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Role of the junctional epithelium in periodontal innate defense and homeostasis

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Background and Objective: The junctional epithelium provides the front-line defense against periodontal bacterial infection. The migration of neutrophils into the junctional epithelium might represent a protective reaction against bacterial infections. However, neutrophils penetrate into the junctional epithelium even under sterile conditions. In this study, we analyzed and compared the number of neutrophils and the cytokine expression related to neutrophil migration in the junctional epithelium in conventional and germ-free mice.

Material and Methods: Germ-free and conventional ICR mice were used at 12 wk of age. Frozen sections were used for the detection of Gr-1, macrophage inflammatory protein-2 (MIP-2/CXCL2) and proliferating cell nuclear antigen-positive cells in the two groups of mice. Laser capture microdissection and RT-PCR analysis were used to evaluate the expression of keratinocyte-derived chemokine (KC/CXCL1), MIP-2, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) mRNAs in the two groups of mice.

Results: Morphometric examination indicated an increase in the area of the junctional epithelium upon bacterial infection. Immunohistochemical studies also detected an increased number of neutrophils in the junctional epithelium upon bacterial infection. Higher up-regulation of KC and MIP-2 were detected in the junctional epithelium of conventional mice than in germ-free mice, whereas the expression of *Il-1*β and *Tnf*α mRNAs was not affected.

Conclusion: Junctional epithelium cells constitutively expressed several types of chemokines and cytokines and the expression of chemokines was augmented by bacterial infection. Therefore, the constitutive expression of cytokines in junctional epithelium might be related to the morphological and functional homeostasis of the junctional epithelium in addition to the defense against the bacterial infection.

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Periodontitis is initiated and maintained by a microbial biofilm that forms the dental plaque (1). The junctional epithelium at the dentogingival junction has a central role in the defense of periodontal tissue against microbial challenge. The junctional epithelium is characterized as a highly permeable, nonkeratinized squamous epithelium with wide intercellular spaces (2). Under normal conditions, this epithelium is constantly exposed to pathogenic microbial flora that can elicit inflammation and thus cause tissue destruction.

Neutrophils are key components of the host defense against bacterial infection. In early and chronic periodontal lesions, neutrophils are the most abundant type of leukocyte within the periodontal tissues (3). The density of neutrophils within the epithelium increases towards the more superficial layers in close relationship with the presence of subgingival plaque bacteria (4). Thus, neutrophils play a pivotal role in the maintenance of the host–parasite homeostasis in bacterial invasion, and the loss of the neutrophilic barrier function is associated with severe periodontal disease (5).

Early histological studies of clinically healthy tissue also demonstrated the infiltration of neutrophils within the junctional epithelium (6). It was also reported that leukocyte accumulation in the junctional epithelium should not be regarded unequivocally as a first step toward severe gingivitis and periodontitis but rather as a normal constituent of the host defense system that is not associated with a destructive process (7). Thus, a relationship between the host and commensal bacteria may provide a stimulus for the expression of inflammatory mediators (8). However, several studies demonstrated the migration of neutrophils within the junctional epithelium of germ-free mice (9–12).

Neutrophil recruitment follows a gradient of specific chemokines secreted in response to bacterial products or proinflammatory cytokines. The CXC chemokine is the most abundant and potent chemoattractant for neutrophils, and it is well known that interleukin (IL)-8 is the most potent human CXC chemokine. IL-8 can be produced by various cells, including leukocytes, fibroblasts, endothelial cells and keratinocytes, in response to both endogenous and exogenous stimuli (13). Although homologues of some human chemokines (e.g. IL-8/CXCL8) are probably missing in some rodents (e.g. mice), other potent neutrophil chemoattractants exist in the mouse, such as keratinocyte-derived chemokine (KC/CXCL1) and macrophage inflammatory protein-2 (MIP-2/CXCL2) (14,15).

