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The distribution and expression of S100A8 and S100A9 in gingival epithelium of mice

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Objective and Background: Gingival epithelium protects against bacterial infection by producing antimicrobial peptides such as calprotectin. Calprotectin consists of proteins S100A8 and S100A9. Although *in vitro* assay has shown that neutrophils and gingival epithelial cells express calprotectin, the expression of S100A8 and S100A9 and colocalization of both S100 proteins in gingival tissue *in vivo* are not fully understood. The aim of this study was to investigate the distribution of S100A8 and S100A9 expression in gingival epithelium of mice in the presence and absence of infection.

Materials and Methods: A quantitative analysis of S100A8 and S100A9 mRNA in junctional epithelium (JE) and oral gingival epithelium (OGE) of both germ-free mice and conventional mice was performed using laser microdissection and real-time polymerase chain reaction (PCR). Confirmation of S100A8 and S100A9 mRNA expression in the JE was conducted by fluorescent immunohistochemistry.

Results: Real-time PCR analysis indicated that S100A8 and S100A9 expressions were mainly detected in JE and only slightly or not detected in OGE. Levels of both S100A8 and S100A9 mRNA expression in JE of conventional mice were significantly higher than those in JE of germ-free mice. Additionally, fluorescent immunohistochemistry showed that S100A8 expression was observed in the JE of both conventional and germ-free mice, whereas S100A9 was expressed in the JE of conventional but not germ-free mice.

Conclusion: S100A8 protein is expressed in JE cells of mice in the presence and in the absence of infection with oral bacteria. S100A9 expression in JE cells in the presence of microflora is significantly increased compared with the absence of microflora, which suggests that S100A9 expression may be induced by infection of microflora. The production of calprotectin in gingival epithelial cells may be mediated through S100A9 induction by bacterial infection.

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Gingival epithelium functions as a barrier against many microbes (1). In addition to physically separating the mucosal microflora from the connective tissues and circulation, oral epithelium produces innate immune-effector molecules against infecting microbes. To prevent microbial infection, oral epithelial cells protect themselves by producing antimicrobial peptides (AMPs), including calprotectin, defensin, secretory leukocyte protease inhibitor (SLPI), adrenomedullin, and cathelicidin (LL37) (2).

Calprotectin is a major cytosolic protein derived from granulocytes and detected in monocytes, macrophages, and epithelial cells. Calprotectin constitutes about 50% of granulocyte cytosolic protein and comprises two units, proteins S100A8 and S100A9 (3–6). Calprotectin is also known as one of the AMPs having zinc-chelating antimicrobial activity (6). Calprotectin inhibited the growth of *Porphyromonas gingivalis* and its adhesion to epithelial cells (7). Suryono *et al.* (8) reported that calprotectin was detected in the marginal epithelium and connective tissue from patients with periodontal disease, but not healthy people. Kido *et al.* (9) found calprotectin in human dental calculus. Furthermore, calprotectin has been detected in gingival crevicular fluid (GCF) (10). Calprotectin levels in GCF from patients with periodontitis were significantly higher than those found in healthy subjects and were positively correlated with several clinical parameters (11,12). These data suggest that calprotectin, the complex of S100A8 and S100A9 may protect against infection by periodontopathic bacteria.

S-100A8 and S100A9 belong to a family of low-molecular-weight proteins found in vertebrates and are characterized by two calcium-binding sites of the helix–loop–helix (EF-hand type) conformation (13). To date, the S100 protein family includes about 23 members, including S100A8 and S100A9 (14). S100A8 (also known as migration inhibitory factor-related protein 8 or calgranulin-A) may function in the inhibition of casein kinase and as a cytokine. Altered expression of this protein is associated with the disease cystic fibrosis (15). Protein S100A9 [migration inhibitory factor-related protein 14 (MRP-14) or calgranulin-B] complexes with protein S100A8 to regulate myeloid cell function by binding to Toll-like receptor-4 (TLR-4) and the receptor for advanced glycation end products (16). This complex (calprotectin) also broadly regulates vascular inflammation and contributes to the biological response to vascular injury by promoting leukocyte recruitment (17). Levels of calprotectin are markedly increased in plasma, feces, and synovial fluid from patients with bacterial infections and inflammatory diseases such as rheumatoid arthritis, septicemia, inflammatory bowel disease, and cystic fibrosis (5,18,19). However, there are few reports about each expression of S100A8 and S100A9 *in vivo*.

