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Irsogladine Maleate Regulates Gap Junctional Intercellular Communication-Dependent Epithelial Barrier in Human Nasal Epithelial Cells

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Abstract The airway epithelium of the human nasal mucosa acts as the first physical barrier that protects against inhaled substances and pathogens. Irsogladine maleate (IM) is an enhancer of gastric mucosal protective factors via upregulation of gap junctional intercellular communication (GJIC). GJIC is thought to participate in the formation of functional tight junctions. However, the effects of IM on GJIC and the epithelial barrier in human nasal epithelial cells (HNECs) remain unknown. To investigate the effects of IM on GJIC and the tight junctional barrier in HNECs, primary cultures of HNECs transfected with human telomerase reverse transcriptase (hTERT-HNECs) were treated with IM and the GJIC inhibitors oleamide and 18β-GA. Some cells were pretreated with IM before treatment with TLR3 ligand poly(I:C) to examine whether IM prevented the changes via TLR3-mediated signal pathways. In hTERT-HNECs, GJIC blockers reduced the expression of tight junction molecules claudin-1, -4, -7, occludin, tricellulin, and JAM-A. IM induced GJIC activity and enhanced the expression of claudin-1, -4, and JAM-A at the protein and mRNA levels with an increase of barrier function. GJIC blockers prevented the increase of the tight junction proteins induced by IM. Furthermore, IM prevented the reduction of JAM-A but not induction of IL-8 and TNF-α induced by poly(I:C). In conclusion, IM can maintain the GJIC-dependent tight junctional barrier via regulation of GJIC in upper airway nasal epithelium. Therefore, it is possible that IM may be useful as a nasal spray to prevent the disruption of the epithelial barrier by viral infections and exposure to allergens in human nasal mucosa.

Keywords Irsogladine maleate · GJIC · Tight junctions · Barrier function · Human nasal epithelial cells · TLR3

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Introduction

The airway epithelium of the human upper respiratory mucosa acts as the first physical barrier that protects against inhaled substances and pathogens (Holgate 2007). The nasal epithelium is a highly regulated and impermeable barrier formed by tight junctions (Takano et al. 2005; Holgate 2007; Kojima et al. 2013). Tight junctions are formed by not only the integral membrane proteins claudins, occludin, tricellulin, and junctional adhesion molecules (JAMs), but also many peripheral membrane proteins, including the scaffold PDZ-expression proteins zonula occludens (ZO) and non-PDZ-expressing proteins (Tsukita et al. 2001; Sawada et al. 2003; Schneeberger and Lynch 2004; Kojima et al. 2009). These tight junction proteins are regulated by various cytokines and growth factors via distinct signal transduction pathways (Gonzalez-Mariscal et al. 2008; Kojima et al. 2009, 2013).

Gap junctional intercellular communication (GJIC) coordinates cellular functions essential for sustaining tissue homeostasis and is thought to play a crucial role in development, cell growth, and cell differentiation (Loewenstein 1979; Yamasaki and Naus 1996; Trosko and Ruch 1998). Gap junctional channels composed of

connexins mediate reciprocal exchanges of ions and small molecules of less than 1 kDa, including second messengers such as cyclic adenosine monophosphate (cAMP), inositol 1,4,5-trisphosphate (IP_3), and Ca^{2+} between adjacent cells (Saez et al. 1986; Kumar and Gilula 1996). Furthermore, GJIC is thought to participate in the formation of functional tight junctions (Kojima et al. 2002, 2007; Morita et al. 2004). In human nasal epithelial cells (HNECs) in vitro, both GJIC and the barrier function are upregulated by coculture with human nasal fibroblasts (Koizumi et al. 2007).

Irsogladine maleate (IM) is an enhancer of gastric mucosal protective factors, which is often prescribed in Japan, Korea, and China (Hiraishi et al. 2010). IM upregulates GJIC of rabbit gastric epithelial cells and rat pancreatic acinar cells (Ueda et al. 1991; Ito et al. 1997). Furthermore, it reverses the TNF-α-induced disruption of the gingival epithelial barrier by regulating E-cadherin and claudin-1 (Fujita et al. 2012). However, the effects of IM on the airway epithelium including human nasal mucosa remain unknown.

In HNECs in vivo and in vitro, tight junction proteins occludin, JAM-A, tricellulin, claudin-1, -4, -7, -8, -12, -13, -14, and ZO-1 and -2 with well-developed tight junction strands and mRNAs for all ten known human toll-like receptors (TLRs) have been detected (Takano et al. 2005; Koizumi et al. 2008; Ohkuni et al. 2009, 2011). Human telomerase reverse transcriptase (hTERT)-transfected HNECs (hTERT-HNECs) that express tight junctions and TLRs similarly in vivo can be used as an indispensable, stable model for studying the regulation of the nasal epithelial barrier and the epithelial response (Kurose et al. 2007; Kamekura et al. 2009; Ogasawara et al. 2010). We previously reported that the TLR3 synthetic analog polyinosinic–polycytidylic acid [poly(I:C)] reduced the expression of JAM-A and induced the secretion of proinflammatory cytokines IL-8 and TNF-α via distinct signal transduction pathways in hTERT-HNECs (Ohkuni et al. 2011).