The expression of proinflammatory cytokines occurs within the junctional epithelium in humans and conventional rats that have normal microflora (16–18). Thus, neutrophil infiltration and expression of inflammatory mediators are considered part of the innate host defense mechanism triggered by the host–parasite interaction. Neutrophil accumulation has also been reported in the junctional epithelium of germ-free animals (9–11). Unique characteristics of the junctional epithelium not caused by host–parasite interactions were also reported in recent studies (19,20).

Previous reports have demonstrated changes in the expression of proinflammatory cytokines within the gingival tissue when comparing germ-free and conventional mice (21). IL-1β was identified as differentially expressed in the periodontal tissues of germ-free and conventional mice. However, the basal expression level of proinflammatory cytokines and chemokines was not measured in the junctional epithelium of germ-free animals. Thus, little is known about the contribution of the oral commensal inflammatory state in the junctional epithelium.

The aim of this study was to examine the inflammatory responses of the junctional epithelium in a germ-free condition and to evaluate the contribution of commensal bacteria by assessing the number of neutrophils and the expression of proinflammatory cytokines and chemokines in the junctional epithelium of germ-free and conventional mice.

Material and methods

The experimental protocol used was reviewed and approved by the Animal Care Committee of Showa University.

Animals

Forty, 4-wk-old male IQI/JIC (ICR) germ-free mice ($n = 40$) were purchased from Clea Japan (Tokyo, Japan). These mice were divided into germ-free and conventional groups. Twenty mice were reared for 8 wk under conventional conditions. Germ-free and conventional mice at 12 wk of age were used for the following experiments. In each group, 15 mice were used for histological and immunohistochemical analyses, and five were used for the examination of mRNA expression. To assess whether there was adequate conventionalization of the germ-free mice, oral effusion samples were cultured using a thioglycolate test (data not shown).

Tissue preparations

For the histological and immunohistochemical studies, the mandibles were dissected and fixed with 4% paraformaldehyde for 6 h at 4°C. After decalcification with 10% ethylenediaminetetraacetate (EDTA) for 2 wk at 4°C, specimens were embedded in OCT compound (Sakura, Torrance, CA, USA). Frontal serial frozen sections (of 10 μm thickness), parallel to the long axis of the first molars, were cut at the central position of the mesiodistal width and collected on glass slides. Every third section was stained with hematoxylin and eosin to determine the area of junctional epithelium by light microscopy. The area of junctional epithelium was measured in μm^2 using PALM MB IV RoboSoftware 4.2 (Carl Zeiss, Tokyo, Japan). The other sections were processed for immunohistochemical procedures.

The preparation of tissue for laser capture microdissection was performed according to the previously described protocol (20). Briefly, the whole heads of the animals were removed immediately after death, embedded in OCT compound and then fast-frozen in isopentane cooled in liquid nitrogen. Frozen samples prepared for laser capture microdissection were sliced using a cryomicrotome (Microm, Woodstock, CT, USA) into sections of 14 μm thickness, and each section was fixed to a slide to which a thin film (Meiwafosis, Osaka, Japan) had been attached with silicon adhesive (GE Toshiba Silicone, Osaka, Japan). The sections were stored at −40°C until use.

Immunohistochemistry

The primary antibodies used included rat monoclonal anti-mouse Gr-1 IgG (BD Pharmingen, Tokyo, Japan), mouse monoclonal anti-mouse proliferating cell nuclear antigen (PCNA) IgG (BD Pharmingen) and goat polyclonal anti-mouse MIP-2 IgG (R&D Systems, Minneapolis, MN, USA). After washing serial sections in phosphate-buffered saline, they were immersed in phosphate-buffered saline containing 1% H_2O_2 for 30 min to block endogenous peroxidase activity and then incubated with normal goat serum or normal horse serum for 30 min at room temperature. The sections were incubated with the primary antibody for 24 h at 4°C. After the sections were washed thoroughly with phosphate-buffered saline, they were incubated with goat anti-rat, goat anti-mouse and horse anti-goat secondary IgG for 60 min respectively, and then incubated for 30 min with the avidin–biotin–peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). The color was developed with 3,3'-diaminobenzidine tetrahydrochloride in Tris buffer plus H_2O_2 (DAB Reagent set; KPL, Gaithersburg, MD, USA). The sections were counterstained with methyl green (Methyl Green Histological Staining Reagent; Dako, Tokyo, Japan). The negative control used normal rat serum or normal goat serum rather than the primary and secondary antibodies.

