Irsogladine maleate regulates neutrophil migration and E-cadherin expression in gingival epithelium stimulated by Aggregatibacter actinomycetemcomitans

Tsuyoshi Fujita¹, Akiyoshi Kishimoto¹, Hideki Shiba¹, Kouichi Hayashida¹, Mikihiro Kajiya¹, Yuushi Uchida¹, Shinji Matsuda¹, Katsuhiro Takeda¹, Kazuhisa Ouhara¹, Hiroyuki Kawaguchi¹, Yoshimitsu Abiko², Hidemi Kurihara³

¹) Department of Periodontal Medicine, Division of Frontier Medical Science, Hiroshima University Graduate School of Biomedical Sciences
²) Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo

Corresponding author: Tsuyoshi Fujita, Department of Periodontal Medicine, Division of Frontier Medical Science, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3, Kasumi, Minami-ku, Hiroshima, JAPAN, 734-8553

Phone: +81-82-257-5663, FAX: +81-82-257-5664

Email: tfuji@hiroshima-u.ac.jp
Abstract

Irsogladine maleate (IM) counters Aggregatibacter actinomycetemcomitans-induced reduction of the gap junction intercellular communication and the expression of zonula occludens-1, which is a major tight junction structured protein, in cultured human gingival epithelial cells (HGEC). In addition, IM obviates the A. actinomycetemcomitans-induced increase in interleukin (IL)-8 levels in HGEC. Thus, by regulating the intercellular junctional complex and chemokine secretion in HGEC, IM may be useful to prevent periodontal disease. To clarify the effects and regulatory mechanism of IM in vivo and in vitro, we examined the expression of E-cadherin and neutrophil chemotaxis induced by A. actinomycetemcomitans under IM pretreatment. Immunohistochemical studies revealed that A. actinomycetemcomitans application to the gingival sulcus decreased the number of cells positive for E-cadherin and increased those positive for cytokine-induced neutrophil chemoattractant-2α (CINC-2α) in rat gingival epithelium. However, in IM-pretreated rats, A. actinomycetemcomitans application had little effect on CINC-2α and E-cadherin in gingival epithelium. In cultured HGEC, real-time PCR and
Western blotting showed that IM and the ERK inhibitor PD98059 abolished the *A. actinomycetemcomitans*-induced increase in CXCL-1 and IL-8 in HGEC. On the other hand, IM, PD98059, and the p38 MAP kinase inhibitor SB203580 recovered the decrease in E-cadherin expression. In addition, conditioned medium from *A. actinomycetemcomitans*-stimulated HGEC enhanced human neutrophil chemotaxis, compared to that from un-stimulated HGEC or that from *A. actinomycetemcomitans*-stimulated HGEC under IM pretreatment. Furthermore, IM down-regulated the p38 MAP kinase and ERK phosphorylations induced by *A. actinomycetemcomitans*. In conclusion, IM may control *A. actinomycetemcomitans*-induced gingival inflammation by regulating neutrophil migration and E-cadherin expression in gingival epithelium.

Keywords: Irsogladine maleate, *Aggregatibacter actinomycetemcomitans*, E-cadherin, neutrophil migration, CXC-chemokine, gingival epithelial cells
1. Introduction

Periodontitis is an inflammatory condition caused by colonization of the gingival sulcus by periodontopathogenic bacteria. *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) is a facultative gram-negative anaerobic coccobacillus that has the capacity to ferment many sugars, including glucose and fructose, and is strongly implicated as a causative organism in periodontitis [1-3]. Several virulence factors from *A. actinomycetemcomitans* have been identified, including lipopolysaccharide, leukotoxin, cytolethal distending toxin, collagenase, and outer membrane protein [4-8]. In periodontitis, gingival epithelial cells actively contribute to inflammatory processes as they represent the first line of defense against microbial attack. Epithelial cells function as a mechanical barrier against invasion by pathogenic organisms and promote intercellular communication through cell-cell junction complexes, for example, tight junctions, adherens junctions, and gap junctions [9-12]. In addition, epithelial cells produce inflammatory cytokines and anti-microbial peptides. Therefore, the interaction between epithelial cells and *A. actinomycetemcomitans* has been suggested to play a significant role in the initiation of periodontitis.
Irsogladine maleate (IM) is known to enhance gap junctional intercellular communication in cultured rabbit gastric epithelial and pancreatic cancer cells [13, 14], and is clinically used as an anti-gastric ulcer agent. Our previous studies have shown that IM counters *A. actinomycetemcomitans*-induced reduction of the gap junction intercellular communication and the expression of zonula occludens-1, which is a major tight junction structured protein, and obviates the *A. actinomycetemcomitans*-induced increase in interleukin (IL)-8 levels in the culture of human gingival epithelial cells (HGEC) [11, 12]. Furthermore, IM counters the IL-1β-induced suppression in gap junctional intercellular communication in HGEC [15]. Since IM seems to regulate inflammatory responses induced by the bacterial attack and cytokine stimulation of human epithelial cells, it may be a candidate preventive medicine for periodontal disease.

