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Original Article

DsRNA induction of microRNA-155 disrupt tight junction barrier by modulating claudins

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Conflict of Interest
The authors have no financial conflicts of interest.

ABSTRACT

Background: The impaired barrier function of the airway epithelium due to RNA virus infection is closely related to the development and exacerbation of allergic airway inflammation.
Objective: In this study, we investigated the roles of microRNAs on the mechanisms of double-stranded RNA (dsRNA)-induced epithelial barrier dysfunction.
Methods: 16HBE14o- human bronchial epithelial cells were grown to confluence on Transwell inserts and exposed to poly-I:C. We studied epithelial barrier function by measuring transepithelial electrical resistance and paracellular flux of fluorescent markers and structure of tight junctions by immunofluorescence microscopy.
Results: Poly-I:C treated 16HBE14o- cells increased paracellular permeability. Knockdown of Toll-like receptor 3 and TRIF abrogated these effects. The expression of microRNA-155 (miR-155) was increased by poly-I:C in dose-dependent manner. Transfection of mir155 mimics into 16HBE14o- cells increased permeability and inhibited tight junction formation. Transfection of miR-155 inhibitor suppressed poly-I:C-induced barrier disruption. Poly-I:C treatment significantly decreased the expression of claudin members—claudin-1, -3, -4, -5, -9, -11, -16, -18 and -19. Transfection of miR-155 mimics showed similar changing expression pattern of claudin members with those of poly-I:C treatment.
Conclusion: These results suggest that RNA virus infection can impair the epithelial barrier disruption mechanism by down-regulation of claudin members through the induction of miR-155.

Keywords: dsRNA; Airway epithelial cells; Tight junction; Virus; Claudins

INTRODUCTION

The airway epithelial barrier is formed by intercellular binding components, including adherent junctions and tight junctions (TJs) [1, 2]. TJs located at the most apical part of adjacent airway epithelial cells contribute significantly to permeability barrier function [1, 2]. TJs regulate the selective passage of ions and solutes through the paracellular space and prevent paracellular migration of allergen and pathogens and their products from lumen to submucosal tissues. Thus, vulnerability of epithelial permeability barrier results in active

Author Contributions

Conceptualization: Hisato Hiranuma, Yasuhiro Gon, Shuichiro Maruoka, Kenji Mizumura. Data curation: Hisato Hiranuma, Yasuhiro Gon, Shuichiro Maruoka, Yutaka Kozu. Formal analysis: Hisato Hiranuma, Shiho Yamada, Asami Fukuda, Yusuke Kurosawa, Shimizu Tetsuo, Yoshiko Nakagawa. Funding acquisition: Yasuhiro Gon, Shuichiro Maruoka. Methodology: Hisato Hiranuma, Yasuhiro Gon, Shuichiro Maruoka. Project administration: Yasuhiro Gon, Shuichiro Maruoka. Visualization: Hisato Hiranuma, Shuichiro Maruoka, Shiho Yamada, Asami Fukuda, Yusuke Kurosawa. Writing - original draft: Hisato Hiranuma, Yasuhiro Gon. Writing - review & editing: Hisato Hiranuma, Yasuhiro Gon, Shuichiro Maruoka.

transport of allergens. It might be incorporated to the pathogenesis of allergic disorders or chronic inflammatory diseases. Bronchial asthma is a chronic inflammatory disorder of the airways characterized by inflammation, airway hyperresponsiveness, and reversible airflow obstruction [3, 4].

Infection with RNA viruses in the airway such as rhinovirus and respiratory syncytial virus (RSV), is known to be a major cause of the exacerbation of asthma. Furthermore, It is considered that such virus infections might be involved in the development of allergy [5, 6]. In RNA virus infection, double-stranded RNA (dsRNA) is naturally produced during virus replication in host cells. DsRNA is recognized by Toll-like receptor 3 (TLR3) on endosomes and induces the production of cytokines and chemokines, including RANTES (regulated upon activation, normal T cell expressed and presumably secreted) and thymic stromal lymphopoietin linked to eosinophilic inflammation [7-9]. DsRNA has reported to induce the disruption of TJ barrier through the activation of TLR3 signal [10, 11]. Therefore, after virus infection, environmental factors such as allergen, viruses, and airborne pollutants can undisturbedly enter the airway mucosa through disrupted airway epithelial barrier. It could potentially lead to development of asthma.

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression at the posttranscriptional level and have been as a critical regulatory factor in the mammalian immune system. Recently, miRNAs have been documented to play vital roles in TLR immunity and been as the fine-tuners of TLR signaling [12-14].

