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The Cytobiological Differences Between Two Odontogenic Cyst-lining Keratinocytes

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Purpose: Odontogenic cysts are classified into a developmental group, including follicular cysts (FC) and keratocysts, and an inflammatory group including radicular cysts (RC). In clinical cases, we frequently encounter RC and FC. The purpose of this study was to investigate the cytobiological differences between two odontogenic cyst-lining keratinocytes using a cytobiological approach from the aspect of metabolic function and the degree of maturation of the epithelium.

Materials and methods: Samples of odontogenic cyst-lining keratinocytes and oral keratinocytes collected at surgery, and of cultured oral keratinocytes, were analyzed (1) by immunohistochemical staining of granulocyte macrophage colony stimulating factor (GM-CSF), human beta defensin-2 (HBD-2) and chemokine receptor 6 (CCR6) expressing cell (Langerhans cell, helper T cell and suppressor T cell) antibodies, (2) by reverse transcription-polymerase chain reaction (RT-PCR) to determine the expression of GM-CSF and HBD-2 mRNA and (3) by gas chromatography to evaluate the composition of fatty acids (16:0, 18:2, 20:4) in the cell membranes of the keratinocytes.

Results: 1. Immunohistochemical staining indicated that HBD-2 and GM-CSF expression were higher in RC than in FC. 2. The same results were obtained from the RT-PCR analysis. 3. The % composition of palmitic acid (16:0) was significantly higher in the RC-lining keratinocytes ($38.62 \pm 5.86\%$) and in the FC-lining keratinocytes ($30.37 \pm 1.38\%$) than in the normal gingiva ($23.00 \pm 1.40\%$). The % composition of essential fatty acids (18:2+20:4) was significantly higher in the FC-lining keratinocytes ($26.20 \pm 3.55\%$) than in the RC-lining keratinocytes ($20.50 \pm 8.17\%$).

Conclusion: The present study demonstrated definite cytobiological evidence of the differences between RC and FC.

INTRODUCTION

Odontogenic cysts are the most common form of cystic lesions that affect the maxillofacial region. In clinical cases, we frequently encounter radicular cysts (RC) and follicular cysts (FC). They normally arise from tooth-forming epithelial residue. The epithelium originating from odontogenic cysts and the developmental environment are classified into various types. RC may arise from the epithelium rests of Malassez because of bacterial infection (26). In contrast, FC may arise from reduced enamel epithelium without inflammation (12).

It is predicted that there are differences in the cytobiological features between the cyst-lining keratinocytes of RC and FC. Therefore, it may be possible that the phenotype in the epithelium is different between RC and FC. However, there have so far been no reports which have analyzed these differences.

The phospholipids of the cellular membrane are essential for cellular function and morphological maintenance (2, 16). Fatty acids, the major component of these phospholipids, are key factors in the regulation of cell proliferation and differentiation (21). Among the fatty acids constituting the cell membrane, palmitic acid (16:0) is a basic unit in the membranes of all human cells. Palmitic acid (16:0) is first produced during the pentose phosphate cycle, then 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. Major essential fatty acids (EFAs) such as linoleic acid (18:2) and arachidonic acid (20:4) cannot be synthesized by the human body and must be obtained from dietary sources (27). Arachidonic acid (20:4) is essential as a source of leukotrienes and prostaglandins. EFAs are therefore indispensable for keratinocyte differentiation; also the glucose metabolism shifts to 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 EFAs.

The determination of the percent composition of palmitic acid (16:0) in the membranes of keratinocytes makes it possible to measure the capacity for energy metabolism, i.e., the glucose metabolism capability of cells, which is a parameter of their proliferative activity. As a parameter of the differentiation of keratinocytes, the percent composition of total linoleic acid (18:2) and arachidonic acid (20:4), essential fatty acids in cell membranes of keratinocytes, was analyzed. The accumulation of linoleic acid (18:2) is another important component of differentiation (24).

Kuroki et al described that it was very important to analyze the composition of fatty acids in order to investigate energy metabolism in keratinocytes (8). However, there has been no report describing the membrane phospholipid composition of the epithelial cells lining odontogenic cysts.