Similarly in gingival tissue, although *in vitro* assay has shown that neutrophils and gingival epithelial cells express calprotectin, the expression of S100A8 and S100A9 and colocalization of both S100 proteins in gingival tissue are not fully understood. In this study, we investigated S100A8 and S100A9 expression in gingival epithelium from both conventional and germ-free mice.

Material and methods

Samples

Six female, 28-d-old, conventional and germ-free ICR mice (CLEA Japan, Inc., Tokyo, Japan) were used. The mice were euthanized using diethyl ether anesthesia. Immediately after death, whole heads of the animals were removed, and the nasal parts were dissected for tissue processing. From each mouse, about 100 sections were made for laser microdissection, and 50 sections for immunohistochemistry were made. For laser microdissection and real-time RT-PCR analysis, junctional epithelium (JE) and oral epithelium were collected from about 50 sections. For immunohistochemical analysis, about 10 sections were stained. Figures showed staining results. This study was approved by the Animal Research Committee of Showa University (#18088).

Laser microdissection

Dissections from the pharynx side of whole mice heads were perpendicularly embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Torrance, CA, USA) 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 laser microdissection (LMD) were sliced using a cryomicrotome (−25°C; Microm, Woodstock, CT, USA) at 16-μm thickness (20). For total RNA extraction from target areas, the frozen sections were placed at room temperature for 2–3 min and fixed in zinc-fix (21) for 3 min. The frozen sections were stained with an LCM Staining Kit (Ambion, Austin, TX, USA). After air-drying, the sections were microdissected with a PALM MicroBeam (P.A.L.M., Bernried, Germany) using a 337-nm nitrogen laser. The JE and oral gingival epithelium (OGE) were dissected as target areas. In each sample, the microdissected area was estimated to be approximately 0.5–0.6 mm^2 (about 10,000 μm^2 per sample).

Real-time semiquantitative polymerase chain reaction

Total RNA was extracted from each population of laser-microdissected cells using the RNeasy Plus Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed in 20-μL volumes using a High Capacity RNA-to-cDNA MasterMix (Applied Biosystems, Carlsbad, CA, USA). Polymerase chain reaction (PCR) was performed using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems), and the analysis was performed using the sequence detection software supplied with the instrument. Each reaction mixture contained 10 μL TaqMan Gene Expression Master Mix (Applied Biosystems), 1 μL TaqMan Gene Expression Assay (Applied Biosystems), and 2 μL template cDNA supplemented with RNase-free water to a final volume of 20 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 glyceraldehyde-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 for S100A8 and S100A9 were normalized against the GAPDH value for each sample to normalize relative levels of expression (delta C_t Method). Each sample was run in triplicate (dissections and RT-PCR were all repeated three times per each target area). The mean of the triplicates was used in the semiquantitative analysis.

Immunohistochemical staining and antibodies

The mandibles from dissected whole heads were resected and fixed with 4% paraformaldehyde for 6 h and decalcified in 10% EDTA for 2 wks at 4°C. Dissected tissues were perpendicularly embedded in OCT compound and then immediately fast-frozen in isopentane cooled in liquid nitrogen. The specimens were serially sectioned at 5 μm in the buccal–lingual direction and stained with hematoxylin and eosin or were used for immunofluorescent staining. For immunofluorescent staining, frozen sections were air-dried for 10 min and, after washing with Tris-buffered saline, were preincubated with blocking solution (Dako, Tokyo, Japan) for 10 min to prevent non-specific binding. After removal of the blocking solution, the sections were incubated with an anti-S100A8 goat polyclonal antibody (1 : 1000 dilution, 0.2 μg/mL, catalog no. AF3059; R&D Systems, Minneapolis, MN, USA), an anti-S100A9 rat monoclonal antibody (1 : 300 dilution, 3.3 μg/mL, catalog no. ab105472; Abcam, Cambridge, MA, USA), and an anti–Gr-1 rat monoclonal antibody (1 : 100 dilution, 0.6 μg/mL, catalog no. 550291; BD Pharmingen, Tokyo, Japan) for 1 h at room temperature. After washing in Tris-buffered saline, the sections were incubated for 1 h at room temperature with a secondary antibody: anti-goat immunoglobulin G (IgG) conjugated with Alexa 488, anti-rabbit IgG Alexa 594, or anti-rat IgG Alexa 594 of donkey origin (1 : 100 dilution; Molecular Probes, Grand Island, NY, USA). After counterstaining with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI, 1 : 5000 dilution; Dojindo, Kumamoto, Japan), all of the specimens were examined and photographed (Nikon A1 Confocal Microscope System, Tokyo, Japan).

