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Lipopolysaccharide induces rapid loss of follicular dendritic cell-secreted protein in the junctional epithelium

A. Oshiro[1,2], S. Iseki[3], M. Miyauchi[4], T. Terashima[5], Y. Kawaguchi[2], Y. Ikeda[1], T. Shinomura[1]

[1]Tissue Regeneration, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Tokyo, Japan, [2]Oral Health Promotion, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Tokyo, Japan, [3]Molecular Craniofacial Embryology, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Tokyo, Japan, [4]Oral and Maxillofacial Pathobiology, Hiroshima University, Hiroshima, Japan and [5]Maxillofacial Anatomy, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Tokyo, Japan

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Background and Objective: We have previously reported that mRNA encoding follicular dendritic cell-secreted protein (FDC-SP) is expressed specifically in the junctional epithelium at the gingival crevice. Other tissues, such as tonsil, prostate gland and trachea, also express high levels of FDC-SP. These tissues participate in a range of functions closely related to innate immunity. Therefore, it is hypothesized that FDC-SP plays a crucial role in close association with the host defense system within the gingival crevice. Accordingly, the main aim of this study was to investigate the expression and localization of FDC-SP in and around the junctional epithelium and to observe the dynamic changes of FDC-SP in experimental inflammation.

Material and Methods: We examined, immunohistochemically, the expression of FDC-SP in the junctional epithelium using a specific antibody raised in rabbit after immunization with a synthetic peptide derived from the hydrophilic region of FDC-SP. Experimental inflammation was induced in the upper molars of Wistar rats by applying bacterial lipopolysaccharide (LPS; 5 mg/mL in sterile saline) for 1 h.

Results: We confirmed that FDC-SP is present in the junctional epithelium in a pattern that is consistent with the expression of FDC-SP mRNA. Of special interest is that no FDC-SP was detectable in the junctional epithelium 3 h after transient topical treatment with LPS.

Conclusion: The presence of FDC-SP in the junctional epithelium and its loss after LPS treatment strongly support our hypothesis of FDC-SP playing a crucial role in close association with the host defense system within the gingival crevice.

Tamayuki Shinomura, PhD, Tissue Regeneration, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan
Telefax: +81 3 5803 5450
e-mail: t.shinomura.trg@tmd.ac.jp

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Previously, we showed by *in situ* hybridization analyses that follicular dendritic cell-secreted protein (FDC-SP) is expressed specifically in the junctional epithelium adjacent to the gingival crevice (1). The gingival crevice is a very unique structure that surrounds the eruption site of each tooth, creating a space between the tooth and the gingiva that can be a hotbed for bacterial growth, resulting in an increased susceptibility to infection (2). It is reasonable to suggest that to overcome this potential problem, the surrounding tissue would have

developed a host defense system. Although secretion of immunoglobulin A has been implicated as one of the defense mechanisms in the gingival crevice, antimicrobial defense is likely to be a coordinated multifactorial system. Many studies have examined the role of the crevicular epithelium in relation to periodontal disease (3,4). As a result, the concept of an active, complex response by the junctional epithelia to bacterial attack has emerged where the junctional tissue activity secretes various cytokines and chemokines, including tumor necrosis factor-alpha, interleukin-1 and interleukin-8 (5,6), thus activating the immune defense system.

The normal functions of the junctional epithelium are to attach the gingiva to the enamel surface (thus mechanically reducing bacterial invasion) and to provide additional immunological activities leading to periodontal protection against pathogens (2). In this context, a study of FDC-SP that is expressed specifically at the junctional epithelium may offer new insights into additional mechanisms for this defense. At present, the function of FDC-SP in this region is still unknown, although its structural similarity to statherin and histatin (7,8) suggests that FDC-SP could have some important role in the inhibition of bacterial infection. Further study of FDC-SP will probably shed novel insights into mechanisms involved in periodontal defense. In this work, we first confirmed the expression and localization of FDC-SP by specific immunohistochemistry of normal tissue and then examined changes in its localization following lipopolysaccharide (LPS)-
induced inflammation of the junctional epithelium.