A role for miRNAs in the innate immune response was demonstrated when miRNAs such as microRNA-146a, microRNA-155 (miR-155), and microRNA-21 were shown to become induced in response to TLR4 signaling in monocytes [15-18]. A previous study has shown that MyD88 would be down-regulated by miR-155 and suppress *Helicobacter pylori*-induced inflammation. Previously, miR-155 has been found to play vital roles in the regulation of the immune response [19]. MicroRNA-155 activates the response of T cells to viral infection, and this activation is regulated by type I interferon signaling [20]. Recently, miR-155 has been as an important controller of TLR3 signaling via the targeting of adaptor molecules, downstream regulators and cytokines, such as TAB2, IKK-ε, and RIP [13]. Furthermore, miR-155 has been reported to negatively regulate TLR3 expression [21]. Therefore, we hypothesized that miR-155 is involved in virus-induced epithelial barrier impairment in the airway.

MATERIALS AND METHODS

Cells and cell culture

A differentiated SV40-transformed human bronchial epithelial cell line (16HBE14o-; hereafter referred to as 16HBE cells) was a generous gift from Dr. D.C. Gruenert (University of California, San Francisco, CA, USA). 16HBE cells were grown in minimum essential medium supplemented with 10% (v/v) fetal bovine serum and used at passages 10–40. 16HBE cells were seeded on Transwell inserts (Costar, Corning, NY, USA) at a density of 1×10^5 cells/cm^2.

Transepithelial electrical resistance measurements

TER measurement using 16HBE cells has been established in previous studies based on paracellular transport [11, 22, 23]. These were seeded on Transwell inserts at 1×10^5 cells/cm^2. The integrity of the cell monolayer was evaluated by TER using a Millicell-ERS system

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(Millipore Corp., Bedford, MA, USA) according to the manufacture's instruction. TER (ohms × cm^2) was calculated using the equation: (TER sample–TER blank) × surface area (cm^2).

Apparent permeability coefficient

Fluorescein isothiocyanate-labeled dextran (FITC-dextran; 4 kDa) was obtained from Sigma Chemical Company (St. Louis, MO, USA). The permeability of a cell monolayer was determined by FITC-dextran fluxes across this layer. A solution that contained 4-kDa FITC-dextran (1 mg/mL) was added to the apical compartment of a Transwell insert. Samples (200 μL) were removed from the basal compartments at 60 minutes after adding FITC-dextran and FITC-dextran intensity was measured using a PTI fluorometer set for excitation at 492 nm and emission at 520 nm. An apparent permeability coefficient (Papp) was obtained using the equation: Papp (cm/sec) = dQ/dt(V/AC 0), where dQ/dt was the permeability rate (μg/sec), C0 was the initial concentration in the upper chamber (μg/mL), V was the volume of the upper chamber (cm^3), and A was the membrane surface area (cm^2) [24, 25].

Real-time polymerase chain reaction

16HBE cells were cultured for 3 days with or without ds RNA, polyinosinic-polycytidylic acid (poly-I:C) (Sigma-Aldrich, St. Louis, MO, USA).

Real-time polymerase chain reaction (PCR) analysis was performed using an Ambion Fast SYBR Green Cells-to-CT Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol. Using the protocols for an Applied Biosystems 7300 Fast Real- Time PCR system and SYBR green PCR master mix (Life Technologies), PCR was performed in 96-well plates with denaturing at 95 °C for 20 seconds, followed by 40 cycles of annealing at 95°C for 3 seconds and amplification at 60°C for 30 seconds. We set the initial threshold and determined the Ct value. We evaluated the RNA level of each sample using the comparative determination method ($\Delta\Delta C_T$ method). Specific primers used in the present study are shown in **Table 1**.

