Regulation of IL-8 by Irsogladine maleate is involved in abolishment of *Actinobacillus actinomycetemcomitans*-induced reduction of gap-junctional intercellular communication

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Abstract

Our previous report has shown that Irsogladine maleate (IM) counters and obviates the reduction in gap junction intercellular communication (GJIC) and the increase in IL-8 levels, respectively, induced by outer membrane protein 29 from *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) in cultured human gingival epithelial cells (HGEC). In addition, IM suppresses the increase in the secretion of IL-8 caused by whole live *A. actinomycetemcomitans*. These findings implicate the modulation of IL-8 levels by IM in abolishment of the reduction of GJIC in HGEC. Tight junctions are also responsible for cell-cell communication. Zonula occludens protein-1 (ZO-1) is a major tight junction protein. To investigate the regulatory mechanism of intercellular communication mediated by IM, in the present study, we focused on the involvement of IL-8 in *A. actinomycetemcomitans*-induced change in GJIC and ZO-1 expression in HGEC. IM countered the *A. actinomycetemcomitans*-induced reduction in levels of Connexin (CX) 43, suggesting that it could abolish the *A. actinomycetemcomitans*-induced reduction in GJIC in HGEC. CXCR-1 is a receptor of IL-8. The simultaneous addition of *A.*

actinomycetemcomitans and anti-CXCR-1 antibody also abrogated the repression of GJIC and CX43 expression by *A. actinomycetemcomitans* in HGEC, although the anti-CXCR-1 antibody was less effective than IM. IM inhibited the IL-8-induced reduction in CX43 levels and GJIC in HGEC. IM countered the *A. actinomycetemcomitans*-induced reduction in the expression of ZO-1, although anti-CXCR-1 antibody did not influence the decrease in ZO-1 mRNA levels caused by *A. actinomycetemcomitans*. Furthermore, IL-8 had little effect on the mRNA levels of ZO-1. These findings suggest that IL-8 mediates the *A. actinomycetemcomitans*-induced reduction of GJIC and CX43 expression in HGEC. The regulation of IL-8 levels by IM in HGEC is partially involved in abrogation of the reduction of GJIC and CX43 expression by *A. actinomycetemcomitans*. Furthermore, the regulatory effect of IM on the expression of CX43 and ZO-1 is different.

Keywords; IL-8; Irsogladine maleate; *Actinobacillus actinomycetemcomitans*; gap-junctional intercellular communication; human gingival epithelial cells.

1. Introduction

Periodontitis is an inflammatory condition caused by the colonization of the gingival sulucus by periodontopathogenic bacteria, including *Actinobacillus actinomycetemcomitans* (*A. actinomycetemcomitans*) and *Porphyromonas gingivalis (P. gingivalis)*. The gingival epithelium is the primary barrier facing this bacterial challenge. Epithelial cells function as a mechanically protective barrier against invasion by pathogenic organisms of epithelial cell-cell junction complexes such as gap junctions and tight junctions [1-3]. The interaction between epithelial cells and the bacteria plays an important role in the initial stage of inflammation.

Gap junctions, whose structural proteins are connexins, are clusters of transmembranous hydrophilic channels that allow the direct exchange of molecules up to 1200 Da in weight, including ions, sugars, and small peptides, between adjacent cells [4, 5]. Gap junctional intercellular communication (GJIC) plays a critical role in cellular coordination in tissue homeostasis.

Irsogladine maleate (IM) has been clinically used as an anti-gastric ulcer agent. IM has been reported to enhance GJIC in cultured rabbit gastric epithelial cells and pancreatic cancer cells [6, 7]. These findings caused us to examine the use of IM for treatment of periodontal disease. Outer membrane protein (OMP) 29 from *A. actinomycetemcomitans* is a virulent factor that plays a role in the onset of periodontal disease [8]. We have demonstrated that IM counters an OMP29-induced reduction of GJIC and connexin (CX) 43 levels in HGEC [3]. In addition, IM obviates the increase in interleukin (IL)-8 levels induced by live *A. actinomycetemcomitans* or OMP29 in HGEC [3]. However, little is known about the IM-mediated regulatory effect on GJIC through cytokines. We hypothesized that IL-8 might be involved in the regulation of GJIC by IM in HGEC.

Tight junctions constitute a barrier both to the passage of ions and molecules through the paracellular pathway and to the movement of proteins and lipids between the apical and basolateral domains of the plasma membrane [9]. Occludin and Zonula occludins protein-1 (ZO-1) are tight

junction proteins [10]. A previous study has shown that *P. gingivalis* degrades oculudin in Mandin-Darby canine kidney cells [1]. However, there is no report about the effect of IM on the expression of ZO-1 in epithelial cells exposed to microorganisms.

In the present study, to investigate the regulatory mechanism of intercellular communication mediated by IM, we focused on the involvement of IL-8 in *A. Actinomycetemcomitans*-induced modulation of GJIC and CX43 expression in HGEC.