In the present study, we investigated the effects of IM on the tight junction barrier of HNECs using hTERT-HNECs. In hTERT-HNECs, IM induced GJIC activity and enhanced the GJIC-dependent epithelial barrier with an increase of tight junction proteins. Furthermore, IM prevented the reduction of JAM-A induced by poly(I:C).

Materials and Methods

Reagents and Antibodies

IM was a gift from Nippon Shinyaku Co., Ltd. (Kyoto, Japan). Recombinant human IL-8 and TNF-α were purchased from PeproTech EC (London, UK). Polyinosine–polycytidylic acid [poly(I:C)] was purchased from InvivoGene (San Diego, CA). Polyclonal rabbit anti-JAM-A, anti-claudin-1, -4, and -7, anti-occludin, and anti-tricellulin antibodies and monoclonal mouse anti-claudin-1, -4, and anti-occludin antibodies were obtained from Zymed Laboratories (San Francisco, CA). A polyclonal rabbit anti-actin antibody and the gap junction intercellular communication blockers 18β-glycyrrhetinic acid (18β-GA) and oleamide were obtained from Sigma-Aldrich. Alexa Fluor 488 (green) and Alexa Fluor 594 (red) conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Molecular Probes Inc. (Eugene, OR). HRP-conjugated polyclonal goat anti-rabbit immunoglobulins were purchased from Dako A/S (Glostrup, Denmark). The ECL Western blot system was obtained from GE Healthcare UK, Ltd. (Buckinghamshire, UK).

Cell Culture and Treatments

The cultured HNECs were derived from the mucosal tissues of patients who underwent inferior turbinectomy at the Sapporo Hospital of Hokkaido Railway Company or the KKR Sapporo Medical Center Tonan Hospital. Informed consent was obtained from all patients, and this study was approved by the ethics committees of Sapporo Medical University, the Sapporo Hospital of Hokkaido Railway Company, and the KKR Sapporo Medical Center Tonan Hospital.

The procedures for primary culture of HNECs were as reported previously (Koizumi et al. 2008). Some primary cultured HNECs were transfected with the catalytic component of telomerase, the human catalytic subunit of the telomerase reverse transcriptase (hTERT) gene as described previously (Kurose et al. 2007; Kamekura et al. 2009; Ohkuni et al. 2009, Ogasawara et al. 2010). The cells were plated on 35 or 60 mm culture dishes (Corning Glass Works, Corning, NY), which were coated with rat tail collagen (500 μg of dried tendon/ml 0.1 % acetic acid). They were cultured in serum-free bronchial epithelial cell basal medium (BEBM, Lonza Walkersville, Inc., Walkersville, MD) supplemented with bovine pituitary extract (1 % v/v), 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 μg/ml amphotericin B, 0.1 ng/ml retinoic acid, 10 μg/ml transferrin, 6.5 μg/ml triiodothyronine, 0.5 μg/ml epinephrine, 0.5 ng/ml epidermal growth factor (Lonza Walkersville, Inc.), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) and incubated in a humidified 5 % CO_2:95 % air incubator at 37 °C. In this experiment, second and third passaged cells were used.

The HNECs were treated with 0.1–10 μg/ml IM or 100 μM oleamide and 10 μM 18β-GA. Furthermore, some

cells were pretreated with 10 μg/ml IM at 30 min before treatment with 25 μg/ml poly(I:C).

Gap Junctional Intercellular Communication (GJIC) Assay

GJIC was assayed using the scrape-loading/dye transfer technique of El-Fouly et al. (1987). The cells were rinsed with PBS, scrapeloaded using a razor blade, and immersed in a 0.05 % solution of the gap junction-permeable dye, Lucifer yellow (Polysciences, Inc., Warrington, PA) in PBS. After being left in the dye solution for 2 min at room temperature, the cells were rinsed with PBS several times and examined under a fluorescent microscope (Olympus, Tokyo, Japan). The extent of GJIC was calculated as the total number of Lucifer yellow-labeled cells per 350 μm.

RNA Isolation and RT-PCR

Total RNA was extracted and purified from hTERT-transfected HNECs using TRIzol reagent (Invitrogen). One microgram total RNA was reverse transcribed into cDNA using a mixture of oligo (dT) and Superscript II RTase under the recommended conditions (Invitrogen). Each cDNA synthesis was performed in a total volume of 20 μl for 50 min at 42 °C and terminated by incubation for 15 min at 70 °C. PCR containing 100 pM primer pairs and 1.0 μl of the 20 μl total RT reaction was performed in 20 μl of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, and 0.5 U of *Taq* DNA polymerase (Takara Bio, Inc., Shiga, Japan), employing 25, 30, or 35 cycles with cycle times of 15 s at 96 °C, 30 s at 55 °C, and 60 s at 72 °C. Final elongation time was 7 min at 72 °C. Seven microliters of the 20 μl total PCR reaction was analyzed by 1 % agarose gel electrophoresis with ethidium bromide staining and standardized using a GeneRuler TM 100 bp DNA ladder (Fermentas, Ontario, Canada). To provide a quantitative control for reaction efficiency, PCR reactions were performed with primers coding for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). Primers used to detect G3PDH and claudin-1, -4, and JAM-A are indicated in Table 1.

