Protease-activated receptor 2 mediates interleukin-8 and intercellular adhesion molecule-1 expression in response to Aggregatibacter actinomycetemcomitans

Introduction: We investigated the mechanisms by which extracts of Aggregatibacter actinomycetemcomitans affect the inflammatory response in gingival epithelial cells.

Methods: Human gingival cells (Ca9-22) were cultured in bacterial extracts prepared from A. actinomycetemcomitans ATCC 29522. The cells were pretreated with protease inhibitors or transfected with small interfering RNA (siRNA) specific for protease-activated receptor 2 (PAR-2).

Results: The pretreatment of cells with serine protease inhibitors significantly inhibited A. actinomycetemcomitans extract-induced expression of interleukin-8 (IL-8) and intercellular adhesion molecule-1 (ICAM-1) at both the messenger RNA and protein levels. In addition, A. actinomycetemcomitans extract-induced IL-8 and ICAM-1 expression was significantly decreased in PAR-2/siRNA-transfected cells.

Conclusions: A. actinomycetemcomitans extract-induced IL-8 and ICAM-1 expression in gingival epithelial cells is mediated by PAR-2.

Key words: Aggregatibacter actinomycetemcomitans; intercellular adhesion molecule-1; interleukin-8; protease-activated receptor 2
periodontal disease. However, the mechanisms by which extracts of *A. actinomyctetemcomitans* affect gingival epithelial cells are unknown. Lourbakos et al. (16) have reported that a protease produced by *Porphyromonas gingivalis* activates protease-activated receptor-2 (PAR-2) in oral epithelial cells and induces the secretion of proinflammatory cytokines. We hypothesized that gingival epithelial cells use PAR-2 to mediate gene expression for IL-8 and ICAM-1 in response to challenge with *A. actinomyctetemcomitans*. We therefore tested whether PAR-2 is involved in the recognition of extracts of *A. actinomyctetemcomitans* by cultured gingival epithelial cells.

**Materials and methods**

**Cell culture**

The human gingival carcinoma cell line Ca9-22 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and cultured in minimum essential medium (Asahi Technoglass, Tokyo, Japan) containing 10% fetal bovine serum (Asahi Technoglass) and 1% penicillin–streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO2.

**Bacterial extracts and reagents**

Bacterial extracts of *A. actinomyctetemcomitans* ATCC 29522 (serotype b) were prepared as described previously (12). Enzymatic activity of the extracts was assayed using a synthetic substrate (Boc-Phe-Ser-Arg-MCA; Sigma, St Louis, MO). We evaluated the effect of preincubation (10 min) with 3 μM cystatin (a cystein protease inhibitor; Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF; a serine protease inhibitor; Sigma).

Polymyxin B (Sigma) was mixed with *A. actinomyctetemcomitans* extracts for 30 min. Then, the extracts were added to cells cultures. The PAR-2-activating peptide (SLIGKV-NH2) was obtained from Tocris Bioscience (Ellisville, MO).

**RNA interference**

Transfection for targeting endogenous PAR-2 was conducted using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) and small interfering RNA (siRNA; final concentration, 20 or 100 nM; Sigma), according to the manufacturer’s instructions. The sequence of the target for PAR-2 was 5’-GGAUGUG GAACCUGUUUA-3’. AllStars Negative Control siRNA was purchased from Qiagen (Valencia, CA).

**Enzyme-linked immunosorbent assay**

The concentration of IL-8 in each culture supernatant was determined by enzyme-linked immunosorbent assay using commercially available kits (R&D Systems, Minneapolis, MN) and following the manufacturer’s instructions.

**Real-time polymerase chain reaction and reverse transcription-polymerase chain reaction (RT-PCR)**

Cells were seeded at 1.0 × 10^5 cells/well in six-well plates. After 96 h of incubation, the medium was replaced with either fresh medium containing bacterial extract or fresh medium alone (control), and the cells were grown for an additional 6 h at 37°C in a humidified atmosphere of 5% CO2. After treatment, the cells were harvested using 0.25% trypsin/ethylene diamine tetra acetic acid (EDTA) and resuspended in phosphate-buffered saline (PBS).