2. Materials and Methods

2.1. Preparation of cells

Healthy gingival tissues, which had been dissected surgically during the extraction of wisdom teeth and are usually discarded, were collected with the patients' informed consent. HGEC were isolated as previously described [2, 11]. Briefly, the gingiva was treated with 0.025% trypsin and 0.01% EDTA overnight at 4℃ and was divided into the epithelium and connective tissues. The suspension of HGEC was centrifuged at 120*g* for 5 min, and the pellet was suspended in MCDB153 medium (pH 7.4) (Kurabo, Osaka, Japan) containing 30 μg/ml bovine pituitary extract, 0.1ng/ml human epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 50 μg/ml gentamycin (Medium A). The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO_2/95% air at 37℃. When the cells reached subconfluence, they were harvested and subcultured.

2.2. Culture of A. actinomycetemcomitans

A. actinomycetemcomitans Y4 were grown in Todd-Hewitt broth (BBL, Cockeysville, MD) supplemented with 1% yeast extract (Difco Laboratories, Detroit, MI) at 37℃ for 2 days. After cultivation, whole cells were harvested by centrifugation and washed three times in phosphate-buffered saline (pH 7.4). The washed *A. actinomycetemcomitans* were suspended in MCDB medium (pH 7.4) containing 10 μg/ml insulin, 5 μg/ml transferrin, 10 μg/ml 2-mercaptoethanol, 10 μM 2-aminoethanol, and 10 nM sodium selenite (medium B) [2, 3, 11].

2.3. RNA preparation

HGEC in cultures at the fourth passage were harvested, seeded at a density of 40 x 10^4 cells/60 mm plastic tissue culture plate coated with type I collagen, and maintained in 5 ml of medium A. After 10 days of culture, these cells were washed three times with phenol red-free Hank's solution (pH7.4). Confluent HGEC were exposed to *A. actinomycetemcomitans* at 1

x10^8 cells/ml or IL-8 at 1-50 ng/ml for the indicated times before the end of incubation on day 11 in 5 ml of medium B in the absence or presence of IM (supplied by Nippon Shinyaku, Kyoto, Japan) at 1 μM or mouse anti-human CXCR-1 antibody (Sigma, St. Louis, MO) at 0.3 μg/ml. Total RNA was extracted using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan) and quantified by spectrometry at 260 and 280 nm.

2.4. Real time PCR

Standard cDNA synthesis was performed with 1 μg of mRNA extract in a total volume of 20 μl (Roche, Tokyo, Japan). Real time PCR was performed with an ABI 7700 system (Applied Biosystems, Tokyo, Japan). The TaqMan probe, sense primers, and anti-sense primers used for detection of human CX43 and ZO-1 are listed in Table 1. Commercially available human GAPDH (Applied Biosystems) was used for quantitative PCR.

2.5. Measurement of GJIC between HGEC

HGEC in cultures at the fourth passage were harvested, seeded at a density of 40 x 10^4 cells/60 mm plastic tissue culture plate coated with type I collagen, and maintained in 5 ml of medium A. After 10 days of culture, these cells were washed three times with phenol red-free Hank's solution (pH7.4). HGEC of confluent cultures were exposed to *A. actinomycetemcomitans* Y4 at 1 x 10^8 cells/ml or IL-8 at 10 ng/ml in the absence or presence of IM at 1 μM or mouse anti-human CXCR-1 antibody at 0.3 μg/ml in 5 ml of medium B for 12 h. Microinjection of a 10% Lucifer yellow CH (Sigma) solution in 1 M LiCl into HGEC under a phase-contrast microscope was carried out by a method described previously [12]. Five minutes after the microinjection, the extent of the dye's transfer was recorded under a fluorescence microscope (Nikon, Tokyo, Japan). GJIC was assessed by counting the number of Lucifer yellow-fluorescent cells per microinjection.

2.6. Western blotting for CX43 and ZO-1

HGEC in cultures at the fourth passage were harvested, seeded at a density of 40 x 10^4 cells/60 mm plastic tissue culture plate coated with type I collagen, and maintained in 5 ml of medium A. After 10 days of culture, these cells were washed three times with phenol red-free Hank's solution (pH7.4). Then, HGEC of confluent cultures were exposed to *A. actinomycetemcomitans* Y4 at 1 x 10^8 cells/ml or IL-8 at 10 ng/ml in the absence or presence of IM at 1 μM or mouse anti-human CXCR-1 antibody at 0.3 μg/ml in 5 ml of medium B for 12 h. Cells were lysed in 500 μl of 20% SDS with 1% PMSF as sample buffer. Samples were resolved on a 12% SDS-polyacrylamide gel by electrophoresis under non-reducing conditions and electrophoretically transferred onto membranes (Bio-Rad Laboratories, CA). The membranes were blocked with 3% non-fat dried milk for 1 h and then reacted with anti-human CX43 antibody (Zymed, South San Francisco, CA), anti-human ZO-1 antibody (Zymed), or anti-human β-actin antibody (Zymed) overnight. The membrane was incubated with HRP-conjugated sheep anti-mouse IgG (Amersham Biosciences) in Tris-buffered saline for 1 h

at room temperature. Immunodetection was performed according to the manual supplied with the ECL Plus Western blotting detection reagents (Amersham Biosciences).