Human beta-defensin-2 (HBD-2) is a cysteine-rich cationic antimicrobial peptide. HBD-2 is poorly expressed in normal epithelial cells and is induced when the epithelial cells are stimulated by microorganisms or cytokines (1, 3, 6, 13, 19).

Granulocyte macrophage colony stimulating factor (GM-CSF) is a glycoprotein produced by a variety of cell types, including T and B lymphocytes, macrophages, keratinocytes, eosinophils, neutrophils, and endothelial cells. In most cases, its secretion requires the stimulation of producing cells with cytokines, antigens, microbial products or inflammatory agents (4, 10, 11).

The expression of HBD-2 and GM-CSF indicate the level of activation of the defense system of the epithelium. There have been no studies which have compared between RC and FC-lining epithelium. Therefore, we hypothesized that the composition of fatty acids and the expression of HBD-2 and GM-CSF would differ between RC and FC, and we have investigated these factors in the present study.

In addition, we clarify the differences in the cytobiological features between these two odontogenic cyst-lining keratinocytes by comparing their defense mechanism and metabolic function.

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MATERIALS AND METHODS

1. Materials

The Institutional Review Board of Kobe University Hospital approved this study and informed consent was obtained from all patients involved.

Samples of odontogenic cysts (5RCs and 4FCs), obtained at oral surgery from 9 patients (age range, 9-84 years: mean, 38.6 years) consenting to 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 oral mucosa data obtained from our previous study were also used (8).

2. 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 hours, 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. The tissue was separated at the epidermal-dermal junction, so that epithelial basal-layer cells remained on the lamina propria side (5, 14). The components of the suprabasal cells thus separated were then 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 minutes. Cell pellets were either used for primary cell culture or frozen at -80°C until 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°C in the presence of 5%CO₂ at a low calcium concentration (0.06mM). 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 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 × 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 75μl of 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 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 50μg 17:0 was then added (internal standard), and the sample was heated for 3.5 hours at 80°C to form fatty acid methyl esters (FAMES). The FAMES were resuspended in 100μl chloroform, filtered through a 0.45-μm filter, evaporated, and resuspended in 50-150μl (as determined by the quantity of protein) filtered chloroform for an analysis. A total of 0.1μl chloroform was injected for the analysis.

4. Analysis of FAMES

The FAMES were analyzed using a Shimadzu gas chromatograph (GC) model GC-14B (Shimadzu, 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 kept isothermic for 18.5 min. The flame ionization detector output of the gas chromatograph was digitized and evaluated with a C-R8A Shimadzu Chromatopac device (Shimadzu).

5. Evaluation of immunohistological staining

We classified the RC-lining epithelium into three types; the region of the thin epithelium, the region of the thick epithelium and the region of expansion of the intercellular space. Similarly, we classified FC-lining epithelium into two types; the region without infiltration of inflammatory cells and the region with infiltration of inflammatory cells (Fig.1).

All specimens were fixed with 10% formalin and embedded in a paraffin block. Immunohistochemical staining of the sections for HBD-2, GM-CSF and CCR6 expressing cells were carried out according to the standard procedure. Sections (5- μ m thick) were incubated at 37°C 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 0.01M citrate buffer (pH 6.0) using a microwave oven at 98°C for 15 min. After cooling, the sections were washed with Tris-buffered saline with Tween20 (TBST). The sections were then incubated with the primary polyclonal antibody at a dilution of 1:1000 for HBD-2 (PEPTIDE INSTITUTE, INC., Osaka, Japan), GM-CSF (Dako, Glostrup, Denmark), CD1a (Dako), CD4 (Nichirei, Tokyo, Japan) and CD8 (Nichirei). After washing with TBST, signals were detected with 3,3-Diaminobenzidine. Finally, specimens were counterstained with hematoxylin–eosin (HE), dehydrated, and mounted.