Table 1. Specific primers for real-time polymerase chain reaction

Primers	Forward 5′→3′	Reverse 5′→3′
E-cadherin	AAGGTGACAGAGCCTCTGGATAGA	CATTCCCGTTGGATGACACA
Occludin	CGAGGAGTGGGTTAAAAATGTGT	ACTGTCAACTCTTTCCACATAGTCAGA
ZO-1	CGGTCCTCTGAGCCTGTAAG	GGATCTACATGCGACGACAA
ZO-2	GAGGAGTAGGAGCAGGAGCA	GGAGCCAACCTGACAGCTC
JAM-A	CCGTGTGGAGTGGAAGTTTGA	TCACCCGGTCCTCATAGGAA
JAM-B	GCCTGCAAAACCCCAAAGAA	GAGACACTCCGACCCAGTTT
CLDN 1	GGATTTACTCCTATGCCGGCGACAACA	CTCTGCGACACGCAGGACATCCA
CLDN 3	CATCACGTCGCAGAACATCTG	TCGTACACCTTGCACTGCATCT
CLDN 4	AGCCTTCCAGGTCCTCAACT	AGCAGCGAGTCGTACACCTT
CLDN 5	CCTGGACCACAACATCGTGA	AGCACCGAGTCGTACACTTT
CLDN 6	ATGCAGTGCAAGGTGTACGA	CCAGCAAGGTAGACCAGCAA
CLDN 7	AGGCATAATTTTCATCGTGG	GAGTTGGACTTAGGGTAAGAGCG
CLDN 9	ATGCAGTGCAAGGTGTACGA	ATCAGGCCAAGGTCGAAAGG
CLDN 11	CCCGGTGTGGCTAAGTACAG	GATTGTGGGAGTCCATCCCC
CLDN 16	TGGGAATGCGTCACAAATGC	GGATGCTCCGCAAGTATGGA
CLDN 18	TACACATTTGGTGCGGCTCT	TAAAACGTCTGGTTGCAGCG
CLDN 19	GCATTGACAGGTGTGCTTGG	CAGCCCGACTCAGTGTCATC
TRIF	TCTGTAGAAGATACCACCTCTCCAAAT	AGCTGAACAAGGAGTAGATGAAGGA
MyD88	GGCTGCTCTCAACATGCGA	TGTCCGCACGTTCAAGAACA
GAPDH	GTCGGAGTCAACGGATTTGG	GGCAACAATATCCACTTTACCAGAG

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MicroRNA-155 miRNA mimics and inhibitors

MicroRNA-155 miRNA mimics and modified single-stranded RNA inhibitors purchased from QIAGEN. After 24 hours, transfected cells were transferred to a Transwell chamber, cultured with medium and assessed for cell monolayer barrier function by measuring TER and Papp.

Small interfering RNA transfection

Small interfering RNA (siRNA) for TLR3 (Hs_TLR3_1803), TRIF (Hs_TICAMI_6929), and MyD88 (Hs_MYD88_1539) were purchased from Sigma-Aldrich, and siRNA for control from Invitrogen (Waltham, MA, USA). Transfection procedure was previously described [22]. In brief, 16HBE cells were expanded in 6 well plates to 50% confluence and then transduced these siRNAs for 24 hours by Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's procedure. The transfection efficiency by transfection of siTLR3, siTRIF, and siMyD88 was 76.6%, 87.8%, and 44.9%, respectively.

Statistical analysis

Results are given as means ± standard deviations. Results for different experimental groups were compared using Student *t* tests or 1-way analysis of variance and Dunnett multiple comparisons tests. $p < 0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism Software (La Jolla, CA, USA).

RESULTS

Effect of poly-I:C treatment on miR-155 expression

Previously, we have reported that a synthetic analog of dsRNA, poly-I:C treatment disrupt tight junction barrier in airway epithelial cells [11]. As we shown previously, poly-I:C treatment decreased TER (**Fig. 1A**) and increased FITC-dextran influx (**Fig. 1B**) in a dose-dependent manner. To evaluate the roles of TLR3 signaling pathways on the epithelial barrier integrity, we performed knockdown of TLR3 and its adaptor molecules, MyD88 and TRIF by transfection of their specific siRNAs. Transfection with TLR3-specific siRNA suppressed the poly-I:C-induced increase in dextran permeability, revealing the critical role of TLR3