Evaluation of the area of the junctional epithelium and the number of Gr-1 and PCNA-positive cells in the junctional epithelium

The area of the junctional epithelium of the hematoxylin and eosin-stained section was measured in μm^2 using PALM MB IV RoboSoftware 4.2 (Carl Zeiss).

Neutrophils and proliferating cells within the junctional epithelium were evaluated by counting the number of Gr-1-positive and PCNA-positive cells. The results were expressed as the number of positively stained cells in the junctional epithelium divided by the area of the junctional epithelium.

Laser capture microdissection

Laser capture microdissection was performed according to the previously described procedure (20). For the extraction of only junctional epithelium using laser capture microdissection, the fresh-frozen sections were fixed in zinc-fix (22) for 3 min. The sections were stained with a laser capture microdissection staining kit (Ambion, Austin, TX, USA) in RNase-free water. After air-drying, the junctional epithelium and the oral gingival epithelium of the germ-free and conventionalized mice were microdissected using a PALM-MB laser microdissection system (Carl Zeiss, Tokyo, Japan) with a 337-nm nitrogen laser. The microdissected area was estimated to be approximately 13,000 μm^2 per sample (Fig. 1).

Quantitative real-time PCR analysis

Total RNA was extracted from the laser-microdissected samples using the RNeasy Micro Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Complementary DNA (cDNA) was generated by reverse transcription using a High Capacity RNA to cDNA Master Mix (Applied Biosystems, Tokyo, Japan). Quantitative PCR analysis (TaqMan) was performed on an ABI Step One (Applied Biosystems) sequence detection instrument following the manufacturer's instructions. For TaqMan analysis, each reaction mixture contained 15 μL of TaqMan® Gene Expression Master Mix (Applied Biosystems), 1 μL of each sense and antisense primer (50 pmol/μL) and 4 μL of template cDNA supplemented with RNase-free water to a final volume of 30 μL. TaqMan® Gene Expression Assays (Applied Biosystems) were used for the detection of cytokines with specific primers [accession numbers: KC Mm004338 59_gl; MIP-2 Mm00436450_ml; tumor necrosis factor-α (TNF-α) Mm004432 58_ml; and IL-1β Mm01336189_ml; respectively]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The temperature profile consisted of 10 min at 95°C for enzyme activation, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. The cycle counts were normalized against

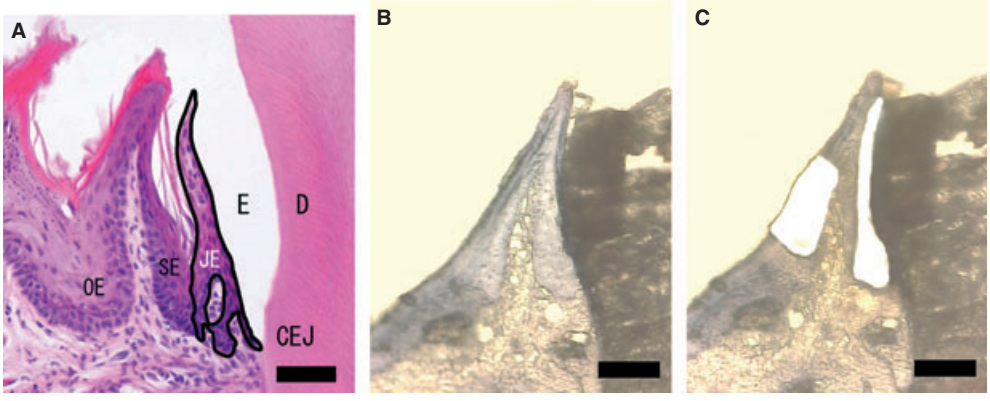

Fig. 1. Preparation of the sampling areas of gingival tissues. (A) Histology of the mouse gingival tissues. CEJ, cemento–enamel junction; D, dentin; E, decalcified enamel space; JE, junctional epithelium; OE, oral gingival epithelium; SE, sulcular epithelium. (B) Frozen section prepared for laser microdissection. (C) Section after laser microdissection. Scale bar = 50 μm.