The number of Langerhans cells (LCs), and CD4- and CD8-labeled T cells which had infiltrated in the epithelium per mm² was counted. Correlations among CD1a-, CD4- and CD8-labeled cells were calculated by the Pearson product-moment correlation coefficient (*r*). Data were analyzed using Pearson product-moment correlation coefficient to determine statistical differences. Labeling indices for HBD-2 and GM-CSF immunostaining were calculated by counting the number of positive cells per mm² (ie, cells with strong positive staining: 3 points, weak positive staining: 1 point).

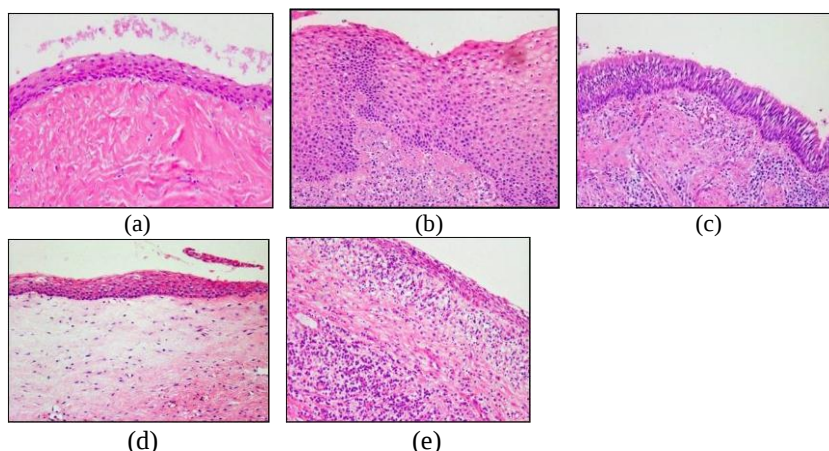


Figure 1. Classification of the keratinocytes in the epithelium lining the RC (a, b, c) and FC (d, e).

(a) The region of the thin epithelium, (b) the region of the thick epithelium, (c) the region of the expansion of the intercellular space, (d) the region without inflammation and (e) the region with inflammation.

6. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the RC and FC-lining epithelium using ISOGEN (Nippon Gene, Tokyo, Japan), 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'-CCAGCCATCAGCCATGAGGGT-3' (sense) and 5'-ACGCCTAAGTCTTTCCCCAGG-3' (antisense) for HBD-2, 5'-GCTGCTGAGATGAATGAAAC-3' (sense) and 5'-GAACAGTAGGGGAAACTGA-3' (antisense) for GM-CSF, and 5'-CGGAGTCAACGGATTTGGTCGTAT-3' (sense) and 5'-AGCCTTCTCCATGGTGGTGAAGAC-3' (antisense) as a housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 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°C, 1 min at 50°C, and 1 min at 72°C. The amplified samples were visualized on 1% agarose gels stained with ethidium bromide and photographed under UV light.

7. Statistical analysis

The Stat-View-J-4.5 software program was used for all statistical analyses that were carried out on all data for each group by means of one-way analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) method. Data were presented as the means $\pm$ standard error (SE). A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

1. The percent composition of major fatty acids in cellular phospholipids of RC, FC and normal oral mucosal keratinocytes

Table I shows the % composition of fatty acids constituting the cell membrane of RC, FC and oral mucosal keratinocytes in the entire epithelial layer. The % composition of palmitic acid (16:0) was significantly higher in RC-lining keratinocytes ($38.62 \pm 5.86\%$) than in FC-lining keratinocytes ($30.37 \pm 1.38\%$) (Fig.2). The % composition of essential fatty acids (linoleic acid, 18:2 + arachidonic acid, 20:4) was significantly higher in FC-lining keratinocytes ($26.20 \pm 3.55\%$) than in RC-lining keratinocytes ($20.50 \pm 8.17\%$) (Fig.3). Although the % composition of linoleic acid (18:2) was significantly higher in the FC-lining keratinocytes ($13.10 \pm 3.64\%$) than in the RC-lining keratinocytes ($8.26 \pm 0.66\%$), the % composition of arachidonic acid (20:4) was not significantly different between the RC-lining keratinocytes and the FC-lining keratinocytes.

