00±1.40%) than in the epidermis (17.54±0.37%). 2. Immunohistochemical staining showed GLUT-1 protein in the skin to be expressed only in the bulge region of hair follicles and in the epidermal basal layer, and observed nearly throughout all epithelial cell layers in the oral mucosa. 3. PAS-positive cells were observed among differentiation-enhanced cells in the upper prickle layer in the oral mucosa. 4. The same results were obtained from RT-PCR and a Western blotting analysis.

Conclusion: The present study demonstrated definite cytobiological evidence that the oral mucosa surpasses the skin in regard to its wound healing capacity.

INTRODUCTION

The damaged epidermis is regenerated by two mechanisms: one involves the activation of epidermal keratinocytes in the wound margin, and the other involves the proliferation, simultaneously with this keratinocyte activation, of hair follicle cells, their migration into the skin, and their transformation to epidermal keratinocytes.

Phospholipids of the cellular membrane are essential for both the cellular function and morphological maintenance. Fatty acids, the major component of these phospholipids, are key factors in the regulation of cell proliferation and differentiation. Among the fatty acids known to constitute the cell membrane, palmitic acid (16:0) is a basic unit in the membranes of all human cells. During glucose metabolism, the core stage of epithelial cell energy metabolism, glucose is, by transporters, incorporated into the cytoplasm as a membrane protein, while palmitic acid (16:0) is first produced during the pentose phosphate cycle, then

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by the production of many fatty acids. For the maintenance and proliferation of human cells, the storage of palmitic acid (16:0) as an energy source is therefore indispensable.

Hair follicle cells store a very large amount of palmitic acid (16:0) that, in a static environment, is used for hair formation. Once the epidermis is damaged, then rapid cell proliferation becomes necessary, and the abundant palmitic acid (16:0) stored in hair follicle cells is used for wound epithelialization, i.e., wound healing in a dynamic environment (5, 11, 12, 14).

The oral mucosa shows earlier wound healing than does the skin, because "early wound healing" of the oral mucosa has been studied in terms of the "moisture environment in the oral cavity" but not from other aspects. The energy metabolism, mainly that of palmitic acid (16:0), in the transformation of hair follicle cells into epidermal keratinocytes is a mechanism of epithelialization — one profile of the wound healing phenomena. In this study, to clarify the "healing capacity" of wounds of the oral mucosa in comparison to those of the skin, the wound healing mechanism of the former was evaluated by a cytobiological approach from the aspect of energy metabolism in oral keratinocytes.

MATERIALS AND METHODS

Materials

Samples of the oral mucosa, the scalp, the abdomen, and the dorsum of body, obtained during oral or plastic surgery from patients consenting to participate in this study, were directly used as human stratified squamous epithelial samples (*in vivo*) or as cultured keratinocytes after primary cell culture (*in vitro*) . In addition, the data of hair follicle cells obtained from a previous study were used (4).

Separation of human stratified squamous epithelium and primary cell culture

To obtain keratinocytes, samples were permeated and digested with 0.05% trypsin plus 0.02% EDTA solution at room temperature for 7 hr, and then mixed with the same volume of 0.05% trypsin inhibitor plus 0.02% EDTA solution for trypsin inactivation. Only keratinocytes were mechanically detached from the tissue by abrasion, while the tissue was separated at the epidermal-dermal junction, so that epithelial basal-layer cells remained on the lamina propria side. The components of the suprabasal cell thus separated were frozen for lipid analysis. The basal-layer cells were gently separated from subepithelial tissue in Epilife® medium (M-EPI-500-CA; Cascade Biologics Inc., Portland OR), and the cell suspension was centrifuged at 1500 rpm for 5 min. Cell pellets were either used for a primary cell culture or they were frozen at -80^{o}C until they underwent a lipid analysis. For the primary cell culture, the cells were cultured in Epilife® medium supplemented with Human Keratinocyte Growth Supplement-V2 (HKG-2; Cascade Biologics Inc., Portland OR) at 37^{o}C in the presence of 5% CO_2 at a low calcium concentration (0.06 mM). The medium was replaced at 3-day intervals.