Material and methods

Preparation of antibodies against mouse FDC-SP

A polyclonal antibody that specifically recognizes mouse FDC-SP was raised in rabbit after immunization with the synthetic peptide (LPVPKDQEREKRSASD) derived from the hydrophilic region of mouse FDC-SP (7). The rabbit antiserum was subjected to affinity chromatography on Sepharose 4B coupled to the glutathione *S*-transferase (GST) fusion protein, as described below. The bound antibody was then eluted with 3 M KSCN in phosphate-buffered saline (PBS) and dialyzed against PBS. In this article, the purified antibody is referred to as anti-mouse FDC-SP.

Fusion proteins and immunoblot analyses

Three cDNA fragments encoding hydrophilic regions of mouse, rat and human FDC-SP were separately inserted into the GST gene-fusion vectors, pGEX-2T and pGEX-3X (GE Healthcare, Buckinghamshire, England, UK). Each construct was then transfected into *Escherichia coli* BL21(DE3). The resulting fusion proteins produced in *E. coli* were termed GST–mouse FDC-SP, GST–rat FDC-SP and GST–human FDC-SP for GST fusion proteins with FDC-SP from mouse, rat and human, respectively. Partial amino acid sequences of each fusion protein are shown in Fig. 1A.

For the preparation of the affinity column described above, GST–mouse FDC-SP was purified from a crude cell lysate by passing though a Glutathione Sepharose 4B column, according to the manufacturer's instructions (GE Healthcare). The purified protein was then coupled to cyanogen bromide-activated Sepharose 4B gel (GE Healthcare).

The specificity of anti-mouse FDC-SP was confirmed by immunoblot analysis, as follows. Crude cell lysate from bacteria expressing different GST fusion proteins, as described above, was prepared by boiling the bacterial pellets for 10 min in a sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 62.5 mM Tris–HCl (pH 6.8), 10%

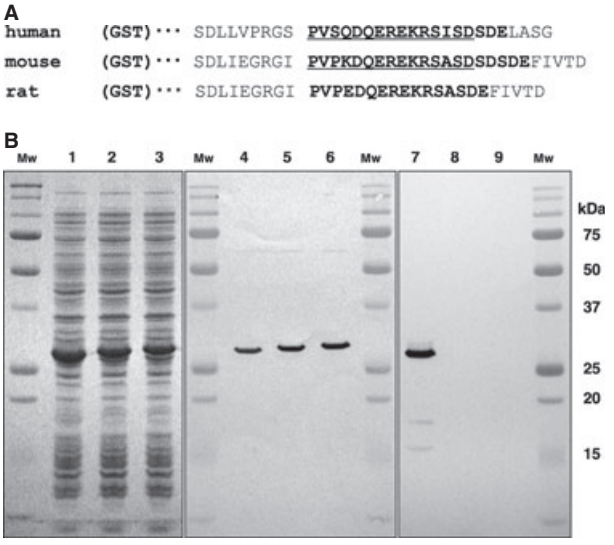

Fig. 1. Characterization of polyclonal antibody against mouse follicular dendritic cell-secreted protein (FDC-SP). (A) Partial (C-terminal) amino acid sequences of the glutathione *S*-transferase (GST) fusion constructs of human, mouse and rat FDC-SP are indicated. Residues shown in bold are derived from each FDC-SP sequence, and the underlined sequences indicate synthetic peptides used for immunization. (B) To confirm the specificity of anti-mouse FDC-SP, crude extracts of bacteria containing human (lanes 1, 4 and 7), mouse (lanes 2, 5 and 8) and rat (lanes 3, 6 and 9) GST–FDC-SP fusion proteins were electrophoresed on 12% SDS-polyacrylamide gels and analyzed by western blotting. Identical protein expression in all samples was confirmed by staining with Coomassie Blue (lanes 1–3). Immunostaining of the blot membranes was performed with anti-mouse FDC-SP prepared in this study (lanes 4–6) and with anti-human FDC-SP described previously (lanes 7–9) (1). The lane marked Mw contains colored protein molecular weight markers (Precision Plus Protein Standards; BioRad, Hercules, CA, USA).

