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Irsogladine maleate counters the interleukin-1β-induced suppression in gap-junctional intercellular communication but does not affect the interleukin-1β-induced zonula occludens protein-1 levels in human gingival epithelial cells

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Background and Objective: Irsogladine maleate counters gap junctional intercellular communication reduction induced by interleukin-8 or *Actinobacillus actinomycetemcomitans* in cultured human gingival epithelial cells. Interleukin-1β is involved in periodontal disease. Little is known, however, about the effect of interleukin-1β on intercellular junctional complexes in human gingival epithelial cells. Furthermore, irsogladine maleate may affect the actions of interleukin-1β. In this study, we examined how interleukin-1β affected gap junctional intercellular communication, connexin 43 and zonula occludens protein-1, and how irsogladine maleate modulated the interleukin-1β-induced changes in the intercellular junctional complexes in human gingival epithelial cells.

Material and Methods: Human gingival epithelial cells were exposed to interleukin-1β, with or without irsogladine maleate. Connexin 43 and zonula occludens protein-1 were examined at mRNA and protein levels by real-time polymerase chain reaction and western blotting, respectively. Gap junctional intercellular communication was determined using the dye transfer method. The expression of zonula occludens protein-1 was also confirmed by immunofluorescence.

Results: Interleukin-1β decreased connexin 43 mRNA levels, but increased zonula occludens protein-1 mRNA levels. Irsogladine maleate countered the interleukin-1β-induced reduction in gap junctional intercellular communication and

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connexin 43 levels. However, irsogladine maleate did not influence the increased zonula occludens protein-1 levels.

Conclusion: The effect of interleukin-1β on gap junctional intercellular communication and tight junctions of human gingival epithelial cells is different. The recovery of gap junctional intercellular communication by irsogladine maleate in the gingival epithelium may be a normal process in gingival epithelial homeostasis.

Interleukin-1β has a deleterious role in inflammatory diseases, including rheumatoid arthritis, Crohn's disease, psoriasis and periodontal disease. Interleukin-1β is present in gingival fluid (1,2) and in periodontally inflamed gingival tissues (3–7). The excessive production of interleukin-1β by macrophages, lymphocytes and cells present in the periodontal tissue appears to be capable of stimulating gingival epithelial cells and fibroblasts in an autocrine or paracrine manner to induce the production of other cytokines, matrix-degrading enzymes and prostaglandin E_2. These mediators may be responsible for periodontal destruction, leading to loss of attachment. Thus, interleukin-1β has been suggested to play a key role in the pathogenesis of periodontal disease (7,8). Interleukin-1β also regulates cell–cell communication in the human airway epithelium (9). Regulating the function of interleukin-1β may be useful for the prevention of periodontal disease.

Epithelial cells function as a mechanically protective barrier against invasion by pathogenic organisms through epithelial cell–cell junction complexes, such as gap junctions and tight junctions (10–12). A gap junction channel is formed by connexons that consist of six connexins. Gap junctions mediate the reciprocal exchange of ions and small molecules of less than 1200 Da, including ions, sugars and small peptides, between adjacent cells (13,14). Gap junctional intercellular communication plays a critical role in cellular co-ordination in tissue homeostasis. Tight junctions constitute a barrier to the passage of both ions and molecules through the paracellular pathway. Zonula occludens protein-1, a major tight junction structural protein, is thought to contribute to the organization of proteins within the tight junction plaque (15,16). We have shown that *Actinobacillus actinomycetemcomitans* suppresses both gap junctional intercellular communication and connexin 43 expression in human gingival epithelial cells (12). Furthermore, *A. actinomycetemcomitans* decreased zonula occludens protein-1 levels in human gingival epithelial cells (17). On the other hand, interleukin-8 suppressed gap junctional intercellular communication and reduced connexin 43 levels without changing zonula occludens protein-1 expression (17). Little is known, however, about the effect of interleukin-1β on the expression of junctional complexes in gingival epithelial cells, although interleukin-1β plays a role in the exacerbation of periodontal disease.