3. Lipid analysis

Biopsy samples were rinsed twice with calcium-free phosphate buffered saline (PBS), scraped into methanol and then were extracted at a ratio of methanol: chloroform: 0.1 M KCl of 1:2:1.5 in 50% methanol; the organic phase was re-extracted with 2.5 μ volume of 0.1 M KCl in 50% methanol. Each of the protein precipitates was measured by the modified Lowry protein assay. The extracted fraction was then suspended in 75 μ l of chloroform: methanol (1:1) after evaporation under a nitrogen stream, applied to a thin-layer chromatography (TLC) plate, and separated in one direction using a mixture of chloroform, methanol and

glacial acetic acid (90:8:1). After TLC chromatography of the cell lipids, the area of the plate containing the phospholipids was scraped off 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 hr at 80°C to form fatty acid methyl esters (FAMEs). The FAMEs were resuspended in 100 μl chloroform, 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 0.1 μl chloroform was then injected for analysis.

4. Analysis of fatty acid methyl esters (FAMEs)

FAMEs were analyzed using a Shimazu gas chromatograph (GC) model GC-14B (Shimazu, Kyoto, Japan) equipped with a J and W Scientific (Folsom, CA) fused silica Megabore DB225 and a 0.53- μm diameter column. The FAMEs were 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 then were kept isothermic for 18.5 min. The flame ionization detector output of the gas chromatograph was digitized and evaluated using a C-R8Ashimadzu Chromatopac. (Shimazu, Kyoto, Japan).

5. Histological and immunohistological studies

All specimens were fixed with 10% formalin and embedded in a paraffin block. Immunohistochemical staining of the section for Glut-1, PAS and PAS digested with diastase were carried out according to the standard procedure. Sections 5-µm thick were incubated in 37°C for overnight and then deparaffinized with xylene and rehydrated, and endogenous peroxidase activity was blocked for 15 min in 80% methanol + 0.6% hydrogen peroxide. Antigen retrieval methods were carried out for all sections before immunostaining in 0.01-mol/L citrate buffer (pH 6.0) using a microwave oven at 98°C for 15 min. After cooling the sections were then washed with TBST. The sections were then incubated with the primary polyclonal antibody at a dilution of 1:1000 for Glut-1 (RB-9052-R7, Thermo Fisher Scientific Inc.,). After washing with TBST, signals were detected with DAB. Finally, the specimens were counterstained with hematoxylin–eosin (HE), dehydrated, and mounted.

6. Protein extraction and Western blotting

Total protein was extracted from the epidermis and the buccal mucosal epithelium using PRO-PREP (iNtRON BIOTECHNOLOGY, Seoul, Korea), according to the manufacturer's protocol. The samples were separated by SDS-PAGE on 12% minigels under reducing conditions. Separate proteins were transferred onto PVDF membrane (Amersham, Buckinghamshire, UK) and incubated in blocking buffer for 1 hr at room temperature. The membranes were incubated with polyclonal antibody to GLUT-1 (Affinity BioRegends, Co, USA) in blocking buffer for 1 hr at room temperature, and incubated with secondary ECL^{TM} anti-mouse IgG, Horseradish Peroxidase linked whole antibody for 1 hr at room temperature. The Specific antigen-antibody complex was visualized with an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK) to X-ray film.

7. RNA extraction and RT-PCR analysis

Total RNA was extracted from the epidermis and the buccal mucosal epithelium using ISOGEN (Nippon Gene, Tokyo), according to the manufacturer's protocol. The harvested RNA was then reverse-transcribed and amplified using the TaKaRa RNA PCR kit (Takara Shuzo, Otsu, Japan). The cDNA was amplified using the following specific primers:

5'-CCCGCTTCCTGCTCATCAA-3' (sense) and 5'-GACCTTCTTCTCCCGCATCATC-3' (antisense) for GLUT-1, in a mixture containing PCR Buffer and TaKaRa Ex Taq HS (Takara Shuzo, Otsu, Japan). PCR was carried out in a ZYMOREACTOR II AB-1820 (ATTO, Tokyo, Japan) for 50 cycles of 1 min at 95^{O}C, 1 min at 50 ^{O}C, and 1 min at 72 ^{O}C. The amplified samples were visualized on 1% agarose gel stained with ethidium bromide and photographed under UV light.