glycerol, 130 mM dithiothreitol and 0.01% Bromophenol Blue). The resultant samples were then subjected to electrophoresis on 12% SDS–polyacrylamide gels, according to the method of Laemmli (9). Proteins in the gel were then blotted onto Hybond-P membranes, according to the manufacturer's instructions (GE Healthcare). After blotting, the membranes were stained with anti-mouse FDC-SP and with anti-human FDC-SP, as described previously (1).

Immunohistochemistry

Six-week-old male ICR mice and 7-week-old male Wistar rats (Charles River Japan, Yokohama, Japan) were used for immunohistochemical analyses. All experimental procedures were approved by the Institutional Committee of Animal Care and Use at the Tokyo Medical and Dental University, and were performed in conformance with the Guidelines for Animal Experimentation at Tokyo Medical and Dental University.

The animals were killed with an overdose of ethyl ether. Mandibles, maxillae and prostates were dissected and fixed in 4% paraformaldehyde at 4°C overnight. The isolated mandible and maxillae tissues were then decalcified, *en bloc*, in neutralized 10% EDTA at 4°C for 1–2 wk. Finally, all tissue samples were embedded in paraffin or optimal cutting temperature compound (Tissue-Tek; Miles Scientific, Naperville, IL, USA), and then paraffin sections (5 μm thick) for light microscopy analyses and frozen sections (12 μm thick) for electron microscopy analyses were prepared according to standard protocols.

Immunostaining was carried out using a VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA, USA). Briefly, endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol. The nonspecific-binding sites for antibodies were blocked with 5% skim milk in PBS. Then, the sections were incubated, overnight at room temperature, with the following polyclonal antibodies: affinity-purified anti-mouse FDC-SP, anti-pan-keratin (PROGEN GmbH, Heidelberg, Germany), anti-pan-cadherin (Sigma Chemical, St Louis, MO, USA) or anti-myeloperoxidase (DAKO, Glostrup, Denmark). In addition, a polyclonal rabbit IgG (DAKO) and an anti-human FDC-SP were used as negative controls. After rinsing with PBS, the sections were incubated for 30 min with biotin-conjugated antibody against rabbit IgG (Millipore Corp., Billerica, MA, USA). Immunoreactivity was visualized using a VECTASTAIN ABC kit and SIGMAFAST™ 3,3′-diaminobenzidine (DAB) tablets (Sigma Chemical). Finally, the specimens were counterstained with 1% methyl green or Mayer's hematoxylin, dehydrated and mounted.

For the immunoelectron microscopy study, the frozen sections treated with the ABC complex (Vector) described above were further fixed in 1% glutaraldehyde at 4°C for 10 min. The sections were then stained for peroxidase activity. In brief, the sections were preincubated in DAB solution containing 1% dimethylsulfoxide. After 30 min, the medium was exchanged for DAB-H_2O_2 solution, and incubation was continued at room temperature for 10 min. The sections were then post-fixed with 2% OsO_4 solution for 1 h, dehydrated through a graded series of ethanol and acetone, and flat-embedded in Epon 812 (TAAB Laboratories, Aldermaston, UK). Ultrathin sections were prepared using a diamond knife, collected on grids and examined by transmission electron microscopy (H-7100; Hitachi, Tokyo, Japan), after double staining with uranyl acetate and lead citrate solution.