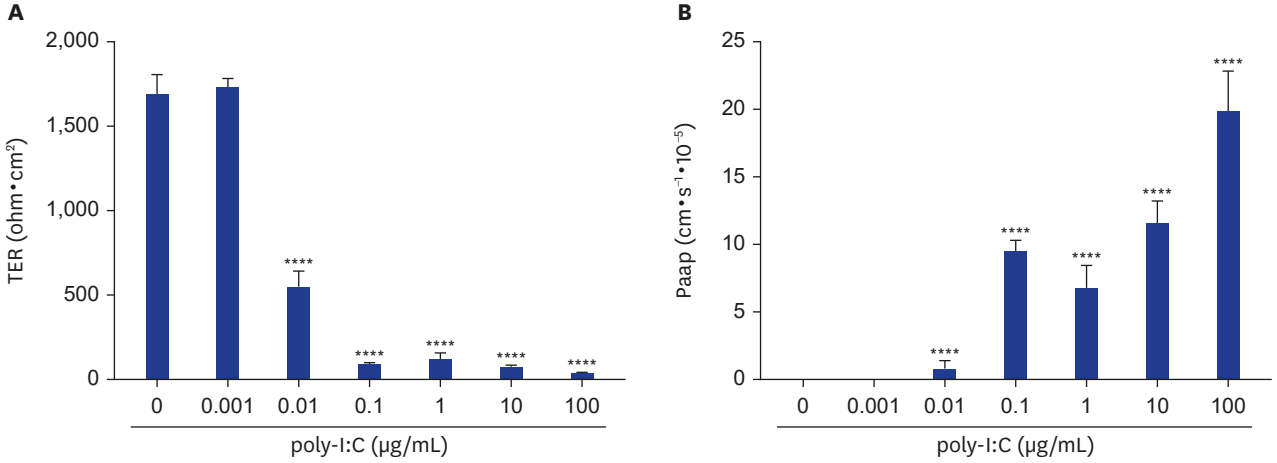

Fig. 1. Poly-I:C decrease transepithelial electrical resistance (TER) and increased fluorescein isothiocyanate (FITC)–dextran permeability. 16HBE cells were cultured on Transwell chamber with indicated dose of poly-I:C for 48 hours and then measured TER (A) and FITC–dextran permeability (B). Data are mean + standard deviation. Significance was determined by 1-way analysis of variance and Dunnetts multiple comparisons test. ****$p < 0.0001$. Paap, apparent permeability coefficients

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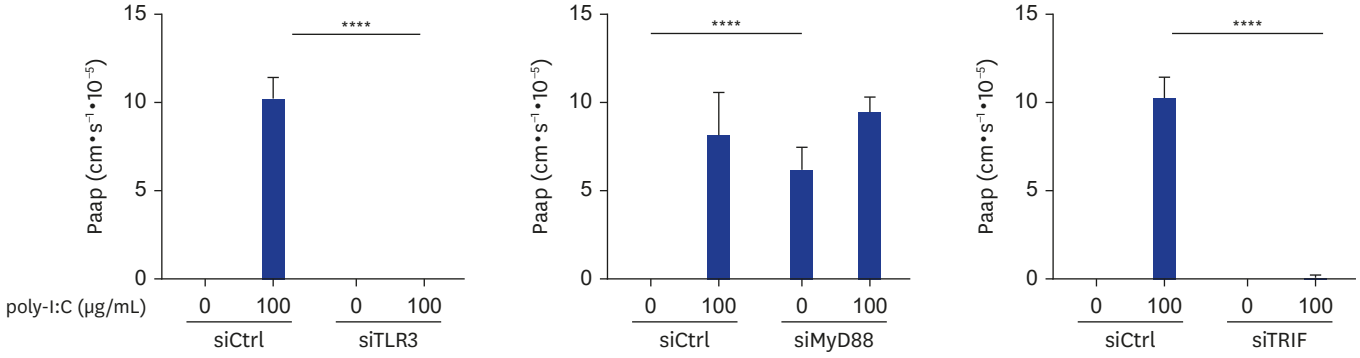

Fig. 2. Roles of Toll-like receptor 3 (TLR3), MyD88, and TRIF in poly-I:C-induced paracellular barrier impairment. 16HBE cells transfected with control siRNA (siCtrl) or TLR3-specific siRNA (siTLR3) were treated with poly-I:C and subjected to the fluorescein isothiocyanate (FITC)–dextran permeability assay. The vertical axis represents apparent permeability coefficients (Paap). Cells transfected with siCtrl- or MyD88-specific siRNA (siMyD88) were treated with poly-I:C and subjected to the FITC–dextran permeability assay (middle). Cells transfected with siCtrl- or TRIF-specific siRNA (siTRIF) were treated with poly-I:C and subjected to the FITC–dextran permeability assay (right). Data are mean + standard deviation. Significance was determined by unpaired t test. $^{****}p < 0.0001$.

activation in the epithelial barrier disruption (**Fig. 2**, left). It has been reported that the intracellular TLR3 signal transduction involves pathways mediated by MyD88 and TRIF as adapter molecules. Transfection with MyD88-specific siRNA increased dextran permeability in untreated 16HBE cells (**Fig. 2**, middle). The poly-I:C-induced increase in dextran permeability was suppressed by transfection with TRIF-specific siRNA (**Fig. 2**, right), but not MyD88-specific siRNA (**Fig. 2**, middle).

Next, we investigated the effect of poly-I:C on the expression of miR-155 in 16HBE cells. Poly-I:C treatment significantly increased the expression of miR-155 in 16HBE cells in a dose-dependent manner at 24 hours (**Fig. 3**). We investigated the roles of miR-155 in airway epithelial barrier integrity. To study the role of miR-155 in epithelial barrier integrity, we employed the transfection of a miR-155 mimicking RNA (miR-155 -M) sharing targets with endogenous miR-155, into 16HBE cells. Transfection of miR-155 -M showed the decrease of TER (**Fig. 4A**) and the increase of dextran permeability (**Fig. 4B**) on the basal levels in 16HBE cells. Furthermore, the transfection of an antisense miR-155 inhibitory RNA (miR-155 -I) into