2.7. Statistical analysis

Comparisons between groups were analyzed with Student's *t*-test or ANOVA.

3. Results

Exposure of HGEC to live *A. actinomycetemcomitans* decreased CX43 mRNA levels in a time-dependent manner with a maximal effect at 12 h (data not shown). Confluent HGEC were therefore cultured under stimulative conditions for 12 h in the subsequent experiments. IM at 1 μM countered the decrease in CX43 mRNA levels in HGEC exposed to *A. actinomycetemcomitans* (Fig-1A). A typical three-band pattern for CX43, a non-phosphorylated form (NP) and two slower migrating phosphorylated forms (P1 and P2), was detected by immunoblotting [13]. NP and P1, or all three bands, were thought to migrate very closely in the present and previous studies [3]. Western blotting showed that IM countered the *A. actinomycetemcomitans*–induced decrease in CX43 levels (Fig-1B). Mouse anti-human CXCR-1 antibody inhibited the *A. actinomycetemcomitans*-induced reduction of CX43 expression at the mRNA and protein levels and GJIC (Fig-2A, B, C), although the effect was weaker than that of IM. Incubation with IL-8 at 10 ng/ml decreased CX43 mRNA expression in a time-dependent manner, and the effect was seen at 4 h and reached a maximum at 12 h (Figure 3-A). Dose-course

experiments showed that IL-8 decreased CX43 mRNA expression, and the effect reached a plateau at 1 ng/ml (Figure-3-B). IM countered the IL-8-induced decrease in CX43 levels (Fig-4-A, B). The simultaneous addition of IM at 1 μM into the cultures abrogated the reduction of GJIC induced by IL-8 (Fig-4C).

Although IM countered the *A. actinomycetemcomitans*-induced reduction in the expression of ZO-1 at the mRNA and protein levels in HGEC (Fig5-A,B), mouse anti-human CXCR-1 antibody did not (Fig5-C). Furthermore, IL-8 did not influence the ZO-1 mRNA expression (Fig 5-D).

4. Discussion

In the present study, IM countered the *A. actinomycetemcomitans*-induced reduction of CX43 levels in HGEC. GJIC is inhibited in HGEC exposed to *A. actinomycetemcomitans* is inhibited [3]. IM counters the OMP29-induced reduction in GJIC and CX43 levels in HGEC [3]. From the present findings and previous results, it is thought that IM also counters the *A. actinomycetemcomitans*-induced reduction of GJIC in HGEC.

IL-8 binds two types of receptors, CXCR-1 and CXCR-2. CXCR-1 is the major receptor of the two. Therefore, we added anti-CXCR-1 antibody to the cultures to examine the involvement of IL-8 secreted by HGEC in the regulation of GJIC by IM. The antibody countered the *A. actinomycetemcomitans*-induced decrease in CX43 levels and GJIC in HGEC. Although *A. actinomycetemcomitans* may produce IL-8-like molecules which can bind to the CXCR-1 receptor, mouse anti-human IL-8 antibody also inhibited the decrease in levels of CX43 mRNA induced by *A. actinomycetemcomitans* (Unpublished data). Furthermore, IM countered the suppression of GJIC induced by exogenous IL-8. Exposure of HGEC to *A. actinomycetemcomitans* caused a decrease in GJIC and an increase in the secretion of IL-8 [3]. These findings suggest that IM recovers intercellular communication through gap junctions by down-regulating IL-8 expression. However, in addition to IL-8, other molecules, for example cAMP, may participate in the regulation of GJIC and CX43 expression, since the effect of anti-CXCR-1 antibody on CX43 production was weaker than that of IM, and

the rise in cAMP levels caused by IM contributes to the enhancement of GJIC [3].

It has been recently reported that lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* decreases CX43 expression and increases IL-8 secretion in nasal epithelial cells [14], suggesting that the IL-8 is involved in the decrease in CX43 in nasal epithelial cells. On the other hand, LPS from *A. actinomycetemcomitans* did not affect GJIC in HGEC. Thus, the effect of LPS on GJIC depends on its source or the cell.

Preliminary studies have shown that treatment with IM enhances GJIC in HGEC in the absence of *A. actinomycetemcomitans*, without influencing the expression of CX43 and ZO-1 mRNA. These findings suggest that the increase in GJIC caused by IM in HGEC may be independent of the increase in CX43 levels and dependent on the phosphorylation of CX43 in the absence of *A. actinomycetemcomitans*.