LPS treatment

Under intraperitoneal anesthesia with 20% ethylcarbamate (1 mg/g of body weight), the right and left upper molars of rats were treated for 1 h with LPS (5 mg/mL in sterile saline) from *E. coli* (Sigma Chemical), as previously described (6). Three rats each were killed, by an overdose of ethyl ether, at 0, 1, 3 and 10 h and at 1, 3 and 7 d after treatment with LPS. As an untreated control, three rats that did not receive the LPS treatment were also killed. Tissue samples were resected *en bloc* from the right and left upper molar regions, and then analyzed by immunohistochemistry, as described above.

Results

Specificity of anti-FDC-SP

We previously reported that a high level of expression of mRNA encoding FDC-SP was present in the junctional epithelium adjacent to the gingival crevice (1). So, in this work, we examined the expression and the localization of FDC-SP, in and around the junctional epithelium, by immunohistochemistry. We first developed a specific antibody against a highly antigenic region of the mouse FDC-SP sequence, as described in the "Material and methods". The specificity of this polyclonal antibody was first examined by immunoblotting against crude extracts from bacterial cells expressing different recombinant fusion proteins (Fig. 1B). The results clearly showed that anti-mouse FDC-SP specifically recognized mouse FDC-SP without reacting with other proteins in the extract, but the mouse antibody also recognized rat and human FDC-SPs whose amino acid sequences differ slightly from that of mouse (Fig. 1A). On the other hand, anti-human FDC-SP (1), used as a control, recognized human FDC-SP only; there was no interspecies cross-reactivity (Fig. 1B).

Specific expression of FDC-SP in the junctional epithelium

To confirm the expression of FDC-SP in the junctional epithelium adjacent to the gingival crevice, we stained the bucco-lingual sections of the molars from normal mouse mandible with anti-mouse FDC-SP. As shown in Fig. 2A, only the junctional epithelia were specifically stained with the antibody. This staining pattern was completely identical to the expression pattern of FDC-SP mRNA, which we previously obtained by *in situ* hybridization analyses (1). In contrast, both junctional epithelium and oral epithelium (stratified squamous epithelia)

