Expression of Cytokeratin in Experimentally Created Inflammatory Cyst *in Vivo* and *in Vitro*

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Abstract

The purpose of this study was to investigate the behavior of epithelial lining derived from Malassez's epithelial rest (MER) cells in experimentally created inflammatory cysts *in vivo* and *in vitro*. Porcine MER cells were cultured *in vitro* with or without interleukin (IL)-1 β (1 ng/ml) or IL-6 (1 ng/ml). Cell proliferation was assessed and expression levels of CK19 and CK13 mRNA determined using RT-PCR. *In vivo*, a cavity was created in the first molar of Sprague-Dawley male rats and tissue repair observed using immunohistochemical methods. *In vitro*, treatment with IL-1 β or IL-6 increased proliferation of MER cells and decreased expression of CK19 mRNA, but increased CK13 mRNA at day 1 ($p < 0.05$). *In vivo*, at 2 weeks, CK19-positive epithelial cells were observed adjacent to the cementum, in the cystic lesion, and in connective tissue. At 3 weeks, they were only detected in cells adjacent to the connective tissue. Cells positive for CK13 were observed throughout the epithelium, except in cells adjacent to connective tissue at weeks 2 and 3. Exposure to IL-1 β and/or IL-6 induced proliferation and differentiation of MER cells.

Key words: Histology—Epithelium/Epithelia—Cyst—Inflammation—
Cell proliferation

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Introduction

Inflammation due to dental pulpitis gives rise to cysts in root furcations or apical areas of the dentition. The epithelial lining of such cysts is derived from Malassez's epithelial rest (MER) cells¹⁴. As residual cells from Hertwig's epithelial root sheath, they form an odontogenic epithelium which remains in the periodontal ligament^{9,16}, occurring as strands located close to the surface of the cementum. Normally, MER cells remain in the resting phase of the cell cycle. They are present in the periodontal ligament throughout life, and do not disappear unless a tooth root is lost. They constitute an immature odontogenic epithelium which is positive for cytokeratin 19 (CK19), but not cytokeratin 13 (CK13), which stains for matured epithelial cells⁸. The characteristic properties of this epithelium are not known, but some of its functions are required to maintain the width of the periodontal ligament space and for cementum formation^{4,15}. Furthermore, it has been suggested that inflammatory stimulation, such as by bacterial infection, induces proliferation of MER cells, which can then lead to the formation of dental cysts⁷.

Inflammatory cytokines, and particularly IL-1 and IL-6, which are well known to play a role in pathological change in cells and tissues, induce proliferation of epithelial cells and the formation of cysts¹⁰. One earlier study using RT-PCR found that IL-1 and IL-6 mRNA was expressed in the epithelial lining of human radicular cyst¹¹. Taken together, this suggests that these inflammatory cytokines play an important role in the formation of the epithelial lining. Few studies have investigated cell behavior in the epithelial lining in terms of cell proliferation and differentiation, however. The purpose of this study was to investigate whether exposure to either IL-1 β or IL-6 *in vitro* induced proliferation and/or differentiation of MER cells. Moreover, cysts were created experimentally in rat molars to investigate proliferation and differentiation of MER cells based on expression of CK19 and CK13 using histochemical and immunohisto-

chemical staining *in vivo*.

Materials and Methods

1. *In vitro* study

1) Cell culture

Porcine MER cells were provided by Professor Abiko of the Department of Dental Science of the Health Sciences University of Hokkaido. The cells were frozen and stored until used. They were cultured in 75-cm² tissue culture flasks with minimum essential medium α (MEM) (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and penicillin-streptomycin (Wako, Osaka, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO₂ according to the method of Brunette *et al*¹.

2) Cell proliferation assay

The experimental group comprised MER cells cultured in 1 ml MEM containing either 1 ng IL-1 β or IL-6; the control group comprised MER cells cultured in 1 ml MEM alone. The concentration of cytokines was set in accordance with earlier reports^{5,13}. The culture medium was changed every 3 days. The MER cells (1×10^4 cells) were seeded in 24-well plates and cultured as detailed above. Cell proliferation was evaluated using a C-chip hemocytometer (NanoEnTek, Seoul, Korea) at days 1, 3, 7, and 14.