Previous studies have shown that irsogladine maleate enhances gap junctional intercellular communication in cultured rabbit gastric epithelial cells and pancreatic cancer cells (18,19), counters the reduction in gap junctional intercellular communication and the connexin 43 and zonula occludens protein-1 levels in human gingival epithelial cells stimulated by *A. actinomycetemcomitans* or outer membrane protein 29 from *A. actinomycetemcomitans* (12,17), and obviates the increase in interleukin-8 levels in human gingival epithelial cells stimulated by *A. actinomycetemcomitans* and outer membrane protein 29 (12). Furthermore, irsogladine maleate is used clinically as an antigastric ulcer agent. Irsogladine maleate may affect the expression of intercellular junctional complexes in interleukin-1β-stimulated human gingival epithelial cells.

In the present study, we investigated the effect of interleukin-1β on gap junctional intercellular communication and on the connexin 43 and zonula occludens protein-1 levels in human gingival epithelial cells, and we studied the effect of irsogladine maleate on interleukin-1β-induced changes in gap junctional intercellular communication and on the expression of connexin 43 and zonula occludens protein-1.

Material and methods

Preparation of cells

Healthy gingival tissues, which had been surgically dissected through the process of wisdom tooth extraction and are usually discarded, and that were periodontally healthy with no pericoronitis around the wisdom tooth extracted, were collected after obtaining the patients' informed consent. Human gingival epithelial cells were isolated from three healthy gingivae from three different volunteers, as previously described (11,20). Briefly, the gingiva was treated with 0.025% trypsin and 0.01% EDTA overnight at 4°C and was divided into epithelium and connective tissues. The suspension of human gingival epithelial cells was centrifuged at 120 *g* for 5 min and the pellet obtained was suspended in MCDB153 medium (pH 7.4) (Kurabo, Osaka, Japan) containing 30 μg/mL of bovine pituitary extract, 0.1 ng/mL of human epidermal growth factor, 5 μg/mL of insulin, 0.5 μg/mL of hydrocortisone and 50 μg/mL of 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°C. When the cells reached subconfluence, they were harvested and subcultured.

Determination of appropriate concentration and incubation time of interleukin-1β

Human gingival epithelial cells in cultures at the fourth passage were harvested, seeded at a density of 40×10^4 cells per 60-mm plastic tissue culture plates coated with type I collagen and maintained in 5 mL of medium A. After 10 d of culture, these cells were washed three times with phenol red-free Hank's solution (pH 7.4). To determine the appropriate concentration and time for exposure of interleukin-1β to human gingival epithelial cells in the following experiment, dose- and time-course experiments were carried out. Confluent human gingival epithelial cells were exposed to interleukin-1β at 1–50 ng/mL for 1–24 h before the end of incubation on day 11 in 5 mL of MCDB 153 medium containing 10 μg/mL of insulin, 5 μg/mL of transferrin, 10 μg/mL of 2-mercaptoethanol, 10 μM 2-aminoethanol and 10 nM sodium selenite (medium B).

Real-time PCR

Human gingival epithelial cells were cultured as described in 'Determination of appropriate concentration and incubation time of interleukin-1β'. In the dose-course experiment with irsogladine maleate (supplied by Nippon Sinyaku, Kyoto, Japan), irsogladine maleate at 1 μM abolished the *A. actinomycetemcomitans*-induced increase of interleukin-8 in human gingival epithelial cells at the maximal effect (12). Therfore, irsogladine maleate at 1 μM was used in the following experiments. Confluent human gingival epithelial cells were pretreated with or without 1 μM irsogladine maleate for 30 min and were then exposed to various concentrations of interleukin-1β for the indicated times before the end of incubation on day 11 in 5 mL of medium B. Total RNA was extracted using Isogen (Wako Pure Chemical Industries, Osaka, Japan) and quantified by spectrometry at 260 and 280 nm. First, standard cDNA synthesis was performed with 1 μg of mRNA extract in a total volume of 20 μL (Roche, Tokyo, Japan). Then, real-time PCR was performed with an ABI 7700 system (Applied Biosystems, Tokyo, Japan). The Taq-Man probe, sense primers and antisense primers used for the detection of human connexin 43, zonula occludens protein-1, claudin-1 and occuldin are listed in Table 1. Commercially available human glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems) was used for quantitative PCR.

