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

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**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

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*Aggregatibacter actinomycetemcomitans*, a gram-negative capnophilic coccobacillus, is a major pathogen in the initiation and progression of periodontitis (1–8). Periodontal infection with *A. actinomycetemcomitans* can occur if the pathogen is able to breach the initial line of host defense in the periodontium, largely represented by epithelial cells (9–11). The proinflammatory cytokines interleukin-8 (IL-8) and intercellular adhesion molecule-1 (ICAM-1) are upregulated in gingival epithelial cells following challenge with bacterial extracts of *A. actinomycetemcomitans*. The bacterial components responsible for the inhibition of epithelial cell growth and enhanced expression of IL-8 and ICAM-1 are heat-sensitive molecules (12). In addition, *A. actinomycetemcomitans* is able to degrade host tissue proteins (13, 14), and this activity has been identified in bacterial cellular material and culture medium (14). Furthermore, protease from *A. actinomycetemcomitans* inhibits the proliferation of human gingival epithelial cells, and the heat-inactivated protease is similar to that in the controls (12, 15). Protease is secreted *in vivo* by *A. actinomycetemcomitans* in the plaque of periodontal pockets and also during the invasion of periodontal tissues (15). Consequently, it may function as a major virulence factor in the development of

periodontal disease. However, the mechanisms by which extracts of *A. actinomycetemcomitans* 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. actinomycetemcomitans*. We therefore tested whether PAR-2 is involved in the recognition of extracts of *A. actinomycetemcomitans* 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% $CO_2$.

### Bacterial extracts and reagents

Bacterial extracts of *A. actinomycetemcomitans* 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. actinomycetemcomitans* extracts for 30 min. Then, the extracts were added to cells cultures. The PAR-2-activating peptide (SLIGKV-$NH_2$) 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 GAACCUGUUUAA-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 $1.0 \times 10^5$ 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% $CO_2$. 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 RNeasy 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

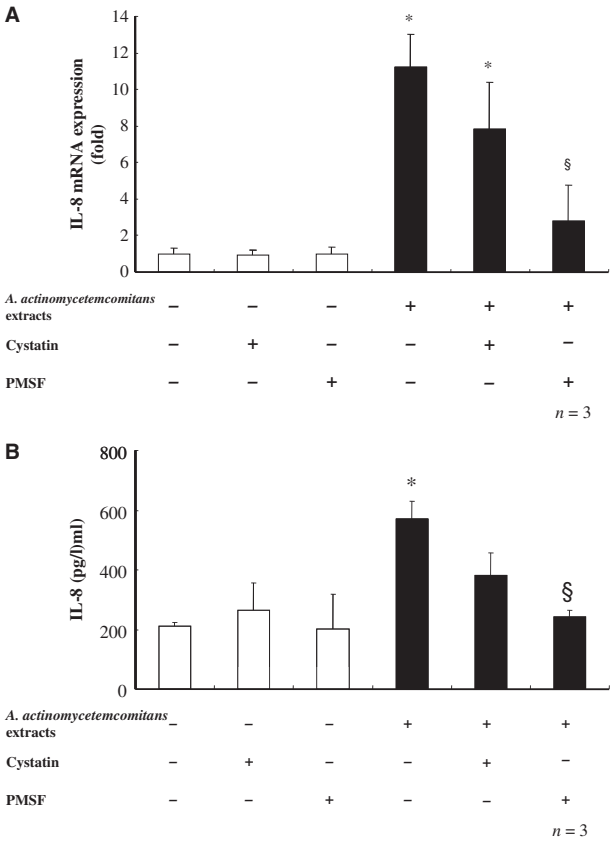


*Fig. 1.* Effects of protease inhibitors on *Aggregatibacter actinomycetemcomitans* extract-induced expression of interleukin 8 (IL-8). (A) Cells were preincubated with protease inhibitors and then stimulated with *A. actinomycetemcomitans* extract (3 μg/ml) for 6 h. Real-time polymerase chain reaction 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. actinomycetemcomitans* 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′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, reverse 5′-CATGTGGGCCATGAGGTCCACCAC-3′, 983 base pairs (bp); PAR-1, forward 5′-TGTGAACTGATCATGTTTATG-3′, reverse 5′-TTCGTAAGATAAGAGATATGT-3′, 708 bp; PAR-2, forward 5′-TGGATGAGTTTTCTGCATCTGTCC-3′, reverse 5′-CGTGATGTTCAGGGCAGGAATG-3′, 1066 bp; PAR-3, forward 5′-ATAACGTTTAAGAGACGGGACT-3′, reverse 5′-TAGCAGTAGATGATAAGCACA-3′, 858 bp; PAR-4, forward 5′-GACGAGAGCGGGAGCACC-3′, reverse 5′-CCCGTAGCACAGCAGCATGG-3′, 725 bp. The PCR products were analysed using 1% agarose gel electrophoresis with ethidium bromide.