Total RNA was extracted from the cells using an RNase Mini Kit (Qiagen, Valencia, CA). The complementary DNA was synthesized using a Ready-To-Go T-Primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ). The primer and probe sets for ICAM-1, IL-8, and PAR-2 were obtained from Applied Biosystems (Tokyo, Japan). Real-time PCR was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using the following parameters: 50°C for 2 min, 95°C for 10 min; 40 cycles of denaturation

![Fig. 1. Effects of protease inhibitors on *Aggregatibacter actinomyctetemcomitans* extract-induced expression of interleukin 8 (IL-8).](image-url)

(A) Cells were preincubated with protease inhibitors and then stimulated with *A. actinomyctetemcomitans* extract (3 μg/ml) for 6 h. Real-time polymerase chain reaction was used to monitor the levels of IL-8 messenger RNA, which were normalized to those of glyceraldehyde 3-phosphate dehydrogenase. The relative differences between the control and experimental groups are expressed as relative increases, with the control set at 100%. The data are shown as the mean ± SD of three independent experiments. Each experiment was run in duplicate. 

(B) Cells were preincubated with protease inhibitors and stimulated with *A. actinomyctetemcomitans* extract (3 μg/ml) for 24 h. Culture supernatants were assayed for IL-8 using enzyme-linked immunosorbent assay. The data are shown as the mean ± SD of three independent experiments. Each experiment was run in duplicate. *P < 0.01 vs. control; §P < 0.01 vs. untreated.
at 95°C for 15 s; and primer extension at 60°C for 1 min. The data are shown as the mean ± SD of three independent experiments. Each experiment was duplicated. RT-PCR was performed using Ready to Go U Prime Synthesis beads (Pharmacia, Uppsala, Sweden) and PuReTaq Ready-To-Go PCR beads (GE Healthcare, Chalfont St Giles, UK) for the determination of expression of PARs. The PCR conditions for PARs were denaturation at 95°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 3 min for 40 cycles. The PCR conditions for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 1 min for 40 cycles.

The following primers were used (16): GAPDH, forward 5'-TGAAGGTGCGGATCAACGGATTCTGTGCATC-3', reverse 5'-TGGTGGGCCCATTGAGGGTCCACCAC-3', 983 base pairs (bp); PAR-1, forward 5'-ATGTGGGCCATGAGGTCCACCAC-3', reverse 5'-TGTGAACTGATCATGTTTATG-3', 708 bp; PAR-2, forward 5'-TGGATGAGGTCCACACC-3', reverse 5'-CGTGATGTCCAGACCCAG-3', 1066 bp; PAR-3, forward 5'-ATAACGTTCCCAGATCTGTC-3', reverse 5'-CCCGTAAGGATGATAAGCACA-3', 858 bp; PAR-4, forward 5'-GACGAGAGCAGCCGGGATG-3', reverse 5'-CCCCTACAGACCCAGGATG-3', 752 bp. The PCR products were analysed using 1% agarose gel electrophoresis with ethidium bromide.

Flow cytometry

Cells were seeded at 1.0 × 10^5 cells/well in six-well plates as described above. After 96 h of incubation, the medium was replaced with either fresh medium containing bacterial extract or fresh medium alone (control), and the cells were incubated for an additional 24 h at 37°C in a humidified atmosphere of 5% CO₂. After treatment, the cells were harvested using 0.25% trypsin/EDTA and resuspended in PBS (pH 7.4). The cells were then incubated with monoclonal anti-human ICAM-1 antibody (fluorescein isothiocyanate-conjugated CD54; Beckman Coulter, Fullerton, CA) and anti-human PAR-2 antibody (phycoerythrin-conjugated PAR-2; R&D Systems, Minneapolis, MN) at 20 µl antibody/10⁶ cells. The suspension was incubated with gentle shaking for 30 min at 4°C in the dark. After each step, the cells were washed and resuspended in PBS. Flow cytometry was performed using EXPO32 (Beckman Coulter). The data shown are representative of three independent experiments.