Measurement of gap junctional intercellular communication between human gingival epithelial cells

Human gingival epithelial cells were cultured as described in 'Determination of appropriate concentration and incubation time of interleukin-1β'. Confluent human gingival epithelial cells, which had been pretreated with or without 1 μM irsogladine maleate for 30 min, were exposed to 10 ng/mL of interleukin-1β for 12 h before the end of incubation on day 11 in 5 mL of medium B. Microinjection of 10% Lucifer yellow CH (Sigma, St Louis, MO, USA) solution in 1 M LiCl into human gingival epithelial cells through a phase-contrast microscope was carried out using the method described previously (21). Five minutes after the microinjection, the extent of dye transfer was recorded under a fluorescence microscope (Nikon, Tokyo, Japan). Gap junctional intercellular communication was assessed by counting the number of Lucifer yellow-fluorescent cells per microinjection.

Western blotting for connexin 43 and zonula occludens protein-1

Human gingival epithelial cells were cultured as described in 'Measurement of gap junctional intercellular communication between human gingival epithelial cells'. Cells were lysed in 500 μL of 20% sodium dodecyl sulfate, containing 1% phenylmethanesulfonyl fluoride, as the sample buffer. Samples were resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis under nonreducing conditions and electrophoretically transferred onto membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 3% nonfat dried milk for 1 h and then reacted overnight with mouse antihuman connexin 43 or zonula occludens protein-1 immunoglobulin (Zymed, South San Francisco, CA, USA) or with mouse antihuman β-actin immunoglobulin (Zymed). The membrane was incubated with horseradish peroxidase-conjugated sheep antimouse immunoglobulin G (Amersham Biosciences, Bucks, UK) 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).

Confocal laser-scanning microscopic analysis

Human gingival epithelial cells in cultures at the fourth passage were harvested, seeded onto a glass coverslip coated with type I collagen in 35-mm plastic tissue culture plates and

Table 1. Primers and probes for real-time polymerase chain reaction used in this study

CX43	Sense	5′-AGGGAAAGAGCGACCCTTACC-3′
	Antisense	5′-GGTGAGGAGCAGCCATTGAA-3′
	Taq Man probe	5′-CCCTGCCAAAGACTGTGGGTCTCAAA-3′
ZO-1	Sense	5′-GTAACCATTTTTGGACCAATAGCTG-3′
	Antisense	5′-GCCAGAGCTACGTTGGTCAGTT-3′
	Taq Man probe	5′-CGTCTCGTGGTCACTCTTTG-3′
Claudin-1	Sense	5′-ACCCCAGTCAATGCCAGGTA-3′
	Antisense	5′-TGGATAGGGCCTTGGTGTTG-3′
	Taq Man probe	5′-TCTCTGCCTTCTGGGAGGTGCCCTAC-3′
Occuldin	Sense	5′-TCCCATGGCATACTCTTCCAAT-3′
	Antisense	5′-AACGAGGCTGCCTGAAGTCA-3′
	Taq Man probe	5′-TGGTTCAGGAGCTTCCATTAACTTCG-3′

CX43, connexin 43; ZO-1, zonula occludens protein-1.