Exposure to *A. actinomycetemcomitans* resulted in a reduction of ZO-1 expression as well as GJIC in HGEC. IM also countered *A.*

actinomycetemcomitans-induced decrease of ZO-1. However, IL-8 and anti-CXCR-1 antibody did not influence ZO-1 expression in HGEC. These findings suggest that IL-8 is not involved in the regulation of ZO-1 in HGEC. Taken together, tight junctions and gap junctions seem to be regulated by IM in different pathways.

Intercellular communication via gap junctions composed of CX32 enhances the function of tight junctions [15]. The C-terminal domain of CX43 or CX45 binds to the N-terminal domain of ZO-1 in cardiac myocytes, in mink lung epithelial cells, in epithelial MDCK cells, and in other cells [16-19]. The interaction between CX43 and ZO-1 may regulate each other's expression in HGEC.

IL-8 enhances GJIC between fibroblasts in granulation tissue [20]. IL-1β decreases the expression of CX32 in rat hepatocytes [21] and increases CX43 levels and GJIC in human articular chondrocytes [22]. IL-1β and IL-8 did not affect ZO-1 expression in human fetal astrocytes or human airway epithelial cells [23, 24]. To our knowledge, there has been no study of GJIC and inflammatory cytokines in epithelial cells. In the present study,

IL-8 decreased CX43 levels and GJIC in HGEC, but did not affect ZO-1 mRNA expression. In addition, IL-1β decreased CX43 mRNA level, but increased ZO-1 mRNA level in HGEC (Unpublished data). Thus, the difference in the response to cytokines may be dependent on the type of cell.

Increased IL-8 levels are responsible for a delay in burn wound closure [25]. On the other hand, IL-8 enhances re-epithelialization and wound closure [26]. IL-8 seems to play a role in chronic leukocyte recruitment and tissue destruction [27-29]. In this study, IL-8 decreased GJIC and CX43 expression in HGEC, suggesting that in addition to its more traditional role in inflammation, IL-8 impairs cell-cell communication to be involved in diseases.

In conclusion, IL-8 mediates the *A. actinomycetemcomitans*-induced disruption of GJIC and CX43 expression. The regulation of IL-8 by IM in HGEC may partially elicit abrogation of the reduction in GJIC and CX43 expression induced by *A. actinomycetemcomitans*. Furthermore, the regulatory effect of IM on the expression of CX43 and ZO-1 is diverse.

Acknowledgements

We thank the Nippon Shinyaku Co., Ltd, for donating IM.

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Figure legends

Figure 1. Effect of IM on the expression of CX43 in HGEC exposed to *A. actinomycetemcomitans*

HGEC were seeded and maintained as described in materials and methods. Confluent cultures were exposed to *A. actinomycetemcomitans* at 1 x 10^8 cells/ml in the absence or presence of IM at 1 μM for 12 h before the end of incubation on day 11. (A) CX43 mRNA levels: CX43 mRNA expression was analyzed by Real time PCR. Values are the mean ±SD of three cultures. **Differs significantly (*t*-test, $P<0.01$). (B) CX43 levels: CX43 and β-actin were analyzed by Western blotting with mouse anti-human CX43 polyclonal antibody and mouse anti-human β-actin monoclonal antibody, respectively. The lower band shows non-phosphorylated CX43 (NP) and the lower molecular-weight form of phosphorylated CX43 (P1), while the higher band shows the higher molecular-weight form of phosphorylated CX43 (P2).

Figure 2. Effect of mouse anti-human CXCR-1 antibody on the expression of CX43 and GJIC in HGEC exposed to *A. actinomycetemcomitans*

HGEC were seeded and maintained as described in materials and methods. Confluent cultures were exposed to *A. actinomycetemcomitans* at 1 x 10^8 cells/ml in the absence or presence of mouse anti-human CXCR-1 antibody at 0.3 μg/ml for 12 h before the end of incubation on day 11. (A) CX43 mRNA levels: CX43 mRNA expression was analyzed by Real time PCR. Values are the mean ±SD of three cultures. **Differs significantly (*t*-test, P<0.01). (B) CX43 levels: CX43 and β-actin were analyzed by Western blotting with mouse anti-human CX43 polyclonal antibody and mouse anti-human β-actin monoclonal antibody, respectively. Only one band was seen, since NP, P1, and P2 migrated very closely. (C) GJIC: GJIC was determined with the dye transfer method. The microinjected cell in each group is marked with an arrowhead. The graph shows the number of Lucifer yellow-fluorescent cells. Values are the mean ±SD of three cultures. **Differs significantly (*t*-test, P<0.01).

Figure 3. Effect of IL-8 on the CX43 mRNA expression in HGEC

(A) Time-course of the effect of IL-8 on CX43 mRNA levels. Confluent cultures of HGEC were exposed to IL-8 at 10 ng/ml for the indicated time before the end of incubation on day 11. (B) Dose-course experiment with IL-8. Confluent cultures were exposed to IL-8 at 1, 5, 10, and 50 ng/ml for 12h before the end of incubation on day 11. CX43 mRNA expression was analyzed by Real time PCR. Values are the mean ±SD of three cultures. **Differs significantly (ANOVA, $P<0.01$). *Differs significantly (ANOVA, $P<0.05$).