3) Quantitative real-time PCR

Total RNA was extracted from the MER cells in each group at day 1 ($n=5$). The samples were homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed to obtain cDNA and amplified in 20- μ l volumes using a QuantiTect Reverse Transcription kit (Qiagen, Germantown, MD, USA). The RT-PCR products were analyzed by quantitative real-time RT-PCR in TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for the target genes *CK19* (XM_003131437.3) and *CK13* (XM_003131436.2). The primer sequences used are shown in Table 1. Reaction conditions consisted of a primary dena-

Table 1 Primers used for RT-PCR in this study.

Primer	Gene name	NCBI Reference Sequence
CK19	Sus scrofa keratin 19, type I (KRT19), mRNA	XM_003131437.3
CK13	Sus scrofa keratin, type I cytoskeletal 13 (LOC100515166), mRNA	XM_003131436.2
GAPDH	G6PDH	Ss03374854_g1

Fig. 1 Experimental design of *in vivo* study.

Floor of pulp chamber was perforated towards alveolar bone using 25 G needle.

turation at 95°C for 20 sec and then 40 cycles at 95°C for 1 sec and at 60°C for 20 sec. Relative mRNA expression levels were determined after normalizing the cycle threshold values for each gene with the internal control (GAPDH). Quantitative RT-PCR analyses were reproduced 3 times.

4) Statistical analysis

A one-way analysis of variance (ANOVA) was used for the cell proliferation assay and to compare mRNA expression levels between the experimental and control groups. A *p*-value of <0.05 was considered to indicate a significant difference.

2. *In vivo* study

1) Animals

This study was conducted in compliance with the Guidelines for the Treatment of Experimental Animals at Tokyo Dental College (Approval no: 283207). Sprague-Dawley male rats, each weighing approximately 100–150 g (Sankyo Lab Service, Tokyo, Japan), were housed in a conventional animal labora-

tory until used for the study.

2) Experimental procedure

After application of general anesthesia with 5 ml/mg Somnopentyl (Kyoritsuseiyaku, Tokyo, Japan), the occlusal surface of the maxillary first molar in each rat was drilled with a steel round burr (ϕ : 1 mm) (MANI, INC, Tochigi, Japan) to expose the coronal pulp tissue. The floor of the pulp chamber was then perforated towards the alveolar bone using a 25 G needle. After perforation, hemorrhaging was controlled by irrigation with sterile water and cotton used to keep it open (Fig. 1).

3) Histological and immunohistochemical observations

The rats were sacrificed at 1 (*n*=3), 2 (*n*=3), or 3 (*n*=3) weeks postoperatively and the maxillary first molars removed with the surrounding alveolar bone by mechanical means. The excised molars were immersed in 10% neutral formalin for 72 hr at room temperature, demineralized with 10% formic acid for 1 week, and then washed with running water before being embedded in paraffin. Paraffin sections approximately 4 μ m in thickness were cut sagittally to the tooth. These sections were then deparaffinized and stained with hematoxylin and eosin. For immunohistochemical staining, antibodies to CK19 (Sigma, St. Louis, MO, USA) and CK13 (Abcam, Cambridge, UK) were used as primary antibodies to observe epithelial cell differentiation. An antibody to Proliferating Cell Nuclear Antigen (PCNA, PC10, DAKO, Santa Clara, CA, USA) was also used as a primary antibody to observe epithelial cell proliferation. The paraffin sections were deparaffinized, microwaved in an Immunosaver (Nisshin EM, Tokyo, Japan), and diluted 1:

Fig. 2 Three observation areas.

- ① Upper area: 1/3 of lesion, continuing from injured cementum.
- ② Middle area: central 1/3 of lesion.
- ③ Bottom area: 1/3 of lesion closest to alveolar bone.

200 for antigen retrieval for 25 min at 80°C. After blocking endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for 30 min at room temperature, the sections were treated with 10% goat serum albumin for 1 hr at room temperature. The sections were incubated with antibodies to CK19 (diluted 1:100), CK13 (diluted 1:100), and PCNA (diluted 1:100) at 4°C overnight. They were then incubated with the MACH 2 Universal HRP Polymer Detection kit (Biocare Medical, Concord, CA, USA) for 30 min. Immunoreactivity was visualized using the Betazoid DAB Chromogen kit (Biocare Medical). The sections were rinsed with phosphate-buffered saline and counterstained with hematoxylin.