An accumulation of activated neutrophils in lesional areas is observed in all diseases, and is thought to be involved in the onset of inflammation. As inflammation progresses, the distraction of periodontal tissue occurs with deeper periodontal pockets. The development of
periodontal disease seems to be related the progression of inflammatory cell infiltration into deeper periodontal tissues [16]. Previous reports showed that IL-8 is present in diseased human periodontal tissues [17-20], and the levels of IL-8 in both periodontal tissue and GCF have been correlated with the disease severity [21]. In addition, the expressions of IL-8 in diseased tissue, especially in gingival epithelium, is correlated with the migration of PMNs [17, 22, 23, 24]. Taken together, as Graves et al. suggested, the development of periodontal diseases may be related to the progression of inflammatory cell infiltration into periodontal tissues [16]. Therefore, the blocking of neutrophil activity and regulation of CXC-chemokines represent candidate therapeutic strategies for inflammation.

The junctional epithelium is located at a strategically important interface at the base of the gingival sulcus. E-cadherin, a subclass of cadherin found in stratified squamous epithelium, plays a crucial role in maintaining the structural integrity and function of both adherens and desmosomal epithelial intercellular junctions [25]. In the junctional epithelium, E-cadherin, which is a key molecule involved in forming adherens junctions and desmosomes [25], is known to play an important role
against bacterial invasion [26-28], although the reduction of E-cadherin was observed in inflamed gingival tissue [26, 29]. In addition, *Porphyromonas gingivalis* or *A. actinomycetemcomitans* decreased E-cadherin expression in cultured gingival epithelial cells [9, 10]. In gastric mucosal epithelium, the disruption of E-cadherin seems to cause epithelial permeability to decrease [30]. Thus, the breakdown of interconnecting epithelial cell adhesions was suggested to lead to the disruption of the epithelial cell barrier function. Recovery of the barrier function may cause prevent bacterial invasion.

Mitogen-activated protein (MAP) kinases play a central role in mediating intracellular signal transduction and regulating cell functions in HGEC [31-33]. Three distinct mammalian MAP kinases have been identified: extracellular signal-regulated kinase (ERK or p44/42 MAP kinase), c-Jun kinase or the stress-activated protein kinase (c-JNK or SAP kinase), and p38 MAP kinase. In this study, to clarify the effect and mechanism of IM, we focused on neutrophil migration, the barrier function, and involvement of MAP kinase in the gingival epithelium on stimulation with *A. actinomycetemcomitans*. 
2. Materials and Methods

2.1. Reagents and antibodies

IM was supplied by Nippon Sinyaku (Kyoto, Japan). Humedia-KB2 medium was obtained from Kurabo (Osaka, JAPAN). Todd-Hewitt broth was obtained from BBL™ (Cockeysville, MD). Yeast extract was from Difco Laboratories (Detroit, MI). SB203580 and PD98059 were purchased from Calbiochem (La Jolla, CA). Histopaque 1119 and Histopaque 1077 were purchased from Sigma (St. Louis, MO). ISOGEN was from Wako Pure Chemical Industries (Osaka, Japan). Goat anti-rat CINC-2α antibody, goat anti-mouse E-cadherin antibody, mouse anti-human E-cadherin antibody were from R&D Systems (Minneapolis, MN). Rabbit anti-phosphor-p38 MAP kinase antibody, rabbit anti-total p38 MAP kinase antibody, rabbit anti-phosphorylated ERK antibody, and rabbit anti-total ERK antibody were from Cell Signaling (Beverly, MA). Mouse anti-human β-actin antibody was from Zymed (Carlsbad, CA). HRP-conjugated sheep anti-rabbit or anti-mouse IgG, and ECL Plus Western blotting detection reagents were obtained from Amersham Biosciences (Arlington Heights, IL).
2.2. Animal experiment

After approval by the animal care committee of Hiroshima University was obtained, a total of twenty-four, 9-week-old, male Fischer 344 rats were used in this study. Under intraperitoneal anesthesia of 20% ethyl carbamate (30 mg/kg body weight), rats were used in this study. IM was dissolved in the 0.5% methyl cellulose and 10 µl of the solvent was injected under the skin of the back (3 mg/kg body weight). After 1 hour of injection of IM or 0.5% methyl cellulose alone, whole live *A. actinomycetemcomitans* Y4 at $1 \times 10^9$ cells/ml or phosphate-buffered saline (PBS) was applied into gingival pocket. Rats were killed at 3 hour after *A. actinomycetemcomitans* application, by an overdose of ethyl ether.

Tissue samples were resected en bloc from the left and right molar regions and fixed with 4% paraformaldehyde solution. They were then decalcified in a 10% ethylenediaminetetraacetate (EDTA) solution in PBS for 14 days at 4°C. The decalcified tissue blocks were embedded in paraffin. Sections (5 µm thick) of the frontal plane parallel to the long axis of the tooth, including the root apex, were cut and collected on glass slides. We divided
the preparations into 3 groups: methyl cellulose pre-injected rats before PBS application, methyl cellulose pre-injected rats before \textit{A. actinomycetemcomitans} application, and IM-injected rats before \textit{A. actinomycetemcomitans} application. The sections were stained with hematoxylin and eosin, and observed under a light microscope.