Western Blot Analysis

hTERT-HNECs were scraped from a 60-mm dish containing 300 μl of buffer (1 mM $NaHCO_3$ and 2 mM phenylmethylsulfonyl fluoride), collected in microcentrifuge tubes, and then sonicated for 10 s. The protein concentrations of the samples were determined using a BCA protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Aliquots of 15 μl of protein/lane for each sample were separated by electrophoresis in 5/20 % SDS polyacrylamide gels (Wako, Osaka, Japan), and electrophoretic transfer to a nitrocellulose membrane (Immobilon, Millipore Co., Bedford, UK) was performed. The membrane was saturated for 30 min at room temperature with blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1 % Tween 20, and 4 % skim milk) and incubated with polyclonal rabbit anti-JAM-A, anti-occludin, anti-tricellulin, anti-claudin-1, -4, -7, and anti-actin antibodies at room temperature for 1 h. The membrane was incubated with HRP-conjugated anti-mouse and anti-rabbit IgG antibodies at room temperature for 1 h. The immunoreactive bands were detected using an ECL Western blot system.

Immunocytochemical Analysis

hTERT-HNECs grown in 35 mm glass-coated wells (Iwaki, Chiba, Japan) were fixed with cold acetone and ethanol (1:1) at −20 °C for 10 min. After rinsing in PBS, the cells were incubated with a polyclonal rabbit anti-JAM-A antibody and monoclonal mouse anti-occludin, claudin-1, and -4 antibodies at room temperature for 1 h. Alexa Fluor 488 (green)-conjugated anti-rabbit IgG and Alexa Fluor 592 (red)-conjugated anti-mouse IgG (Invitrogen) were used as secondary antibodies. The specimens were examined using a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany).

Measurement of Transepithelial Electrical Resistance (TER)

hTERT-HNECs were cultured to confluence on inner chambers of 12 mm Transwell inserts with 0.4 μm pore-size filters (Corning Life Sciences). TER was measured using a cellZscope (nanoAnalytics, Germany), a computer-controlled automated multiwell device (12 wells). The values are expressed in standard units of ohms per square centimeter and presented as the mean ± SD.

Enzyme-Linked Immunosorbent (ELISA) Assay

The concentrations of human IL-8 and TNF-α in cell culture supernatants of hTERT-HNECs at 24 h after treatment were measured using ELISA kits for human IL-8 and TNF-α (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Data Analysis

Signals were quantified using Scion Image Beta 4.02 Win (Scion Co., Frederick, MA). Each set of results shown is representative of at least three separate experiments.

Table 1 Primers of RT-PCR

Gene	Forward primer	Reverse primer	Product size (bp)
Claudin-1	AACGCGGGGCTGCAGCTGTTG	GGATAGGGCCTTGGTGTTGGGT	593
Claudin-4	AGCCTTCCAGGTCCTCAACT	AGCAGCGAGTCGTACACCTT	249
Claudin-7	AGGCATAATTTTCATCGTGG	GAGTTGGACTTAGGGTAAGAGCG	252
JAM-A	GGTCAAGGTCAAGCTCAT	CTGAGTAAGGCAAATGCAG	582
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452

Results

GJIC Blockers Oleamide and 18β-GA Reduce the Expression of Tight Junction Molecules in HNECs

To investigate whether the expression of tight junction proteins in HNECs was dependent on GJIC, hTERT-HNECs were treated with the GJIC blockers 100 μM oleamide and 10 μM 18β-GA for 24 h. In Western blots, claudin-1, -4, -7, occludin, tricellulin, and JAM-A proteins were decreased after treatment with the GJIC blockers (Fig. 1a, b).

IM Enhances GJIC in HNECs

It is known that IM enhances GJIC in various types of cells (Ito et al. 1997; Fujita et al. 2012). To investigate whether IM enhanced GJIC of HNECs, hTERT-HNECs were treated with 10 μg/ml IM for 24 h, and GJIC was examined using the scrape-loading/dye transfer technique. In the cells treated with 10 μg/ml IM, the dye reached the fifth or sixth cell from the cutting line, whereas in the control, the dye spread was two to three cells thick (Fig. 1c, d).