4) Observation area and PCNA-positive cell ratio assay

Each lesion was divided into 3 areas for analysis of PCNA immunohistochemical staining as shown in Fig. 2. The upper area shows 1/3 of the lesion, continuing from the injured cementum; the middle area, the central 1/3 of the lesion; and the bottom area, the 1/3 of the lesion closest to the alveolar bone. Fig. 2-① shows cells positive for CK19 or CK13. The PCNA ratio was calculated as the ratio of PCNA-positive cells to total number of cells in each of the 3 areas. Measurements in each area were carried out 3 times.

Fig. 3 Proliferation of MER cells.

All groups showed daily increase in number of cells. MER cells treated with IL-1β or IL-6 showed significant increase in proliferation after day 3 compared with untreated control cells. * $p < 0.05$; ** $p < 0.01$

A PCNA positive ratio =

$$\frac{\text{PCNA positive cells}}{\text{All the cells}} \times 100(\%)$$

5) Statistical analysis of PCNA ratio

The data are expressed as the mean $\pm$ standard deviation, and a one-way ANOVA was used for the statistical analysis. A p -value of <0.05 was considered to indicate a significant difference.

Results

1. *In vitro* study

1) Cell proliferation

The proliferation of MER cells in both the experimental and control groups showed a daily increase for 2 weeks (Fig. 3). Although no significant difference was observed between the two groups at 1 day of culture, a significant increase was observed in proliferation in the IL-1β-treated cells in comparison with in the control cells at days 3 ($p < 0.01$), 7 ($p < 0.05$), and 14 ($p < 0.01$). Proliferation of the IL-6-treated cells showed a significant increase compared with in the control cells at days 3 ($p < 0.01$) and 7 ($p < 0.05$), but with only a slight increase at day 14.

2) Expression of CK19 and CK13 mRNA

(1) Expression of CK19 mRNA

Expression of CK19 mRNA in both the

Fig. 4 mRNA expression levels of CK19 by MER cells on day 1.

Expression of CK19 mRNA assessed by RT-PCR showed decrease in both experimental groups compared to in untreated controls. ** $p < 0.01$.

Fig. 5 mRNA expression levels of CK13 by MER cells on day 1.

Expression of CK13 mRNA assessed by RT-PCR showed increase in experimental groups compared to in untreated controls. ** $p < 0.01$.

IL-1 β and IL-6-treated MER cells was significantly lower than that in the controls at day 1 ($p < 0.01$) (Fig. 4). No significant difference was observed between the IL-1 β and IL-6-treated MER cells.

(2) Expression of CK13 mRNA

Expression of CK13 mRNA in both the IL-1 β and IL-6-treated MER cells was significantly higher than that in the controls at day 1 ($p < 0.01$) (Fig. 5). No significant difference was observed between the IL-1 β and IL-6-treated MER cells.

2. In vivo study

1) Histological observations

At 1 week postoperatively, food debris had filled in the experimentally created lesion and leukocytes, mainly consisting of neutrophils, had infiltrated the surrounding connective tissue (Fig. 6-a, b). The MER cells had proliferated, migrated, and attached to the cementum surface, extending deeper into the connective tissue. At 2 weeks postoperatively, the lesion was completely covered with proliferating epithelium, which had formed a cystic lesion (Fig. 6-c). Proliferation of fibroblasts and capillaries was observed in the wall of the cyst. At 3 weeks postoperatively, the epithelial lining of the cyst wall was thicker than at 2 weeks (Fig. 6-d).

2) Immunohistochemical observations

(1) CK19 expression

The MER cells in the periodontal ligament were positive for CK19 (Fig. 7-a). At 1 week postoperatively, no CK19-positive cells were clearly observed among the epithelial cells (Fig. 7-b). At 2 weeks postoperatively, CK19-positive cells were observed among cells attached to the cementum, cystic space, and connective tissues (Fig. 7-c). At 3 weeks postoperatively, cells that were attached to the connective tissues were positive for CK19, but other cells were not (Fig. 7-d).

(2) CK13 expression

The MER cells in the periodontal ligament were negative for CK13 (Fig. 8-a). At 1 week postoperatively, no CK13-positive cells were observed among the epithelial cells (Fig. 8-b). At 2 weeks, CK13-positive cells were observed among all epithelial cells, except basal cells (Fig. 8-c). At 3 weeks, CK13-positive cells were observed in the same manner as at 2 weeks (Fig. 8-d).

(3) PCNA-positive cells

Upper area:

At 1 week postoperatively, PCNA-positive cells were observed among cells attached to the cementum, cystic space, and connective tissues (Fig. 9-a). However, PCNA-positive cells were mostly observed among cells attached to the connective tissues at weeks 2 and 3, and the number of PCNA-positive cells

Fig. 6 Representative features in *in vivo* study using HE staining after surgery.