\textbf{2.3. Histomorphometric analysis of polymorphonuclear leukocyte (PMN) infiltration}

For histomorphometric analysis, the number of infiltrated PMNs in the gingival tissue was counted using specimens stained with hematoxylin and eosin. Small, round cells containing a nucleus with three to five lobes were judged as PMNs. The number of PMNs per unit area (0.1x0.1 mm$^2$ glid) was counted and the number of PMNs from 10 units at random was summed. The number of cells were averaged from 12 different sections in each experimental and control group.

\textbf{2.4. Immunohistochemistry for CINC-2$\alpha$ and E-cadherin}

After deparaffinization, the endogenous horseradish peroxidase was
deactivated with 3% hydrogen peroxide in PBS. Then, each section was incubated with 0.2% casein in Tris-HCl buffered saline (TBS, 20 mM Tris HCl, 0.15 M NaCl, pH 7.6) for 30 minutes at room temperature and then with goat anti-rat CINC-2α antibody and goat anti-mouse E-cadherin antibody for 24 hours at 4°C. After being rinsed with PBS, the sections were incubated with biotinylated anti-goat IgG for 30 minutes. The section were rinsed with PBS, incubated with the peroxidase-conjugated streptavidin for 30 minutes, and then rinsed with PBS. The color was developed with 0.025% 3,3’-diaminobenzidine tetrahydrochloride in TBS plus hydrogen peroxide. The specimens were counterstained with methyl green, dehydrated, and then mounted.

2.5. Culture of A. actinomycetemcomitans

A. actinomycetemcomitans Y4 was grown in Todd-Hewitt broth supplemented with 1% yeast extract at 37°C for 2 days. After cultivation, whole cells were harvested by centrifugation and washed three times in PBS (pH 7.4). Some of the washed A. actinomycetemcomitans were suspended in Humedia-KB2 medium (pH 7.4) containing 10 μg/ml insulin, 5 μg/ml
transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 nM sodium selenite.

2.6. Preparation of cells

Healthy gingival tissues, which had been surgically dissected through the process of wisdom tooth extraction and which were to be discarded, were collected with patients’ informed consent. HGEC were isolated as previously described [11, 12, 34]. Briefly, gingival tissues were treated with 0.025% trypsin and 0.01% EDTA overnight at 4°C, and divided into the epithelium and connective tissues. The HGEC suspension was centrifuged at 120 x g for 5 minutes, and the pellet was suspended in Humedia-KB2 medium containing 10 μg/ml insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 μM sodium selenite, 50 μg/ml bovine pituitary extract, 100 units/ml penicillin, and 100 μg/ml streptomycin (medium A). The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO₂/95% air at 37°C. When the cells reached subconfluence, they were harvested and subcultured.
2.7. Preparation of conditioned medium (CM) for neutrophil migration

HGEC in cultures at the fourth passage were harvested, seeded at a density of 4 x 10^4 cells/35-mm plastic tissue culture plates coated with type I collagen, and maintained in 2 ml of medium A. After 10 days of culture, these cells were washed three times with phenol red-free Hank’s solution (pH 7.4). Confluent HGEC were exposed to heat-killed A. actinomycetemcomitans Y4 at 1x10^8 cells/ml in the presence or absence of IM at 1 μM for 6, 12, or 24 h before the end of incubation on day 11 in 2 ml of Humedia-KB2 medium containing 10 μg/ml insulin, 5 μg/ml transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol, 10 nM sodium selenite, 100 units/ml penicillin, and 100 μg/ml streptomycin (medium B). CM was obtained and stored at -20°C.

2.8. Human neutrophil migration

Peripheral venous blood was collected into vacutainer tubes containing 25 units/ml of heparin. Neutrophils were isolated by Ficoll-Hypaque density centrifugation, as previous described [35, 36].
Briefly, 3 ml of Histopaque 1119 and 1.5 ml of Histopaque 1077 were layered in 15 ml polystyrene culture tubes. Peripheral blood (4.5 ml) was layered on the separating medium, and the tubes were centrifuged at 500 X g for 30 min. After the neutrophil fraction was collected and contaminating erythrocytes were lysed, then the isolated cells were washed twice with PBS without magnesium and calcium. The preparations were >95% neutrophils. Neutrophil migration was measured by the Transwell insert method using a 24-well microchemotaxis chamber in which a 5-µm pore-sized filter (Costar, Corning, NY) separates the upper and lower chambers. Isolated human neutrophils were placed into the upper chamber at 1x10⁶ per well in PBS. In the lower chambers, CM from A. actinomycetemcomitans-stimulated HGEC was added as a chemoattractant. Neutrophils were allowed to migrate toward the soluble attractants in the lower chambers for 30 min at 37°C in a humidified atmosphere (5% CO₂). Migrating cells were collected and counted by microscopy. All experiments were performed at least three times.