IM Enhances the Expression of Tight Junction Molecules in HNECs

To investigate the effects of IM on expression of tight junction molecules in HNECs, hTERT-HNECs were treated with 0.1–10 μg/ml IM for 24 and 48 h. In Western blots, proteins of claudin-1 and -4 were increased from 24 h, and JAM-A protein was increased at 48 h in a dose-dependent manner (Fig. 2a–d). No changes of claudin-7, occludin, and tricellulin proteins were observed throughout the treatment. In RT-PCR at 48 h after treatment with 10 μg/ml IM, mRNAs of claudin-1, -4, and JAM-A were increased from 0.1 μg/ml IM (Fig. 2c, d). In immunostaining at 48 h after treatment with 10 μg/ml IM, the expression of claudin-1, -4, -7, occludin, and JAM-A at the membranes was strongly observed compared to the control (Fig. 2e).

IM Enhances Tight Junctional Barrier Function in HNECs

To investigate the effects of IM on the tight junction barrier function of HNECs, hTERT-HNECs were treated with 1 and 10 μg/ml IM, and transepithelial electric resistance (TER) was measured using a cellZscope. The TER was increased by 1 μg/ml IM at 48 h after the treatment compared to the control, whereas no changes of the TER values were observed at 24 h after the treatment (Fig. 3).

GJIC Blockers Oleamide and 18β-GA Prevent Increases of Tight Junction Molecules Induced by IM in HNECs

To investigate whether the effects of IM on tight junction molecules in HNECs were dependent on GJIC, hTERT-HNECs were pretreated with the GJIC blockers 100 μM oleamide and 10 μM 18β-GA 30 min before treatment with 10 μg/ml IM for 48 h. In Western blots, the GJIC blockers prevented upregulation of claudin-1, -4, and JAM-A proteins induced by treatment with IM (Fig. 4a, b).

IM Prevents Reduction of JAM-A But Not Induction of IL-8 and TNF-α Induced by Poly(I:C)

We previously demonstrated that the TLR3 ligand poly(I:C) reduced expression of the tight junction protein JAM-A and induced secretion of proinflammatory cytokines IL-8 and TNF-α in HNECs (Ohkuni et al. 2011). To investigate whether IM prevented the effects of poly(I:C) in HNECs, hTERT-HNECs were pretreated with 0.1–10 μg/ml IM 30 min before treatment with 25 μg/ml poly(I:C). In Western blots, IM prevented the downregulation of JAM-A protein induced by poly(I:C) in a dose-dependent manner (Fig. 5a, b). In immunostaining, the expression of JAM-A and occludin in part disappeared at cell borders of some cells after treatment with poly(I:C) and 10 μg/ml IM prevented the change (Fig. 5c). In ELISA, poly(I:C) induced secretion of IL-8 and TNF-α, but IM did not affect upregulation of the secretion (Fig. 5d).

