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Irsogladine maleate regulates epithelial barrier function in tumor necrosis factor-α-stimulated human gingival epithelial cells

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Background and Objective: As epithelial cells function as a mechanical barrier, the permeability of the gingival epithelial cell layer indicates a defensive capability against invasion by periodontal pathogens. We have reported the expression of claudin-1 and E-cadherin, key regulators of permeability, in the gingival junctional epithelium. Irsogladine maleate (IM) is a medication for gastric ulcers and also regulates *Aggregatibacter actinomycetemcomitans*-stimuated chemokine secretion and E-cadherin expression in gingival epithelium. In this study, we have further investigated the effects of IM on the barrier functions of gingival epithelial cells under inflammatory conditions.

Material and Methods: We examined the permeability, and the expression of claudin-1 and E-cadherin, in human gingival epithelial cells (HGECs) stimulated with tumor necrosis factor (TNF)-α, with or without IM.

Results: TNF-α increased the permeability of HGECs, and IM abolished the increase. TNF-α reduced the expression of E-cadherin in HGECs, and IM reversed the reduction. In addition, immunofluorescence staining showed that TNF-α disrupted claudin-1 expression in HGECs, and IM reversed this effect.

Conclusion: The results suggest that IM reverses the TNF-α-induced disruption of the gingival epithelial barrier by regulating E-cadherin and claudin-1.

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Periodontitis is an inflammatory condition caused by the colonization of periodontopathogenic bacteria, such as *Porphyromonas gingivalis* or *Aggregatibacter actinomycetemcomitans*, in the gingival sulcus. The gingival junctional epithelium is located at a strategically important interface at the bottom of the gingival sulcus and contributes actively to inflammatory processes because it represents the first line of defense against microbial attack (1–3). As epithelial cells function as a mechanical barrier (4,5), disruption of the gingival epithelial cell layer allows periodontopathogenic bacteria to invade periodontal tissue, leading to periodontal disease. Therefore, regulation of the barrier function may prevent bacterial invasion.

Although epithelial cells are generally interconnected by tight junctions, adherence junctions, desmosomes and gap junctions, previous studies have shown that the junctional epithelium is interconnected only by a few desmosomes, and occasionally by gap junctions, and has wide intercellular spaces (1,2). However, we recently found that claudin-1, a tight junction

structured protein, was expressed in the healthy junctional epithelium of Fischer 344 rats (6). A previous report showed that claudin-1-deficient mice died within 1 d of birth and exhibited severe defects in the permeability of the epidermis (7). Cells over-expressing claudin-1 showed increased transepithelial electrical resistance (8). Therefore, claudin-1 may play an important role in the barrier function of the junctional epithelium, in spite of the absence of tight junctions. E-cadherin, a key protein involved in the formation of desmosomes and adherens junctions, is known to regulate the permeability of epithelial cells (5,9). In the gastric mucosal epithelium, the disruption of E-cadherin seems to cause epithelial permeability to increase (10). Immunohistochemical staining has shown that E-cadherin is expressed in the healthy junctional epithelium of humans and rats (6,11) and that its level is decreased in diseased tissue (11,12). Therefore, E-cadherin plays an important role against bacterial invasion in the gingival junctional epithelium.

Irsogladine maleate (IM) is known to enhance gap junctional intercellular communication in cultured rabbit gastric epithelial and pancreatic cancer cells (13,14), and is used clinically as an anti-gastric ulcer agent. Our previous study showed that IM inhibits *A. actinomycetemcomitans*-induced inflammatory responses in the gingival epithelium by suppressing neutrophil migration *in vivo* and *in vitro* (12). In addition, IM rescued the *A. actinomycetemcomitans*-induced reduction in E-cadherin *in vivo* and *in vitro* (12). Furthermore, IM countered the reduction of gap junctional intercellular communication in cultures of human gingival epithelial cells (HGECs) stimulated with *A. actinomycetemcomitans* or interleukin (IL)-1β (15,16). As IM seems to regulate the inflammatory responses induced by bacterial attack and cytokine stimulation in the human gingival epithelium, it may be a candidate preventive medicine for periodontal disease.

Junctional epithelium, which is originally derived from the reduced enamel epithelium, may be replaced over time by a junctional epithelium formed by basal cells originating from the oral gingival epithelium (17). In fact, after gingivectomy, a new junctional epithelium is formed from the basal cells of the oral gingival epithelium (18–22). These findings show that the origin of the junctional epithelium is the same as that of the oral gingival epithelium, suggesting that the cultured gingival epithelial cells used in this study possess some characteristics of junctional epithelial cells.