Table I. The percent composition of major fatty acids in the cellular phospholipids of RC, FC and gingival keratinocytes (percent total lipids)

Fatty acids	RC	FC	gingiva
14 : 0	0	0	1.20±0.16
16 : 0	38.62±5.86	30.37±1.38	23.00±1.40
18 : 0	23.02±9.94	19.80±2.67	16.90±2.00
16 : 1	0.06±0.02	0	4.30±0.85
18 : 1	17.68±4.97	22.88±2.44	20.30±1.80
18 : 2	8.26±0.66	13.10±3.64	14.50±2.57
20 : 4	12.24±6.40	13.05±1.79	9.30±1.43
n	5	4	4

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				

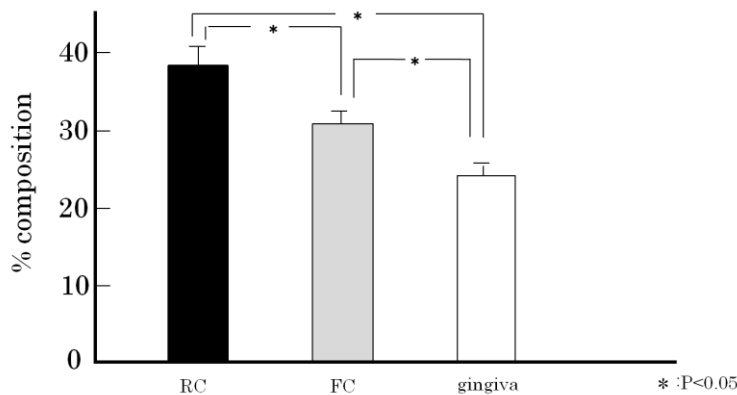


Figure 2. A comparison of the palmitic acid (16:0) composition between keratinocytes.

The percent composition of palmitic acid (16:0) was significantly higher in RC(38.62 ±5.86) and FC(30.37 ±1.38) than in the normal gingiva (parakeratinization) (23.00 ± 1.40).

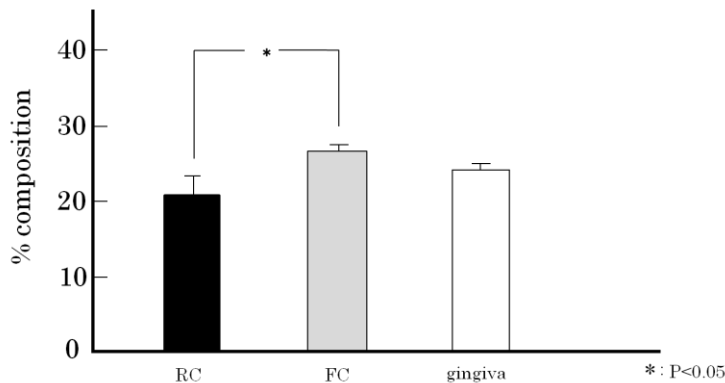


Figure 3. A comparison of the essential fatty acid composition (linoleic acid (18:2) + arachidonic acid (20:4)) between RC and FC keratinocytes.

The percent composition of essential fatty acids was significantly higher in FC keratinocytes (26.20 ± 3.55) than in RC keratinocytes (20.50 ± 8.17)

2. Expression of HBD-2 and GM-CSF proteins in the epithelium lining RC and FC

The expression of the HBD-2 and GM-CSF proteins was strongly associated with inflammation in RC and FC. Interestingly they were not expressed in the basal cell layer. In the thin types of the RC-lining epithelium, HBD-2 was weakly expressed throughout most of the epithelial layers. In the thick types, HBD-2 expression was strongly observed in the upper epithelial layers. In the expansion of intercellular space types, HBD-2 expression was strongly observed in the uppermost epithelial layers. In FCs, HBD-2 expression was observed only in the uppermost epithelial layers in the region without inflammation, but was observed throughout most of the epithelial layers in the region with inflammation (Fig.4). In every RC type, the GM-CSF protein was expressed strongly and its expression was unrelated to the degree of inflammation. In FCs, the GM-CSF protein was only expressed in inflammatory type cysts (Fig.5).