(a) Epithelial cells were directly attached to injured cementum surface and extended to deeper area at 1 week postoperatively.

(b) Extended image of (a).

(c) Lesion was completely covered with proliferating epithelium and had formed cystic lesion at 2 weeks postoperatively.

(d) Maximum thickness of epithelial lining of cyst reached at this point. Fibroblasts and capillaries were observed at 3 weeks postoperatively in cyst wall.

*: Intracystic space, C: Cementum, E: Epithelium.

Fig. 7 Immunohistochemical staining for CK19 at area ① of Fig.2.

(a): MER cells in periodontal ligament were positive.

(b), (c) and (d): Immunohistochemical staining of CK19 in upper area.

(b): Epithelial cells (arrows) in area ① of Fig.2 were not clearly positive for CK19 at 1 week postoperatively.

(c): CK19-positive cells were mostly seen in area where cells were attached to connective tissue at 2 weeks postoperatively.

(d): CK19-positive cells were only seen in cells that were attached to connective tissue at 3 weeks postoperatively.

C: cementum, E: epithelium, IS: Intracystic space.

Fig. 8 Immunohistochemical staining for CK13 at area ① of Fig. 2.

(a): MER cells in periodontal ligament were negative.

(b), (c) and (d): Immunohistochemical staining of CK13 in upper area.

(b): At 1 week postoperatively, epithelial cells were not positive.

(c): At 2 weeks postoperatively, CK13-positive cells were found throughout epithelial lining, except for in basal cells.

(d): At 3 weeks postoperatively, all cells in epithelial lining except for basal cells were positive for CK13, as seen at 2 weeks postoperatively.

C: Cementum, E: Epithelium. IS: Intracystic space.

attached to the cementum and cystic space decreased week by week (Fig. 9-b, c). The PCNA-positive cell ratio among cells attached to the cementum was higher than in the other areas at week 1 ($p < 0.01$). The PCNA-positive cell ratios at weeks 2 and 3 among cells attached to the connective tissues were significantly higher than those on the side of the cystic space (Fig. 10). The PCNA-positive ratio at each time period among cells attached to the cementum was significantly higher than that among cells in the cystic space (at 2 weeks, $p < 0.01$; at 3 weeks, $p < 0.05$).

Middle area:

At 1 week postoperatively, no epithelial cells had formed in the middle area. Cells positive for PCNA were mainly observed among those attached to the connective tissues at both 2 and 3 weeks postoperatively (Fig. 9-d, e). At 2 and 3 weeks postoperatively, PCNA-positive cells that were attached to the connective tissues and side of the cystic space increased week by week (Fig. 11). The PCNA-positive cell ratios at weeks 2 and 3 among cells attached to the connective tissues were significantly higher than on the side of the cystic space. However, no significant differ-

ences were observed between the PCNA-positive cell ratios at weeks 2 and 3 in the same area.

Bottom area:

Cells positive for PCNA were observed in the same manner as in the middle area (Fig. 9-f, g). At 1 week postoperatively, no epithelial cells had formed in the bottom area. At 2 and 3 weeks postoperatively, PCNA-positive cells attached to the connective tissues and side of the cystic space increased week by week (Fig. 12). At 2 and 3 weeks postoperatively, the PCNA-positive cell ratio among cells attached to the connective tissues was significantly higher than that on the side of the cystic space. The PCNA-positive cell ratio among cells on the side of the cystic space was not significantly different from that at 2 and 3 weeks.

Discussion

One study reported expression of both IL-1 β and IL-6 in the epithelial lining of human radicular cysts, concluding that these cytokines were the most important factors in

Fig. 9 Immunohistochemical staining for PCNA.

(a): At 1 week postoperatively, PCNA-positive cells were attached to cementum, and cystic space and connective tissues were observed in upper area.

(b, c): At 2 and 3 weeks postoperatively, PCNA-positive cells attached to connective tissues had increased and PCNA-positive cells attached to cementum and side of cystic space had decreased clearly in upper area.

(d, e): At 2 and 3 weeks postoperatively, PCNA-positive cells attached to side of cystic space and connective tissues were observed in middle area. Moreover, PCNA-positive cells increased in cells attached to connective tissues.