Statistical analysis

Data were analysed for significance using Dunnett’s pairwise comparison t-test.

Results

We used protease inhibitors to investigate the role of Aggregatibacter actinomycetemcomitans protease in IL-8 and ICAM-1 expression. The pretreatment of cells with the serine protease inhibitor PMSF significantly
inhibited *Aggregatibacter actinomycetemcomitans* extract-induced IL-8 and ICAM expression at the messenger RNA (mRNA) and protein levels (Figs 1 and 2). In contrast, the cysteine protease inhibitor cystatin inhibited ICAM-1 mRNA, but had less of an effect on ICAM-1 mean fluorescence intensity and no significant effect on IL-8 expression. These results suggest that it is the serine protease in *A. actinomycetemcomitans* extracts that mainly contributes to IL-8 and ICAM-1 expression. The enzyme activity of *A. actinomycetemcomitans* extracts was 0.89 ± 0.03 U/ml.

To investigate to what extent *A. actinomycetemcomitans* lipopolysaccharide contributed to the expression of IL-8 and ICAM-1, polymyxin B (100 and 10 µg/ml) was mixed with the extracts, and the mixtures were added to cell cultures. Pretreatment with polymyxin B caused a slight reduction in IL-8 expression (Fig. 3A and B) but no reduction in ICAM-1 expression (Fig. 3C and D).

**Discussion**

The oral epithelium is directly exposed to periodontal bacteria, and the products of inhibited *A. actinomycetemcomitans* extract-induced IL-8 and ICAM expression at the messenger RNA (mRNA) and protein levels (Figs 1 and 2). In contrast, the cysteine protease inhibitor cystatin inhibited ICAM-1 mRNA, but had less of an effect on ICAM-1 mean fluorescence intensity and no significant effect on IL-8 expression. These results suggest that it is the serine protease in *A. actinomycetemcomitans* extracts that mainly contributes to IL-8 and ICAM-1 expression. The enzyme activity of *A. actinomycetemcomitans* extracts was 0.89 ± 0.03 U/ml.

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RT-PCR analysis of RNA extracted from Ca9-22 cells revealed the presence of PAR-2 (Fig. 4). Following on from this, we examined the involvement of PAR-2 in *A. actinomycetemcomitans* extract-induced IL-8 and ICAM-1 expression and used siRNA to block these expressions. The transfection of cells with PAR-2-specific siRNA resulted in the inhibition of PAR-2 expression (Fig. 5). In the transfected cells, *A. actinomycetemcomitans* extract-induced IL-8 and ICAM-1 expression was significantly inhibited by PAR-2-specific siRNA (Fig. 6). To further support the role of PAR-2 in regulating *A. actinomycetemcomitans* extract-induced IL-8 and ICAM-1 expression, we conducted the study to determine whether a specific agonist to PAR-2 can similarly induce IL-8 and ICAM-1 expression. As shown in Fig. 7, significant induction of IL-8 and ICAM-1 was observed in cells stimulated with 10 µM PAR-2 agonist. These findings suggest that the *A. actinomycetemcomitans* extract-induced expression of IL-8 and ICAM-1 in Ca9-22 cells is mediated by the activation of PAR-2.
Fig. 6. Effects of protease-activated receptor 2 (PAR-2) silencing on interleukin-8 (IL-8) and intercellular adhesion molecule-1 (ICAM-1) expression. Cells were transfected with 20 or 100 nM PAR-2 small interfering RNA (siRNA) or 100 nM negative control siRNA. Six hours after transfection, the medium was changed to complete medium with or without *Aggregatibacter actinomycetemcomitans* extract. IL-8 expression was evaluated 6 h post stimulation for messenger RNA (A) and 24 h post stimulation for culture supernatant (B). ICAM-1 expression was evaluated 6 h post stimulation for messenger RNA (C) and 24 h post stimulation for flow cytometry (D). The data are shown as the mean ± SD of three independent experiments. Each experiment was run in duplicate. *P < 0.01 vs. control; §P < 0.01 vs. *A. actinomycetemcomitans* extract treated.