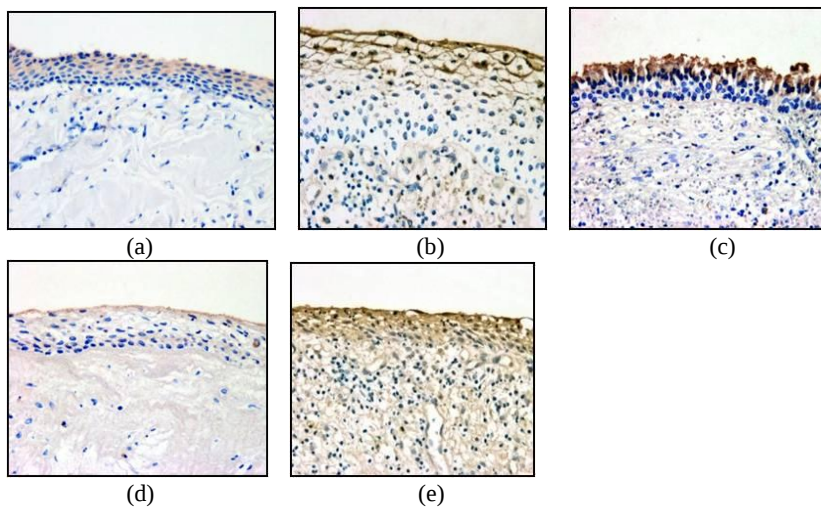


Figure 4. The expression of HBD-2 protein in the epithelium lining RC and FC.

(a) In the thin types of RC-lining epithelium, HBD-2 expression was weakly observed throughout most of the epithelial layers. (b) In the thick types of epithelium, HBD-2 expression was strongly observed in the upper epithelial layers. (c) In the expansion of intercellular space types, HBD-2 expression was strongly observed primarily in the upper epithelial layers. (d) In the region without inflammation, HBD-2 expression was observed only in the uppermost epithelial layers. (e) In the region of inflammation, HBD-2 expression was observed throughout most of the epithelial layers.

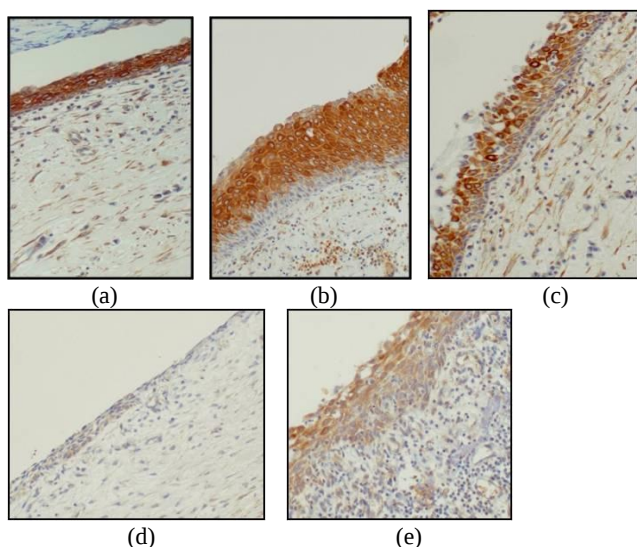


Figure 5. The expression of the GM-CSF protein in the epithelium lining RC and FC.

(a)(b)(c) In RCs, the expression of the GM-CSF protein was similarly strong, and was not related to the degree of inflammation. (d) In the region without inflammation, no GM-CSF protein was expressed. (e) In the region with inflammation, the GM-CSF protein was expressed.

3. Infiltration of CCR6 expressing cells in the epithelium

LCs, one of the CCR6 expressing cell types, infiltrate extensively when there is inflammation associated with RC and FC-lining epithelium, but the number of cells was significantly lower than those present in the normal gingiva (Fig.6). The infiltration of CD4-labeled T cell in the RC-lining epithelium was higher than that in the FC-lining epithelium, however the infiltration of CD8-labeled T cells in the FC epithelium was higher than that in the RC epithelium (Fig.7). That is, the CD4-labeled T cells infiltrate in the intraepithelium in the presence of inflammation. The number of LCs infiltrating into the intraepithelium remarkably increased in the lining epithelium stimulated by inflammation, but was still much lower than that seen in the normal gingiva. In the thin and thick regions, the CD4-labeled T cells and CD8-labeled T cells infiltrate the intraepithelium at the same ratio. In the region of expansion of the intercellular space, the total number of T cells significantly increased but the number of CD8-labeled T cells decreased. That is, only the number of CD4-labeled T cells increased. In the inflammatory FC, the total number of T cells increased compared with non-inflammatory FC, because the number of CD8-labeled T cells increased.