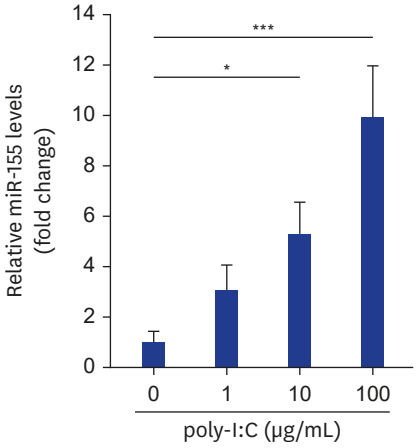

Fig. 3. Induction of microRNA-155 (miR-155) induced by poly-I:C. 16HBE cells were cultured on Transwell chamber with indicated dose of poly-I:C for 48 hours and then measured miR-155 by real-time polymerase chain reaction. Data are mean + standard deviation. Significance was determined by 1-way analysis of variance and Dunnett multiple comparisons test. $^{*}p < 0.05$. $^{***}p < 0.001$.

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16HBE cells significantly abrogated the decrease of TER (**Fig. 4C**) and the increase of dextran permeability (**Fig. 4D**) induced by poly-I:C on 16HBE cells.

Transfection of miR-155 -M and miR-155 -I did not affect to TLR3, MyD88, and TRIF expression (**Fig. 5A**). Transfection of both miR-155 -M or miR-155 -I did not affect to cell growth and viability (**Fig. 5B**).

Effect of miR-155 on the expression of adherence and tight junction molecules

To identify the mechanisms of miR-155 regulated-tight junction function, we investigated the change of mRNA expression profile of adherence junction and tight junction molecules by the treatment with dsRNA and miR-155 -M. Real-time PCR analysis revealed that miR-155 -M transfection significantly reduced the mRNA expression levels of CLDN3, CLDN5, CDLN16, and CLDN19, but not E-cadherin, Occludin, ZO-1, ZO-2, JAM-1, JAM-2, CLDN1, CLDN4, CLDN6, CLDN7. Interestingly, the change of adherence and tight junction molecules mRNA profile by