the cycle count for GAPDH for each sample to normalize the relative level of expression. Three technical replicates per sample and gene were performed, and the mean value was calculated. The relative gene-expression levels were calculated using the comparative ΔCt method. The results were expressed as mean ± standard deviation.

Statistical analysis

Data analysis was performed using statistical software (SPSS, Tokyo, Japan). Statistical analysis was performed using the Student's two-tailed unpaired *t*-test for comparison between the two groups. The level of significance was defined as $p < 0.05$ or $p < 0.01$.

Results

Estimation of conventionalization of germ-free mice

To determine the conventionalization of the germ-free mice under the 8-wk period of conventional breeding, oral effusion samples were cultured using a thioglycolate test and the results indicated the growth of bacteria. No apparent growth of bacteria was detected in the samples of primary germ-free mice (data not shown).

Histological changes of the junctional epithelium upon bacterial infection

The junctional epithelium in the germ-free and conventional mice showed a nonkeratinized epithelium with wide spaces between the cells (Fig. 2A and 2B). Morphometrical study indicated a significant increase of the junctional epithelium area by the bacterial infection (Fig. 2A, 2B and 2E), which might be a result of the increase in the number of epithelial cells in the junctional epithelium of the conventional mice. The accumulation of inflammatory cells beneath the junctional epithelium was not detected in germ-free or conventional mice. Furthermore, no apparent apical migration of the junctional epithelium was detected in conventional mice.

The PCNA-positive cells were located in the apical portion of the junctional epithelium in the germ-free mice (Fig. 2C). The PCNA-positive cells were also located in the basal layer of the gingival outer epithelium and in the sulcular epithelium (Fig. 2C). In conventional mice, the PCNA-positive cells were distributed within the junctional epithelium and localized in the basal layer of the gingival outer epithelium and the sulcular epithelium (Fig. 2D). The number of PCNA-positive cells per unit area in the junctional epithelium was not significantly different between the two groups (Fig. 2F).

Localization and number of neutrophils in the junctional epithelium

Gr-1-positive cells were localized within the junctional epithelium and in the subepithelial connective tissue in the germ-free mice (Fig. 3A). The same localization of Gr-1-positive cells was detected in conventional mice (Fig. 3B). However, the number of these cells was significantly increased by the bacterial infection (Fig. 3B and 3C). Furthermore, the Gr-1-positive cells showed a unique localization within the junctional epithelium: these cells were located in the layers peripheral to the sulcular epithelium and to the enamel (Fig. 3B).

Expression of cytokine and chemokine mRNAs in junctional epithelium

In germ-free mice, quantitative RT-PCR was utilized to determine the basal expression level of inflammatory cytokines and chemokines in the junctional epithelium. The junctional epithelium in germ-free mice constitutively expressed *KC*, *Mip2*, *Tnf*α and *Il-1*β mRNAs. The expression of these mRNAs was always higher in the junctional epithelium than in the outer epithelium (Fig. 4). In junctional epithelium, The *KC* and *Mip2* mRNAs were significantly up-regulated by the bacterial infection, whereas no significant differences were detected in the expression of *Tnf*α and *Il-1*β mRNAs between germ-free and conventional mice (Fig. 5).