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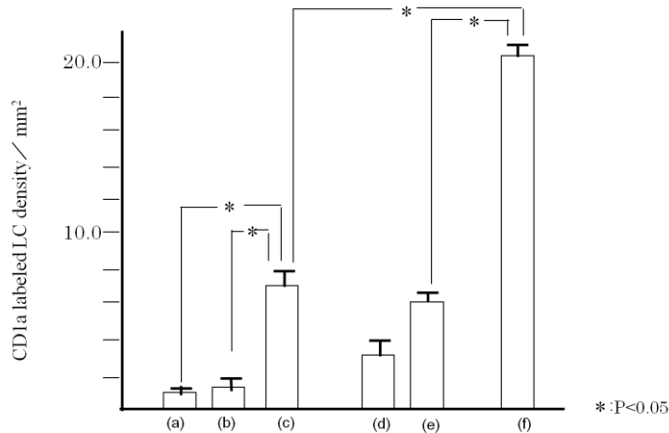


Figure 6. Infiltration of LCs in the epithelium lining the RC and FC. The number of LCs infiltrating in lining epithelium increased with inflammation, but was lower than that of the normal gingiva. (a) The region of the thin epithelium, (b) the region of the thick epithelium, (c) the region of expansion of the intercellular space, (d) the region without inflammation, (e) the region with inflammation and (f) normal gingiva.

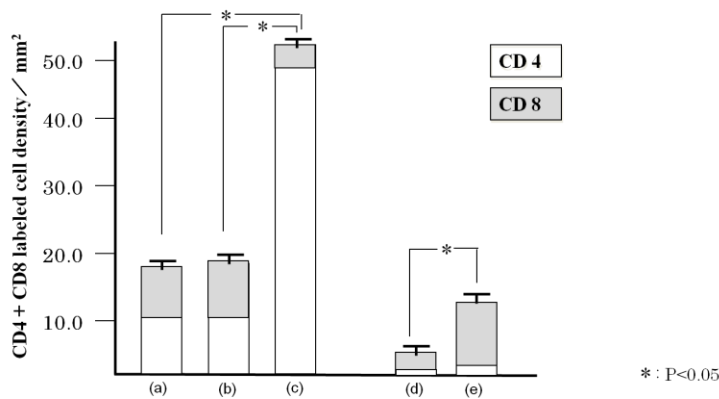


Figure 7. Infiltration of CD4-labeled T cells and CD8-labeled T cells in RC and FC.

The infiltration of CD4-labeled T cells in RC was increased compared to that in FC. In contrast, the infiltration of CD8-labeled T cells in FC was higher than that in RC. (a) The region of the thin epithelium, (b) the region of the thick epithelium (c) the region of expansion of the intercellular space, (d) the region without inflammation and (e) the region with inflammation.

4. RT-PCR analysis of HBD-2 and GM-CSF expression

A slightly higher HBD-2 expression was detected in the RC-lining epithelium than in the FC-lining epithelium. In addition, a higher GM-CSF expression was detected in RC than in FC. GAPDH was detected at the same level in all samples (Fig.8).