Evaluation of the area of junctional epithelium and the number of S100A8-, S100A9- and Gr-1-positive cells in junctional epithelium

The area of JE of the hematoxylin and eosin section was measured in μm^2 using PALM MB IV ROBOSOFTWARE 4.2 (Carl ZEISS, Tokyo, Japan). Neutrophils and gingival epithelial cells within the JE were evaluated by counting the number of S100A8-, S100A9- and Gr-1–positive cells. The results were expressed as the number of positively stained cells in JE divided by the area of JE.

Statistical analysis

All values are expressed as mean ± standard deviation (SD). The statistical significance of differences between groups was analyzed by the Mann–Whitney U test. $p < 0.05$ was considered statistically significant.

Results

S100A8 and S100A9 mRNA expression in gingival tissue

To investigate expression of S100A8 and S100A9 in gingival tissue, we performed LMD and real-time PCR analysis. We examined conventional and germ-free mice because the existence of resident microbiota may change expression of AMPs. The gingival epithelia are classified according to three types: OGE, oral sulcular epithelium (OSE), and JE. We extracted RNA from JE and OGE using LMD and compared expression of S100A8 and S100A9 in JE and OGE from both conventional and germ-free mice. To our surprise, both S100A8 and S100A9 were mainly detected in JE and only slightly or not detected in OGE. Levels of both S100A8 and S100A9 mRNA in JE of conventional mice were higher than in JE of germ-free mice (Fig. 1).

S100A8 and S100A9 expression in gingival epithelium of germ-free mice by fluorescence immunohistochemistry

To confirm expression of S100A8 and S100A9 mRNA, we performed fluorescence immunohistochemistry analysis and counted S100A8-,

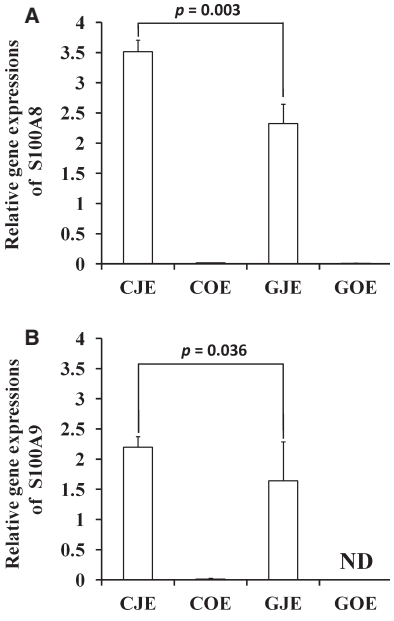

Fig. 1. Real-time reverse transcriptase-polymerase chain reaction comparison of mRNA expression of S100A8 and S100A9 in junctional epithelium (JE) and oral gingival epithelium of conventional and germ-free mice. CJE, JE of conventional mice; GJE, JE of germ-free mice; COE, oral epithelium of conventional mice; GOE, epithelium of germ-free mice; ND; not detectable. *$p < 0.05$.