Figure 4. Effect of IM on the expression of CX43 and GJIC in HGEC exposed to IL-8

HGEC were seeded and maintained as described in materials and methods. Confluent cultures were exposed to IL-8 at 10 ng/ml in the absence or presence of IM at 1 μM for 12 h before the end of incubation on day 11. (A) CX43 mRNA levels: CX43 mRNA expression was analyzed by Real time PCR.

Values are the mean ±SD of three cultures. **Differs significantly (*t*-test, P<0.01). (B) CX43 levels: CX43 and β-actin were analyzed by Western blotting with mouse anti-human CX43 polyclonal antibody and mouse anti-human β-actin monoclonal antibody, respectively. The lower band shows NP and P1, and the higher band shows P2. (C) GJIC: GJIC was determined with the dye transfer method. The microinjected cell in each group is marked with an arrowhead. The graph shows the number of Lucifer yellow-fluorescent cells. Values are the mean ±SD of three cultures. **Differs significantly (*t*-test, P<0.01).

Figure 5. Change in ZO-1 expression in HGEC

Confluent cultures of HGEC were exposed to *A. actinomycetemcomitans* at 1 x 10^8 cells/ml or IL-8 at 1-50 ng/ml in the absence or presence of IM at 1 μM or mouse anti-human CXCR antibody at 0.3 μg/ml for 12 h before the end of incubation on day 11.

(A) ZO-1 mRNA levels in HGEC exposed to *A. actinomycetemcomitans* in the

absence or presence of IM: ZO-1 mRNA expression was analyzed by Real time PCR. Values are the mean ±SD of three cultures. **Differs significantly (*t*-test, P<0.01).

(B) ZO-1 levels in HGEC exposed to *A. actinomycetemcomitans* in the absence or presence of IM: ZO-1 and β-actin were analyzed by Western blotting with mouse anti-human ZO-1 polyclonal antibody and mouse anti-human β-actin monoclonal antibody, respectively.

(C) ZO-1 mRNA levels in HGEC exposed to *A. actinomycetemcomitans* in the absence or presence of anti-CXCR antibody: ZO-1 mRNA expression was analyzed by Real time PCR. Values are the mean ±SD of three cultures. **Differs significantly (*t*-test, P<0.01).

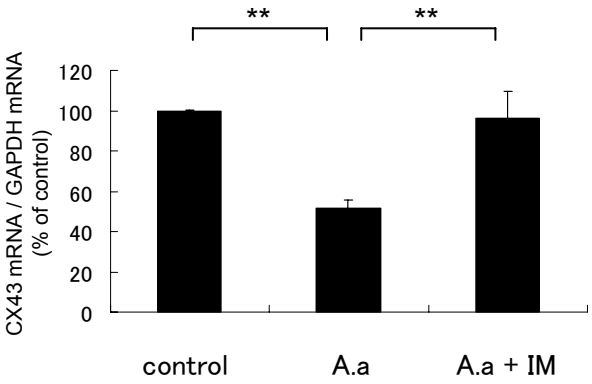
(D) Effect of IL-8 on the expression of ZO-1 mRNA in HGEC

ZO-1 mRNA expression was analyzed by Real time PCR. Values are the mean ±SD of three cultures (ANOVA, no significance)

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

Cx43
Sense: 5'-AGG GAA AGA GCG ACC CTT ACC-3'
Anti-sense: 5'-GGT GAG GAG CAG CCA TTG AA-3'
Taq Man probe: 5'-CCC TGC CAA AGA CTG TGG GTC TCA AA-3'
ZO-1
Sense: 5'-GTA ACC ATT TTT GGA CCA ATA GCT G-3'
Anti-sense: 5'-GCC AGA GCT ACG TTG GTC AGT T-3'
Taq Man probe: 5'-CGT CTC GTG GTT CAC TCT TTG-3'

(A)


**
**
CX43 mRNA / GAPDH mRNA
(% of control)
120
100
80
60
40
20
0
control
A.a
A.a + IM

(B)

