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Comprehensive analysis of gene expression in the junctional epithelium by laser microdissection and microarray analysis

Y. Hayashi[1,2], T. Matsunaga[2], G. Yamamoto[2], K. Nishii[1,2], M. Usui[1], M. Yamamoto[1], T. Tachikawa[2]

Departments of [1]Periodontology and [2]Oral Pathology and Diagnosis, Showa University School of Dentistry, Ohta-ku, Tokyo, Japan

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Background and Objective: The junctional epithelium attaches to the tooth enamel at the dentogingival junction. The attachment mechanisms of the junctional epithelium have been studied histologically, but the molecular functions of the junctional epithelium have not been elucidated. The aim of this study was to perform a comprehensive analysis of gene expression in the junctional epithelium and to search for specific genetic markers of the junctional epithelium.

Material and Methods: A comprehensive analysis of genes expressed in the mouse junctional epithelium and oral gingival epithelium was performed using laser microdissection and microarray analysis. To extract high-quality RNA from these tissues, we made frozen sections using a modified film method. Confirmation of the differential expression of selected genes was performed by quantitative real-time PCR and immunohistochemistry.

Results: The modified method produced RNA of sufficient quality for microarray analysis. The result of microarray analysis showed that 841 genes were up-regulated in the junctional epithelium compared with the oral gingival epithelium, and five were increased more than 50-fold in the junctional epithelium. These five genes were secretory leukocyte protease inhibitor (*Slpi*), keratin 17 (*Krt17*), annexin A1 (*Anxa1*), myosin light peptide 6 (*Myl6*) and endoplasmic reticulum protein 29 (*Erp29*). In particular, *Slpi* expression in the junctional epithelium was approximately 100-fold higher than in the oral gingival epithelium by real-time PCR. Additionally, immunohistochemistry indicated that the Slpi protein is highly expressed in the junctional epithelium.

Conclusion: We developed a method for generating fresh-frozen tissue sections suitable for extraction of good-quality RNA. We determined that *Slpi* is characteristically expressed in the junctional epithelium. Our results provide a substantial advance in the analysis of gene expression in the junctional epithelium.

Gou Yamamoto, DDS, PhD, Department of Oral Pathology and Diagnosis, Showa University School of Dentistry, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan
Tel: +81 3 3784 8169
Fax: +81 3 3784 2870
e-mail: gd0021@dent.showa-u.ac.jp

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The epithelium adjacent to a tooth is classified into three types: the oral gingival epithelium, the oral sulcular epithelium and the junctional epithelium (1). The junctional epithelium is a unique epithelium located at a strategically important interface between the gingival sulcus and the underlying soft connective tissues of the periodontium (2). This epithelium contains a non-keratinizing epithelial layer at the free surface and has wider intercellular spaces than other oral mucosal epithelia. These structures may thereby provide a pathway for fluid and transmigrating leukocytes from the gingival connective tissue to the gingival sulcus (3,4), and even for micro-organisms moving in the opposite direction (5,6). Furthermore, recent research suggests that the junctional epithelium is a critical structure contributing to periodontal host defenses against infection (7). However, the particular functions and specific markers of the junctional epithelium have not been identified.

To clarify the function and identify specific markers of the junctional epithelium, some researchers have studied gene expression in the junctional epithelium. Immunohistochemical analysis has shown that cellular adhesion-related genes, such as genes coding for integrin subunits α6β4 (4,8), α2β1, α3β1 and α6β1 (4), laminin-5 (9–11) and intercellular adhesion molecule-1 (12,13), are expressed in the junctional epithelium. However, since these genes are also expressed in other tissues, they may not be thought to be useful as specific markers of the junctional epithelium. Therefore, we performed a comprehensive analysis of the junctional epithelium with laser microdissection (LMD; 14,15) and microarray analysis to investigate genes differentially expressed in the junctional epithelium.