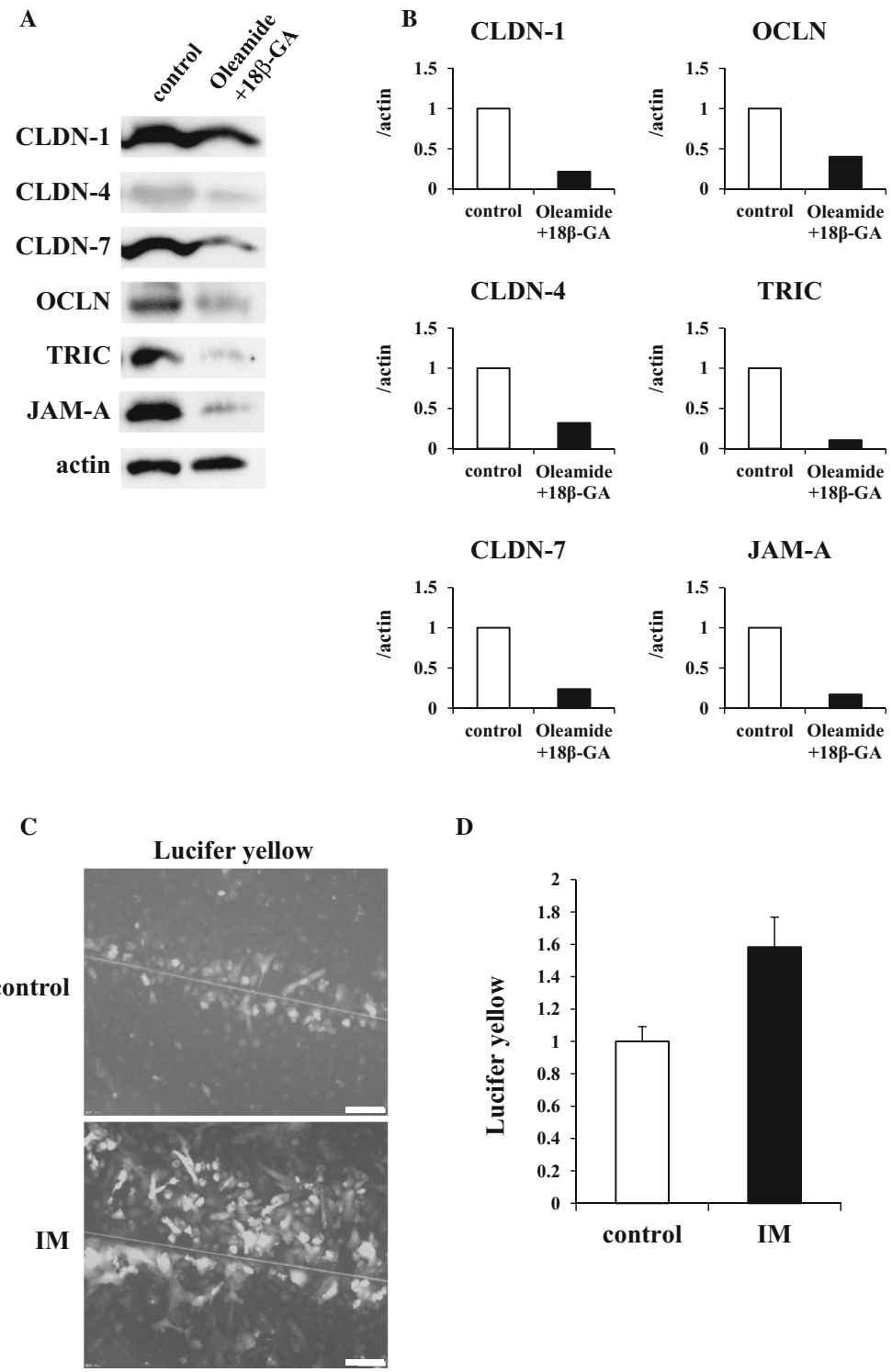
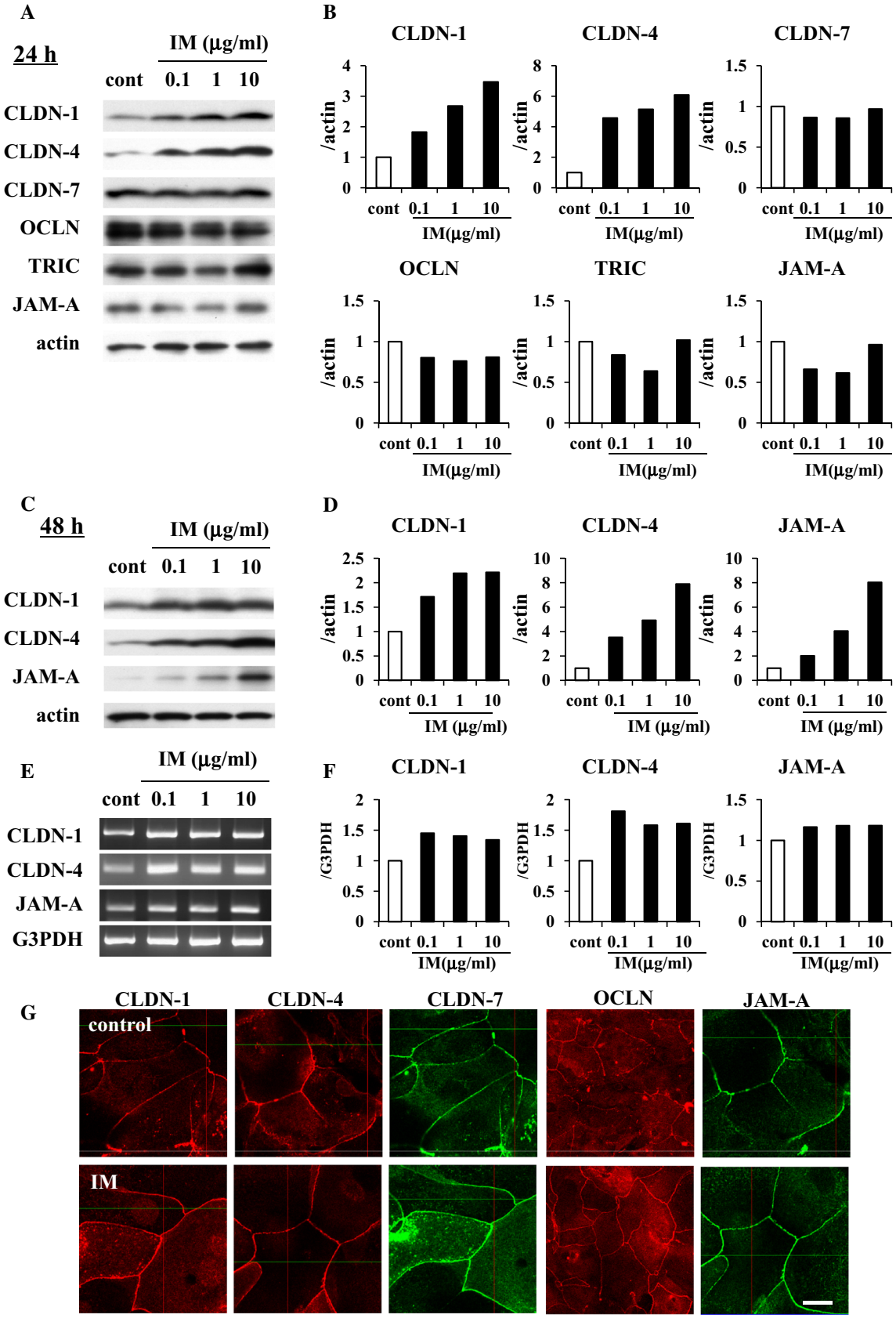