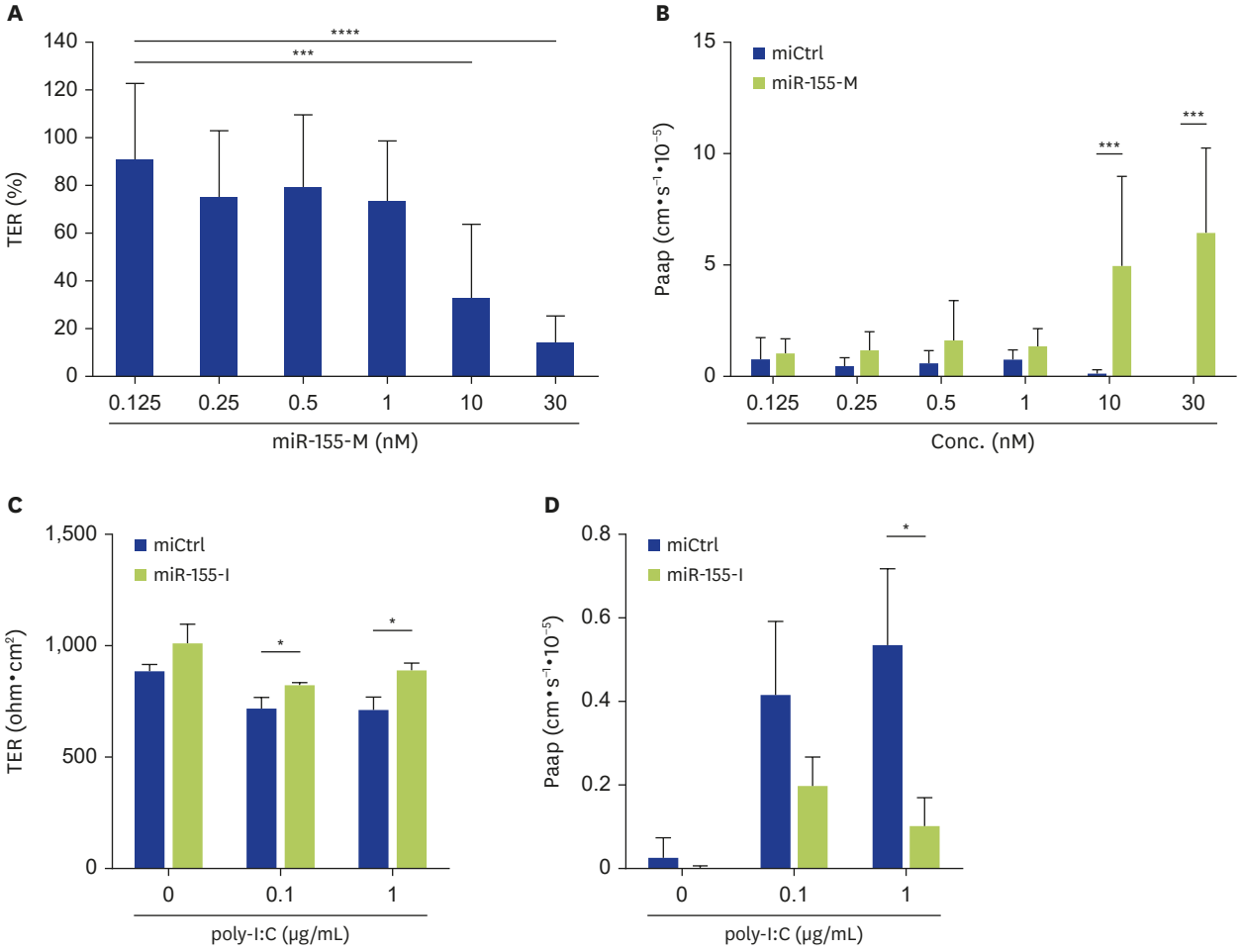

Fig. 4. Role of microRNA-155 (miR-155) on poly-I:C-induced barrier impairment. (A) 16HBE cells were transfected with indicated dose of control miRNA (miCtrl) and miR-155 mimics (miR-155 -M) and then transepithelial electrical resistance (TER) were measured after 48 hours. %TER represents percentage in reference to the control miRNA. (B) Cells were transfected with indicated dose of miCtrl or miR-155 -M and then subjected to the fluorescein isothiocyanate (FITC)–dextran permeability assay at 48 hours. The vertical axis represents apparent permeability coefficients (Paap). (C) Cells transfected with miCtrl or miR-155 -inhibitor (miR-155 -I) were treated with indicated dose of poly-I:C and measured TER at 48 hours. (D) MiCtrl or miR-155 -I cells were treated with poly-I:C and subjected to the FITC–dextran permeability assay at 48 hours. Data are mean + standard deviation. Significance was determined by 1-way analysis of variance and Dunnett multiple comparisons test (A) and unpaired t test (B–D). $^{*}p < 0.05$, $^{***}p < 0.001$.

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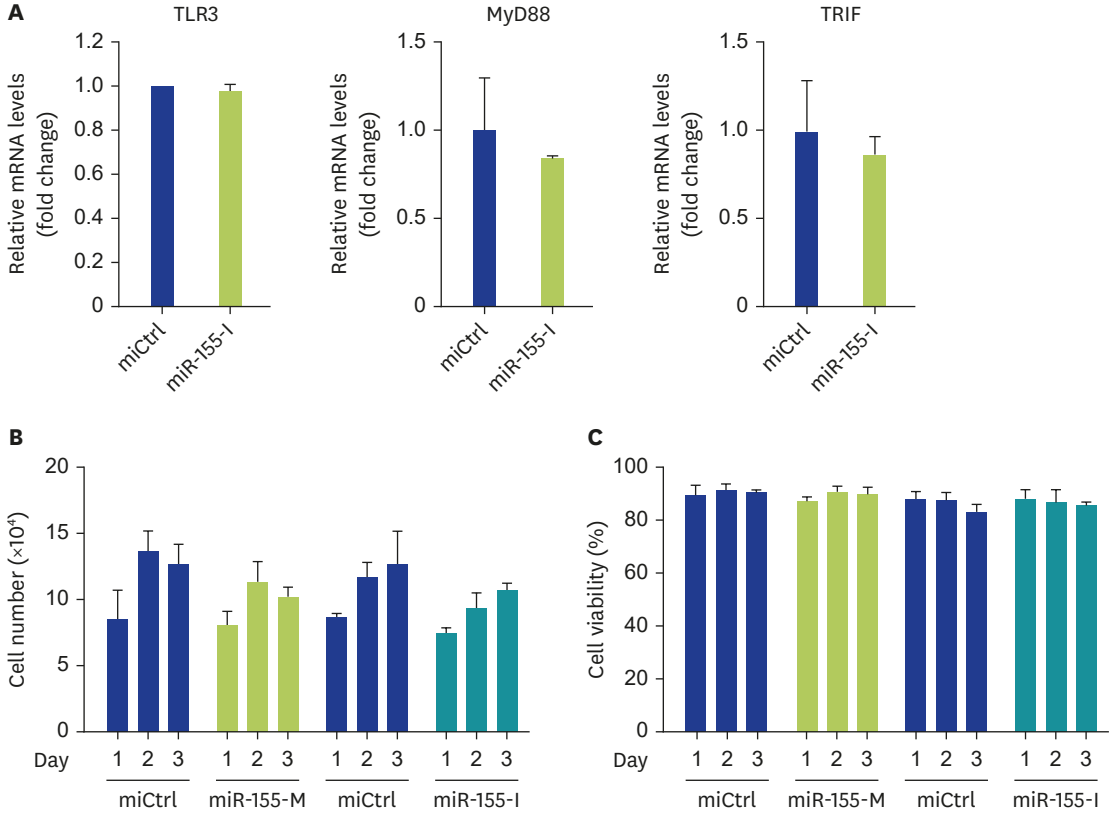