Tumor necrosis factor-α (TNF-α) is a major inflammatory cytokine produced in response to *P. gingivalis* or *A. actinomycetemcomitans* infection (23). It has also been reported that TNF-α induces barrier dysfunction in many types of cells. In this study, to investigate the effect of IM on the gingival epithelial barrier, we examined permeability and junctional protein expression in HGECs following stimulation with TNF-α.

Material and methods

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 the patients' informed consent. HGECs from three donors (two women, 24 and 27 years of age; and one man, 22 years of age) were isolated as previously described (15,16). Briefly, gingival tissues were treated with 0.025% trypsin and 0.01% EDTA overnight at 4°C, and divided into epithelial and connective tissues. The HGEC suspension was centrifuged at 120 *g* for 5 min, and the pellet was suspended in Humedia-KB2 medium (Kurabo, Osaka, Japan) containing 10 μg/mL of insulin, 5 μg/mL of transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol, 10 μM sodium selenite, 50 μg/mL of bovine pituitary extract, 100 units/mL of penicillin and 100 μg/mL of streptomycin (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.

A Simian virus-40 (SV40) antigen-immortalized gingival epithelial cell line, OBA-9, was kindly provided by Dr Shinya Murakami (Osaka University Graduate School of Dentistry, Osaka, Japan) and maintained in Medium A (24). OBA-9 cells were used for immunofluorescence staining.

RNA preparation and real-time PCR

Cultured HGECs were harvested at the fourth passage, seeded in 35-mm plastic tissue-culture plates coated with type I collagen and maintained in 2 mL of Medium A. Confluent HGECs were pretreated for 1 h with or without 1 μM IM (supplied by Nippon Shinyaku, Kyoto, Japan) and then exposed for 24 h to 50 ng/mL of TNF-α (R&D Systems, Minneapolis, MN, USA) in 2 mL of Humedia-KB2 medium containing 10 μg/mL of insulin, 5 μg/mL of transferrin, 10 μM 2-mercaptoethanol, 10 μM 2-aminoethanol, 10 μM sodium selenite, 100 units/mL of penicillin and 100 μg/mL of streptomycin (Medium B). Total RNA was extracted using ISOGEN (Wako Pure Chemical Industries, Osaka, Japan) and quantified by spectrometry at 260 and 280 nm. First-strand 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 SYBR Green (Roche). The sense and antisense primers for human E-cadherin, claudin-1 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA are listed in Table 1.

Western blotting

To analyze the expression of claudin-1, E-cadherin and β-actin, HGECs were cultured as described above. Confluent HGECs, which had been pretreated for 1 h with or without 1 μM IM, were exposed to 50 ng/mL of TNF-α for 24 h in 2 mL of Medium B. The cells were lysed in 200 μL of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol and 0.01% Bromophenol Blue). The samples were resolved on a 10%

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

Claudin-1
Sense: 5′-GCG CGA TAT TTC TTC TTG CAG G-3′
Antisense: 5′-TTCGTACCTGGCATT GACTGG-3′
E-cadherin
Sense: 5′-TTC TGC TGC TCT TGC TGT TTC-3′
Anti-sense: 5′-AGT CAAAGT CCT GGT CCT CTT-3′
GAPDH
Sense: 5′-AAC GTG TCA GTG GTG GAC CTG-3′
Antisense: 5′-AGT GGG TGT CGC TGT TGA AGT-3′

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

SDS-polyacrylamide gel by electrophoresis under nonreducing conditions and electrophoretically transferred onto membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked for 1 h with 5% nonfat dried milk and reacted overnight with mouse anti-human E-cadherin IgG (R&D Systems), rabbit anti-claudin-1 IgG (Invitrogen, Carlsbad, CA, USA) and mouse anti-human β-actin IgG (Invitrogen). Then, the membrane was incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit or goat anti-mouse IgG (R&D Systems) 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 (GE Healthcare, Bucks., UK).