### Flow cytometry

Cells were seeded at $1.0 \times 10^5$ 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% $CO_2$. 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 $\mu$l antibody/$10^6$ 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 *A. actinomycetemcomitans* protease in IL-8 and ICAM-1 expression. The pretreatment of cells with the serine protease inhibitor PMSF significantly

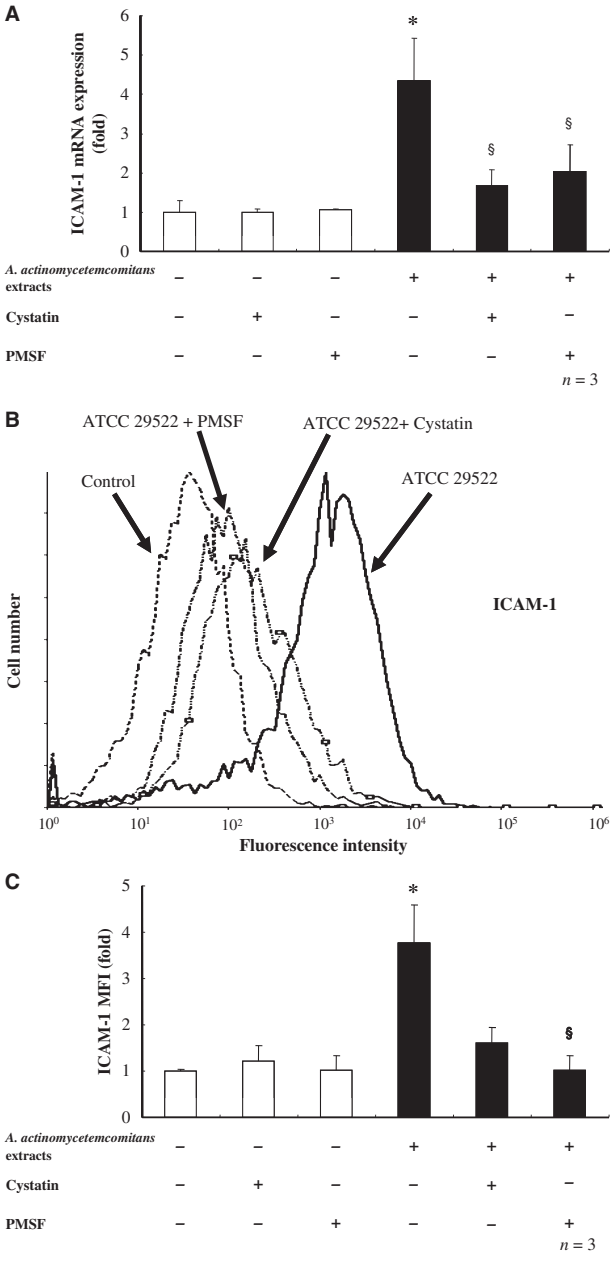


*Fig. 2.* Effects of protease inhibitors on *Aggregatibacter actinomycetemcomitans* extract-induced expression of intercellular adhesion molecule-1 (ICAM-1). (A) Cells were incubated with medium that contained *A. actinomycetemcomitans* extract (3 $\mu$g/ml) for 6 h. Real-time polymerase chain reaction was used to monitor the levels of ICAM-1 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) Representative individual fluorescence intensity profile of ICAM-1 in flow cytometry. (C) Cells were preincubated with protease inhibitors and then stimulated with *A. actinomycetemcomitans* extract (3 $\mu$g/ml) for 24 h. Flow cytometry was used to monitor the levels of ICAM-1. The mean fluorescence intensity (MFI) of ICAM-1 expression is 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.