Fig. 1 **a** Western blots for CLDN-1, -4, -7, OCLN, TRIC, and JAM-A in hTERT-HNECs after treatment with 100 μM oleamide and 10 μM 18β-GA. *18β-GA* 18β-glycyrrhetinic acid. **b** The corresponding expression levels of (**a**) are shown as *bar* graphs. *CLDN* claudin, *OCLN* occludin, *TRIC* tricellulin. **c** GJIC activity examined using the scrape-loading/dye transfer (SL/DT) technique in hTERT-HNECs at 24 h after treatment with 10 μg/ml IM. *IM* irsogladine maleate. **d** Bar graph of GJIC activity of (**c**) measured using the SL/DT technique, as reported in "Materials and Methods" section

Discussion

In the present study, we first found that, in upper airway epithelial HNECs, IM induced GJIC activity and enhanced GJIC-dependent epithelial barrier with an increase of tight junction proteins. Furthermore, IM prevented the reduction of JAM-A, but not the induction of IL-8 and TNF-α, induced by TLR3 synthetic analog poly(I:C).

GJIC is thought to participate in the formation of functional tight junctions (Kojima et al. 2007). Cx32-mediated GJIC induces the expression and function of tight junctions at the transcriptional level in an hepatic cell line


A
24 h
IM (μg/ml)
cont 0.1 1 10
CLDN-1
CLDN-4
CLDN-7
OCLN
TRIC
JAM-A
actin
B
CLDN-1
CLDN-4
CLDN-7
OCLN
TRIC
JAM-A
/actin
cont 0.1 1 10
IM(μg/ml)
C
48 h
IM (μg/ml)
cont 0.1 1 10
CLDN-1
CLDN-4
JAM-A
actin
D
CLDN-1
CLDN-4
JAM-A
/actin
cont 0.1 1 10
IM (μg/ml)
E
IM (μg/ml)
cont 0.1 1 10
CLDN-1
CLDN-4
JAM-A
G3PDH
F
CLDN-1
CLDN-4
JAM-A
/G3PDH
cont 0.1 1 10
IM(μg/ml)
G
CLDN-1
CLDN-4
CLDN-7
OCLN
JAM-A
control
IM

◀**Fig. 2** Western blots for CLDN-1, -4, -7, OCLN, TRIC, and JAM-A in hTERT-HNECs at 24 h (**a**) and 48 h (**c**) after treatment with 0.1–10 μg/ml IM. The corresponding expression levels of (**a**) and (**c**) are shown as bar graphs of (**b**) and (**d**). (E) RT-PCR for CLDN-1, -4, and JAM-A in hTERT-HNECs at 48 h after treatment with 0.1–10 μg/ml IM. **f** The corresponding expression levels of **e** are shown as bar graphs. **g** Immunocytochemistry of CLDN-1, -4, -7, OCLN, and JAM-A in hTERT-HNECs at 48 h after treatment with 10 μg/ml IM. *Bar* 10 μm. *IM* irsogladine maleate, *CLDN* claudin, *OCLN* occludin, *TRIC* tricellulin

(Kojima et al. 2002). Cx26-mediated GJIC suppresses paracellular permeability of human intestinal epithelial cell line Caco-2 (Morita et al. 2004). In HNECs in vitro, both GJIC and barrier function are upregulated by coculture with human nasal fibroblasts (Koizumi et al. 2007). However, the specific transcriptional factors and second messengers via which GJIC can induce tight junctions are not yet known.

IM is clinically used as an anti-gastric ulcer agent that prevents gastric mucosal damage without inhibiting gastric secretion (Uchida et al. 2005). It upregulates GJIC of rabbit gastric epithelial cells and rat pancreatic acinar cells in part via upregulation of the cyclic adenosine monophosphate (cAMP) concentration (Ueda et al. 1991; Ito et al. 1997). In the present study, in HNECs, GJIC blockers decreased expression of claudin-1, -4, -7, occludin, tricellulin, and JAM-A. IM induced expression of claudin-1, -4, and JAM-A, but not claudin-7 and tricellulin together, at the transcriptional level with an increase of the epithelial barrier function. Furthermore, GJIC blockers prevented the increase of the tight junction proteins induced by IM. In HNECs in vitro, tight junction molecules and the barrier function are upregulated by various stimuli via distinct signal transduction pathways and the responses differ among the tight junction molecules (Kojima et al. 2013). Thus, in HNECs, it appears that IM can enhance expression