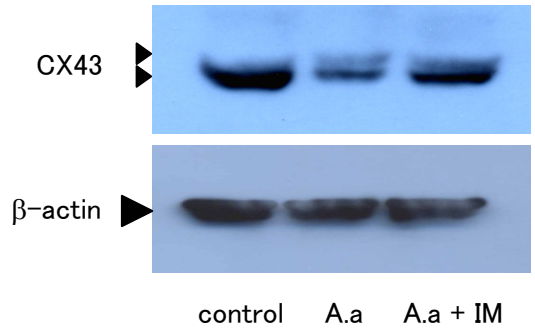

CX43
β-actin
control
A.a
A.a + IM

Fig-1

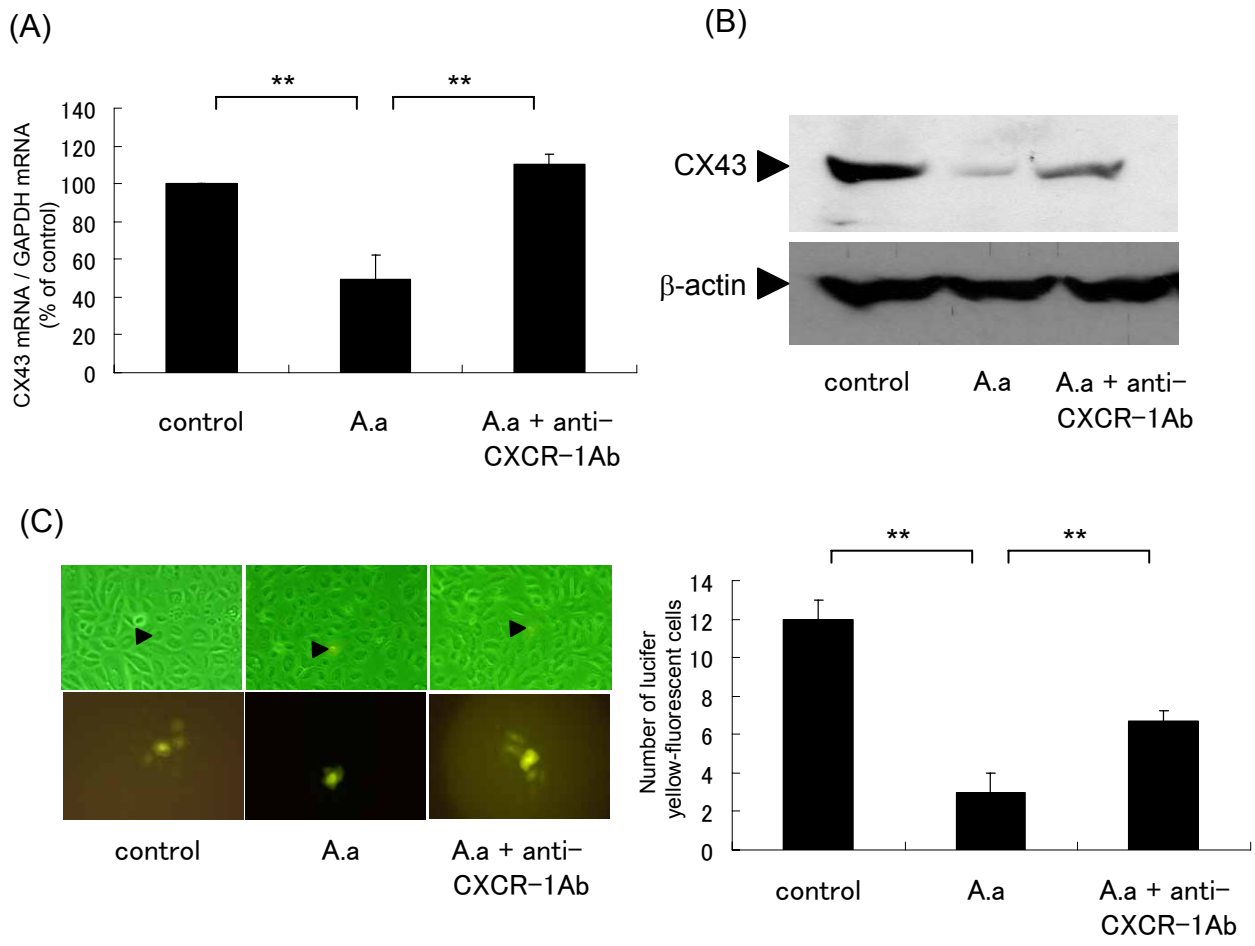
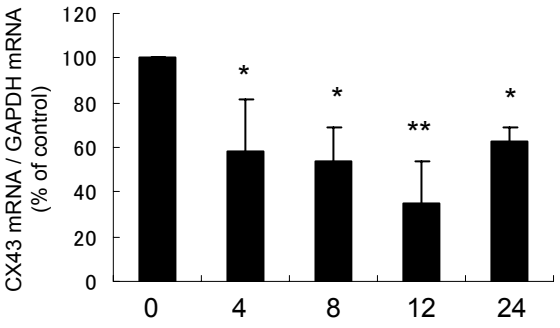

(A)
**
**
CX43 mRNA / GAPDH mRNA (% of control)
140
120
100
80
60
40
20
0
control
A.a
A.a + anti−CXCR−1Ab
(B)
CX43
β-actin
control
A.a
A.a + anti−CXCR−1Ab
(C)
control
A.a
A.a + anti−CXCR−1Ab
**
**
Number of lucifer yellow-fluorescent cells
14
12
10
8
6
4
2
0
control
A.a
A.a + anti−CXCR−1Ab