2.9. RNA preparation
HGEC in cultures at the fourth passage were harvested, seeded at a density of 4 x 10^4 cells/35-mm plastic tissue culture plates coated with type I collagen, and maintained in 2 ml of medium A. After 10 days of culture, these cells were washed three times with phenol red-free Hank’s solution (pH7.4). To study the involvement of p38 MAP kinase and ERK, SB203580 (p38 MAP kinase inhibitor) and PD98059 (ERK inhibitor) were used. Confluent HGEC were pretreated with IM (1 μM), SB203580 (10 μM), and PD98059 (10 μM) for 1 h, and then exposed to heat-killed *A. actinomycetemcomitans* Y4 at 1x10^8 cells/ml or 5x10^8 cells/ml in the presence or absence of IM for 12 or 24 h before the end of incubation on day 11 in 2 ml in medium B. Total RNA was extracted using ISOGEN and quantified by spectrometry at 260 and 280 nm.

2.10. Determination of CXCL-1, CXCL-8 (IL-8), and E-cadherin mRNA

First standard cDNA synthesis was performed with 1 μg of total RNA extract in a total volume of 20 μl (Roche, Tokyo, Japan). Real-time PCR was performed with a Lightcycler system using SYBER green (Roche). The sense and anti-sense primers for human CXCL-1, IL-8, E-cadherin, and
GAPDH mRNA are listed in Table 1.

2.11. ELISA for CXCL-1 and IL-8

HGEC were cultured as described in the section entitled 2.9. RNA preparation. Confluent HGEC, which had been pretreated with SB203580 (10 μM), PD98059 (10 μM) for 1 h, were exposed to heat-killed *A. actinomycetemcomitans* Y4 at 1 x10^8 cells/ml in the presence or absence of IM at 1 μM for 12 h before the end of incubation on day 11 in 5 ml of medium B. The concentrations of CXCL-1 and IL-8 in the medium were determined using CXCL-1 and IL-8 ELISA kits, respectively (Biosource International, Camarillo, CA, U.S.A.).

2.12. Immunoblotting for E-cadherin, β-actin, phosphorylated p38 MAP kinase, total p38 MAP kinase, phosphorylated ERK, and total ERK

To analyze E-cadherin and β-actin expressions, HGEC were cultured as described in the section entitled 2.9. RNA preparation. Confluent HGEC, which had been pretreated with SB203580 (10 μM), PD98059 (10 μM), and IM (1 μM) for 1 h, were exposed to heat-killed *A. actinomycetemcomitans* Y4
at 5x10^8 cells/ml for 24 h before the end of incubation on day 11 in 2 ml of medium B. To detect phosphorylated p38 MAP kinase, total p38 MAP kinase, phosphorylated ERK, and total ERK, confluent HGEC were exposed to *A. actinomycetemcomitans* Y4 in the presence or absence of IM for 10 or 20 min. Cells were lysed in 200 µl of SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromphenol blue). Samples were resolved on a 10% SDS-polyacrylamide gel by electrophoresis under non-reducing conditions and electrophoretically transferred onto membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 5% non-fat dried milk for 1 h and then reacted with mouse anti-human E-cadherin antibody, mouse anti-human β-actin antibody, rabbit anti-phosphor-p38 MAP kinase antibody, rabbit anti-total p38 MAP kinase antibody, rabbit anti-phosphorylated ERK antibody, or rabbit anti-total ERK antibody, overnight. The membrane was incubated with HRP-conjugated sheep anti-rabbit or anti-mouse IgG in TBS for 1 h at room temperature. Immunodetection was performed according to the manual supplied with the ECL Plus Western blotting detection reagents.
2.13. DNA microarray

Microarray analysis was conducted using GeneChip Human Genome U133 Plus 2.0 (ca. 8,000 probe sets, Affymetrix, Santa Clara, CA). RNA samples were reverse-transcribed, and biotin-labeled cRNA probes were synthesized by in vitro transcription. Subsequently, the probes were hybridized to the human genome (GeneChip Fluidics Station, Affymetrix) using the provided instructions. Chip performance, background levels, and the presence or absence of signals were assessed using the supplied instructions (Microarray Suite Software, Affymetrix). Each chip in a given set was normalized by adjusting the probe intensity. The presence and absence of signals were reevaluated, and intensity normalization was performed across all arrays.

2.14. Statistical analysis

Comparisons between groups were analyzed with Student’s t-test or ANOVA.
3. Results

3.1. Histological findings of rat gingival epithelium

We divided the preparations into 3 groups: non-applied control rats, *A. actinomycetemcomitans*-applied rats without IM, and IM-injected rats before *A. actinomycetemcomitans* application. HE staining indicates that *A. actinomycetemcomitans* application caused the dilatation of intercellular spaces, and the severe infiltration of PMNs into the gingival epithelium (figure 1-B). On the other hand, in IM-injected rats before *A. actinomycetemcomitans* application, the gingival epithelium showed the minimal migration of PMNs through intercellular spaces (figure 1-C). Figure 1-D shows the number of PMNs that infiltrated into the gingival epithelium from that 12 samples. PMNs migrating into the gingival epithelium were seen in small numbers in the non-applied group. Although the application of *A. actinomycetemcomitans* significantly increased the number of PMNs that infiltrated into the gingival epithelium, IM-pretreatment before *A. actinomycetemcomitans* application inhibited the increase in PMNs induced by *A. actinomycetemcomitans* (figure 1-D).
3.2. Localization of CINC-2α in rat gingival epithelium

Figure 2 illustrates the localization pattern of CINC-2α protein. Positive reactions for CINC-2α were seen in a small number of gingival epithelial cells in control rats. Intense staining for CINC-2α was seen in the *A. actinomycetemcomitans*-applied gingival epithelium (figure 2), although IM pretreatment inhibited the *A. Actinomycetemcomitans*-induced increase in positive reactions for CINC-2α in gingival epithelial cells.