maintained in 2 mL of medium A. After 10 d of culture, these cells were washed three times with phosphate-buffered saline (pH 7.4). Then, confluent human gingival epithelial cells, which had been pretreated with or without 1 μM irsogladine maleate for 30 min, were exposed to 10 ng/mL of interleukin-1β for 12 h at the end of incubation on day 11 in 5 mL of medium B. After incubation, the medium was removed and the cells were washed three times with phosphate-buffered saline. The cells on coverslips were immersed for 10 min in 3.5% formaldehyde and 0.2% Triton X-100 in phosphate-buffered saline. They were washed three times with phosphate-buffered saline. Blocking was performed by immersing the coverslips in Tris-buffered saline containing 0.2% casein and 0.1% Triton X-100. After a 30-min incubation at 37°C, the blocking solution was removed and the coverslips were washed twice with phosphate-buffered saline. To stain actin, Alexa Fluor 594-phalloidin (Invitrogen, Carlsbad, CA, USA) was added and incubated at room temperature for 15 min. For staining of zonula occludens protein-1, human gingival epithelial cells were labeled with mouse antihuman zonula occludens protein-1, followed by Alexa Fluor 488 antimouse immunoglobulin G. Fluorescence signals were detected with a Zeiss LSM 510 laser-scanning confocal microscope (Zeiss Microimaging, Thornwood, NY, USA).

Statistical analysis

Comparisons between groups in dose- and time-course experiments of interleukin-1β exposure to human gingival epithelial cells were analyzed using analysis of variance. Comparisons among groups of control, interleukin-1β treatment, and interleukin-1β + irsogladine maleate treatment, were analyzed using the Student's *t*-test.

Results

Figure 1 shows the mRNA expression of connexin 43 and zonula occludens protein-1 in interleukin-1β-stimulated human gingival epithelial cells by real-time PCR analysis. Exposure to interleukin-1β resulted in a decrease of the connexin 43 mRNA levels (Fig. 1-A,B). Dose- and time-course experiments showed that the decrease occurred at 10 and 50 ng/mL of interleukin-1β, and at 12- and 24-h exposure times, respectively. On the other hand, interleukin-1β increased the mRNA levels of zonula occludens protein-1 in a dose- and time-dependent manner (Fig. 1C,D). Furthermore, interleukin-1β enhanced the mRNA levels of claudin-1 and occuldin in a dose- and time-dependent manner (data not shown). From these results, experiments were conducted with treatment with interleukin-1β at 10 ng/mL for 12 h. Irsogladine maleate countered the interleukin-1β-induced decrease in connexin 43 mRNA levels (Fig. 2A). A typical three-band pattern for connexin 43 – a nonphosphorylated form and two phosphorylated forms (P1 and P2) that migrated more slowly than the nonphosphorylated form – was detected by western blotting (22). The nonphosphorylated form and the P1 phosphorylated form were thought to migrate very closely in the present study (Fig. 2B). Western blotting showed that interleukin-1β inhibited connexin 43 expression (Fig. 2B), and irsogladine maleate countered the interleukin-1β-induced decrease in connexin 43 levels (Fig. 2B). Gap junctional intercellular communication in human gingival epithelial cells exposed to interleukin-1β at 10 ng/mL was inhibited (Fig. 2C). However, the simultaneous addition of 1 μM irsogladine maleate to the cultures countered the reduction of the gap junctional intercellular communication induced by interleukin-1β (Fig. 2C). Interleukin-1β increased the zonula occludens protein-1 levels and irsogladine maleate did not affect the interleukin-1β-induced increase in the zonula occludens protein-1 level (Fig. 3A). Figure 3B shows the results of an immunofluorescence analysis of human gingival epithelial cells for zonula occludens protein-1 expression. Zonula