Microarray analysis requires a large amount of highly pure RNA. Although the periodontal tissues, including the junctional epithelium and tooth, can be chemically fixed and demineralized to cut sections, the chemicals may cause degradation and denaturation of the RNA in the sample. Recently, an adhesive film method has been developed (16), in which tissues are rapidly fresh-frozen, freeze-dried and cut into thin sections. These sections can be used directly in enzyme chemistry, immunohistochemistry and *in situ* hybridization (17). In the present study, we modified this method to use a special thin film so that we were able to collect large amounts of high-purity RNA from fresh-frozen sections.

The purpose of the present study was to investigate the characteristic gene expression profile for junctional epithelium with suitable methods. As a result, we were able to clarify an important factor to be related with special immune system in junctional epithelium.

Material and methods

Samples

Female, 28-d-old, conventional and germ-free Institute of Cancer Research mice (Clea, Tokyo, Japan) were used. Germ-free and conventional mice were used for immunohistochemical study, and other experiments were performed using conventional mice. The mice were killed by diethyl ether anesthesia. Immediately after killing, the whole heads of the animals were removed and the nasal part dissected for tissue processing. This study was approved by the Animal Research Committee of Showa University (#18088).

Preparation of frozen fixed samples

Dissected tissues were perpendicularly embedded in OCT compound (Sakura, Torrance, CA, USA) from the pharyngeal side and then immediately fast-frozen in isopentane cooled in liquid nitrogen. The specimens were then made into frozen blocks and stored at −80°C. Frozen samples prepared for LMD were sliced using a cryomicrotome (Microm, Woodstock, CT, USA) at 16 μm thickness (Fig. 1), and each tissue section was affixed to a slide to which an original thin film (Meiwafosis, Osaka, Japan) had been attached with silicon adhesive (GE Toshiba Silicone, Tokyo, Japan). Sliced samples were stored at −40°C. Our original film has two major advantages. One is that the film can be sterilized by dry heat to remove RNase because it is resistant to heat. The other is that there are few damages and contaminations because the film can be used directly as a film for LMD.

Laser microdissection

For total RNA extraction using LMD, the frozen sections were placed at room temperature for 2–3 min and fixed in zinc-fix (18) for 3 min. The frozen sections were stained with an LCM Staining Kit (Ambion, Austin, TX, USA) with RNase-free water. After

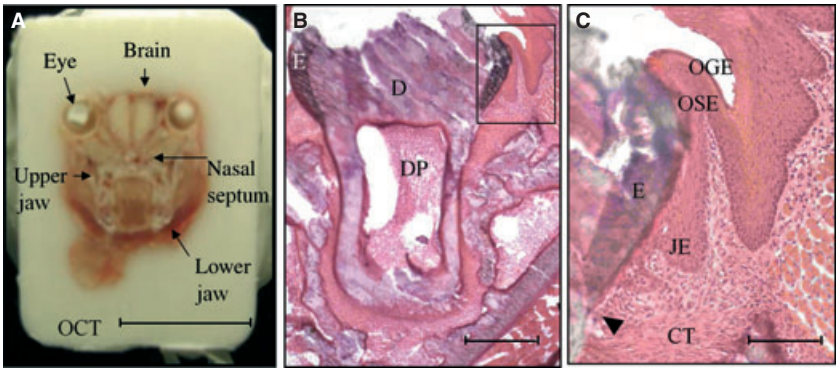

Fig. 1. Preparation of frozen, fixed samples. (A) The whole head of an Institute of Cancer Research mouse was removed and dissected for tissue processing. (B) Frozen samples prepared for laser microdissection were sliced using a cryomicrotome at 16 μm thickness. (C) The anatomical relationship between the hard tissue (tooth enamel) and the soft tissue (junctional epithelium, JE) was preserved. Arrowhead indicates the cemento-enamel junction. Abbreviations: CT, connective tissue; D, dentin; DP, dental pulp; E, enamel; OCT, OCT compound; OGE, oral gingival epithelium; and OSE, sulcular epithelium. Scale bar indicates A, 100 mm; B, 300 μm; C, 150 μm.

air-drying, the sections were microdissected with a PALM Micro Beam (PALM, Bernried, Germany) using a 337 nm nitrogen laser. The junctional epithelium and the oral gingival epithelium were dissected as target areas (Fig. 2). In each sample, the microdissected area was estimated to be approximately 0.5–0.6 mm^2 (about 20,000 μm^2 per sample).