Immunofluorescence staining

Fourth-passage HGECs, or OBA-9 cells, were seeded on glass coverslips coated with type I collagen in 35-mm plastic tissue-culture plates, and maintained in 2 mL of Medium A. Confluent HGECs or OBA-9 cells, which had been pretreated for 1 h with or without 1 μM IM, were exposed to 50 ng/mL of TNF-α for 24 h in 2 mL of Medium B. After incubation, the cells on the coverslips were washed and immersed for 10 min in 3.5% formaldehyde and 0.2% Triton X-100 in phosphate-buffered saline. Blocking was performed by immersing the coverslips in Tris-buffered saline, containing 0.2% casein and 0.1% Triton X-100, for 30 min at 37°C. After blocking, the coverslips were washed twice with phosphate-buffered saline. For staining of claudin-1 or E-cadherin, HGECs or OBA-9 cells were labeled with rabbit anti-claudin-1 IgG or mouse anti-human E-cadherin IgG (Takara, Shiga, Japan), followed by Alexa Fluor 488-conjugated anti-rabbit or anti-mouse IgG. Fluorescence signals were detected using a Zeiss LSM 510 laser scanning confocal microscope (Zeiss Microimaging, Thornwood, NY, USA) or a fluorescence microscope, BZ-9000 (Keyence, Osaka, Japan). The immunofluorescence intensity was quantitatively measured.

Transepithelial electrical resistance

HGECs were seeded on cell-culture polyethylene terephthalate membrane inserts (ThinCerts, 0.4 μm pore size; Greiner Bio-One, Frickenhausen, Germany) placed in a 24-well tissue culture plate and maintained in 800 μL of Medium A. Confluent HGECs were pretreated for 1 h with or without 1 μM IM, and then exposed to 10, 50 or 100 ng/mL of TNF-α for 0, 12, 24, or 31 h in Medium B. The transepithelial electrical resistance (TER) of HGECs was measured using a Millicell-ERS (Millipore, Billerica, MA, USA).

Fluorescein–dextran conjugate transport assay

To examine cell permeability, we also used a fluorescein–dextran conjugate

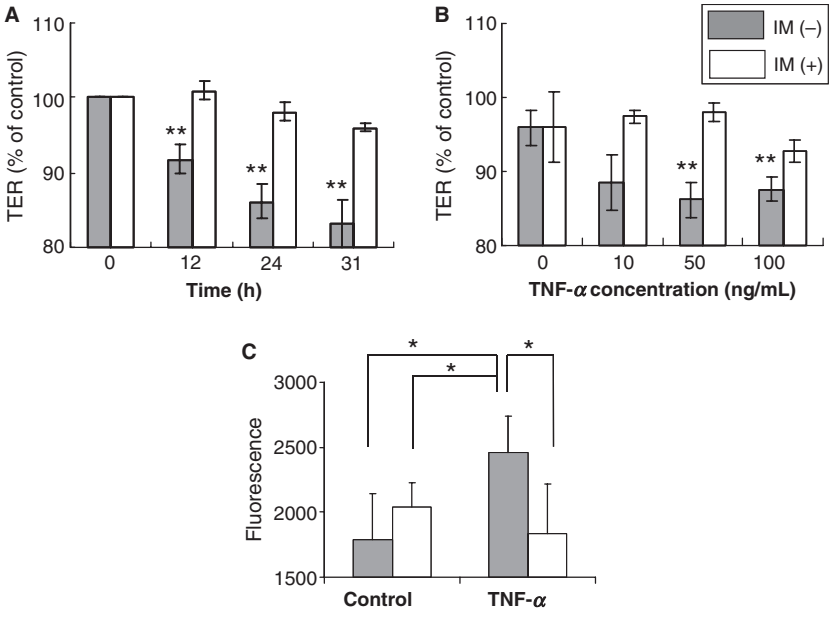

Fig. 1. Effect of irsogladine maleate (IM) on the permeability of the human gingival epithelial cell (HGEC) layer exposed to tumor necrosis factor-α (TNF-α). (A, B) Transepithelial electrical resistance (TER) of the HGEC layer. Confluent HGECs on the cell-culture polyethylene terephthalate membrane insert were pretreated for 1 h with or without 1 μM IM and then exposed to 50 ng/mL of TNF-α for the indicated times (A) or to 0–100 ng/mL of TNF-α for 24 h (B). The TER of HGECs was measured using a Millicell-ERS. **Significant difference from control (0 h) (Student's *t*-test, $p < 0.01$). (C) Fluorescein–dextran conjugate transport assay. Confluent HGECs on cell-culture polyethylene terephthalate membrane inserts were pretreated for 1 h with or without 1 μM IM and then exposed to 50 ng/mL of TNF-α for 24 h. Fluorescein–dextran conjugate was then added to the upper chamber to give a final concentration of 10 μg/mL. Four hours after the addition of fluorescein–dextran conjugate, the medium was collected from the lower chamber and the concentration was measured using a fluorescence microplate reader with excitation at 485 nm and emission at 535 nm. *Significant difference (Student's *t*-test, $p < 0.05$).