3.3. Localization of E-cadherin in rat gingival epithelium

Immunohistochemical studies showed that the expression of E-cadherin was intensive in the junctional epithelium at cell-cell contacts from uninfected control rats and *A. Actinomycetemcomitans*-applied rats under IM pretreatment (figure 3-A,C,D,F). However, in *A. actinomycetemcomitans*-applied gingival epithelium, weaker staining for E-cadherin was observed in junctional epithelium (figure 3-B, E).

3.4. Human neutrophil migration for the CM from *A. actinomycetemcomitans*
**actinomycetemcomitans**-stimulated HGEC in vitro

CM from *A. actinomycetemcomitans* stimulated HGEC enhanced human neutrophil chemotaxis compared to the unstimulated condition in an *A. actinomycetemcomitans*-stimulated, time-dependent manner (figure 4-A). Next, to examine the involvement of CXC-chemokines in neutrophil chemotaxis in this model, we examined the inhibition assay using antibodies against CXCR-1 or -2, which are major receptors of CXC-chemokines. The co-incubation of either anti-CXCR-1 or anti-CXCR-2 antibody with HGEC inhibited human neutrophil chemotaxis for the CM from *A. actinomycetemcomitans*-stimulated HGEC. Furthermore, CM from HGEC co-treated with *A. actinomycetemcomitans* and IM did not induce neutrophil chemotactic activity (figure 4-B).

3.5. Expressions of CXCL-1 and CXCL-8 in cultures of HGEC

The exposure of *A. actinomycetemcomitans* at 5 x 10^8 cells/ml to HGEC increased the levels of CXCL1 and IL-8, and the addition of IM to the culture abolished the *A. actinomycetemcomitans*-induced increase at the mRNA and protein levels (figure 5-A,B,C,D). Furthermore, the increases in
CXCL-1 and IL-8 mRNA levels caused by *A. actinomycetemcomitans* were significantly inhibited by pretreatment with PD98059 at 10 μM for 12 h. However, SB203580 had a little effect on the increase in the mRNA expression of CXCL-1 and IL-8 induced by *A. actinomycetemcomitans* (figure 5·A, B). In addition, IM and PD98059 abrogated the increase in CXCL-1 and IL-8 secretion induced by *A. actinomycetemcomitans* (figure 5·C, D), although SB203580 had a little effect on the CXCL-1 secretion in HGEC (data not shown).

**3.6. Expressions of E-cadherin in cultures of HGEC**

*Real-time* PCR and Western blotting showed that the addition of *A. actinomycetemcomitans* at 5 x 10^8 cells/ml to HGEC decreased the expression of E-cadherin at the mRNA and protein levels. However, IM, SB203580, and PD98059 recovered the reduction in E-cadherin induced by *A. actinomycetemcomitans* at the mRNA and protein levels (figure 6·A, B).

**3.7. Action of IM on p38 MAP kinase and ERK in A. actinomycetemcomitans-stimulated HGEC**
The exposure of heat-killed *A. actinomycetemcomitans* to HGEC induced the phosphorylations of p38 MAP kinase and ERK, and the addition of IM inhibited both phosphorylations induced by *A. actinomycetemcomitans*. Neither *A. actinomycetemcomitans* nor IM affected total ERK and total p38 MAP kinase (figure 7-A, B).

### 3.8. DNA microarray analysis

We compared the gene expression patterns among each group (unstimulated HGEC vs. *A. actinomycetemcomitans*-stimulated HGEC, and *A. actinomycetemcomitans*-stimulated HGEC vs. *A. actinomycetemcomitans*-stimulated HGEC under IM treatment). Table 2 indicates that CXCL-1, CXCL-2, CXCL-3, CXCL-6, and CXCL-8 were down-regulated by IM in *A. actinomycetemcomitans*-stimulated HGEC.
4. Discussion

In the present study, we demonstrated that IM inhibited the *A. actinomycetemcomitans*-induced inflammatory response in gingival epithelium by suppressing neutrophil migration *in vivo and in vitro*. In addition, IM recovered the *A. actinomycetemcomitans*-induced reduction in E-cadherin, suggesting enhancement of the barrier function of gingival epithelium.