Extraction of total RNA and quality control

Total RNA was extracted from the junctional epithelium and the oral gingival epithelium using an RNeasy Micro Kit (Qiagen, Valencia, CA, USA) with complete removal of genomic DNA by RNase-free DNase I treatment (Qiagen). The RNA quality and size distribution were analyzed using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies, Carlsbad, CA, USA). The RNA concentration of each sample was 479 (oral gingival epithelium) and 1189 pg/μl (junctional epithelium). Total RNA sample volume was 12 μl.

In vitro transcription and RNA amplification

To study the global gene expression profiles of the junctional epithelium and the oral gingival epithelium, RNA amplification was necessary. In this study, the Ramp Up plus kit (Genisphere, Hatfield, PA, USA) was used. To produce sufficient amplified RNA (cRNA) from 2 ng total RNA input, two rounds of RNA amplification were performed. All the procedures were carried out according to the manufacturer's instructions. After RNA amplification, the cRNA from the junctional epithelium and the oral gingival epithelium was labeled with Cy3, and purified using RNeasy MinElute spin columns (Qiagen).

Hybridization and slide processing

We used the Agilent 44 K mouse 60-mer oligo microarray (Agilent Technologies), which contains more than 40,000 unique, well-characterized gene/expressed sequence tag features enriched with a comprehensive set of toxicity markers. All the procedures for hybridization and slide and image processing were carried out according to the manufacturer's instructions (for the Agilent 60-mer oligomicroarray processing protocol, see http://www.agilent.com). Three microgram aliquots of contrasting cRNA samples were fragmented and hybridized onto the mouse 44 K oligo microarray slides at 60°C for 17 h. The slides were then sequentially washed, dried and scanned using an Agilent DNA microarray scanner with SureScan technology (Agilent Technologies). Replica experiments were performed with independent hybridization and processing.

Scanning and analysis of results

Feature Extraction 9.5 software (Agilent Technologies) was used with the default analysis parameters for the initial extraction, signal quantification and scaling of the data. The data were analyzed with Genespring7.3 software (Agilent Technologies).

Real-time semi-quantitative PCR

Total RNA was extracted from each population of laser-microdissected cells using the RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription was carried out in 20 μl volumes using a High Capacity RNA to cDNA MasterMix (Applied Biosystems, Carlsbad, CA, USA).

Polymerase chain reactions were performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems), and the analysis was carried out using the sequence detection software supplied with the instrument. Each reaction mixture contained 15 μl TaqMan Universal PCR Master Mix