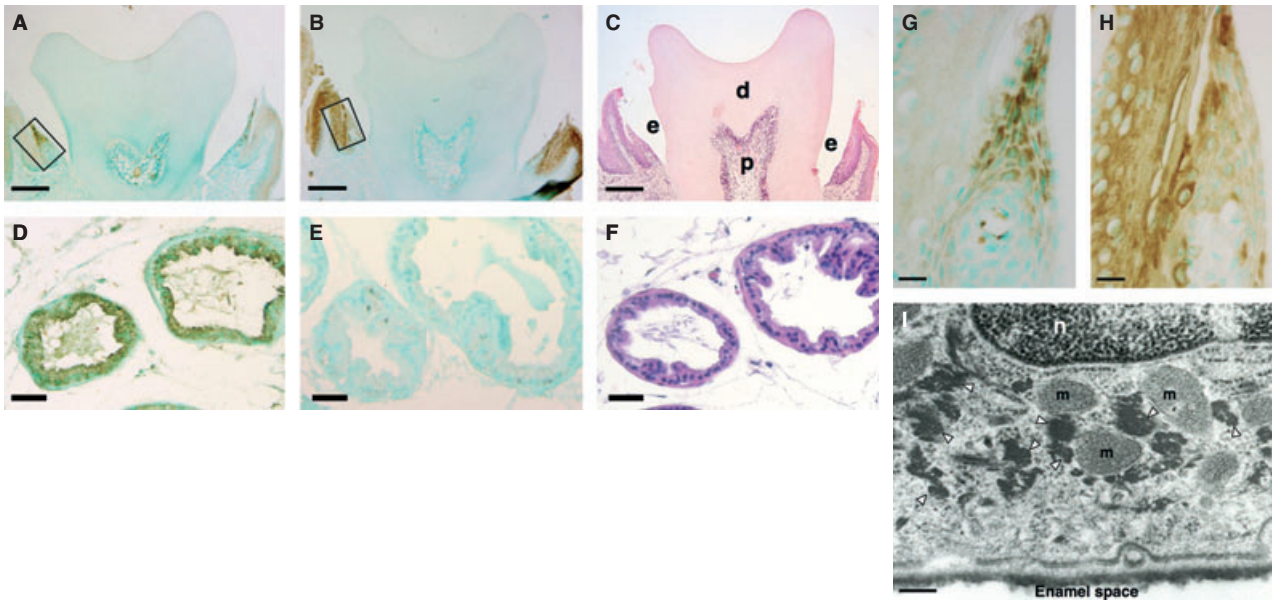

Fig. 2. Expression of follicular dendritic cell-secreted protein (FDC-SP) in the junctional epithelium. Bucco-lingual sections of the first molar of a 6-week-old mouse mandible were stained with anti-mouse FDC-SP (A), anti-pan-keratin (B) and hematoxylin and eosin (C). The expression of FDC-SP was detected specifically in the junctional epithelium (A). To confirm the specificity of immunohistochemical staining, prostate sections were stained with anti-mouse FDC-SP (D), anti-human FDC-SP (E) and hematoxylin and eosin (F). FDC-SP expression was detected only with anti-mouse FDC-SP. Higher magnifications of the rectangular areas in panels A and B are shown in panels G and H, respectively. Methyl green was used as a counterstain (A, B, D, E, G and H). A transmission electron micrograph of the positive area in panel G is shown in panel I. Arrowheads indicate positive signals detected in the cytoplasm as an irregularly shaped electron-dense deposit. d, dentin; e, enamel space; m, mitochondrion; n, nucleus; p, pulp. Bar = 200 μm (A–C), 40 μm (D–F), 20 μm (G and H) and 200 nm (I).

were uniformly stained with anti-pan-keratin (Fig. 2B and 2H). To confirm the specificity of immunohistochemical staining, tissue sections of mouse prostate expressing a high level of FDC-SP were stained with anti-mouse FDC-SP and anti-human FDC-SP, which did not recognize mouse and rat FDC-SP, as shown in Fig. 1B. Not surprisingly, prostate glands were stained with anti-mouse FDC-SP but not with anti-human FDC-SP, as shown in Fig. 2D and 2E. Furthermore, immunohistochemical staining of junctional epithelium with anti-mouse FDC-SP was absent when the antibody was pre-absorbed to the purified GST–mouse FDC-SP (data not shown). Therefore, these data strongly support that staining of FDC-SP is restricted to the junctional epithelium. Furthermore, a higher magnification of the positively stained area shown in Fig. 2A indicates that FDC-SP seems to be localized intracellularly (Fig. 2G). However, this distribution pattern was seemingly contradictory to the fact that FDC-SP has a potential signal sequence for secretion (10). Therefore, we conducted a more detailed observation on the localization of FDC-SP by immunoelectron microscopy. As shown in Fig. 2I, positive signals were detected as irregularly shaped granules in the cytoplasm. In addition, no membrane structures were detected for these granules. Hence, it would seem that FDC-SP is deposited in the cytoplasm of the junctional epithelial cells.

Effects of LPS treatment on the expression of FDC-SP

Previous studies (6,11) have demonstrated that the application of bacterial LPS to the rat gingival sulcus causes inflammatory changes in and around the junctional epithelium. Therefore, we examined whether the expression and the distribution of FDC-SP in the junctional epithelium were affected by the presence of LPS. For this study, we applied LPS onto the upper molars of rats because of their ease of access and we examined whether anti-mouse FDC-SP reacted specifically with the junctional epithelium of normal rats. As shown in Fig. 3A, the junctional epithelium of any site was specifically stained with this antibody. These staining patterns were essentially the same as those of the mouse junctional epithelium shown in Fig. 2A. Furthermore, the positive signals were detected preferentially in cytoplasm, as in the case of the mouse tissue (Fig. 3A-e).