S100A9- and Gr-1–positive cells per area of JE. In germ-free mice, S100A8 was strongly and differentially expressed in the edges of JE (Fig. 2A, 2D and 2H). Because neutrophils exist in JE and express both S100A8 and S100A9, we double-immunostained using Gr-1 antibody, a marker of neutrophils to distinguish between neutrophils and GECs. Surprisingly, most S100A8-positive cells in JE were not consistent with Gr-1–positive cells (Fig. 2B and 2C). Furthermore, to investigate for colocalization of S100A8 and S100A9, we immunostained gingival epithelium of germ-free mice using both S100 antibodies. In contrast with mRNA data, there were very few S100A9-positive gingival epithelial cells in JE of germ-free mice (Fig. 2E and 2H). Contrary to our expectations, most S100A8-positive gingival epithelial cells did not express S100A9 (Fig. 2D –2F and 2H). No immunoreactions was detected without primary antibodies (data not shown). These data suggested that gingival epithelial cells of JE in germ-free mice expressed S100A8 independently of S100A9 and that calprotectin, which is a heterodimer of S100A8 and S100A9, was not expressed in gingival epithelium of germ-free mice.

S100A8 and S100A9 expression in gingival epithelium of conventional mice by fluorescence immunohistochemistry

Similar to results found in germ-free mice, S100A8 expression was detected in the edges of JE of conventional mice (Fig. 3A, 3D and 3H). To distinguish the expression of S100A8 between gingival epithelial cells and neutrophils, we also immunostained with Gr-1 antibody and S100A8 antibody. Although Gr-1–positive neutrophils also expressed S100A8, S100A8-positive cells in the edges of JE did not express Gr-1 (Fig. 3B and

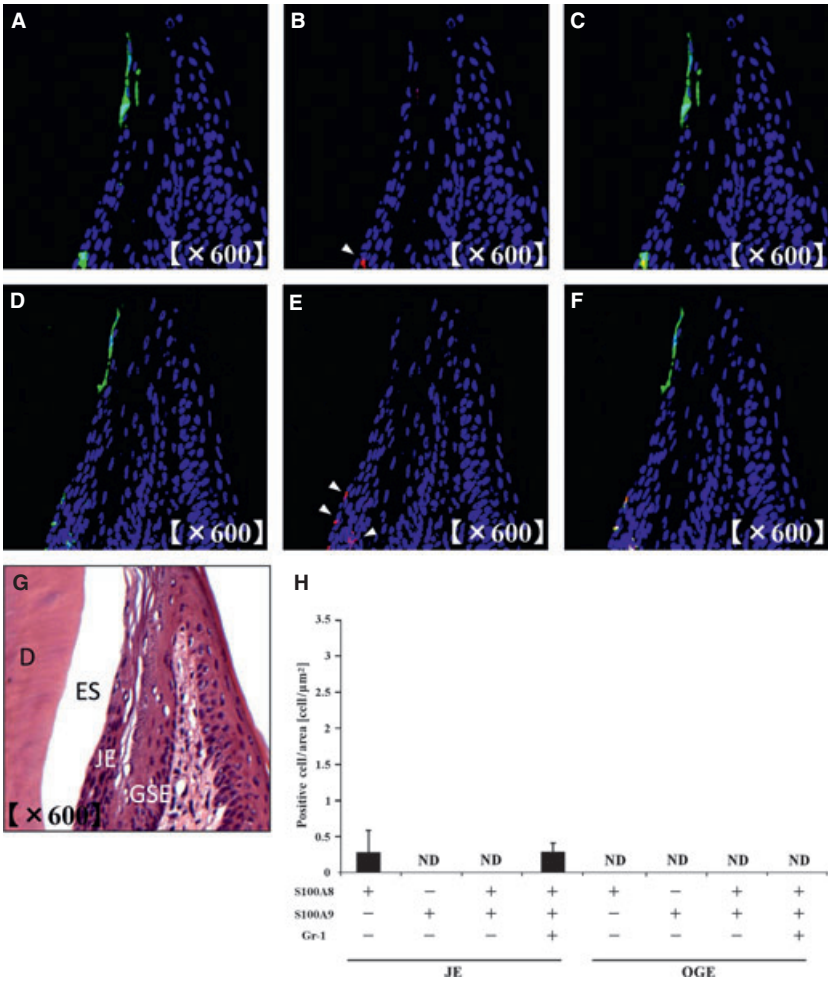

Fig. 2. Immunohistochemical localization of S100A8, S100A9, and Gr-1 in junctional epithelium (JE) of germ-free mice. (A, D) S100A8-positive cells were stained by green fluorescence. (B) Gr-1–positive cells (arrowheads) were stained by red fluorescence. (C) Merged S100A8 and Gr-1. (E) S100A9-positive cells were stained by red fluorescence. Arrowheads indicated neutrophils. (F) Merged S100A8 and S100A9. Cell nuclei stained with DAPI in A–F are shown in blue. (G) Hematoxylin and eosin staining of periodontal tissue. (H) Count of number of S100A8-, S100A9- and Gr-1–positive cells per area of the JE. D, dentin; ES, enamel space; GSE, gingival sulcus epithelium; ND, not detectable.