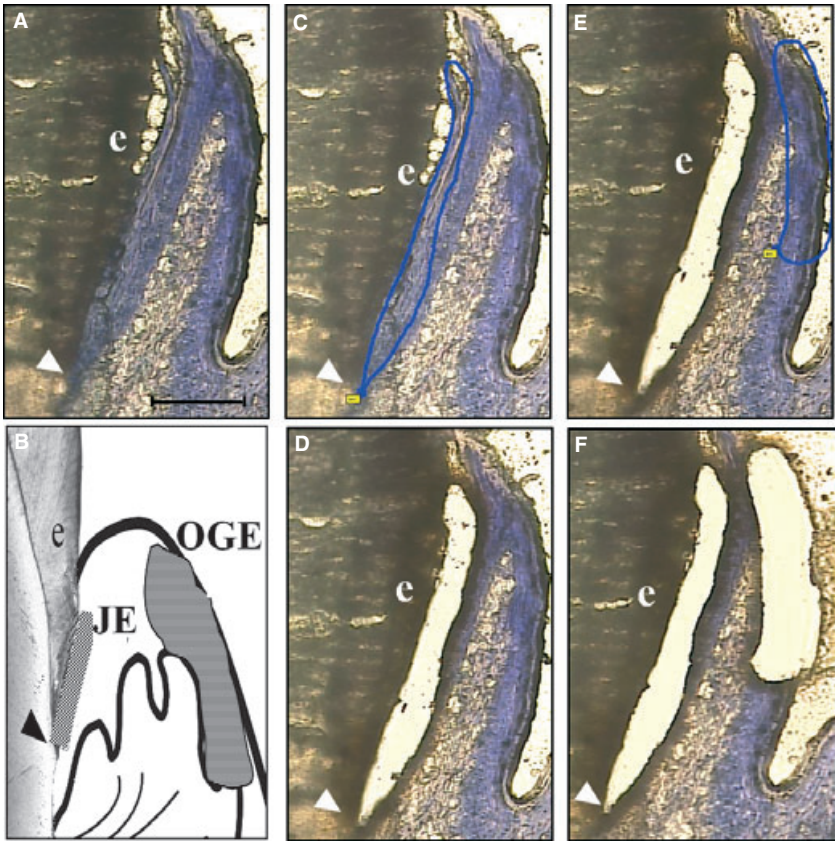

Fig. 2. Cells collected from mouse gingival tissue by laser microdissection. (A,B) Areas of collection. (C,D) Cells of the junctional epithelium. (E,F Cells of the oral gingival epithelium. (C,E) Before laser microdissection. (D,F) After microdissection. Arrowheads indicate the cemento-enamel junction. Abbreviations: e, enamel; JE, junctional epithelium; and OGE, oral gingival epithelium. Scale bar indicates 150 μm.

(Applied Biosystems), 1 μl 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. The primers were positioned to span exon–intron boundaries, reducing the risk of detecting genomic DNA. Each PCR consisted of 10 min at 95°C for enzyme activation, followed by 50 cycles of a denaturation step at 95°C for 15 s and an annealing/extension step at 60°C for 1 min. A negative control (RNase-free water substituted for template cDNA) was included to control for DNA contamination. The housekeeping gene glyceradehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. The calibration standard curve was set up using serial dilutions (1–0.0001) of known quantities of mRNA from a control sample. In addition, a melting curve analysis was performed to ensure the purity and specificity of the amplified PCR product. The expression values of secretory leukocyte protease inhibitor (*Slpi*) and integrin subunit α6 (*Itga6*) were normalized against the *GAPDH* value for each sample to normalize relative levels of expression. Each sample was run in triplicate (the dissections and RT-PCR reactions were all repeated three times for each target area). The mean of the triplicates was used in the semi-quantitative analysis.

Immunohistochemical staining and antibodies

Anti-SLPI (M-110; Santa Cruz Biotechnology, Santa Cruz, CA, USA) is a rabbit polyclonal antibody raised against amino acids 15–124 within a region of mouse Slpi. The fresh-frozen sections were cut using a cryomicrotome (Microm) at 16 μm thickness. The fresh-frozen sections were air-dried for 10 min and fixed with 4% paraformaldehyde for 5 min. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol solution for 10 min. Samples were preincubated with blocking solution (Dako, Tokyo, Japan) for 15 min to block non-specific binding, and then incubated with primary antibody (1:300 dilution) for 1 h at room temperature. Then, the polymer reagent of the Dako ENVISION System (Dako) was applied for 30 min. Thereafter, samples were incubated with a diaminobenzidine substrate kit (Dako) according to the manufacturer's protocol. The sections were rinsed in water, counterstained with hematoxylin, again rinsed in water and mounted. A negative control was performed by replacing the antibody with normal rabbit serum. Slides were then observed by conventional light microscopy.

Results

Quality control of RNA isolated by LMD

To confirm the purity of total RNA from the junctional epithelium and oral gingival epithelium extracted by LMD, we performed an RNA quality assay. We could observe the peaks corresponding to the 18S and 28S RNAs (Fig. 3). This suggested that total RNA from both the junctional epithelium and the oral gingival epithelium were of sufficiently high quality for microarray analysis.