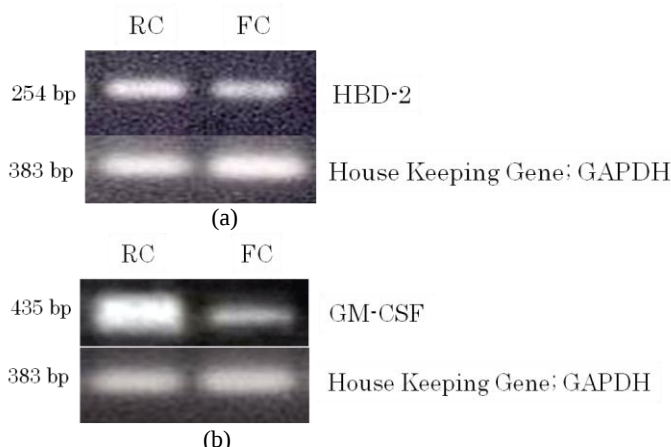


Figure 8. A slightly higher HBD-2(a) expression was detected in RC than in FC. In addition, a higher GM-CSF(b) expression was detected in the epithelium lining RC.

DISCUSSION

In this study, we first clarified the composition of fatty acids and the expression of HBD-2 and GM-CSF between the keratinocytes lining an RC and FC. The results of this study suggest that there are cytobiological differences between these two odontogenic cyst-lining keratinocytes from the aspect of their defense mechanism and metabolic functions.

The percent composition of palmitic acid (16:0) was higher in RC than in FC keratinocytes. The percent composition of linoleic acid (18:2) was higher in FC than in RC keratinocytes, but the percent composition of arachidonic acid (20:4) was not significantly different between the RC and FC keratinocytes. These results suggest that the RC-derived keratinocytes had a much higher level of potential energy, and differentiation of keratinocytes may be related to linoleic acid (18:2). These findings suggest that epithelialization is a phenomenon whereby keratinocytes proliferate while consuming palmitic acid (16:0), and that this proliferation subsequently shifts to differentiation after being provided with EFAs from the diet.

HBD-2 may play an important role in the innate defense against oral microorganisms (13, 23). HBD-2 may promote the adaptive immune response by recruiting dendritic cells (DCs) and T cells to the site of microbial invasion through its interaction with CCR6 (18). HBD-2 has therefore developed the capacity to play important roles in both innate and adaptive immune responses against microbial invasion. HBD-2 expression is markedly increased by antigenic stimulation, such as inflammation and pathogen invasion. In our study, the expression of HBD-2 was also higher in RC than in FC. The results of recent *in vitro* studies have clarified that HBD-2 is a chemokine that targets CCR6 and mobilizes T cells and epithelial DCs (LCs) expressing CCR6 via chemotaxis (18, 25). In other words, in the skin or mucosa, HBD-2 may interact with CCR6 and thus play an important role in the defense of the body against external stimulation by facilitating immunoreactions involving T cells and DCs. Other factors, such as GM-CSF, are also important for the accumulation of LCs *in vivo* and their differentiation *in vitro*. Mature LCs, antigen presenting cells, activate CD4 labeled T cells by major histocompatibility complex (MHC) class II (17, 22).

It has been reported that the infiltration of CD4-labeled T cells is higher than that of CD8-labeled T cells during the active period, but that the infiltration of CD8-labeled T cells

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is higher than that of CD4-labeled T cells during the stable period (7, 9, 15, 20). In the RC, infiltration of CD4-labeled T cells into the intraepithelium was higher than that of CD8-labeled T cells. In the FC, infiltration of CD4-labeled T cells into the intraepithelium is increased compared to that of CD8-labeled T cells. That is, it is predicted that MHC II is activated by means of GM-CSF-LCs in combination with the HBD-CCR6 immune system in an RC. These two cytokines derived from keratinocytes in odontogenic cysts are expressed at much lower levels than in the oral mucosa. Therefore, the odontogenic cyst-lining epithelium may have an immature defense system compared with the normal oral mucosal epithelium.

In fact, the infiltration of LCs in RC and FC is influenced by the degree of inflammation. The number of these cells in RC and FC is much lower than that in the normal gingiva. Therefore, it is predicted that odontogenic cysts have a weaker DC-based immune defense system compared with the normal gingiva.

In conclusion, we have herein demonstrated the fatty acid composition constituting the cell membrane and the expression of HBD-2 and GM-CSF in RC and FC-lining keratinocytes. The results of this study show that there are cytobiological differences between these two odontogenic cyst-lining keratinocytes, thus including differences in their defense mechanism and metabolic functions.

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