Chemokines are a strong inducer of neutrophil chemotaxis and classified into four sub-families depending on the number and spacing of the first conserved cysteine residues in the NH2 terminal region: CXC, CC, XC, and CX3C [37-39]. CXCL-1/GRO-α, CXCL-2/GRO-β, CXCL-3/GRO-γ, CXCL-5/epithelial cell-derived neutrophil attractant (ENA-78), CXCL-6/granulocyte chemotactic protein (GCP2), CXCL7/neutrophil activating protein (NAP2), and CXCL8/IL-8 are potent activators and chemoattractants for neutrophils, and CXCR-1 and CXCR-2 are known as their main receptors [37-39]. Among these chemokines, CXCL-1 and CXCL-8 are known to be stronger neutrophil attractants at sites of inflammation [37]. In the present study, CM from *A.
*actinomycetemcomitans*-stimulated HGEC induced human neutrophil migration. In addition, the levels of CXC-chemokines in the CM were increased by the exposure of *A. actinomycetemcomitans* to HGEC. Furthermore, the blocking of CXCR-1 or CXCR-2 resulted in the inhibition of neutrophil migration to CM from *A. actinomycetemcomitans*-stimulated HGEC. An immunohistochemical study of the rat gingiva showed that the application of *A. actinomycetemcomitans* increased the secretion of CINC-2α and leukocyte migration to gingival epithelium. These data suggest that CXC-chemokines play a critical role in *A. actinomycetemcomitans*-induced neutrophil accumulation in the gingival epithelium.

In the present study, the animal model indicated that IM suppressed the *A. actinomycetemcomitans*-induced increase in CINC-2α expression in rat gingival epithelium, and inhibited leukocyte migration into it. In addition, CM from IM-treated HGEC with *A. actinomycetemcomitans* inhibited neutrophil migration more markedly than that from IM-untreated HGEC with *A. actinomycetemcomitans*. IM inhibited the increased levels of IL-8 and CXCL-1 in CM from *A. actinomycetemcomitans*-stimulated HGEC. Thus, by suppressing CXC-chemokine expression, IM
down-regulates *A. actinomycetemcomitans*-induced neutrophil migration into gingival epithelium. DNA array analysis showed that IM inhibited the increased expression of several CXC-chemokines induced by *A. actinomycetemcomitans* stimulation in HGEC. Using *real-time* PCR, we confirmed that IM inhibited the *A. actinomycetemcomitans*-induced increase in CXCL-2 and CXCL-3 mRNA in HGEC, which is consistent with DNA array data (data not shown). These data suggest that the inhibition of CXC-chemokine expression is one of the characteristics of IM.

For the inhibition of neutrophil migration, there is still argument from host protection to tissue destruction. Neutrophils play an important role in combating infection by phagocytosis and killing infecting bacteria [40]. The neutrophil is the first cell type found in large numbers in early periodontal lesions [23]. Since the chemotaxis of neutrophils to the site of infection is an important step in the immune response induced by chemoattractants, some reports have shown that defective chemotaxis is involved in the initiation of periodontal disease because of a disordered immunological response to bacteria [36, 41, 42]. In contrast, the persistence of a local chronic host response may alter the protective roles of
inflammatory cells and have deleterious effects on tissues [43-45]. In fact, the hyperactivity of neutrophils is associated with periodontal tissue destruction [46, 47]. Further, it has also been reported that chronic inflammatory conditions often result from the aberrant production of chemokines such as IL-8 [48]. Aberrant IL-8 production can lead to chronic inflammatory conditions, as suggested for inflammatory diseases such as rheumatoid arthritis [49, 50]. In addition to several animal models of acute inflammatory disease [51-54], the use of anti-IL-8 neutralizing antibody leads to clinically relevant reductions in the disease activity of patients suffering from palmoplantar pustulosis, a chronic inflammatory skin disease characterized by high-level IL-8 overexpression [55]. Thus, the abrogation of chemokine activity represents a candidate therapeutic strategy for inflammatory disease in gingival tissue. IM, regulating neutrophil migration into gingival tissue, may suppress periodontal inflammation and prevent the initiation of periodontal disease.

In the present study, in vivo and in vitro investigations showed that IM prevented the A. actinomycetemcomitans-induced decrease of E-cadherin in gingival epithelial cells. The epithelium provides an important barrier
against microbial invasion. Epithelial cells are generally interconnected by tight junctions, adherence junctions, desmosomes, and gap junctions, and tight junctions are essential for the tight sealing of cellular sheets, thus controlling paracellular ion flux and maintaining tissue homeostasis [56]. However, previous studies have shown that the junctional epithelium is interconnected by a few desmosomes only, occasionally by gap junctions, and shows wide intercellular spaces [57, 58]. E-cadherin is a key molecule in the formation of adherens junctions and desmosomes [25]. Therefore, it is suggested that the recovery of A. actinomycetemcomitans-induced decrease in E-cadherin resulted in enhancement of the barrier function in junctional epithelium. IM may enhance the epithelial barrier to prevent the invasion of periodontopathogenic bacteria.