Highly expressed genes in the junctional epithelium

Of the 41,534 probe sets arrayed on the Whole Mouse Genome Oligo DNA Microarray, there were 841 genes upregulated in the junctional epithelium compared with the oral gingival epithelium (data not shown). Table 1 shows the five most highly expressed genes in the junctional epithelium, all of which were increased more than 50-fold compared with the oral gingival epithelium. Although *Slpi* (19) and the genes encoding keratin 17 (*Krt17*; 20) and annexin A1 (*Anxa1*; 21) had previously been identified in cells from gingival epithelium, these reports had not shown that these genes were preferentially expressed in the junctional epithelium. Genes encoding myosin light peptide 6 (*Myl6*) and endoplasmic reticulum protein 29 (*Erp29*) had not been previously reported oral gingival epithelium and junctional epithelium. We focused on *Slpi*, the most highly expressed gene within the junctional epithelium, which is a one of the serine protease inhibitors (Table 1).

Real-time PCR analysis

To confirm the validity of the microarray results, real-time quantitative RT-PCR was performed with a set of

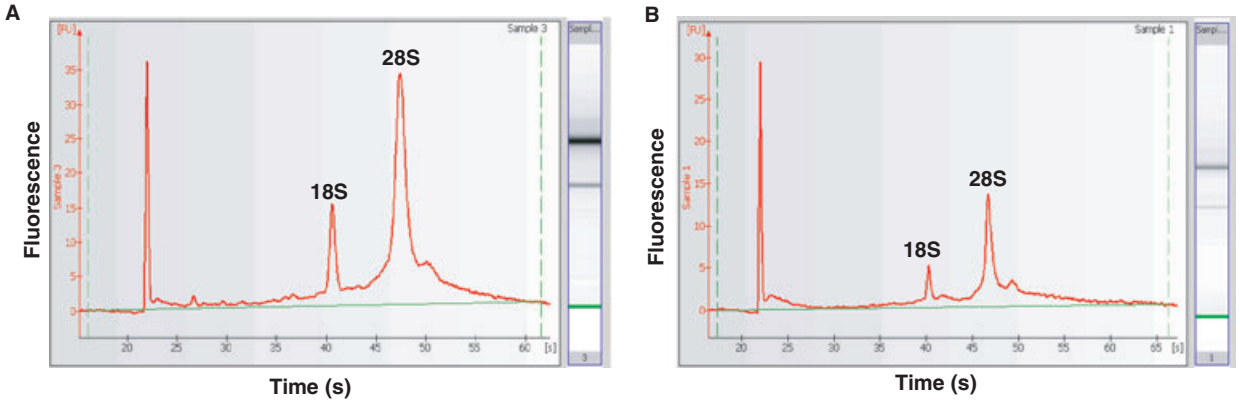

Fig. 3. Quality of total RNA isolated from mouse gingival tissue. (A) Junctional epithelium. (B) Oral gingival epithelium. The RNA quality was measured using an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit.

Table 1. Genes differentially upregulated in junctional epithelium compared with oral epithelium

Reference sequence	Gene symbol	Gene name	Fold change
NM_011414	*Slpi*	Secretory leukocyte protease inhibitor	107.6
NM_010860	*Myl6*	Myosin, light polypeptide 6	104.3
NM_010663	*Krt-17*	Keratin complex 1, acidic, gene 17	93.57
NM_026129	*Erp29*	Endoplasmic reticulum protein	77.97
NM_010730	*Anxa1*	Annexin A1	60.13

mouse-specific primers and template cDNA generated by reverse-transcription PCR. Similar to the microarray results, the level of *Slpi* expression in the junctional epithelium was about 30-fold higher than in the oral gingival epithelium (Fig. 4). In this analysis, we used *Itga6* as a positive control, which has been previously reported to be expressed in the junctional epithelium (8). The level of *Itga6* expression in the junctional epithelium was also significantly higher than in the oral gingival epithelium (Fig. 4). These data suggested that *Slpi* could be a characteristic marker of the junctional epithelium.