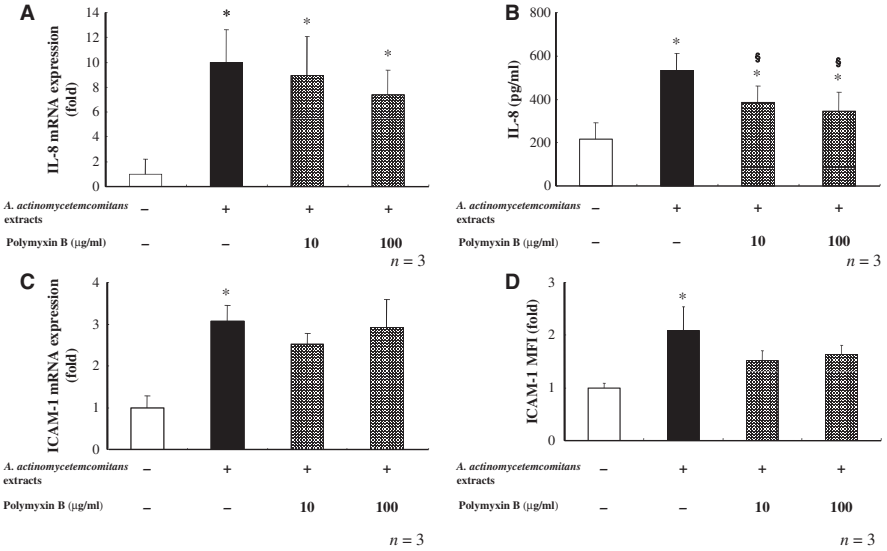


*Fig. 3.* Effects of polymyxin B on interleukin-8 (IL-8) and intercellular adhesion molecule 1 (ICAM-1) expression. Polymyxin B was mixed with *Aggregatibacter actinomycetemcomitans* extracts for 30 min. Then the extracts were added to cells cultures. IL-8 expression was evaluated 6 h post stimulation 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.

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.

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 the 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).

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.

## Discussion

The oral epithelium is directly exposed to periodontal bacteria, and the products of

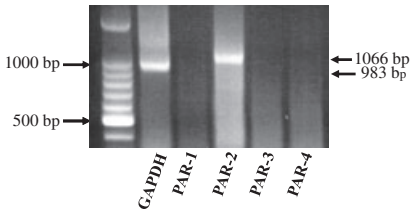


*Fig. 4.* Expression of messenger RNAs encoding protease-activated receptors (PARs) in Ca9-22 cells. RNA isolated from Ca9-22 cells was analysed for PAR transcripts by reverse transcription-polymerase chain reaction (RT-PCR) as described in the Materials and methods. The sizes of the expected products of RT-PCR are shown to the right of panel.

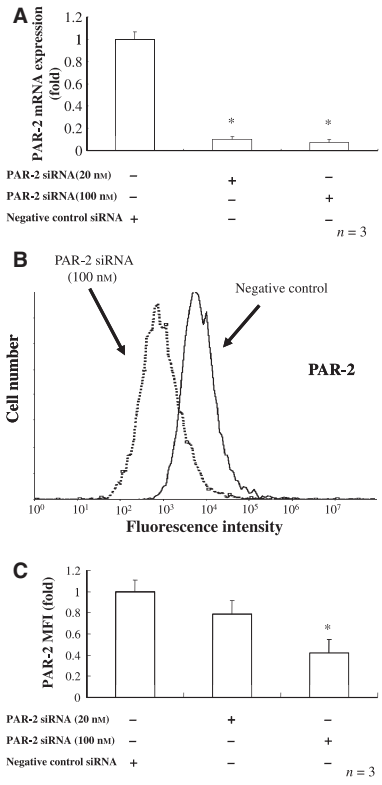


*Fig. 5.* Effects of protease-activated receptor-2 (PAR-2) silencing. Cells were transfected with 20 or 100 nM PAR-2 small interfering RNA (siRNA) or 100 nM negative control siRNA. Six hours after transfection, PAR-2 messenger RNA expression was evaluated using real-time polymerase chain reaction (A). PAR-2 expression was evaluated 24 h post transfection using flow cytometry (B,C). *$P < 0.01$ vs. control.