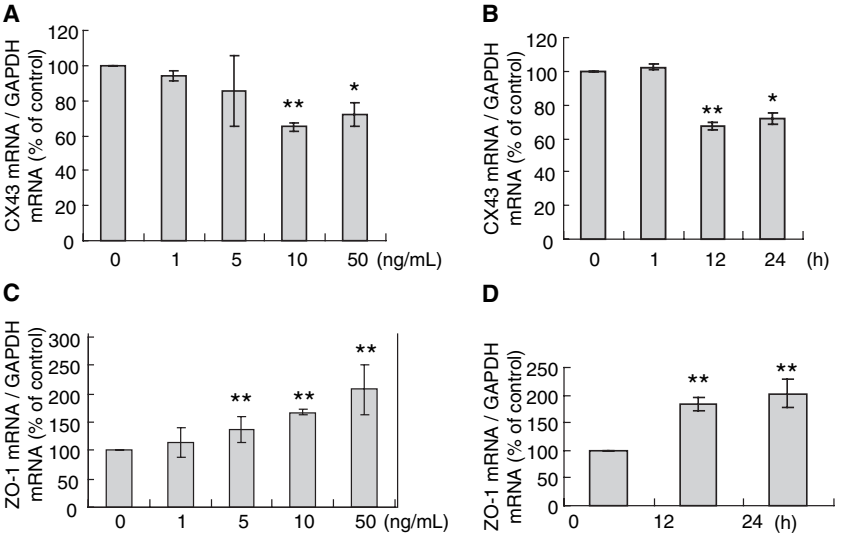

Fig. 1. Dose- and time-course effects of interleukin-1β on connexin 43 and zonula occludens protein-1 mRNA expression in human gingival epithelial cells. (A) Dose-course experiment on the effect of interleukin-1β on connexin 43 mRNA levels. (B) Time-course experiment on the effect of interleukin-1β on connexin 43 mRNA levels. (C) Dose-course experiment on the effect of interleukin-1β on zonula occludens protein-1 mRNA levels. (D) Time-course experiment on the effect of interleukin-1β on zonula occludens protein-1 mRNA levels. Confluent cultures of human gingival epithelial cells were exposed to interleukin-1β at 1, 5, 10 or 50 ng/mL for 12 h (A,C), at 10 ng/mL for 1, 12, or 24 h (B), or at 10 ng/mL for 12 or 24 h (D) before the end of incubation on day 11. The expression of connexin 43 and zonula occludens protein-1 mRNAs was analyzed by real-time polymerase chain reaction. Values are the mean ± standard deviaition of three cultures. **, $p < 0.01$; *, $p < 0.05$; analysis of variance. CX43, connexin 43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; ZO-1, zonula occludens protein-1.