Expression of MIP-2 in the junctional epithelium

Intense immunoreactivity of MIP-2 was detected in the junctional epithelium but not in the oral epithelium of the germ-free mice (Fig. 6A), which agreed with the results of the mRNA expression analysis (Fig. 4). MIP-2 immunoreactivity was detected in the cytoplasm of junctional epithelium cells (Fig. 6A). In conventional mice, the immunoreactivity of MIP-2 was also restricted to the junctional epithelium cells (Fig. 6B). No expression of MIP-2 was observed in the other epithelium (Fig. 6B). These expression patterns were the same in germ-free and conventional mice, whereas the expression of MIP-2 was increased in the conventional mice (Fig. 6A and 2B).

Discussion

Periodontal diseases are characterized by the elicitation of chronic inflammation, which is induced by pathogenic bacteria in the subgingival plaque, resulting in soft-tissue destruction and alveolar bone loss. The junctional epithelium plays a critical role in the host defense against bacterial invasion in this disease.

It has been indicated that the structural features, such as the types of intercellular junctions and cell-adhesion molecules, in the junctional epithelium are different from those of other oral epithelia (2,19,23). However, the physiological function of this difference has not been fully clarified.

Laser capture microdissection and RT-PCR analysis indicated that all factors examined in this study were more highly expressed in the junctional epithelium than in the oral gingival epithelium of germ-free mice. The commensal microflora plays a critical role in postnatal development of the