Fig-2

(A)


CX43 mRNA / GAPDH mRNA
(% of control)
120
100
80
60
40
20
0
*
*
**
*
0
4
8
12
24

(B)

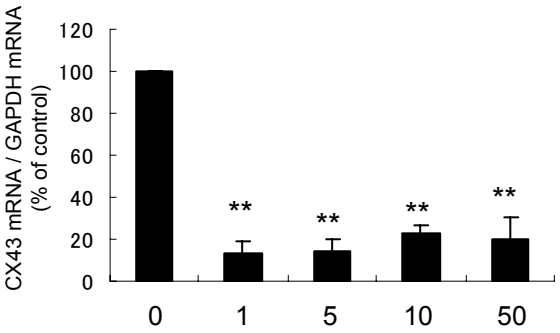

CX43 mRNA / GAPDH mRNA
(% of control)
120
100
80
60
40
20
0
**
**
**
**
0
1
5
10
50

Fig-3

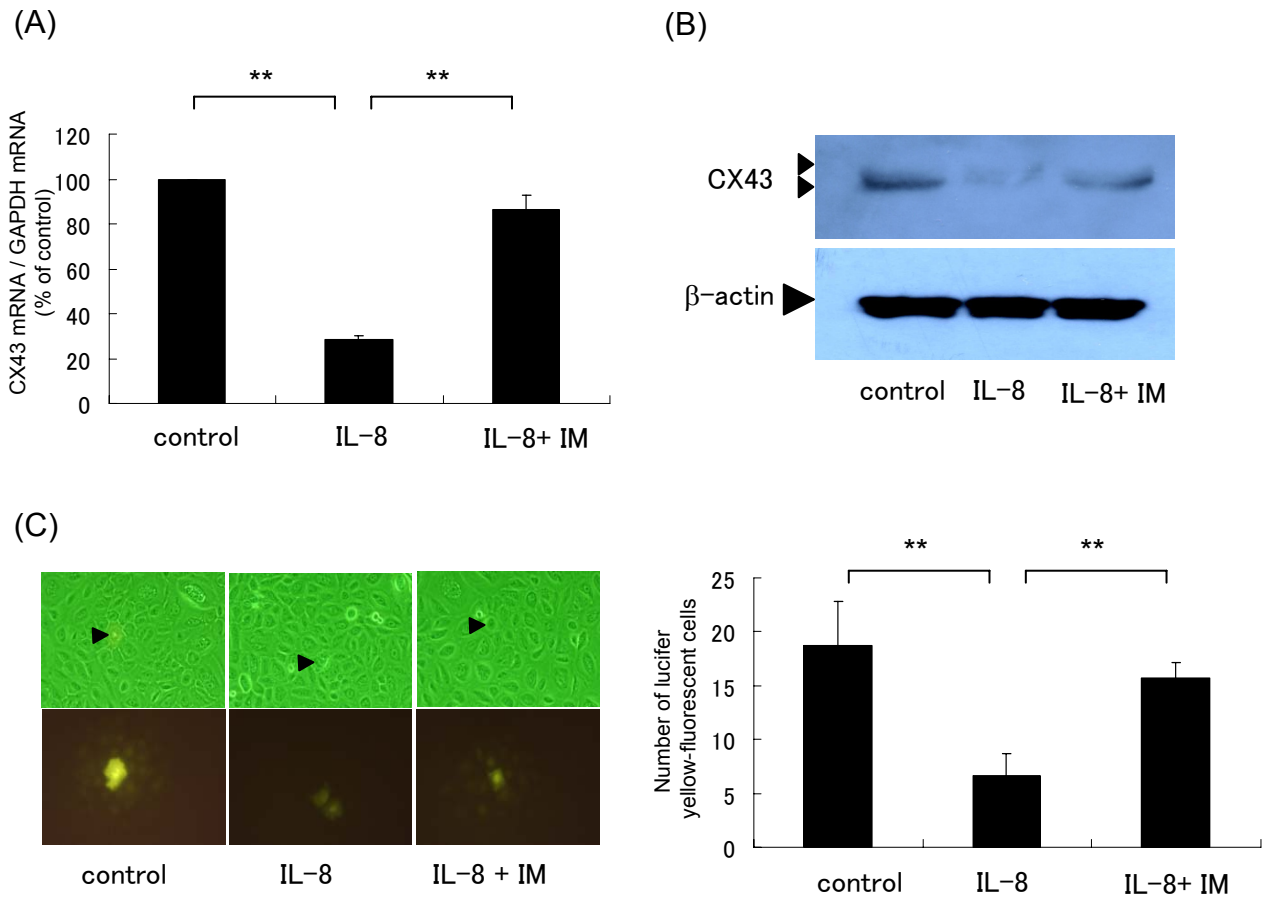

(A)
**
**
CX43 mRNA / GAPDH mRNA
(% of control)
120
100
80
60
40
20
0
control
IL-8
IL-8+ IM
(B)
CX43
β-actin
control
IL-8
IL-8+ IM
(C)
control
IL-8
IL-8 + IM
**
**
Number of lucifer
yellow-fluorescent cells
25
20
15
10
5
0
control
IL-8
IL-8+ IM