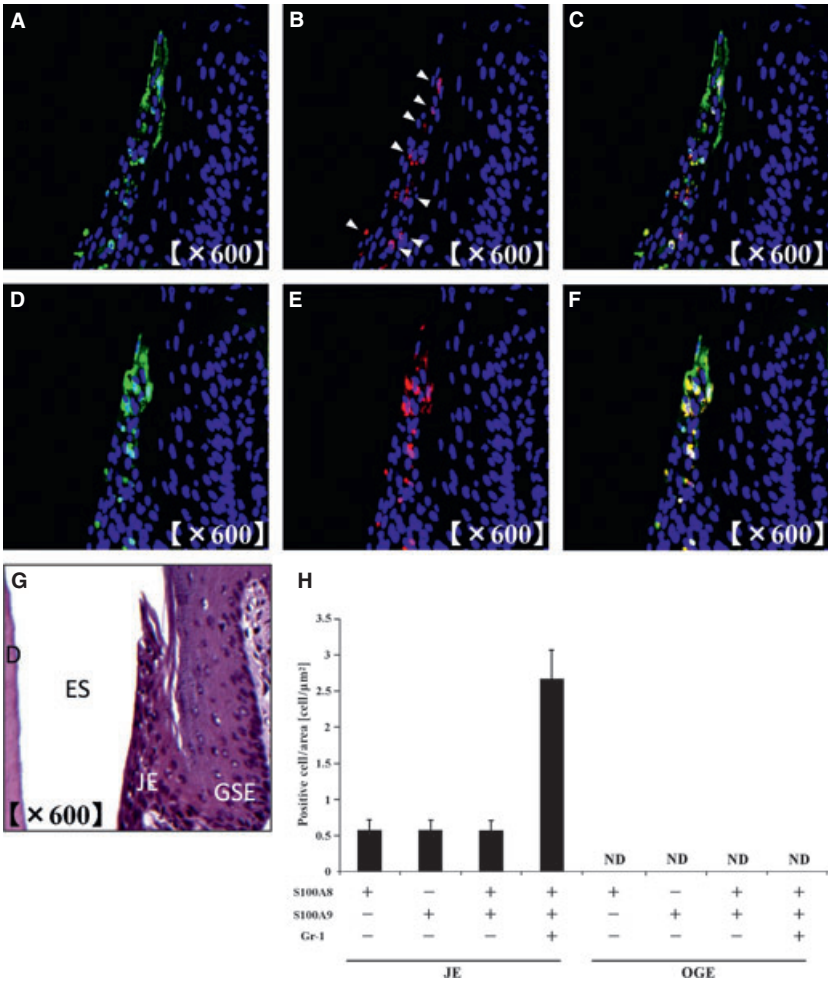

Fig. 3. Immunohistochemical localization of S100A8, S100A9, and Gr-1 in junctional epithelium (JE) of conventional mice. (A, D) S100A8-positive cells were stained by green fluorescence. (B) Gr-1–positive cells (arrowheads) were stained by red fluorescence. (C) Merged S100A8 and Gr-1. (E) S100A9-positive cells were stained by red fluorescence. (F) Merged S100A8 and S100A9. Cell nuclei stained with DAPI in A–F are shown in blue. (G) Hematoxylin and eosin staining of periodontal tissue. (H) Count of number of S100A8-, S100A9- and Gr-1–positive cells per area of the JE. D, dentin; ES, enamel space; GSE, gingival sulcus epithelium; ND, not detectable.