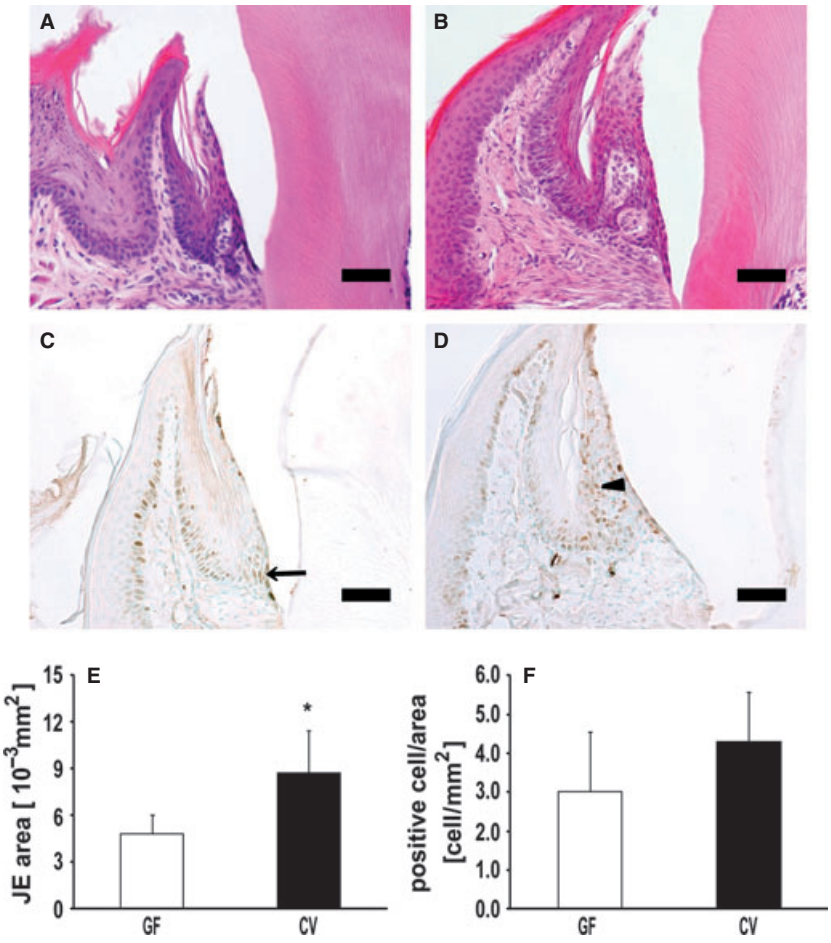

Fig. 2. Histological, immunohistochemical and histomorphometrical examination of gingival tissues. (A) Gingival tissue of germ-free mice. (B) Gingival tissue of conventional mice. Hematoxylin and eosin-stained sections showed the increased junctional epithelium area in conventional mice. (C) In germ-free mice, PCNA-positive cells of the junctional epithelium were located in the apical portion (arrow). (D) In conventional mice, PCNA-positive cells were located throughout the junctional epithelium (arrowhead) and were present in higher numbers than in germ-free mice. (E) The area of the junctional epithelium was significantly increased in conventional mice compared with germ-free mice ($p < 0.01$). The area of the junctional epithelium was the average of 15 different sections in each group. The results are expressed as mean ± standard deviation. (F) The number of PCNA-positive cells per unit area (the number of PCNA-positive cells/the junctional epithelium area) was calculated. The number of cells per unit area was the average of 15 different sections in each group. The results are expressed as mean ± standard deviation. The number of PCNA-positive cells per unit area showed a tendency to increase, whereas there was no significant difference between the two groups in this study. CV, conventional mice; GF, germ-free mice; JE, junctional epithelium. Scale bar = 50 μm.

system, as the physiologically occurring inflammatory response in the gut during early postnatal ontogeny is essential for the development of the immune system and for its appropriate functioning (24). An interaction between commensal bacteria and the innate defense system of the periodontal tissue has also been reported (8). In the present study, immunohistochemistry indicated the migration of neutrophils in the junctional epithelium of germ-free mice. The expression of KC and MIP-2-potent chemokines for the migration of neutrophils in mice (14,15) was also observed. However, the junctional epithelium had a low-level inflammatory response to which there was no bacterial challenge, in contrast to the epithelial tissue from other oral mucosa. Hayashi *et al.* (2010) observed the increased expression of secretory leukocyte protease inhibitor in junctional epithelium compared with oral gingival epithelium (20). Secretory leukocyte protease inhibitor is a single-chain serine protease inhibitor whose expression is induced by cross-talk between epithelial cells and neutrophils (25). Thus, the constitutive expression of these factors and the migration of neutrophils in junctional epithelium might be induced by intrinsic genetic regulation rather than by bacterial infection, for the maintenance of the functional specificity of junctional epithelium.

Histological examination indicated an increase in the area of the junctional epithelium in conventional mice. Immunohistochemistry also indicated an increase in the number of PCNA-positive epithelial cells within the junctional epithelium upon bacterial infection. Celenligil-Nazliel *et al.* (2000) evaluated the relationship between epithelial cell proliferation and inflammation in clinically healthy