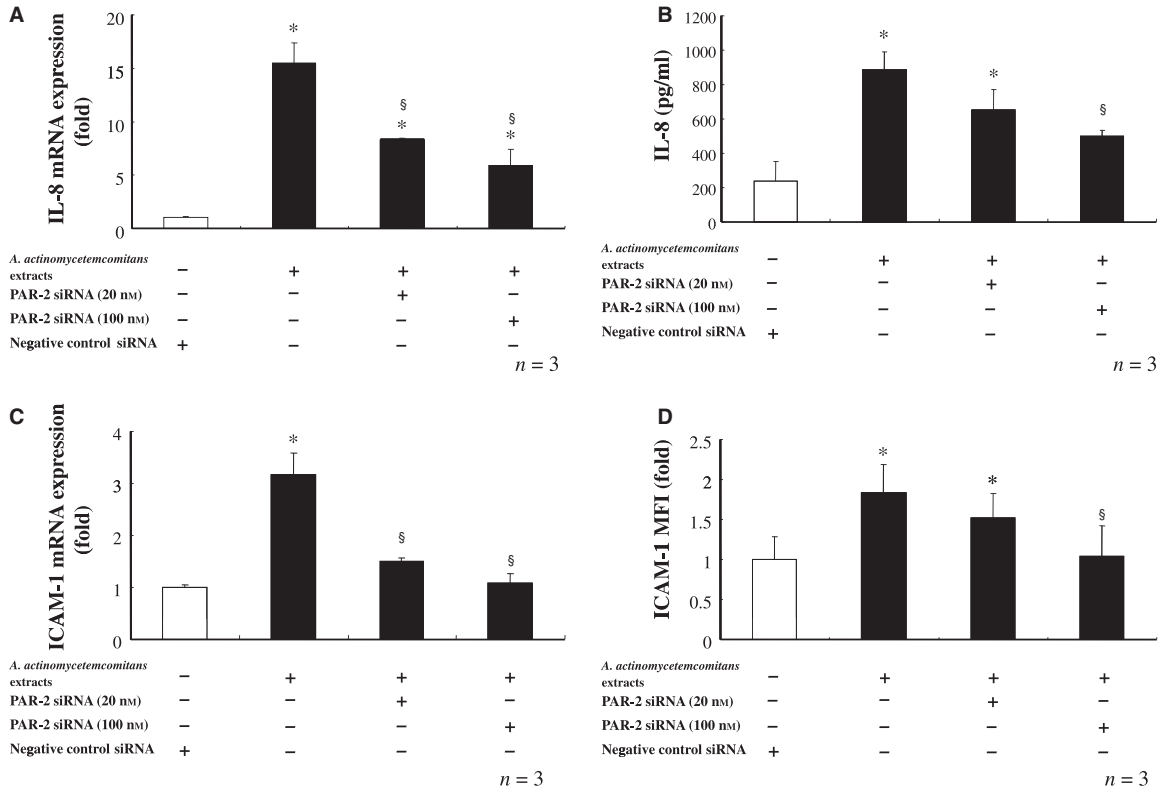


*Fig. 6.* Effects of protease-activated receptor 2 (PAR-2) silencing on interleukin-8 (IL-8) and intercellular adhesion molecule-1 (ICAM-1) expression. Cells were transfected with 20 or 100 nM PAR-2 small interfering RNA (siRNA) or 100 nM negative control siRNA. Six hours after transfection, the medium 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.

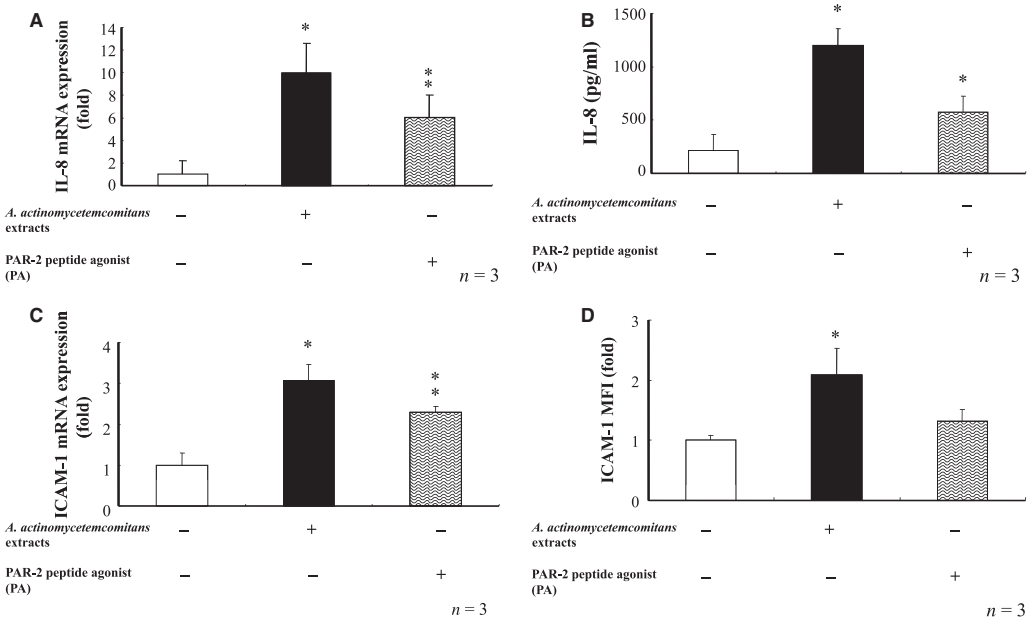


*Fig. 7.* Effects of protease-activated receptor 2 (PAR-2) agonist peptide on interleukin-8 (IL-8) and intercellular adhesion molecule 1 (ICAM-1). Cells were stimulated with 100 $\mu$M PAR-2 agonist peptide (SLIGKV-$NH_2$). IL-8 expression was evaluated 6 h post stimulation for messenger RNA (A) and 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 $\beta$-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.

## Acknowledgements

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