Interestingly, immunoreactivity to FDC-SP in the junctional epithelium was completely lost 3 h after treatment with LPS (Fig. 3B-d) but the immunoreactivity was detected again after a few days (Fig. 3B-g). In contrast, immunoreactivity to cadherin, whose expression was specifically observed in the junctional epithelium (12), was constant at all times, regardless of the presence of LPS (Fig. 3B-q–x). In this experiment, we also monitored for neutrophil invasion to characterize the inflammatory reaction (Fig. 3B-i–p). The numbers of neutrophils detected by anti-myeloperoxidase serum clearly increased within 1 h in the subjunctional

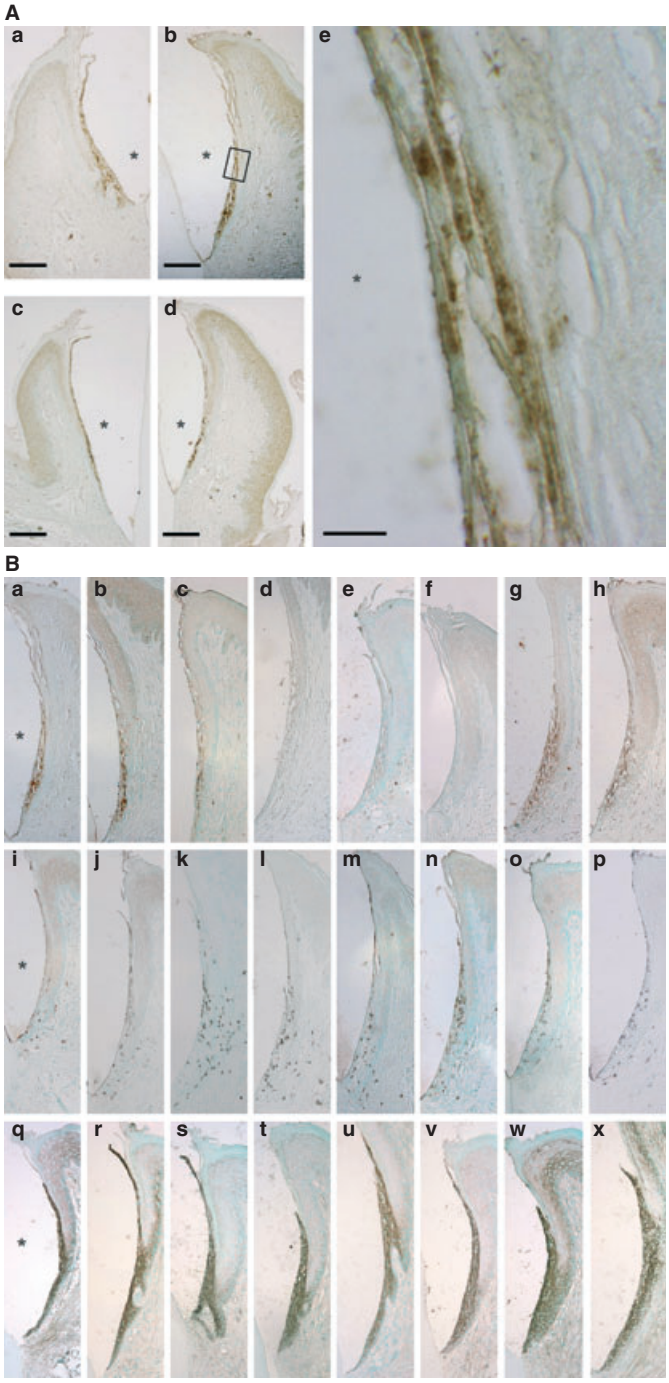

Fig. 3. Effects of lipopolysaccharide (LPS) on the staining of follicular dendritic cell-secreted protein (FDC-SP). (A) Bucco-lingual sections of upper (a, b) and lower (c, d) molars of 7-week-old rats were stained with anti-mouse FDC-SP. A similar intensity of positive staining was present in the junctional epithelium at both buccal (a, c) and lingual (b, d) sides. No immunoreactivity was observed with control immunoglobulin G. A higher magnification of the rectangular area in panel b is shown in panel e. The enamel space is indicated by an asterisk. Bar = 100 μm (a–d) and 10 μm (e). (B) To induce inflammatory conditions in the junctional epithelium, the upper molars were treated with LPS (5 mg/mL) for 1 h. After the treatment, rats were killed at 0 (b, j, r), 1 (c, k, s), 3 (d, l, t) and 10 (e, m, u) h and at 1 (f, n, v), 3 (g, o, w) and 7 (h, p, x) d. The expression of FDC-SP was analyzed by immunostaining with anti-mouse FDC-SP (a–h). Neutrophil invasion was observed using anti-myeloperoxidase (18) to examine the inflammatory reaction (i–p). As a control, cadherin expression was analyzed by anti-pan-cadherin (q–x). Sections of LPS-untreated normal upper molars (a, i, q) are also shown, and the enamel space is indicated by an asterisk.

epithelial area (Fig. 3B-k). Neutrophil changes over time were inversely correlated with the changes of FDC-SP.

Discussion

In this study, immunohistochemical analysis clearly indicated that FDC-SP accumulates in the junctional epithelium in a pattern that is consistent with the expression of FDC-SP mRNA observed by *in situ* hybridization analysis (1). However, contrary to expectations, FDC-SP was accumulated intracellularly, even though it has a prospective signal sequence (10). A similar accumulation pattern was also observed in mouse trachea, where the cell bodies of glandulae tracheales were strongly stained with anti-mouse FDC-SP (data not shown). Recently, Guo *et al.