RESULTS

The percent composition of major fatty acids in cellular phospholipids of gingival, buccal mucosal and epidermal keratinocytes

Table I shows the % composition of the fatty acids constituting the cell membrane of epidermal, oral mucosal and cultured keratinocytes in the entire epithelial layer. The % composition of palmitic acid (16:0) was significantly higher in buccal mucosal keratinocytes (non-keratinization, 27.18±3.74%) and in the gingiva (parakeratinization, 23.00±1.40%) than in the epidermis (orthokeratinization, 17.54±0.37%). Similarly, it was significantly higher in cultured gingival keratinocytes (23.10±0.3%) than in cultured epidermal keratinocytes (18.60±0.2%; Fig.1). The % composition of essential fatty acids (linoleic acid, 18:2 + arachidonic acid 20:4) was significantly higher in epidermal keratinocytes (33.67±1.67%) than in the oral mucosal keratinocytes (buccal mucosal, 23.12±2.6%; gingival, 23.80±2.66%; Table I; Fig. 2).

Table I. The percent composition of major fatty acids in cellular phospholipids of gingival,buccal mucosal and epidermal keratinocytes(percent total lipids)

Fatty acids	buccal	gingiva	epidermis	cultured gingiva	cultured epidermis
14 : 0	0.90±0.51	1.20±0.16	0.94±0.37	3.40±0.10	3.00±0.11
16 : 0	27.18±3.74	23.00±1.40	17.54±0.37	23.10±0.30	18.60±0.20
18 : 0	17.08±3.16	16.90±2.00	15.96±0.75	14.50±0.20	14.80±0.40
16 : 1	3.42±1.15	4.30±0.85	0.95±0.56	9.20±0.20	9.00±0.20
18 : 1	28.25±5.13	20.30±1.80	15.99±2.76	34.10±0.40	39.90±0.70
18 : 2	16.07±2.29	14.50±2.57	27.42±1.74	2.20±0.10	2.00±0.10
20 : 4	7.05±1.18	9.30±1.43	6.25±1.02	3.10±0.10	2.90±0.00
n	6	4	4	3	3

14:0	myristic acid	16:1	palmitoleic acid	18:2	linoleic acid
16:0	palmitic acid	18:1	oleic acid	20:4	arachidonic acid
18:0	stearic acid				