Fig. 5. Effects of microRNA-155 (miR-155) on Toll-like receptor 3 (TLR3)-signaling molecules and cell growth. (A) 16HBE cells transfected with control miRNA (miCtrl) and miR-155 -inhibitor (miR-155 -I) for 48 hours and then analyzed the expression of TLR3, MyD88, or TRIF mRNA by real-time polymerase chain reaction. (B) Cells were transfected with miCtrl, miR-155 -I, miR-155 -mimic (miR-155 -M), and after 48 hours cell number and viability were assessed. Data are mean + standard deviation. Significance was determined by unpaired *t* test (A) 1-way analysis of variance and Dunnett multiple comparisons test (B).

miR-155 -M overexpression was thoroughly similar with the change of their expressions after the treatment with dsRNA (**Fig. 6**).

DISCUSSION

The production of cytokines by airway epithelial cells and the disruption of the epithelial barrier upon infection with viruses are deeply related to the onset of asthma [26, 27]. Infection of the airways, particularly by RNA viruses such as rhinovirus and RSV, has been found to induce the production of cytokines and chemokines by airway epithelial cells via dsRNA. This occurs via a mechanism by which dsRNA activates TLR3 signaling. DsRNA is a ligand for TLR3, and induces the production of eosinophil migration factors such as RNATES via TLR3 [28-30]. DsRNA exacerbates the pathology of asthma by inducing the infiltration of the respiratory tract by eosinophils. In recent years, the possibility that dsRNA contributes to the pathology of asthma by disrupting the epithelial barrier via TLR3 has also been identified [10, 11, 31].

There are 2 pathways by which dsRNA activate TLR3 signaling: via the activation of nuclear factor-kappa B through the adapter molecule, MyD88, and via the activation of IRFs through another adapter molecule, TRIF [32]. In the previous studies, both pathways downstream of TLR3, which involve the adapter molecules MyD88 and TRIF, have been reported as important in the production of cytokines and chemokines due to dsRNA [33-36]. In our

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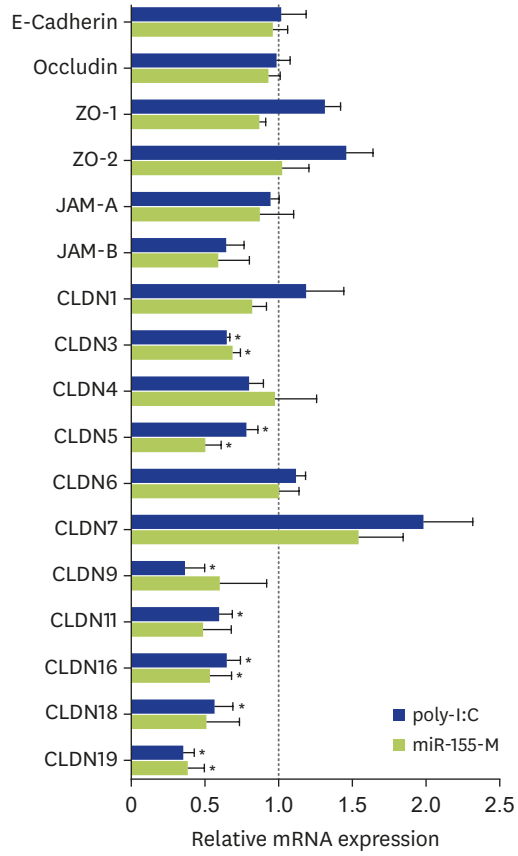

Fig. 6. Comparison of changes in expression levels of adherence junction and tight junction molecules between poly-I:C treatment and microRNA-155 (miR-155) transfection. Real-time polymerase chain reaction analysis of E-cadherin, Zo-1, -2 and -3 and claudin-1, -3, -4, -5, -6, -7, -9, -11, -16, -18 and -19 mRNA. Expression levels are shown as ratios of post-48-hour poly-I:C (blue) or miR-155 -M (green) treatment to pretreatment values. Data are mean + standard deviation. Significance was determined by unpaired *t* test. $^{*}p < 0.05$.