* (13) revealed, by proteome analysis, the presence of a prospective signal sequence in FDC-SP from human saliva. Therefore, the signal sequence of FDC-SP may be a pseudo sequence. An alternative explanation is that FDC-SP is synthesized in the cytoplasm and may enter the secretory pathway post-translationally (14). Further work will be necessary to define the cytoplasmic accumulation of FDC-SP and to reveal the biosynthetic process of FDC-SP in normal conditions and the regulatory mechanisms for its excretion in inflammatory conditions.

At present, the function of FDC-SP in the junctional epithelium is still unclear. However, this region is very important for periodontal protection as a host defense against pathogens (15). As LPS is known to provoke initial periodontal tissue destruction (6,11), we applied it to the junctional epithelium to induce inflammatory conditions. We unexpectedly found that FDC-SP was undetectable in the junctional epithelium within a few hours following LPS treatment, in spite of cadherin expression being unchanged. This loss does not seem to be related to cell death because dead cells were not detected following close analysis of hematoxylin-eosin stained sections (data not shown). We also observed a preferential invasion of neutrophils into the subjunctional

epithelial area within 1 h after LPS treatment, as described previously (6). Therefore, the loss of immunoreactivity to FDC-SP in the junctional epithelium during this tissue's response to an LPS challenge appears to occur in concert with the initial inflammatory response.

Tissues expressing FDC-SP at high levels, such as tonsil, prostate gland and trachea, participate in a range of functions closely related to innate immunity. In this regard, Marshall *et al*. (16) reported that FDC-SP has a significant binding activity to B cells. Additionally, it was reported that LPS treatment increases the B-cell number in the connective tissue subjacent to the junctional epithelium (11). Therefore, FDC-SP may mediate the activation of innate immunity and acquired immunity. In this context, it would be very important to know whether FDC-SP is released into the surrounding tissue or degraded in the junctional epithelium after LPS treatment. At the same time, to determine the molecular signals controlling the accumulation and elimination of FDC-SP, it will be essential to have a full understanding of host defense mechanisms at the junctional epithelium. Toll-like receptor is a candidate for the initial receptor of the LPS signaling pathway (17). However, it has not been shown clearly to be present in the junctional epithelium. Further study is essential.

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