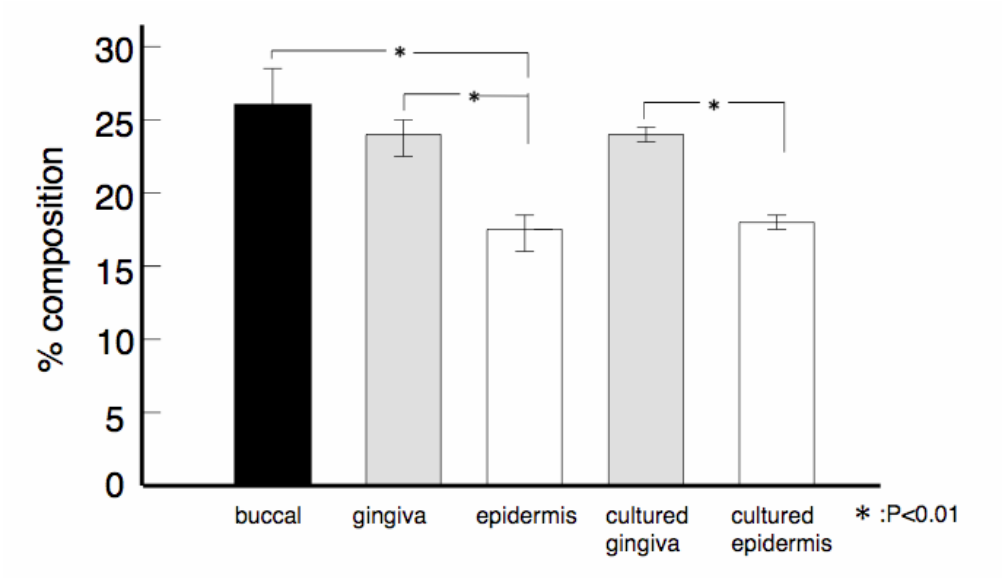

Figure 1 Comparison of palmitic acid (16:0) between keratinocytes. The percent composition of palmitic acid (16:0) was significantly higher in buccal mucosal keratinocytes (non-keratinization; 28.58 ± 5.25) and the gingiva (parakeratinization; 23.00 ± 1.40) than in the epidermis (orthokeratinization; 17.54 ± 0.37). Similar results were obtained with cultured keratinocytes (cultured gingival keratinocytes: 23.10 ± 0.03; cultured epidermal keratinocytes: 18.60 ± 0.20)

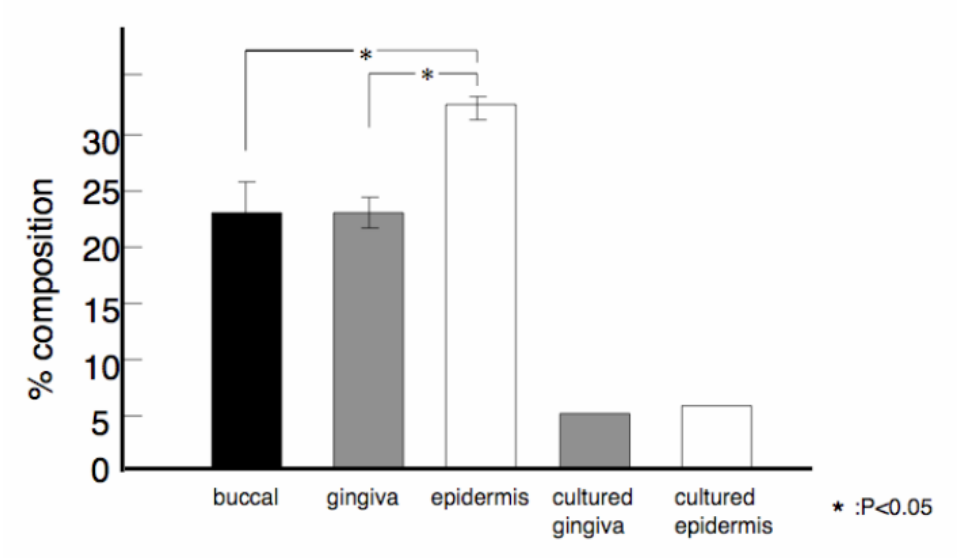

Figure 2 Comparison of essential fatty acids (linoleic acid (18 : 2) ＋Arachidonic acid (20 : 4)) between keratinocytes. The percent composition of essential fatty acids was significantly higher in epidermal keratinocytes (33.67 ± 1.4) than in oral mucosal keratinocytes (buccal mucosa, 22.67 ± 2.4; gingival, 23.80 ± 2.0)

Epithelial cells were classified into basal layer and suprabasal layer keratinocytes (Table II), and the percent composition of palmitic acid (16:0) in basal layer keratinocytes (gingival, 29.7±1.90%; epidermal, 19.10±0.30%) was slightly higher than in suprabasal keratinocytes (gingival, 23.80±0.30%; epidermal, 17.50±0.30%) but the difference was not significant (Fig. 3).

Table II. The percent composition of fatty acids in cellular phospholipids of oral mucosal keratinocytes (gingiva) and epidermal keratinocytes (percent total lipids)

	epidermis		gingiva	
Fatty acids	suprabasal	basal	suprabasal	basal
14 : 0	0.90±0.10	1.10±0.10	1.40±0.20	1.00±0.30
16 : 0	17.50±0.30	19.10±0.30	23.80±0.30	29.70±1.90
18 : 0	16.00±0.20	18.10±0.40	15.90±0.40	19.40±1.10
18 : 1	16.00±0.80	15.70±1.00	17.50±0.70	16.00±0.60
16 : 1	0.90±0.20	0.90±0.00	3.60±0.50	2.20±0.40
18 : 2	27.40±0.50	20.70±0.90	18.6±0.60	15.10±0.90
20 : 4	6.30±0.30	8.40±0.50	6.50±0.20	9.20±1.10
n	4	4	5	5