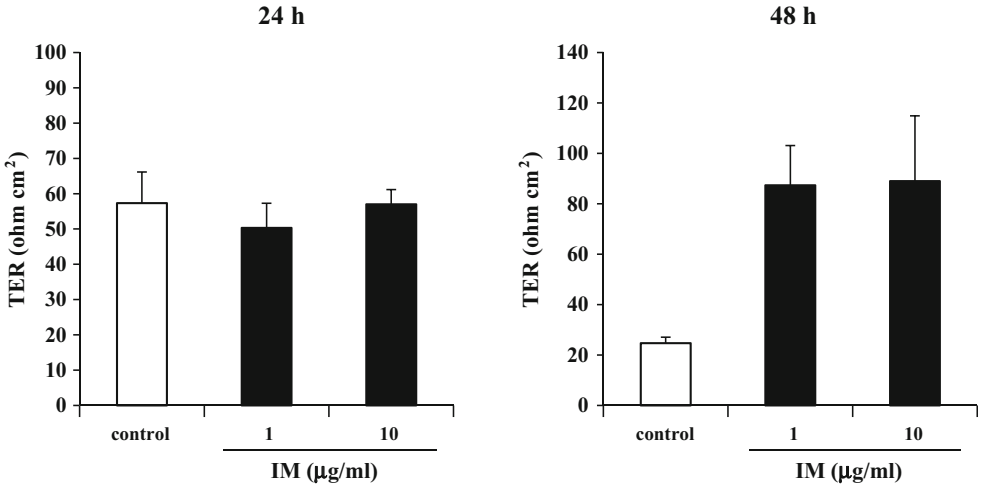


Fig. 3 Barrier function measured by TER in hTERT-HNECs at 24 and 48 h after treatment with 10 μg/ml IM. *IM* irsogladine maleate

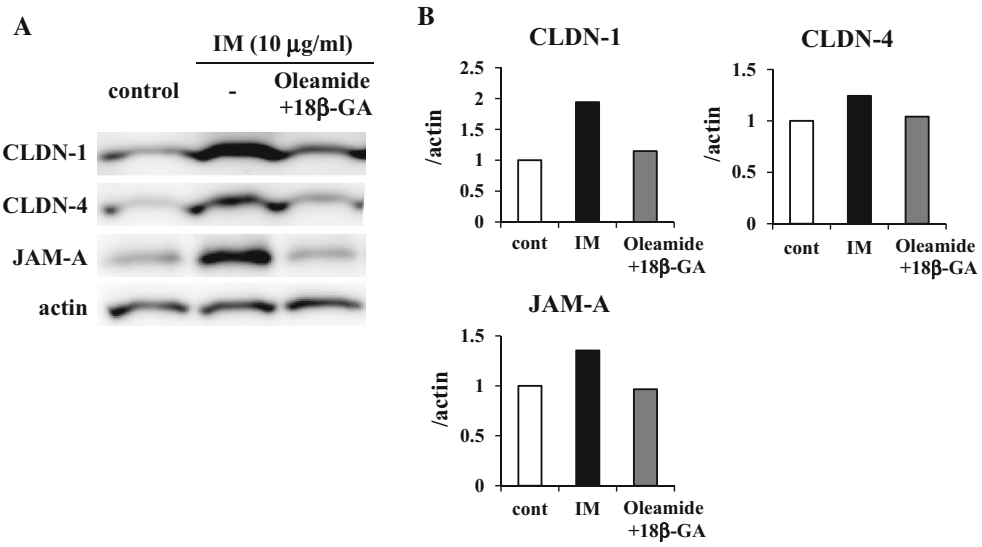


Fig. 4 **a** Western blots for CLDN-1, -4, and JAM-A in hTERT-HNECs pretreated with 100 μM oleamide and 10 μM 18β-GA 30 min before treatment with 10 μg/ml IM for 48 h. *18β-GA* 18β-glycyrrhetinic acid. **b** The corresponding expression levels of **a** are shown as *bar graphs*. *IM* irsogladine maleate, *CLDN* claudin