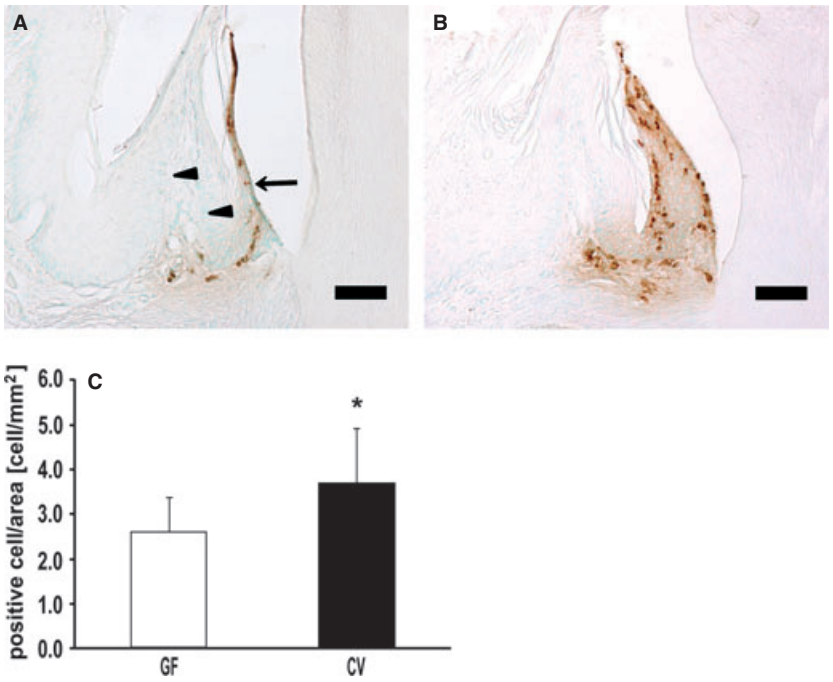

Fig. 3. Immunohistochemical staining of Gr-1-positive cells in the gingival tissue. (A) In germ-free mice, Gr-1-positive cells were localized in the junctional epithelium (arrow) and subepithelial connective tissue, but were not found in the sulcular epithelium or the oral gingival epithelium (arrowheads). (B) In conventional mice, many immunopositive cells were observed in the junctional epithelium compared with the junctional epithelium of germ-free mice. (C) The number of Gr-1-positive cells per unit area (the number of Gr-1-positive cells/the area of the junctional epithelium) of conventional mice was significantly higher than that of germ-free mice ($p < 0.01$). The number of cells per unit area was the average of 15 different sections in each group. The results were expressed as mean ± standard deviation. Scale bar = 50 μm.

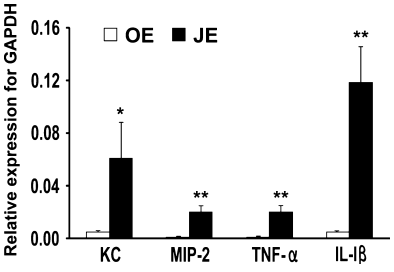

Fig. 4. The gene-expression profile of chemokines and cytokines in the junctional epithelium (JE) and in the oral gingival epithelium (OE) of germ-free mice. The expression values were normalized relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) value. Keratinocyte-derived chemokine (KC), macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were all expressed at significantly higher levels in the junctional epithelium than in the oral gingival epithelium. *$p < 0.05$ and **$p < 0.01$.

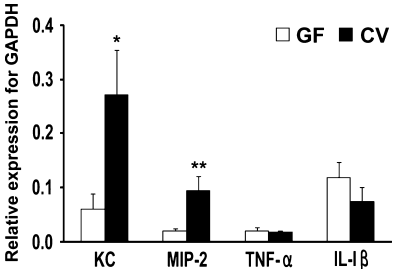

Fig. 5. Comparison of the gene-expression profile of chemokines and cytokines in the junctional epithelium between the germ-free mice (GF) and the conventional mice (CV). The expression values were normalized relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) value. The keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2) mRNAs were significantly up-regulated by the bacterial infection, whereas no significant differences were detected in the expression of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) mRNAs between germ-free and conventional mice. *$p < 0.05$ and **$p < 0.01$.

and inflamed human gingival tissue and found that epithelial cell proliferation and epithelial thickness were associated with gingival inflammation (26). The present study demonstrated that the number of neutrophils and the expression of chemokines (KC and MIP-2) in the junctional epithelium were significantly higher in the conventionalized mice than in the germ-free mice. Similarly, the augmentation of chemokines was induced by bacterial lipopolysaccharide (17,18). Thus, commensal bacteria might be an important factor in the up-regulation of the physiological barrier function of the junctional epithelium.

Intercellular adhesion molecule-1 (ICAM-1) is a critical adhesion molecule for the migration of neutrophils. ICAM-1 is constitutively expressed on the epithelial cells of the junctional epithelium (12,27). Previous studies in a germ-free animal model demonstrated increased expression of carcinoembryonic antigen-related cell adhesion