14:0	myristic acid	16:1	palmitoleic acid	18:2	linoleic acid
16:0	palmitic acid	18:1	oleic acid	20:4	arachidonic acid
18:0	stearic acid				

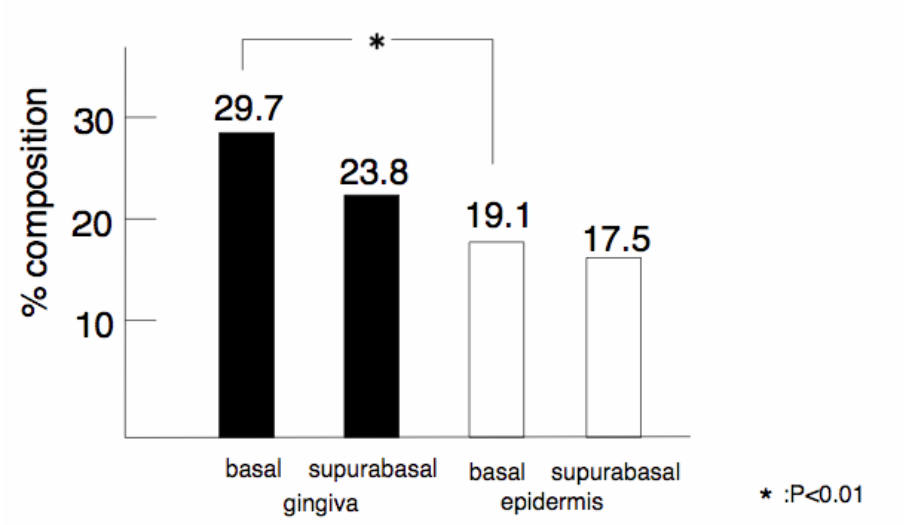

Figure 3 The percent composition of palmitic acid (16:0) of epidermis and gingiva in basal and suprabasal layer cell. The percent composition of palmitic acid (16:0) was slightly higher in basal layer keratinocytes (gingival, 29.70 ± 1.90; epidermis, 19.10 ± 0.30) than in suprabasal keratinocytes (gingival, 23.80 ± 0.30; epidermis, 17.50 ± 0.30) but the difference was not significant.

Expression of GLUT-1 protein in gingival, buccal mucosal epithelium and epidermis

GLUT-1 protein in the epidermis was expressed only in the bulge region of hair follicles and in the epidermal basal layer. In the gingival, its expression was observed almost throughout the entire prickle cell layer, and the stainability decreased with the differentiation gradient. The basal layer lying over the tips of the connective tissue papillae showed the most marked expression, but the tip of the rete-ridge lacked stainability. In the buccal mucosa, unlike in the gingival, particularly marked expression was observed in the prickle cell layer immediately above the basal layer, and the stainability decreased with the differentiation gradient (Fig.4).