3C). Likewise, there were more neutrophils and S100A8-positive gingival epithelial cells in conventional mice than in germ-free mice (Figs 2C, 2H, 3C and 3H). These data indicated that S100A8 was expressed in not only neutrophils, but in gingival epithelial cells of conventional mice and that oral resident microflora may induce S100A8 expression in gingival epithelium. To investigate the expression of calprotectin in JE of conventional mice, we performed double-staining with S100A8 and S100A9 antibodies. In contrast with germ-free mice, S100A9 and S100A8 proteins were expressed in the edges of JE from conventional mice (Fig. 3D, 3E and 3H). S100A8-positive cells were almost as commonly expressed as S100A9, as shown in the yellow stain of Fig. 3F. These data indicated that calprotectin expression in JE may be enhanced by oral microflora.

Discussion

In this study, immunostaining data showed that gingival epithelial cells of mice expressed S100A8 and S100A9 and that expression of both S100 proteins did not coincide. This is the first detailed report regarding the distribution of S100A8 and S100A9 and colocalization of these S100 proteins in gingival epithelium.

Although neutrophils express both S100A8 and S100A9 in gingival epithelium of germ-free mice, gingival epithelial cells express only S100A8, not S100A9 (Fig. 2). As far as we know, there is only one report that each expression of S100A8 and S100A9 was observed in both epithelial cells and neutrophils. Aochi *et al*. reported the intensity of S100A8 and S100A9 expression in skin keratinocytes and neutrophils of patients with psoriasis. They found that both S100A8 and S100A9 protein expressed in keratinocytes and neutrophils in the skin of a patient with psoriasis (22). Their observation is not consistent with our findings in the

term of S100A9 expression. This difference might be caused by two possibilities. One is a difference of bacterial flora between germ-free mice and patients with psoriasis. Another is a difference of sites between JE of gingival tissue and skin epithelia. Either way, our finding is the first report about inconsistency of S100A8 and S100A9 expression in gingival epithelial cells. Hereafter, we will investigate which factors cause the difference between S100A8 and S100A9 expression.

While S100A8 was expressed in gingival epithelial cells of JE both in the presence and absence of bacteria, levels of S100A8 mRNA and protein expression of JE were increased by bacterial presence. S100A9 expression in gingival epithelial cells of JE was induced by resident microbiota, although gingival epithelial cells expressed a small amount of S100A9 protein in the asepsis. S100A8 and S100A9 expressions in gingival epithelial cells might be regulated by presence of bacteria. In monocyte and bronchial mucosal epithelial cells, calprotectin (a heterodimer of S100A8 and S100A9) was induced and released by the lipopolysaccharide–TLR–nuclear factor κB pathway (8,23,24). However, lipopolysaccharides of *P. gingivalis* and *Escherichia coli* did not increase S100A8 and S100A9 expression in gingival epithelial cells (7,25,26). Furthermore, mRNA expression of both S100A8 and S100A9 were not increased by inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α induced by bacterial infection. The mechanism of induction of S100A8 and S100A9 in gingival epithelial cells is not fully understood. Hayashi *et al*. (26) reported that IL-1α upregulated both S100A8 and S100A9 mRNA expression by *in vitro* assay. Lipopolysaccharide treatment increased IL-1α protein levels in JE as shown by macrophages *in vivo* (27). S100A9 expression in the JE of conventional mice may be induced by the lipopolysaccharide–IL-1α pathway. In keratinocytes of human skin, IL-22 and IL-1α increased AMPs, including calprotectin (28–30). Thus, IL-22 is also a candidate for S100A8 and S100A9 induction in gingival epithelial cells.

Whereas this study investigated S100A8 and S100A9 expression in gingival epithelium of mice, Suryono *et al*. (8) previously examined expression of calprotectin in periodontal tissue of both healthy human subjects and patients with periodontal disease. Similar to our findings in conventional mice, calprotectin was weakly expressed in gingival epithelium from the healthy patient. Additionally, calprotectin was marginally detected in the spinous cell layer and strongly expressed in connective tissue sectioned from a patient with periodontal disease (8). In human gingival tissue, the expression of calprotectin was increased in parallel with bacterial infection and inflammation. Our results with immunostaining in conventional mice also indicated that the number of neutrophils (Gr-1–positive cells) increased in the presence of resident microflora (Fig. 3). These results suggest that the bacterial infection or presence increases the levels of calprotectin expression in gingival tissue of both humans and mice, regardless of the intensity of calprotectin expression.