Immunohistochemistry

To confirm the expression of the Slpi protein within the junctional epithelium, we performed immunostaining of the gingival epithelium, including the junctional epithelium and oral gingival epithelium, in the conventional mouse. As shown in Fig. 5A,B, Slpi-positive cells were found along the enamel in the region known anatomically as the junctional epithelium. In contrast, we found few Slpi-positive reactions in the oral gingival epithelium, the external surface of the gingival epithelium (Fig. 5C). The positive staining in the keratinized layer may be regarded as misreaction of Slpi antibody. The expression of Slpi is increased by local protection against microbial, fungal and HIV-1 insults (22). Expression of Slpi in the junctional epithelium of the conventional mouse is possibly determined by genetic factors or influenced by the normal and pathological bacterial flora that occurs in this region. We examined the expression of Slpi in the gingival epithelium of the germ-free mouse to answer this question. Intriguingly, Slpi protein expression was limited in the junctional epithelium of the germ-free mouse, similar to the conventional mouse, except that some positive granules were seen in the intercellular space of junctional epithelium (Fig. 5E,F). These data suggested that Slpi was characteristically expressed in the junctional epithelium.

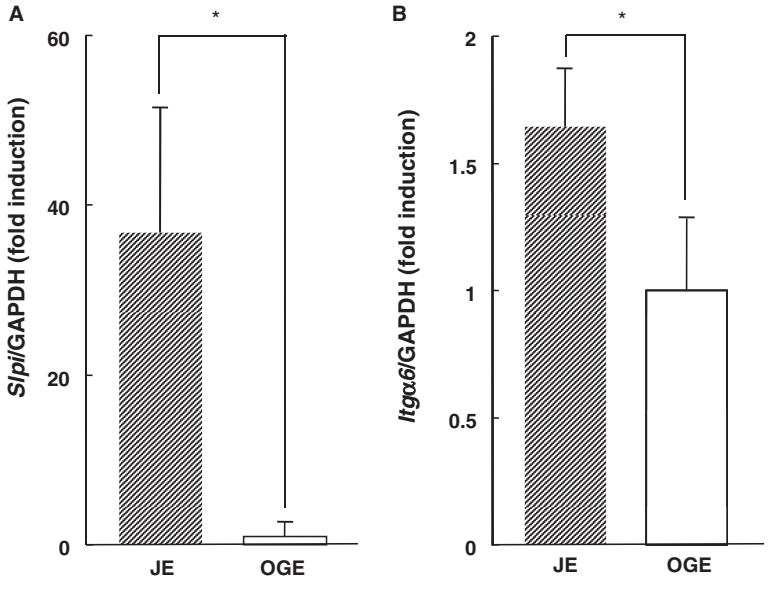

Fig. 4. Real time RT-PCR comparison of mRNA expression levels of secretory leukocyte protease inhibitor (*Slpi*; A) and integrin subunit α6 (*Itga6*; B) in the junctional epithelium (JE) and oral gingival epithelium (OGE). Expression of *Slpi* and *Itg*α6 was higher in the junctional epithelium than in the oral gingival epithelium (*$p < 0.05$).

Discussion

Previous studies of the junctional epithelium have been largely restricted to histological and morphogenetic analyses using demineralized paraffin sections. These sections, however, are not appropriate for microarray analysis using LMD, because the chemicals used in section processing denature the RNA. In contrast, fresh-frozen sections, which are useful for immunohistochemistry, *in situ* hybridization and microarray analysis using LMD, cannot preserve the morphology between the enamel and the junctional epithelium. Although Kinumatsu *et al.* (9) also extracted RNA from the junctional epithelium using LMD, they generated sections of gingival tissue without enamel. In the present study, we developed an adhesive film method for fresh-frozen sections, and report the first success in cutting sections without losing the anatomical relationship between the junctional epithelium and the enamel. We were also able to extract high-quality RNA and perform microarray analysis of the junctional epithelium. This study is the first report of microarray comparison between the junctional epithelium and the oral gingival epithelium.