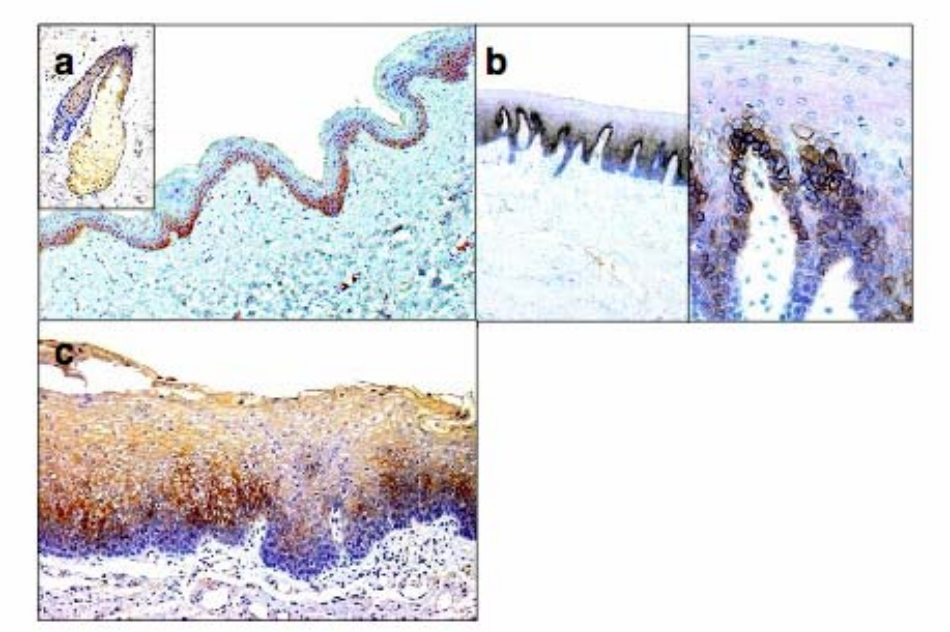

Figure 4 Expression of GLUT-1 protein in gingival, buccal mucosal epithelium and epidermis.
(a) GLUT-1 protein in the skin was expressed only in the bulge region of hair follicles and the epidermal basal layer. (b) In the gingival epithelium, its expression was observed nearly throughout the entire prickle cell layer, and stainability decreased along with the differentiation gradient. The basal cell layer lying over the tips of the connective tissue papillae showed the most marked expression, but the tips of the rete-ridge showed no staining. (c) In the buccal mucosal epithelium, unlike its gingival counterpart, especially marked expression was observed in the prickle cell layer immediately above the basal layer, and stainability decrease along with the differentiation gradient.

PAS and PAS digested with diastase in gingival, buccal mucosal epithelium and epidermis

In the buccal mucosa, PAS-positive cells, present among differentiation-enhanced cells in the upper prickle layer, were digested by diastase, thus suggesting the presence of glycogen in these cells. In the gingival, glycogen was also confirmed in differentiation-enhanced cells in the upper prickle cell layer. PAS-positive cells were not observed in the epidermis (Fig.5).

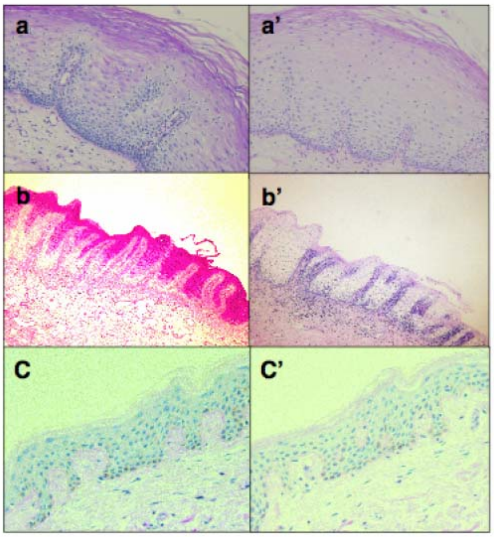

Figure 5 PAS and PAS digested with diastase in gingival and buccal mucosal epithelium. PAS-positive cells were observed among the differentiation-enhanced cells in the upper prickle layer and (a: buccal, b: gingiva) were digested with diastase (a': buccal, b': gingiva). In the epidermis, PAS-positive cells were not observed (c and c').

Western blot analysis of GLUT-1

A prominent GLUT-1 protein band was detected in the buccal suprabasal layer. In the buccal basal layer, on the other hand, an inconspicuous GLUT-1 protein band was detected. No GLUT-1 protein band was detected in the epidermal basal or the suprabasal layer (Fig. 6).

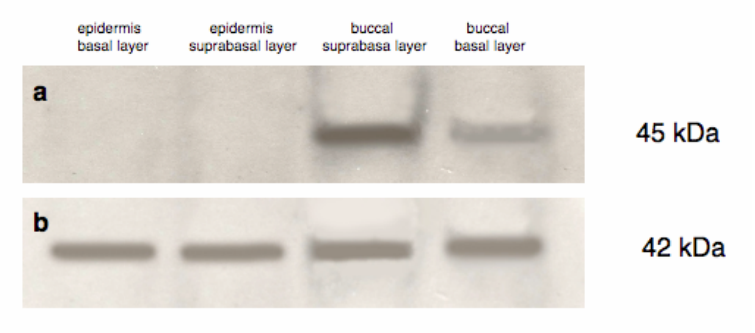

Figure 6 A Western blot analysis of GLUT-1. (a) GLUT-1. (b) β -actin.
A prominent GLUT-1 protein band detected in buccal suprabasal layer. In buccal basal layer, on the other hand, no GLUT-1 protein bands detected. No GLUT-1 protein bands were detected in the epidermal basal and suprabasal layer.