Fig-4

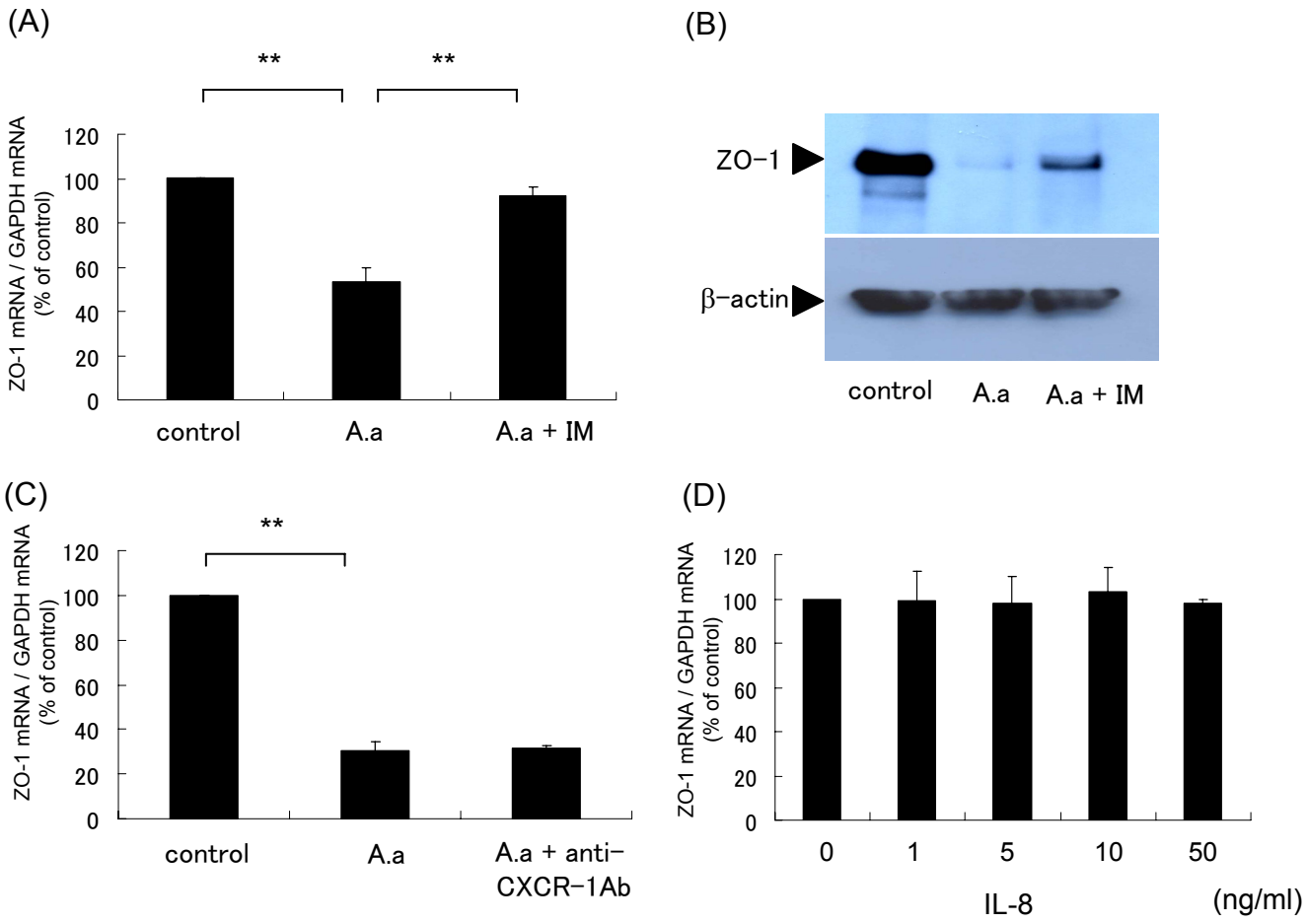

(A)
**
**
ZO-1 mRNA / GAPDH mRNA (% of control)
120
100
80
60
40
20
0
control
A.a
A.a + IM
(B)
ZO-1
β-actin
control
A.a
A.a + IM
(C)
**
ZO-1 mRNA / GAPDH mRNA (% of control)
120
100
80
60
40
20
0
control
A.a
A.a + anti-CXCR-1Ab
(D)
ZO-1 mRNA / GAPDH mRNA (% of control)
120
100
80
60
40
20
0
0
1
5
10
50
IL-8
(ng/ml)

Fig-5