Our microarray analysis showed that the genes expressed more than 50-fold higher in the junctional epithelium than the oral gingival epithelium were *Slpi*, *Myl6*, *Krt17*, *Erp29* and *Anxa1*. Although *Slpi*, *Krt17* and *Anxa1* have already been reported to be expressed in the oral gingival epithelium, *Myl6* and *Erp29* have not. ERp29 is a member of the reticuloplasmin family and is expressed in rat ameloblasts (23,24), but its function is poorly characterized. Since the junctional epithelium cells and ameloblasts are

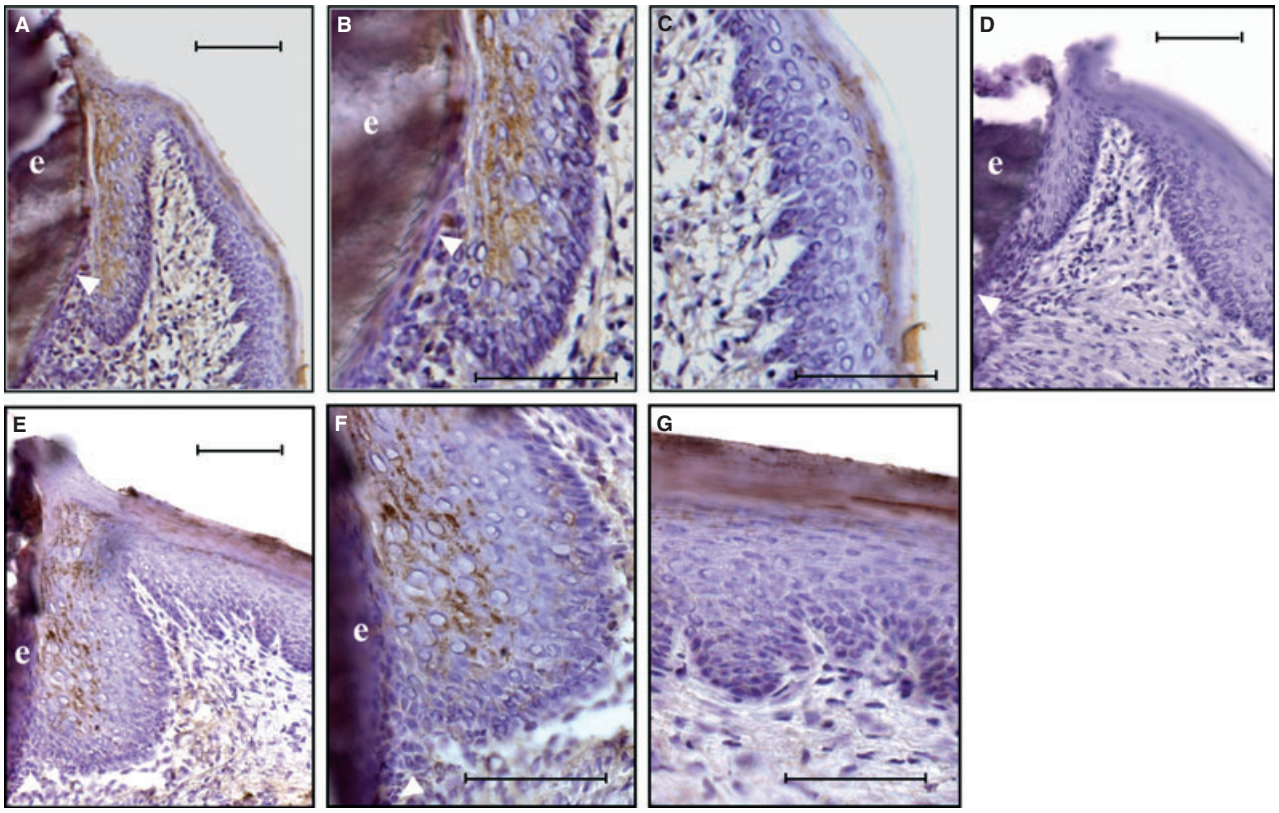

Fig. 5. Immunohistochemical localization of Slpi in a 28-d-old mouse. Sections are shown from conventional (A–C) and germ-free mice (E–G). More staining was observed in the junctional epithelium (B,F) than in the oral gingival epithelium (C,G). (D) Negative control staining. The arrowheads indicate the cemento-enamel junction; e, enamel. Scale bar indicates A, D and E, 150 μm; B, C, F and G, 75 μm.

both derived from the enamel organ, these cells may be thought to express common proteins, including Erp29. *Myl6* encodes a myosin alkali light chain, which is a component of myosin, the ATPase cellular motor protein. Recently, it has been reported that histamine-induced myosin light chain phosphorylation breaks down the barrier integrity of corneal epithelial cells (25). Myl6 may also regulate the barrier integrity of junctional epithelium cells. Further research is necessary to characterize fully the expression and function of both ERp29 and Myl6 in junctional epithelium cells.