RT-PCR analysis of GLUT-1

A high level GLUT-1 expression was detected in the epidermal basal layer and the buccal suprabasal layer. In the epidermal suprabasal layer and the buccal basal layer, on the other hand, a low level GLUT-1 expression was detected (Fig. 7-a). The House Keeping Gene (GAPDH) was detected at the same level in all samples (Fig. 7-b).

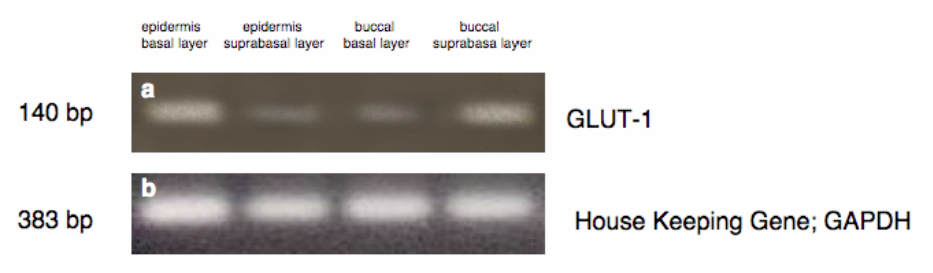

Figure 7 RT-PCR analysis of GLUT-1. (a) GLUT-1. (b) GAPDH.
A high level of GLUT-1 expression was detected in the epidermal basal layer and buccal suprabasal layer. A low level of GLUT-1 expression was detected in the epidermal suprabasal layer and buccal basal layer.

DISCUSSION

Phospholipids of the cellular membrane are essential for cellular function and morphological maintenance. Fatty acids, the major components of these phospholipids, are key factors in the regulation of cell proliferation and differentiation (8). Fatty acids are classified as saturated, monounsaturated, and polyunsaturated. Palmitic acid (16:0) is produced through the glycolytic pathway or the pentose phosphate cycle from glucose incorporated in cells and, subsequently, other saturated or monounsaturated fatty acids are produced from the palmitic acid (16:0). Therefore, palmitic acid (16:0) is a basic unit constituting the cell membrane and an important source of energy supply for cell formation[9]. Glucose provides the energy of 2 ATP/molecules; while energy converted into palmitic acid (16:0) provides 140 ATP/molecule energy (70-fold higher). The percent composition of palmitic acid (16:0) was higher in oral than in epidermal keratinocytes, and per cell, it was markedly higher in hair follicle cells than in keratinocytes from other areas. Nonetheless, the oral mucosal epithelium, particularly the buccal mucosal epithelium, had a thicker layer (at least twice thicker) containing palmitic acid (16:0) and a higher number of cells than the epidermis. The oral mucosal epithelium (both the basal and suprabasal layers) demonstrated a significantly higher percent composition of palmitic acid (16:0) than did the epidermis, but with no difference in its distribution between the two layers (Figs. 1 and 3). These results suggested a much higher energy metabolism in the oral mucosa than in the skin.