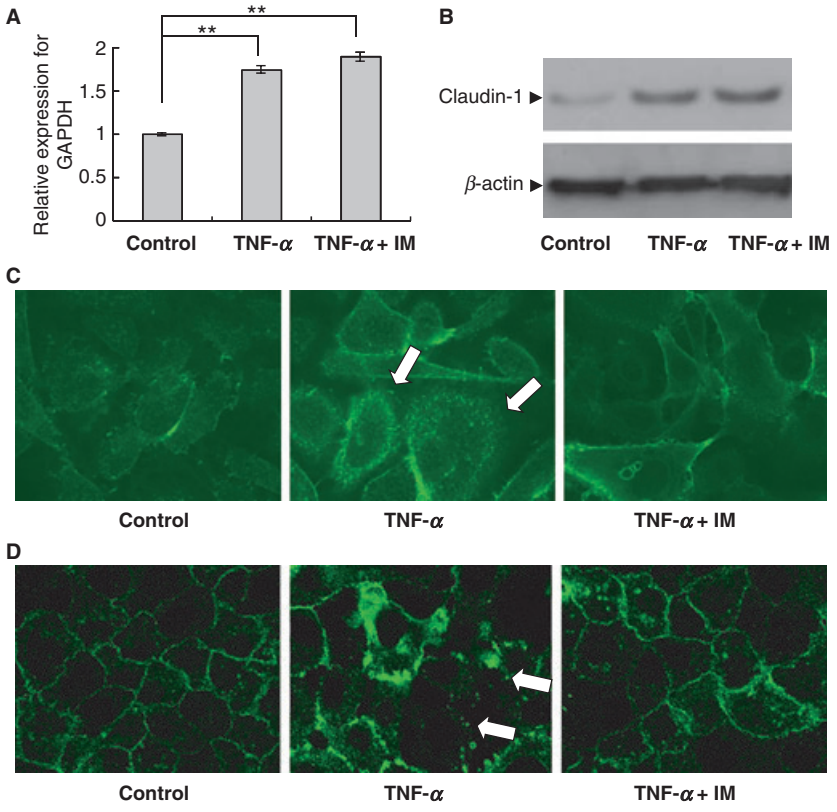

Fig. 2. Effect of irsogladine maleate (IM) on the expression of claudin-1 in human gingival epithelial cells (HGECs) and OBA-9 cells exposed to tumor necrosis factor-α (TNF-α). Confluent HGECs and OBA-9 cells were exposed to 50 ng/mL of TNF-α in the presence or absence of 1 μM IM for 24 h. (A) Claudin-1 mRNA in HGECs was analyzed using real-time PCR. Values are means ± standard deviation of three cultures. **Significant difference (*t*-test, $p < 0.01$). (B) Claudin-1 levels in HGECs were determined by western blotting. The bands are representative of three experiments. (C, D) Representative fluorescence images of the cellular distribution of claudin-1 in HGECs (C) or OBA-9 cells (D) was obtained using microscopy (×640 magnification). Arrows indicate the disruption of claudin-1 scattered in the cytoplasmic compartment. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

transport assay. HGECs were seeded on cell-culture polyethylene terephthalate membrane inserts, as described above. Confluent HGECs were pretreated for 1 h with or without 1 μM IM, and then exposed for 24 h to 50 ng/mL of TNF-α in Medium B. Fluorescein–dextran conjugate (molecular weight = 3000; Molecular Probes, Eugene, OR, USA) was added to the upper chamber to give a final concentration of 10 μg/mL. Four hours after the addition of fluorescein–dextran conjugate, the medium was collected from the lower chamber, and the fluorescence level was measured with excitation at 485 nm and emission at 535 nm using a fluorescence microplate reader (Twinkle LB 970; Berthold Technologies, Bad Wildbad, Germany).

Statistical analysis

Between-group comparisons were analyzed using the Student's *t*-test. Differences were considered significant when the probability value was < 5% ($p < 0.05$).