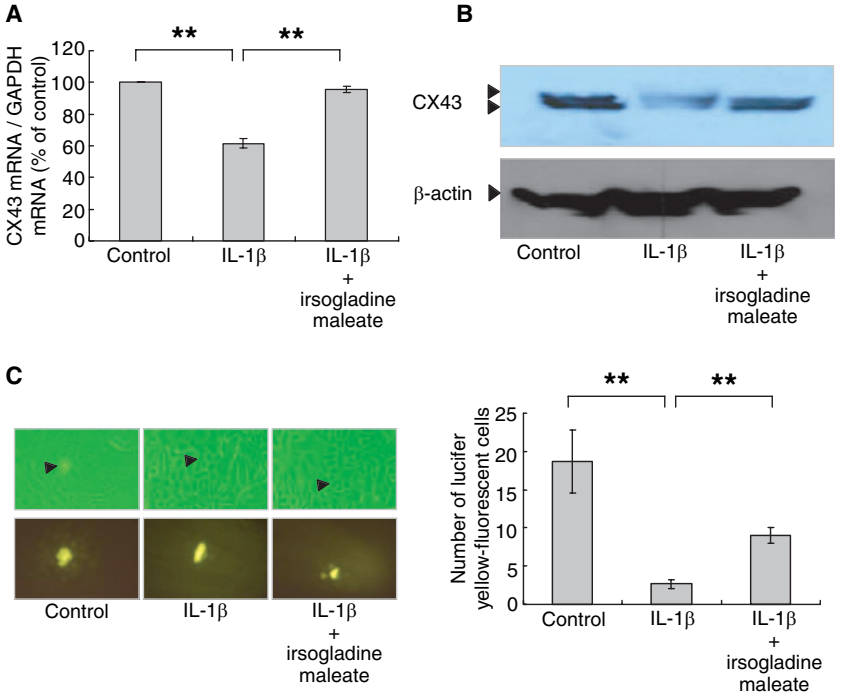

Fig. 2. Effect of irsogladine maleate on connexin 43 levels and gap junctional intercellular communication in human gingival epithelial cells exposed to interleukin-1β. Human gingival epithelial cells at confluence were exposed to 10 ng/mL interleukin-1β in the absence or presence of 1 μM irsogladine maleate for 12 h before the end of incubation on day 11. (A) Connexin 43 mRNA levels: connexin 43 mRNA expression was analyzed by real-time polymerase chain reaction. Values are means ± standard deviation of three cultures. **, $p < 0.01$; *t*-test. (B) Connexin 43 levels: connexin 43 and β-actin were analyzed by western blotting with mouse antihuman connexin 43 polyclonal antibody and mouse antihuman β-actin monoclonal antibody, respectively. The lower band shows nonphosphorylated connexin 43 and the lower-molecular-weight form of phosphorylated connexin 43 (P1), whereas the upper band shows the higher-molecular-weight form of phosphorylated connexin 43 (P2). (C) Gap junctional intercellular communication: gap junctional intercellular communication 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 means ± standard deviation of three cultures. **, $p < 0.01$; *t*-test. CX43, connexin 43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β.

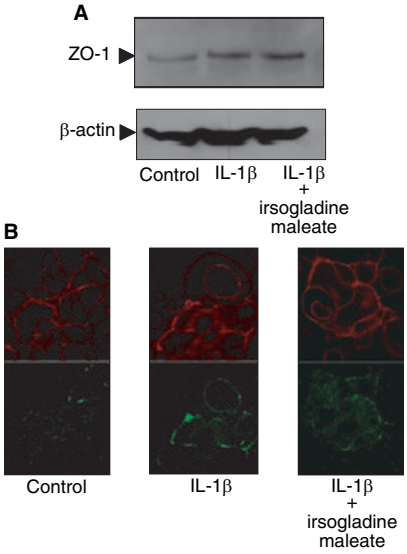

Fig. 3. Effect of irsogladine maleate on the expression of zonula occludens protein-1 in human gingival epithelial cells exposed to interleukin-1β. Human gingival epithelial cells at confluence were exposed to 10 ng/mL interleukin-1β in the absence or presence of 1 μM irsogladine maleate for 12 h before the end of incubation on day 11. (A) Zonula occludens protein-1 levels: zonula occludens protein-1 and β-actin were analyzed by western blotting with mouse antihuman zonula occludens protein-1 polyclonal antibody and mouse antihuman β-actin monoclonal antibody, respectively. (B) Cellular localization of zonula occludens protein-1 in human gingival epithelial cells: fluorescent images were obtained with confocal laser-scanning microscopy. Upper image, β-actin; lower image, zonula occludens protein-1. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; ZO-1, zonula occludens protein-1.

occludens protein-1 showed little intercellular localization in unstimulated conditions. An increase in the level of zonula occludens protein-1 at intercellular junctions occurred in human gingival epithelial cells following exposure to interleukin-1β. The addition of irsogladine maleate to human gingival epithelial cells did not influence the increase in zonula occludens protein-1 caused by interleukin-1β. These findings are consistent with those from western blotting (Fig. 3A).

Discussion

Previous studies have shown that interleukin-1β inhibits gap junctional intercellular communication in human astrocytes and in human myoendothelial cells (23,24), that interleukin-1β does not affect zonula occludens protein-1 expression in human fetal astrocytes or in human airway epithelial cells (25,26) and that interleukin-1β alters the barrier function of tight junctions in retinal pigment epithelial cells, down-regulating occludin expression but up-regulating claudin-1 expression (27). The present study demonstrated that interleukin-1β decreases connexin 43 levels and gap junctional intercellular communication in human gingival epithelial cells. Furthermore, interleukin-1β was found to increase zonula occludens protein-1 levels as well as the synthesis of claudin-1 and occuldin in human gingival epithelial cells. Thus, the response of tight junction structural proteins to interleukin-1β differed among the cells tested.