(f, g): PCNA-positive cells in bottom area were observed in same manner as in middle area.

Upper area: arrows indicate epithelial cells.

C: Cementum, E: Epithelium, IS: Intracystic space.

the proliferation and differentiation of MER cells¹¹⁾. Another study also found IL-1 β and IL-6 in the basal cells of the epithelial lining and in fibroblasts located underneath in human radicular cysts⁶⁾. Meanwhile, another investigation found IL-6 in inflammatory radicular cyst fluid, but not in odontogenic keratocyst or dentigerous cyst. They concluded that epithelial cell proliferation was stimulated directly by bacterial endotoxins from the radicular cyst and also indirectly via stimulation of cytokine synthesis by lymphocytes, monocytes, and fibroblasts in the cystic

wall in *in vivo* experiments¹⁰⁾. In the present *in vitro* study, proliferation of MER cells cultured in medium containing IL-1 β or IL-6 was observed. Taken together, this suggests that, even though neither IL-1 β nor IL-6 was detected in either the fluid or the epithelial lining of the experimentally created radicular cysts in the *in vivo* study, proliferation and differentiation of MER cells results from expression of both IL-1 β and IL-6, leading to the formation of an epithelial lining in the cyst.

It was reported that proliferation of MER cells is usually caused by inflammation-related

Fig. 10 Ratio of PCNA-positive cells in Fig.2-① (upper area).

Ratio of PCNA-positive cells attached to connective tissues increased significantly. However, that ratio decreased significantly in cells on side of cystic space week by week. * $p < 0.05$; ** $p < 0.01$.

Fig. 11 Ratio of PCNA-positive cells in Fig.2-② (middle area).

Two and 3 weeks postoperatively, ratio of PCNA-positive cells attached to connective tissues and side of cystic space increased week by week; however no significant changes were found in PCNA-positive cell ratios between 2 and 3 weeks. ** $p < 0.01$.

factors from the perforated pulp chamber, and that cyst formation occurs in that area^{3,12}. It was also reported that the distribution of both CK19 and CK13 in cyst was similar to that in oral epithelium. And it is known that MER cells in the periodontal ligament are positive for CK19, but negative for CK13⁸. In the present *in vivo* experiment, expression of neither CK19 nor CK13 was detected in the proliferating epithelial cells at 1 week postoperatively. At 2 weeks postoperatively, CK19-

Fig. 12 Ratio of PCNA-positive cells in Fig.2-③ (bottom area).

Two and 3 weeks postoperatively, ratio of PCNA-positive cells attached to connective tissues and side of cystic space increased week by week. * $p < 0.05$; ** $p < 0.01$.

positive cells were observed among cells attached to the connective tissue. At 3 weeks postoperatively, CK19-positive cells were observed in basal cells only of the epithelial lining. In contrast, at 2 and 3 weeks postoperatively, CK13-positive cells were observed opposite the CK19-positive cells. The CK13-positive cells were observed in all the cells of the epithelial lining, except for the basal cells. This suggests that the MER cells started to proliferate at 1 week postoperatively, although at this stage it remains unclear as to whether they yet function as a protective barrier between the external and internal environments.

At 2 weeks postoperatively, the external and internal environments were clearly separated by the proliferating epithelium, which was positive for CK13 inside and positive for CK19 outside, where it was attached to connective tissues. Thus, the epithelial lining was eventually able to exert a protective effect, just as in oral mucosa. In the *in vitro* study, the expression level of CK19 mRNA showed a decrease, but not that of CK13, which showed an increase at day 1 in the culture medium. This also suggests that cultured MER cells differentiate similarly to oral epithelium *in vitro*.

From the viewpoint of proliferation, diffuse PCNA-positive cells were observed in the proliferating epithelium at 1 week, probably because of the effects of inflammatory cyto-

kines. The PCNA-positive cells corresponded to CK19-positive cells at both 2 and 3 weeks postoperatively. This suggests that when MER cells emerge from the resting phase, they start proliferating. The cells which were positive for PCNA, which were located adjacent to the cementum, proliferated at an early time period. This leads to the conjecture that the healing mechanism is the same as that of the long junctional epithelium in periodontal treatment²⁾.

In conclusion, epithelial lining in the present study became the same as normal oral epithelium in terms of the function of the latter as a cover separating the inner from the outer environment.

Conclusion

The results of this study suggest that proliferation and differentiation of MER cells is probably induced by expression of IL-1 β and IL-6.

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