As membrane proteins, members of the glucose transporter family act as carriers for glucose uptake into the cell. Since GLUT-1 as a transporter subtype is expressed in the S phase of the cell cycle and decreases in the G1 phase, its expression can thus be a parameter of the cell energy metabolism and proliferative activity. In an experiment on mice, keratinocytes positive for GLUT-1 have been observed in the wound margin, together with the most marked GLUT-1 expression in areas showing re-epithelialization (5). Therefore, GLUT-1 may be expressed in areas with high cell proliferative activity. In the skin, GLUT-1 positive cells were only observed in the bulge region of hair follicles and in the epidermal basal cell layer. In the oral mucosal epithelium, GLUT-1 positive cells were observed in a wide area of the epithelial layer with a staining gradient associated with differentiation. Therefore, the oral mucosa, even its upper prickle cell layer, may have cell proliferation potential. In the gingiva, GLUT-1 protein expression was the most marked in the basal layer cell lying over the tip of the connective tissue papillae, but was absent at the tip of the rete-ridge in the same basal cell layer. The buccal mucosa, which is also the oral mucosa,

showed no GLUT-1 protein expression in its basal cell layer but marked expression in the lower prickle cell layer. The same results were obtained from RT-PCR and the Western blotting analysis. This suggests that no GLUT-1 protein band was detected in epidermal basal and suprabasal layer in a Western analysis because the amount of GLUT-1 protein in epidermis is very low. These results are of interest considering the association between (1) stem cells as undifferentiated slow-cycling cells and transient amplifying cells with marked proliferative ability, and (2) stem cell localization in the epithelial tissue (3, 6, 7) and GLUT-1 expression in the cell cycle.

Glycogen is a highly branched high-molecular-weight polymer of α-glucose molecules linked by glycosidic bonds. It is produced from glucose, which is incorporated into the cell through GLUT-1, by the action of phosphoglucomutase in the glycolytic pathway and is then temporarily stored in the cell. In the normal oral mucosa, glycogen is contained in non-dividing cells but not in basal layer cells or germ layer cells (2). In this study, PAS staining and diastase-digested PAS staining showed a positive correlation between the distribution of cells containing glycogen with the differentiation gradient and the presence of cells containing glycogen in the upper 2/3 area of the prickle cell layer. In the epidermis, PAS staining was below the detection limit of the experiment and the difference between the oral mucosa and the epidermis was obvious.

A negative correlation was observed between the GLUT-1 protein expression and the glycogen content in normal oral mucosa and precancerous lesions, and a rise in GLUT-1 protein expression indicates an increase in the size of the cancer lesion (10). This suggests an increase in GLUT-1 protein expression when cells transform into proliferative cells, which is consistent with the conclusions reported in previous studies. In this study, GLUT-1 expression decreased with the differentiation gradient, and glycogen storage was observed in the upper 2/3 area of the prickle cell layer. Therefore, non-dividing cells with low proliferative activity in the upper area of the epithelial layer may store latent energy in the form of palmitic acid (16:0) and glycogen and use it as an energy source during dynamic activity at the time of injury. This signifies definite cytobiological evidence that the oral mucosa surpasses the skin in wound healing efficiency.

Tissue grafting is used for large defects in the oral cavity, such as the vascularized free tissue transfer or a pedicled flap. Due to the excellent wound healing in the oral mucosa, when wound healing failure occurs in the absence of hemodynamic problems or dead space, it may therefore be necessary to investigate the level of cell energy metabolism.

The skin was significantly more dependent on essential fatty acids than the oral mucosa (Fig.2), thus suggesting that the skin is more susceptible than the oral mucosa to wound healing failure attributed to malnutrition. Epithelialization, i.e., mucosal wound healing, can be defined as a phenomenon whereby keratinocytes proliferate while consuming palmitic acid (16:0), and this proliferation subsequently shifts to differentiation after acquiring essential fatty acids through the diet.

Severe burns are treated by transplantation of cultured epidermal keratinocytes. Earlier healing would be expected by the transplantation of oral mucosal keratinocytes, which are more conducive than epidermal keratinocytes to wound healing. The differences between oral mucosa and the skin lie in the keratinization pattern, resistance to external pressure, and the existing environment (the moist and the dry). Nonetheless, parakeratinization is observed 3 weeks after the transplantation of cultured oral mucosal sheets to the skin, and orthokeratinization after 4 weeks (4). The transplanted oral mucosa may change into a structure close to the skin. Oral mucosal keratinocytes maintain biological activity longer than epidermal keratinocytes (13). Therefore, the use of oral mucosa with its higher “wound

healing capacity", in comparison to that of skin, is therefore considered to show real promise for future clinical applications.

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