Our previous and present studies have shown that gap junctional intercellular communication and connexin 43 expression were suppressed by exposure of human gingival epithelial cells to *A. actinomycetemcomitans*, outer membrane protein 29 of *A. actinomycetemcomitans*, interleukin-8, or interleukin-1β (12,17). On the

other hand, the level of zonula occludens protein-1 was increased by interleukin-1β, decreased by *A. actinomycetemcomitans* and outer membrane protein 29, and not influenced by interleukin-8, in human gingival epithelial cells. The exposure of human gingival epithelial cells to bacteria and cytokines is suggested to cause the same responses in gap junctional intercellular communication and connexin 43 expression, but not in zonula occludens protein-1 expression. Generally, bacterial infections of gingival epithelial cells are followed by the secretion of cytokines from gingival epithelial cells and gingival fibroblasts. The different responses in junctional complexes to the initial bacterial attack and the subsequent cytokine attack to human gingival epithelial cells may play a role in the onset of periodontal inflammation.

Our previous report showed that interleukin-8 is involved in the *A. actinomycetemcomitans*-induced reduction of gap junctional intercellular communication and connexin 43 in human gingival epithelial cells (17). It might be questionable if interleukin-1β also mediates the *A. actinomycetemcomitans*-induced reduction of gap junctional intercellular communication in human gingival epithelial cells, because interleukin-1β, in addition to *A. actinomycetemcomitans* and interleukin-8, inhibits connexin 43 mRNA expression and gap junctional intercellular communication in human gingival epithelial cells. Although keratinocytes produce pro-interleukin-1β, which is not biologically active, they lack the ability to process pro-interleukin-1β into its active form (28). In addition, keratinocytes cannot actively secrete interleukin-1β via a conventional pathway because interleukin-1β lacks a signal peptide (28). Furthermore, our previous study revealed the increased expression of interleukin-1β mRNA in gingival epithelial cells stimulated with *A. actinomycetemcomitans*, although interleukin-1β could not be detected in the conditioned medium of the human gingival epithelial cells (20). Therefore, interleukin-1β, unlike interleukin-8, may not be involved in the *A. actinomycetemcomitans*-induced reduction in gap junctional intercellular communication.

The present and previous findings demonstrated that irsogladine maleate recovers the whole *A. actinomycetemcomitans*-, outer membrane protein 29-, interleukin-8- and interleukin-1β-induced reduction in gap junctional intercellular communication and connexin 43 levels. Irsogladine maleate abrogates both the bacterial- and cytokine-induced reduction of gap junctional intercellular communication in human gingival epithelial cells, suggesting that irsogladine maleate modulates the suppression of gap junctional intercellular communication in gingival epithelial cells by inflammatory cytokines and periodontopathogenic bacteria, leading to the maintenance of homeostasis of gingival epithelial function. Thus, irsogladine maleate may be a potential therapeutic modality to prevent periodontal disease.

It has been reported that irsogladine maleate up-regulates gap junctional intercellular communication between pancreatic cancer cells via a protein kinase A pathway (19). In addition, we have previously reported that irsogladine maleate recovers the *A. actinomycetemcomitans*-induced reduction in gap junctional intercellular communication by increasing cAMP levels in human gingival epithelial cells (12). Therefore, it may be considered that irsogladine maleate modulates interleukin-1β-induced suppression of gap junctional intercellular communication through a protein kinase A pathway in human gingival epithelial cells. However, the study on signaling cascades by irsogladine maleate, for example, mitogen-activated protein kinase and nuclear factor-κB, needs to be pursued.

In conclusion, interleukin-1β inhibits gap junctional intercellular communication and connexin 43 expression, whereas interleukin-1β promotes zonula occludens protein-1 expression in human gingival epithelial cells. In addition, although irsogladine maleate recovers the interleukin-1β-induced decrease in gap junctional intercellular communication and connexin 43, irsogladine maleate does not influence the increased levels of zonula occludens protein-1 by interleukin-1β in human gingival epithelial cells. To render irsogladine maleate feasible as a medicament to prevent periodontal inflammation, further work, for example *in vivo* studies, is required.

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