The ERK inhibitor as well as IM blocked A. actinomycetemcomitans-induced CXC-chemokine expressions. These data are consistent with our previous study that IM abolishes the increase in IL-8 by outer membrane protein 29 from A. actinomycetemcomitans through ERK in HGEC [34]. In contrast, the p38 MAP kinase inhibitor, ERK inhibitor, and IM recovered the A. actinomycetemcomitans-induced reduction in
E-cadherin expression of HGEC. Previous studies have shown that hypotonic stress induces E-cadherin expression in cultured human keratinocytes through p38 MAP kinase [59]. ERK regulates E-cadherin expression in EGF-induced cell migration [60]. Thus, both p38 MAP kinase and ERK are known to be involved in the regulation of E-cadherin expression. However, as far as we know, there are no reports regarding the involvement of MAP kinase in the regulation of E-cadherin expression by bacterial stimulation. Our present data suggest that p38 MAP kinase and ERK are involved in the reduction of E-cadherin by the exposure of HGEC to *A. actinomycetemcomitans*.

Cyclic AMP and protein kinase A are known as intercellular signalling molecules induced by IM, although there has been no report on the specific receptor of IM. IM enhanced gap junctional intercellular communication by increasing cyclic AMP levels in rabbit gastric epithelial cells [13]. IM also up-regulated gap junctional intercellular communication between human pancreatic cancer cells via protein kinase A [14]. In addition, IM countered the reduction in OMP29-induced gap junctional intercellular communication in HGEC by up-regulating the expression of
cyclic AMP [11]. In the present study, IM inhibited both phosphorylation of ERK and p38 MAP kinases in HGEC stimulated by *A. actinomycetemcomitans*. IM may regulate the expression of E-cadherin and CXC-chemokines upstream of p38 MAP kinase and ERK.

Although further the IM studies are required, the previous and present data suggest therapeutic efficiency of IM in the suppression of periodontal inflammation. IM has been clinically used as a medicament that protects the gastric mucosa. IM, by regulating the physical barrier between epithelial cells and neutrophil migration in gingival epithelial cells, may be useful for the prevention of periodontal disease.

Acknowledgements

We thank the Nippon Shinyaku Co., Ltd., (Kyoto, JAPAN), for donating IM.

This study was supported in part by a Grant-in-aid for the encouragement of Young scientists (B) (No. 20791613) from the Japan Society for the Promotion of Science, Japan.
References


34. Kishimoto A, Fujita T, Shiba H, Komatsuzawa H, Takeda K, Kajiya M, et al., Irsogladine maleate abolishes the increase in interleukin-8 levels caused by outer membrane protein 29 from Aggregatibacter (Actinobacillus) actinomycetemcomitans.

35. Fujita T, Zawawi KH, Kurihara H and Van Dyke TE, CD38 cleavage in fMLP- and IL-8-induced chemotaxis is dependent on p38 MAP kinase but independent of p44/42 MAP kinase. *Cell Signal* 17(2): 167-75, 2005.


Table 1 Primers for real-time PCR used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (Sense)</th>
<th>Reverse (Anti-sense)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL-1</td>
<td>5'-CAA CCC CAA GTT AGT TCA ATC T-3'</td>
<td>5'-CAT GTT GCA GGC TCC TCA-3'</td>
</tr>
<tr>
<td>IL-8</td>
<td>5'-ATG ACT TCC AAG CTG GCC GTG GCT-3'</td>
<td>5'-TCT CAG CCC TCT TCAAAA ACT TCT C-3'</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>5'-TTC TGC TGC TGT TGT TTC-3'</td>
<td>5'-AGT CAA AGT CCT GGT CCT CTT-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AAC GTG TCA GTG GTG GAC CTG-3'</td>
<td>5'-AGT GGG TGT CGC TGT TGAAGT-3'</td>
</tr>
<tr>
<td>Common</td>
<td>Gene Title_Affy</td>
<td>Fold change</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aa/control</td>
</tr>
<tr>
<td>CXCL1</td>
<td>chemokine (C-X-C motif) ligand 1</td>
<td>3.36</td>
</tr>
<tr>
<td>CXCL2</td>
<td>chemokine (C-X-C motif) ligand 2</td>
<td>4.805</td>
</tr>
<tr>
<td>CXCL3</td>
<td>chemokine (C-X-C motif) ligand 3</td>
<td>5.076</td>
</tr>
<tr>
<td>CXCL6</td>
<td>chemokine (C-X-C motif) ligand 6</td>
<td>4.70</td>
</tr>
<tr>
<td>CXCL8</td>
<td>chemokine (C-X-C motif) ligand 8</td>
<td>6.665</td>
</tr>
</tbody>
</table>
Fig. 1

(A) (B) (C)

(D)

Numbers of inflammatory cells

0 50 100 150 200 250

- Aa Aa + IM

** **

Fig. 1
**Fig. 4**

(A) Graph showing the percentage of migrating cells over time (0, 6, 12, and 24 hours) in the presence of CXCR1 and CXCR2 antibodies.

(B) Graph illustrating the effect of CM and antibodies on migrating cells, with significant increases marked at 12 and 24 hours.