Results

IM inhibited the TNF-α-induced increase in the permeability of HGECs

At 50 ng/mL, TNF-α decreased the TER in HGECs, starting 12 h after exposure and continuing at a steady rate (Fig. 1A). In addition, treatment with 50 and 100 ng/mL of TNF-α for 24 h reduced the TER in HGECs (Fig. 1B). However, pretreatment with IM prevented the TNF-α-induced reduction (Fig. 1A and 1B). In addition, using the fluorescein–dextran conjugate transport assay, we confirmed the effect of IM on the permeability of HGECs stimulated by TNF-α. TNF-α increased the concentration of fluorescein–dextran conjugate in the lower chamber, and IM inhibited this increase (Fig. 1C).

E-cadherin and claudin-1 were present in HGECs and OBA-9 cells

Immunofluorescence staining showed junctional localization of claudin-1 and E-cadherin in the primary cultures of HGECs and in immortalized HGECs (OBA-9), and the immunofluorescence staining of OBA-9 cells was stronger than that of HGECs (Figs 2 and 3). Therefore, OBA-9 cells were also used to indicate the disruption of junctional proteins induced by TNF-α.

IM prevented the disruption of claudin-1 and E-cadherin induced by TNF-α in HGECs and OBA-9 cells

At 50 ng/mL, TNF-α increased the expression of claudin-1 at the mRNA and protein levels, and IM did not affect the increased levels of claudin-1 expression (Fig. 2A and 2B). However, immunofluorescence staining showed that 50 ng/mL of TNF-α affected the distribution of claudin-1 in HGECs and OBA-9 cells, and the disrupted claudin-1 proteins were scattered in the cytoplasmic compartment (Fig. 2C and 2D). In addition, IM reversed the TNF-α-induced disruption without changing the levels of mRNA and protein in HGECs and OBA-9 cells. TNF-α suppressed the expression of E-cadherin at the mRNA and protein levels, and IM prevented this decrease (Fig. 3A and 3B). Immunofluorescence staining also indicated that IM recovered the degradation of E-cadherin induced by TNF-α in HGECs and OBA-9 cells (Fig. 3C and 3D).

Discussion

In this study, we demonstrated, for the first time, that IM regulates gingival epithelial permeability by preventing the TNF-α-induced disruption of E-cadherin and claudin-1. At a cellular level, this result supports our previous finding that IM recovered the down-regulated expression of E-cadherin in inflammatory gingival tissue in an animal model.

In the present study, the addition of TNF-α to cultures of gingival epithelial cells increased cell permeability, consistent with previous reports in human airway epithelial cells (25), Caco-2 intestinal epithelial cells (26), human corneal epithelial cells (27) and human corneal endothelial cells (28). The involvement of cytokines in the breakdown of barrier integrity is recognized in many disorders, including pulmonary edema (29) and Crohn's disease (30). The increased permeability by cytokines gives the bacteria and their products the opportunity to enter the junctional epithelium. Therefore, the inhibition of enhancement of cell permeability by IM may protect gingival cells from bacterial invasion.

Although tight junctions, which generally contribute to the regulation of cell permeability, are considered not to exist in the gingival junctional epithelium, we previously found that claudin-1 is present in the gingival junctional epithelium (6). Therefore, we hypothesized that claudin-1 is involved in the permeability of the gingival epithelium in spite of the lack of tight junctions. Although TNF-α unexpectedly increased the expression of claudin-1 mRNA and protein, immunofluorescence staining showed that TNF-α disrupted claudin-1. Similarly, in the human colon carcinoma cell line HT-29/B6, TNF-α mediated claudin-1 internalization into intercellular vesicles while increasing expression levels (31). On the other hand, TNF-α down-regulated claudin-1 expression at the protein level in a rat parotid cell line and in human coronary artery endothelial cells (32,33). These different effects of TNF-α on claudin-1 expression might be a result of the source of cells or of the culture conditions. In addition, IM recovered the disruption of claudin-1 without affecting the increased expression levels. This result is supported by our