Secretory leukocyte protease inhibitor is an 11.7 kDa (107-amino-acid), non-glycosylated, single-chain serine protease inhibitor, produced by secretory cells in the respiratory, genital and lacrimal glands, and by inflammatory cells that include macrophages, neutrophils and B cells (26,27). It is thought that Slpi protects tissue at sites of inflammation by inhibiting host protease activity. In addition, studies in Slpi-deficient mice have revealed other mechanisms by which Slpi protects the host during bacterial infection, by antagonizing bacterial toxins, such as lipopolysaccharide, suppressing production of matrix metalloproteinases and inducing the anti-inflammatory cytokine, interleukin-10. In contrast, arginine-specific gingipains from *Porphyromonas gingivalis*, which are one of the pathogenic factors in periodontal disease, directly reduce Slpi production in human gingival cells (19,28–32). In contrast, in small intestine, Slpi is reported to be positively stained in Paneth cells by immunohistochemistry (33). Paneth cells have a similar function to neutrophils and have an important role in the immune system in the lacuna of the small intestine revealed easily by a pathogen. In Paneth cells, Slpi may protect the intestinal epithelium from various kinds of protease secreted as part of the inflammatory response, and is associated with maintenance of tissue integrity. From these facts, it is expected that Slpi plays a role to protect a host in natural immunity. In this study, we found that *Slpi* mRNA was preferentially expressed in the junctional epithelium and that Slpi protein was localized in all layers of the junctional epithelium. These previous study and our data suggest that the cells of junctional epithelium, as well as inflammatory cells, are one of the sources of Slpi in periodontal pocket-infected periodontitis and have a unique characteristic that is different from other oral epithelia. In other words our results suggest that Slpi is an important factor which maintains epithelial integrity in innate immune response mechanism of junctional epithelium. Reduction of Slpi in junctional epithelium by *Porphyromonas gingivalis* (19) may be responsible for disturbance in the host protective response or acceleration of periodontal tissue destruction.

We showed that Slpi was characteristically expressed in the junctional epithelium using conventional mice (Fig. 5A–C). However, the conventional mice were exposed to microorganisms so that Slpi expression might be reactively increased in the junctional epithelium. For instance, interleukin-1β protein levels in gingival tissue are significantly higher in conventionally reared mice compared with germ-free animals (34). To eliminate that possibility, we examined Slpi protein expression in gingival epithelium of germ-free mice. Germ-free animals that are completely devoid of bacteria are delivered by sterile caesarean section and raised on aseptically sterile food, water and bedding (35). Similar to conventional mice, we could detect Slpi-positive cells in the junctional epithelium of germ-free mice, but precious few in oral gingival epithelium (Fig. 5E–G). In similar research using germ-free animals, CEACAM1, a cell adhesion molecule, was also reported to be preferentially expressed within the junctional epithelium in both conventional and germ-free rats (12). These previous study and our data suggested that the expression of these two genes is determined by genetic factors and not influenced by microbial flora and that they could be markers of the junctional epithelium. However, in our study, the junctional epithelium of germ-free mice had a tendency to show a Slpi-positive reaction in the intercellular space. To elucidate the reason for this, further study would be required.

In conclusion, we developed an adhesive film method to collect a large amount of high-quality RNA for microarray analysis using LMD. We determined that *Slpi*, a serine protease inhibitor, is preferentially expressed in the junctional epithelium. Our method provides a substantial advance in the comprehensive analysis of gene expression in the junctional epithelium.

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