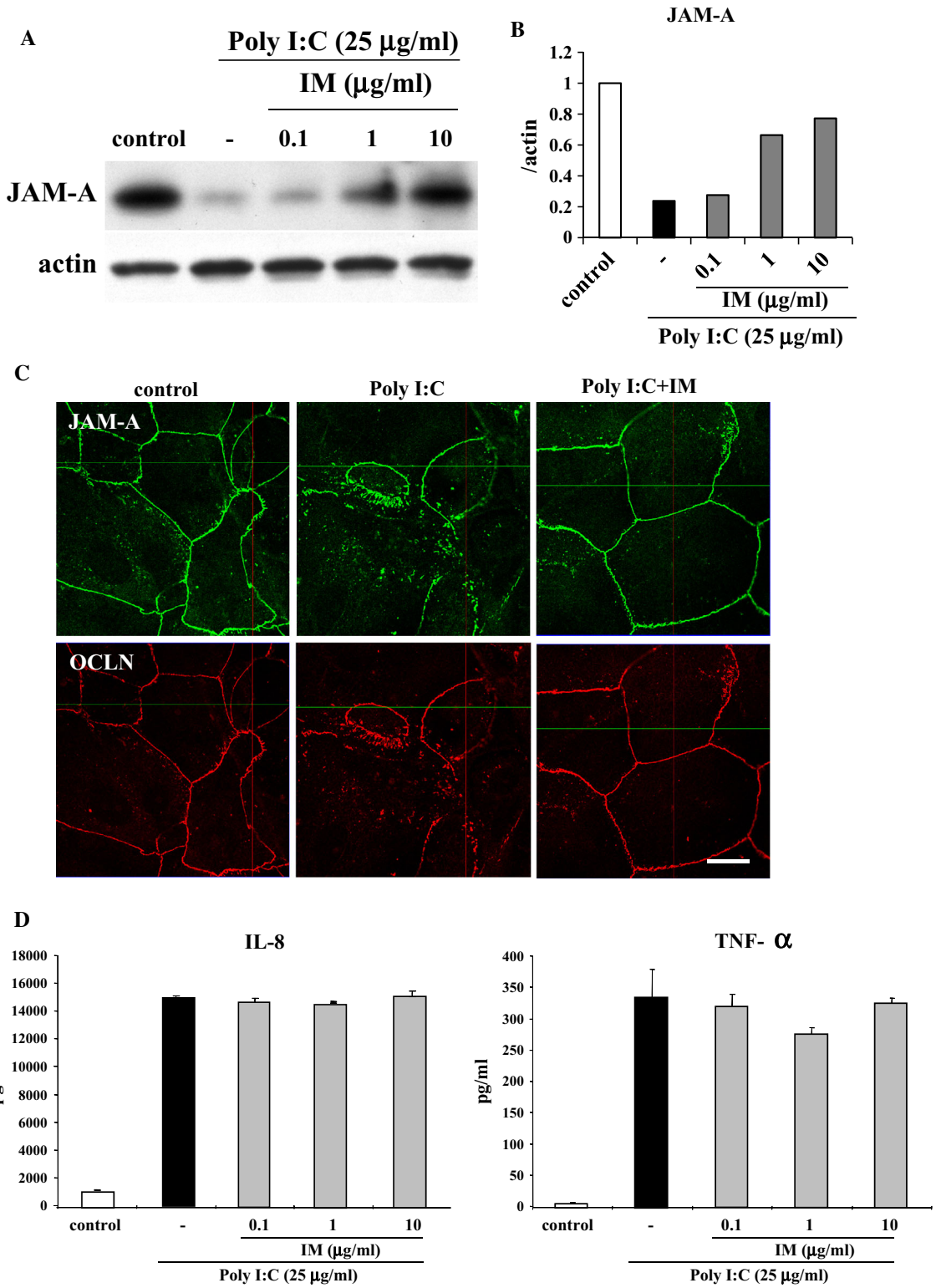


Fig. 5 **a** Western blots for JAM-A in hTERT-HNECs pretreated with 0.1–10 μg/ml IM 30 min before treatment with 25 μg/ml poly(I:C) for 24 h. **b** The corresponding expression levels of **a** are shown as bar graphs. **c** Immunocytochemistry of JAM-A and OCLN in hTERT-HNECs pretreated with 10 μg/ml IM 30 min before treatment with 25 μg/ml poly(I:C) for 24 h. *OCLN* occludin. *Bar* 10 μm. **d** ELISA for IL-8 and TNF-α in hTERT-HNECs pretreated with 10 μg/ml IM 30 min before treatment with 25 μg/ml poly(I:C) for 24 h. *IM* irsogladine maleate

of some tight junction molecules at the mRNA and protein levels and the epithelial barrier via upregulation of GJIC, although the detailed mechanisms remain unknown.

On the other hand, IM reverses the TNF-α-induced disruption of the human gingival epithelial barrier by regulating E-cadherin and claudin-1 (Fujita et al. 2012). It also suppresses the induction of IL-8 production via TLR2 in human gingival epithelial cells (Savitri et al. 2014). We previously reported that when HNECs were stimulated with various ligands of TLRs, TLR3 ligand poly(I:C) reduced the expression of JAM-A and induced secretion of IL-8 and TNF-α via distinct signal transduction pathways including NF-κB in HNECs (Ohkuni et al. 2011). In the present study, IM prevented downregulation of JAM-A induced by poly(I:C) in a dose-dependent manner but did not affect upregulation of the secretion of IL-8 and TNF-α induced by poly(I:C). These findings suggest that IM does not directly affect TLR3-mediated signal pathways and prevents GJIC-dependent change of JAM-A but not the GJIC-independent changes of the proinflammatory cytokines IL-8 and TNF-α.

In conclusion, IM can maintain the GJIC-dependent tight junctional barrier via regulation of GJIC in upper airway nasal epithelium. Therefore, it is possible that IM may be useful as a nasal spray to prevent disruption of the epithelial barrier by viral infections and exposure to allergens in the human nasal mucosa.

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