Fig. 7. Effects of protease-activated receptor 2 (PAR-2) agonist peptide on interleukin-8 (IL-8) and intercellular adhesion molecule-1 (ICAM-1). Cells were stimulated with 100 nM PAR-2 agonist peptide (SLIGKV-NH2). IL-8 expression was evaluated 6 h post stimulation for messenger RNA (A) and 24 h post stimulation for culture supernatant (B). ICAM-1 expression was evaluated 12 h post stimulation for mRNA (C) and 24 h post stimulation for flow cytometry (D). The data are shown as the mean ± SD of three independent experiments. Each experiment was run in duplicate. *P < 0.01, **P < 0.05 vs. control.
these bacteria may play an important role in host defense mechanisms against pathogens (17–19). The upregulation of IL-8 and ICAM-1 by *A. actinomycetemcomitans* may be important in recruiting host immune cells to the site of infection (20–22). *A. actinomycetemcomitans* extract-induced expression of IL-8 and ICAM-1 was significantly inhibited by protease inhibitors. Subsequently, we assessed whether lipopolysaccharide was responsible for the upregulation of IL-8 and ICAM-1. However, the stimulating effect of *A. actinomycetemcomitans* extracts was not inhibited by polymyxin B, suggesting that lipopolysaccharide was not the causative factor for the epithelial stimulation.

We next examined whether the upregulation of IL-8 and ICAM-1 occurred via PAR-2. PARs belong to a recently described family of seven G-protein-coupled transmembrane-domain receptors. PAR activation occurs via the proteolytic cleavage of the N-terminal domain by a proteinase (23–25). To date, four PARs have been identified: PAR-1, PAR-2, PAR-3, and PAR-4 (26–28). Although these receptors have similar mechanisms of activation (29), they may have different biological functions and tissue distribution and are activated by different proteases. In this study, Ca9-22 cells were found to express PAR-2. PAR-2 is expressed throughout the body, especially in the epithelium (e.g. oral epithelial cells), endothelium, fibroblasts (including gingival fibroblasts), osteoblasts, neutrophils, myocytes, neurons, and astrocytes (22, 28, 30–35), and seems to play a critical role in inflammation (36, 37). Moreover, the possible involvement of PAR-2 in periodontitis has been suggested. The transfection of epithelial cells with PAR-2-specific siRNA resulted in an approximately 80% decrease in the level of PAR-2 mRNA. In PAR-2/siRNA-transfected cells, *A. actinomycetemcomitans* extract-mediated upregulation of IL-8 and ICAM-1 was significantly decreased. These results clearly indicate that *A. actinomycetemcomitans* extract-induced IL-8 and ICAM-1 expression is mediated by PAR-2.

In oral epithelial cells, PAR-2 activation by gingipain, a bacterial protease produced by *P. gingivalis*, induces the secretion of the proinflammatory cytokine IL-6, which is a potent stimulator of osteoclast differentiation and bone resorption (16, 32). These studies suggest that PAR-2 activation accounts for the tissue destruction associated with periodontitis. Chung et al. (38) have demonstrated that PAR-2 is involved in the upregulation of β-defensin in gingival epithelial cells. Our results indicate a possible role of PAR-2 in gingival tissues, where its activation can act as a primary inflammatory response against the invasion of bacterial pathogens, and a role for bacterial protease in the pathophysiology of periodontitis.

In conclusion, our in vitro studies showed that protease derived from *A. actinomycetemcomitans* extracts induces cytokine expression in Ca9-22 cells via PAR-2 activation. Further characterization of *A. actinomycetemcomitans* extracts may prove useful in generating potential therapeutic strategies for the modulation of periodontal inflammation.

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**References**