In this study, S100A9 mRNA expression from total RNA in JE of germ-free mice by LMD was detected, while immunohistochemistry analysis did not show S100A9 protein expression in gingival epithelial cells of germ-free mice (Figs 1A, 2E and 2H). Immunohistochemistry with Gr-1 antibody indicated that the JE includes several neutrophils (Fig. 2B and 2H). Taken together, the discrepancy of S100A9 mRNA expression in gingival tissue and protein expression in gingival epithelial cells seems to be due to lacing with neutrophils. We have previously researched JE-specific markers using LMD and microarray analysis (20). We found that SLPI is characteristically expressed in gingival epithelial cells of JE by real-time PC. In the future, it will be necessary to consider the extent to which total RNA of the JE extracted by LMD involves not only gingival epithelial cells but also neutrophils.

GCF can be collected from the gingival sulcus surrounding the teeth and exists as either plasma transudate or, more commonly, as inflammatory exudate. The constituents of the fluid are derived from a variety of sources. Calprotectin is reported to exist in GCF. Additionally, the calprotectin levels in GCF from patients with periodontal disease were significantly higher than those from healthy subjects and were positively correlated with several clinical parameters (11,31). In this study, the area of coexpression of S100A8 and S100A9 (which indicates calprotectin expression) is also increased by infection with resident microbiota. Gingival epithelial cells comprise the gingival sulcus and may be one of the sources of calprotectin detected in GCF. We found that SLPI expressed differentially in the JE previously (20). The levels of SLPI in GCF from patients with periodontal disease were also significantly higher than those from healthy subjects (32). These data suggested that gingival epithelial cells of the JE may be one of the sources of AMPs in GCF against bacterial infection.

We found that expression of S100A8 and S100A9 did not coincide in the gingival epithelial cells of the JE, although every neutrophil in JE expressed both S100 proteins. It is thought that coexpression of S100A8 and S100A9 functions as calprotectin. On the other hand, the function of a single expression of S100A8 or S100A9 in gingival epithelial cells is not known. Expression of S100A8 was consistently observed in the JE adjacent to the gingival sulcus, both in the presence and absence of bacterial infection. The turnover of gingival epithelial cells in this area is known to be very rapid (33). S100A8 has an anti-apoptotic role inhibiting telomerase in skin keratinocytes (34). Thus, S100A8 expressed in the edges of the JE may have an anti-apoptotic role in slowing the turnover of gingival epithelial cells.

In 2006, the concept of an "alarmin" was proposed by the European Molecular Biology Organization (35). An alarmin has four characteristics:

(i) secreted rapidly by cells that fall into necrosis but not apoptosis; (ii) actively secreted by activated immune cells; (iii) activated through the natural immune system receptor, including the dendritic cell; and (iv) promotes reproduction in an organism destroyed by a secondary obstacle through its own aggression and inflammation and contributes to homeostatic maintenance (35). High-mobility group protein 1 (HMGB1) is representative of an alarmin, and S100 proteins are candidates (36). It has been reported that HMGB1 and the complex of S100A8/S100A9 were released in large quantities in periodontal tissue infected with periodontitis (19,32,37). The JE, which expresses S100A8 and S100A9, may become an alarmin reservoir in the presence of periodontal disease.

In conclusion, S100A9 expression in JE in the presence of microflora of conventional mice is significantly increased compared with the absence of microflora of germ-free mice, which suggests that S100A9 expression may be induced by infection of microflora. The coexpression of S100A8 and S100A9, which comprise calprotectin, is found in gingival epithelial cells in the presence of microflora. The production of calprotectin in gingival epithelial cells may be mediated through S100A9 induction by resident microbiota. The differential expression of S100A8 and S100A9 in gingival epithelium may provide valuable information for understanding production of calprotectin in periodontal disease. Our study focused on elucidating the functions of S100A8 and S100A9 in gingival tissue and the mechanism of induction of both S100 proteins.

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