present study, *TLR3* gene knockdown inhibited the impairment of the epithelial barrier by dsRNA. This shows that TLR3 signaling is important in the impairment of the barrier function by dsRNA. Additionally, the knockdown of the *MyD88* gene caused exacerbation of dextran permeability in unstimulated 16HBE cells, and this suggests the possibility that MyD88 itself is important in the constitutive retention mechanism of airway epithelial barrier function. Moreover, because *TRIF* gene knockdown suppressed the induction of barrier disruption by dsRNA, it is clear that signal transmission via TRIF is important in TLR3-mediated disruption of the epithelial barrier. This evidence suggests that MyD88-mediated signaling and TRIF-mediated signaling each play distinct roles in the TLR3-mediated chemokine production and epithelial barrier disruption due to dsRNA.

To present, little has been clarified regarding the role of microRNAs in the TLR3 activation-mediated cellular response. In the present study, we focused on the role of miR-155 in the TLR3-mediated cellular response. This was because previous studies suggest that miR-155 contributes to the regulation of allergic airway inflammation by modulating TH2 responses and allergic airway inflammation [37, 38]. Previously, miR-155 has been found to play vital roles in the regulation of the immune response [39, 40]. MiR-155 activates the response of T cells to viral infection, and this activation is regulated by type I interferon signaling [20]. Furthermore, recent study has shown that miR-155 can directly regulate TLR3 expression [21].

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Meanwhile, miR-155 is related to the suppression of cytokines such as interleukin (IL)-22, IL-10, and interferon-γ [41].

In the present study, we investigated the role of miR-155 on the disruption of the epithelial barrier, which is induced by TLR3 signaling. Upon transfecting with the endogenous miR-155 -mimicking synthetic microRNA increases TER and dextran permeability in 16HBE cells in a dose-dependent manner. Additionally, transfection with miR-155 -inhibitor, which interferes with the function of endogenous miR-155, suppressed the disruption of the epithelial barrier due to dsRNA. From the above, this study has shown that miR-155, which is induced by dsRNA, plays an important role in the disruption of the epithelial barrier caused by dsRNA.

Through stimulation by dsRNA, we investigated the influence of the epithelial barrier on the expression of genes for the component molecules of the adherence junctions and tight junctions [10, 11]. Additionally, if impairment of the epithelial barrier by dsRNA is caused via the induction of endogenous miR-155 by dsRNA, then changes in the expression of genes for the components of the adherence junctions and tight junctions due to insertion of the miR-155 -M gene should be the same as the changes in expression due to dsRNA stimulation. This was another focus for our investigation. Transfection with miR-155 -M suppressed the expression of the claudin family molecules CLDN3, CLDN5, CLDN16, and CLDN19, but not CLDN1, CLDN4, CLDN6, CLDN7, CLDN9, CLDN11, or CLDN18. Claudin-3, -5, -16, -19 have been reported to seal paracellular permeability. In contrast, whether claudin-7 is an anion channel forming or sealing claudin is somewhat controversial [42]. Based on our results, miR-155 mainly decreased the expression of sealing claudins and may induce decreased paracellular permeability. Further research will be needed to address the role of individual claudins or the interaction between claudins upon miR-155-induced airway epithelial barrier dysfunction. Additionally, transfection with miR-155 -M did not suppress E-cadherin, Occludin, ZO-1, ZO-2, JAM-1, or JAM-2. In terms of the effects of stimulation due to dsRNA or transfection with miR-155 -M on the expression of genes, we compared differences between components of the adherence junctions and tight junctions and found the almost same changes in expression patterns. This result strongly suggests that there is a direct relationship between induction by miR-155 -M and the induction of epithelial barrier vulnerability by dsRNA.

To present, miR-155 has been reported to target the suppression of the expression of molecules such as TAB2, IKK-ε, and RIP, which are related to signal transduction in TLRs [13, 21]. It has also been reported to suppress the expression of TLR3 and MyD88. In this study, because MyD88 knockdown exacerbated the permeability of the epithelium, signaling through MyD88 was shown to be important to the epithelial barrier integrity of the respiratory epithelium. The control of the expression of molecules related to TLR3, which targets miR-155, may also be related to the disruption of the barrier by dsRNA. In this study, however, while it was not possible to clarify whether miR-155 directly suppressed the expression of the claudin family molecules, or whether the control of expression was via TLRs or related molecules, we intend to pursue this in future research.

Summarizing our results from above, the disruption of the airway epithelial cells by dsRNA occurs via TLR3/TRIF signaling. Gene suppression by dsRNA-induced miR-155 seems to suppress the function of epithelial tight junctions via the suppression of multiple claudin family molecules. This mechanism is deeply related to airway epithelial injury, which causes viral infections, particularly the onset of respiratory tract inflammation.

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