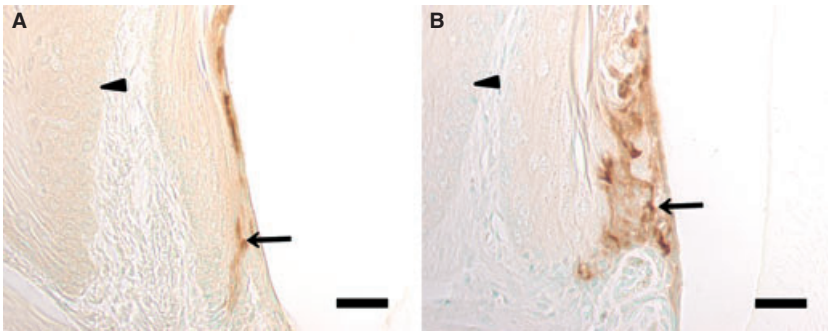

Fig. 6. Macrophage inflammatory protein-2 (MIP-2) expression in the gingival tissues. (A) Germ-free mice. (B) Conventional mice. Intense immunoreactivity for MIP-2 was detected throughout the junctional epithelium (arrows). No expression of MIP-2 was observed in the oral gingival epithelium (arrowheads). These expression patterns were same in both germ-free and conventionalized mice. MIP-2 expression was increased in conventional mice. Scale bar = 25 μm.

molecule 1, a transmembrane cell-adhesion molecule, within the junctional epithelium (10,19). Our previous study indicated the expression of ICAM-1 in the junctional epithelium but not in the oral gingival epithelium of germ-free mice (12). The expression of ICAM-1 in epithelial cells is regulated by proinflammatory cytokines such as IL-1 and TNF-α (28,29). Our study indicated the constitutive expression of these molecules in the junctional epithelium of germ-free mice, whereas the oral sulcular epithelium showed almost no expression of these molecules. Therefore, the expression of ICAM-1 induced by IL-1β and TNF-α in the junctional epithelium might be regulated in an autocrine manner within the junctional epithelium.

It is generally accepted that the expression of IL-1 and TNF-α might be induced by bacterial infection (30,31). Agents such as lipopolysaccharide interact with toll-like receptors expressed on periodontal epithelial cells (32,33), which leads to periodontal disease. The epithelial cells of the mucosal surface, in addition to being able to form a mechanical barrier, can provide signals that are essential for the initiation and amplification of inflammatory responses during the course of bacterial infections. The production of proinflammatory cytokines by gingival epithelial cells may be critical for periodontal protection against bacterial infections.

It has been reported that human gingival epithelial cells induce expression of IL-1β and IL-8 in response to periodontal pathogenic bacteria such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (34,35). An *in vitro* study indicated the induction of IL-8 expression by commensal bacteria in human gingival epithelial cells, whereas there were no significant effects on other cytokines (36). Our study indicates no significant difference in the expression of these molecules in the junctional epithelium between germ-free and conventional mice. The histological examination also indicated no sign of excessive inflammation in the periodontal tissue. Although the bacteria in the oral cavity of the conventional mice in this study were not identified, the results suggest that the commensal bacteria in this study may not elicit an excessive inflammatory response accompanied by tissue destruction in the junctional epithelium.

Recent studies indicated the constitutive expression of follicular dendritic cell-secreted protein (FDC-SP) and odontogenic ameloblast-associated protein in the junctional epithelium (36–38). The genes encoding FDC-SP and odontogenic ameloblast-associated protein appose at the secretory calcium-binding phosphoprotein cluster (39,40). Secretory calcium-binding phosphoproteins interact with several bioactive molecules to regulate cell adhesion, migration, proliferation and tumorigenesis (41,42). Furthermore, the FDC-SP gene lies adjacent to the cluster of C-X-C chemokines (39). These results lead to speculation that the constitutive expression of proinflammatory cytokines and chemokines might be closely associated with the expression of FDC-SP and odontogenic ameloblast-associated protein.

In conclusion, the present study provides evidence that the junctional epithelium expresses high levels of inflammatory mediators and undergoes neutrophil infiltration that is not associated with the host–parasite interaction. These phenomena are enhanced by minimal inflammation induced by commensal bacteria. Homeostatic inflammation in the junctional epithelium might be important for the maintenance of the junctional epithelium and protect against bacterial infection as the front-line defense system.

Acknowledgements

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