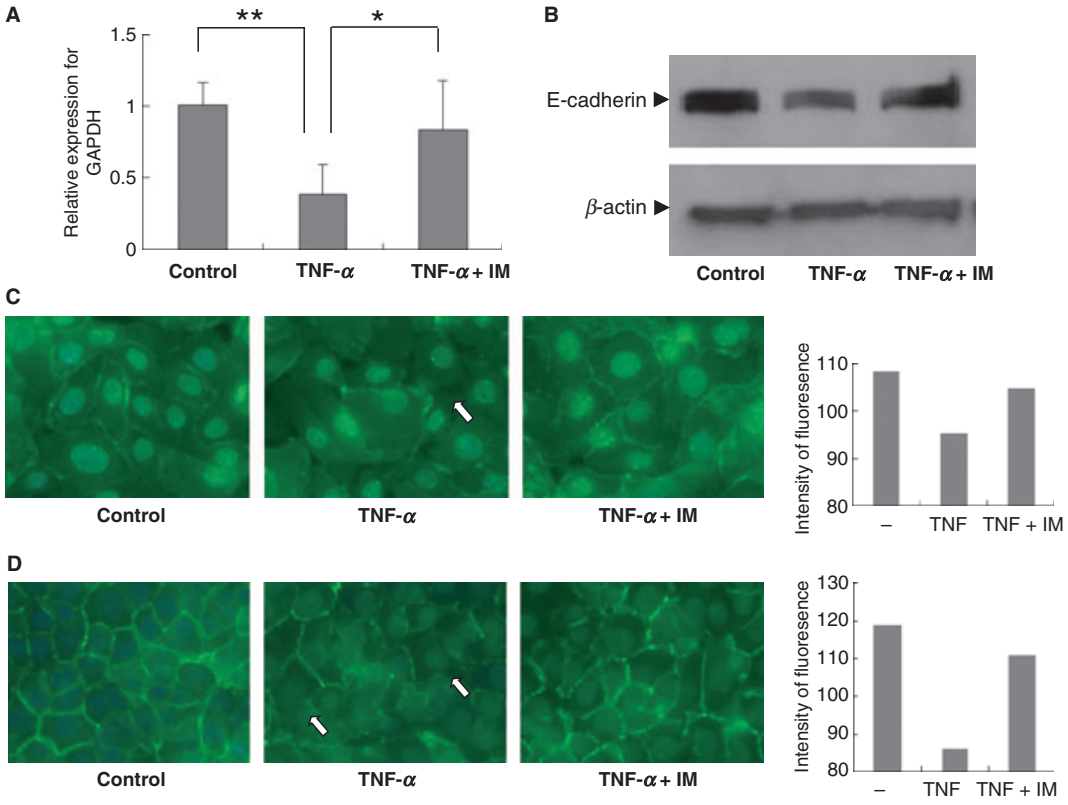

Fig. 3. Effect of irsogladine maleate (IM) on the expression of E-cadherin in human gingival epithelial cells (HGECs) and OBA-9 cells exposed to tumor necrosis factor-α (TNF-α). Confluent HGECs and OBA-9 cells were exposed to 50 ng/mL of TNF-α in the presence or absence of 1 μM IM for 24 h. (A) E-cadherin mRNA in HGECs was analyzed using real-time PCR. The mean value ± standard deviation of six cultures is shown. *Significant difference ($p < 0.05$); **Significant difference ($p < 0.01$) (both Student's *t*-test). (B) E-cadherin levels in HGECs were determined by western blotting. The bands are representative of three experiments. (C, D) Representative fluorescence images of the cellular distribution of E-cadherin in HGECs (C) or OBA-9 cells (D) were obtained using microscopy (×400 magnification). The immunofluorescence intensity was measured quantitatively by histogram function. Arrows indicate the degradation of E-cadherin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

previous finding that IM did not affect the increased levels of ZO-1, a structural protein of tight junctions, induced by IL-1β in HGECs (34). These results may suggest that the total amount of tight junction structured proteins does not affect gingival epithelial cell permeability.

TNF-α suppressed and disrupted E-cadherin in HGECs, consistent with previous reports that TNF-α induces a significant decrease and disruption of E-cadherin in small airway epithelial cells, human bronchial epithelial cells and human nasal epithelial cells (35,36). However, in the present study, IM recovered the reduction of E-cadherin expression induced by TNF-α in HGECs. In addition, our previous report has shown that IM recovered *A. actinomycetemcomitans*-induced reduction of E-cadherin in rat gingival epithelium. As E-cadherin is a key protein in the formation of cell junctions in the gingival junctional epithelium, its recovery may result in enhancement of the epithelial barrier function.

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

Acknowledgements

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