Legend:
- Aa: Antibody to CXCR1
- IM: Antibody to CXCR2
- **: Statistical significance

CM: Concentrated medium

Legend for antibodies:
- - : No antibody
- CXCR1 antibody
- CXCR2 antibody
Fig. 5

(A) Relative expression for GAPDH

(B) Relative expression for GAPDH

(C) CXCL-1 levels (pg/ml)

(D) CXCL-8 levels (pg/ml)

- Aa
- IM
- SB
- PD
Fig. 6

(A) **Relative expression for GAPDH**

- **Aa**
- **IM**
- **SB**
- **PD**

(B) 

- **E-cadherin**
- **β-actin**

**Legend:**
- **-**
- **+**

**Significance:**
- ****

**Notes:**
- Graph shows relative expression levels for GAPDH with treatment conditions indicated for each group.
Fig. 7

(A) Phospho-p38 MAP kinase

(B) Phospho-ERK
**Figure legends**

Figure 1. Histological findings in the gingival tissues. Hematoxylin & eosin staining. Original magnification of gingival tissue representing the (A) control group, (B) *A. actinomycetemcomitans*-applied group, and (C) *A. Actinomycetemcomitans*-applied group under IM pre-treatment. (D) The number of infiltrating PMNs in the gingival tissue. The number of PMNs per a unit area (0.1x0.1 mm² grid) was counted, and the numbers of PMNs from 10 units were summed. The number of cells were averaged from 12 different sections in each experimental and control group. The results are expressed as means ± SD of 12 sections tested for each group. **Differs significantly (t-test, P<0.01).**

Figure 2. Immunohistochemical staining of CINC-2α in the gingival tissues. Original magnification of gingival tissue representing the (A) control group, (B) *A. Actinomycetemcomitans*-applied group, and (C) *A. Actinomycetemcomitans*-applied group under IM pre-treatment.

Figure 3. Immunohistochemical staining of E-cadherin in the gingival tissues. Gingival tissue representing the (A, D) control group, (B, E) *A. Actinomycetemcomitans*-applied group, and (C, F) *A. Actinomycetemcomitans*-applied group under IM pre-treatment. (A, C, E) Original magnification. (B, D, F) Higher magnification.

Figure 4. Human neutrophil migration for CM from *A.
*Actinomyces* sp. stimulated HGEC. (A) Time-course experiment of human neutrophil migration for CM from *A. Actinomyces* sp. stimulated HGEC. Values are means ± SD of three cultures. **Differs significantly (ANOVA, P<0.01).** (B) Antibody blockade of anti-CXCR-1 or anti-CXCR-2 antibody on human neutrophil migration for CM from *A. Actinomyces* sp. stimulated HGEC in the presence or absence of IM. Values are means ± SD for three cultures in one experiment. Three independent experiments were performed and similar results were obtained. **Differs significantly (t-test, P<0.01).**

Figure 5. Effect of IM, SB203580, or PD98059 on the expression of CXCL-1 and IL-8 in HGEC exposed to killed *A. Actinomyces* sp. Confluent HGEC were exposed to killed *A. Actinomyces* sp. at 1x10^8 cells/ml in the presence or absence of IM at 1 μM, SB203580 at 10 μM, or PD98059 at 10 μM for 12 or 24 h before the end of incubation. After 12 h stimulation, the CXCL-1 mRNA level was analyzed by real-time PCR. (B) After 12 h stimulation, the IL-8 mRNA level was analyzed by real-time PCR. (C) After 24 h stimulation, the CXCL-1 level was determined using an ELISA kit. (D) After 24 h stimulation, the IL-8 level was determined using an ELISA kit. Values are means ± SD for three cultures in one experiment. Three independent experiments were performed and similar results were obtained. **Differs significantly (t-test, P<0.01).** *Differs significantly (t-test, P<0.05)*

Figure 6. Effect of IM, SB203580, or PD98059 on the expression of E-cadherin in
HGEC exposed to killed *A. Actinomyces actinomycetemcomitans*. Confluent HGEC were exposed to killed *A. Actinomyces actinomycetemcomitans* at $5 \times 10^8$ cells/ml in the presence or absence of IM at 1 μM, SB203580 at 10 μM, or PD98059 at 10 μM for 24 h before the end of incubation. (A) E-cadherin mRNA was analyzed by real-time PCR. Values are means ± SD for three cultures in one experiment. Three independent experiments were performed and similar results were obtained. **Differs significantly (**$t$-test, P<0.01). *Differs significantly (**$t$-test, P<0.05). (B) E-cadherin levels were determined by Western Blotting. The bands are representative of three independent experiments.

Figure 7. Effect of IM on the *A. Actinomyces actinomycetemcomitans*-induced phosphorylation of ERK or p38 MAP kinase in HGEC.

Confluent HGEC were exposed to *A. Actinomyces actinomycetemcomitans* at $1 \times 10^8$ cells/ml in the presence or absence of IM at 1 μM for the indicated times before the end of incubation. HGEC were lysed in 200 μl of SDS sample buffer and sonicated, as described in the Materials and Methods section. Total ERK, total p38 MAP kinase, phospho-p38 MAP kinase, and phospho-ERK were analyzed by Western blotting. The bands are representative of three independent experiments. The band density of blots from three independent experiments was measured using an imaging densitometer. The graph indicates the ratio of phospho-p38 MAP kinase/total p38 MAP kinase, or phospho-ERK/total ERK. Values are means ± SD for three independent experiments. **Differs significantly (**$t$-test, P<0.01). *Differs significantly (**$t$-test, P<0.05).