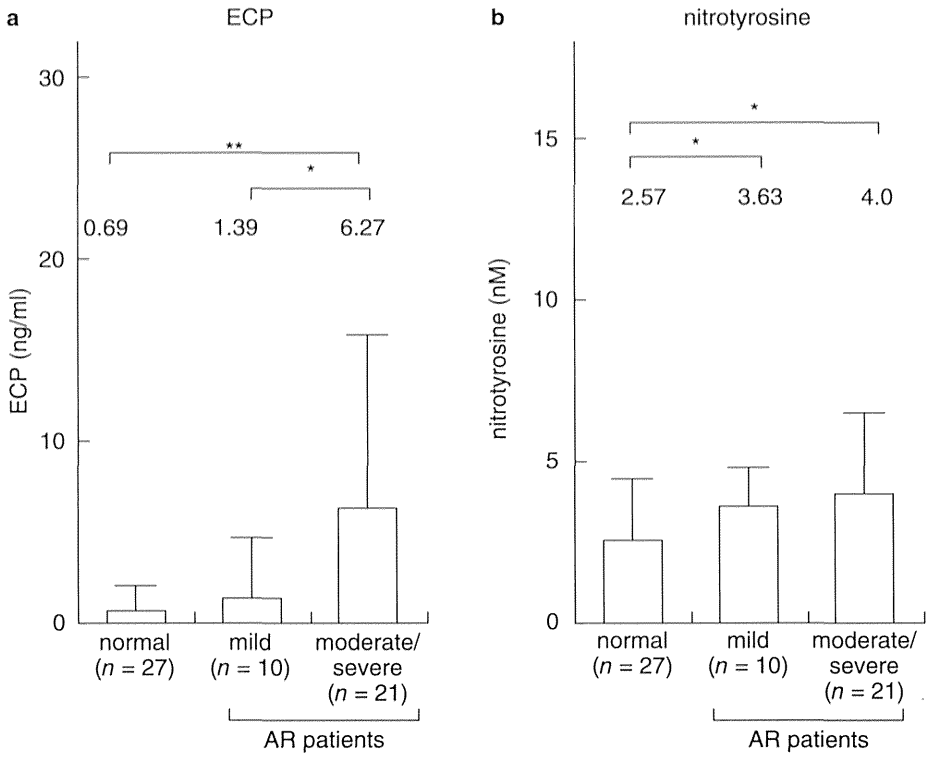

Fig. 5 Comparison of the levels of (**a**) ECP and (**b**) nitrotyrosine in the nasal brushing cells. Error bars = mean values and SD. $*p < 0.05$; $**p < 0.01$.

ELISA OF NASAL BRUSHING CELLS

The ELISA of nasal brushing cells revealed elevated concentrations of ECP derived from activated eosinophils and nitrotyrosine from oxidized NO metabolites in the allergic patients (Fig. 5). The mean ECP level in the moderate/severe group was significantly higher than that in the normal subjects. The mean NT levels in the two AR groups were both significantly higher than that in the normal group.

DISCUSSION

Allergic rhinitis has been thought to be associated with increased NO levels, mainly by the increased inducible nitric oxide synthase (iNOS) expression in the inferior turbinate mucosa.[5,13-15] Several authors have reported increased nasal NO levels in symptomatic AR patients.[12,16-20] Conversely, some studies detected no significant differences between AR patients and control subjects.[21-23] These seemingly conflicting results regarding NO levels may reflect the functional complexity of NO in the human nose. There is a great difference in background NO output between the upper and the lower airways. In the upper airways, there is a high background output, and thus an increase (e.g., allergic rhinitis) tends to be obscured, whereas a decrease (e.g., primary ciliary dyskinesia or chronic rhinosinusitis with nasal polyps) is usually easier to reveal.[8,24] Although NO production in the nasal mucosa of AR patients may be up-regulated, this increase could be counteracted by swelling of the mucosa and secretions that lead to impaired NO diffusion. In addition, the high background levels of NO from constitutive sources may blunt smaller increases in mucosal NO output. We hypothesize that nasal NO is a valid marker for allergic inflammation and that the measurement of local NO levels based on the anatomical features of the human nasal cavity might be useful in distinguishing AR patients from normal subjects. High nasal NO levels detected in the IT area in symptomatic AR patients can directly reflect the persistence of mucosal eosinophilic inflammation in the inferior turbinate.

In this study, we validated two different methods as recommended by the ATS to assess the NO level in the nasal cavity, i.e., nasal FeNO and nasal NO.[1] Nasal FeNO is considered to represent a fraction of endogenous NO with contaminated air passing through the nose at a relatively high flow rate.[7] The exhalation process may be influenced by changes in the airflow physics caused by inter-individual variations in the anatomical structure of the nasal cavity. As for nasal NO measurements, most of the previous reports concluded that nasal NO can be measured with fair reproducibility based on several different approaches proposed thus far.[17,25,26] Qian *et al.* found no significant difference in nasal NO with aspiration flows ranging from 2.2 to 6.2 L/min, which is in agreement with the recommended aspiration by the ATS.[27] In

the present study, the nasal NO measurement was conducted by direct sampling from one side of the nose with an airstream of a constant rate of 3 L/min. The subjects were instructed to breathe orally with their soft palates elevated and to block the communication to the nasal cavity. The method enables us to avoid possible contamination of exhaled NO derived from the lower respiratory tract, and the results obtained are immediately available for clinical assessment.

The results of the present study clearly indicate that increased NO levels near the surface of the inferior turbinate can be a simple and sensitive marker for the diagnosis of AR. In order to avoid an impact on the nasal NO results due to a sizable contribution from the paranasal sinuses, we found it advantageous to perform the monitoring in this designated area. The nasal FeNO levels in this study were significantly different between the AR patients and normal controls. This finding is compatible with our previous study, although the distribution of nasal FeNO levels in the present population was shifted slightly lower.[12] One possible explanation for this change is that the nasal FeNO measurements were carried out for the right and left nasal cavities separately in this study, whereas transnasal exhalation procedure was done bilaterally in our previous study. These results seem to be related to an intimate association between nasal NO in the IT area and nasal FeNO irrespective of allergic diathesis, suggesting that the aerodynamic distribution of NO levels in the human nose is a continuous trait.[28] However, the discriminative power of nasal NO in the IT area in the present study was higher than that obtained with conventional FeNO measurement techniques. We found that the severity of daily nasal symptoms in the AR patients was reflected as an increase in nasal NO levels in the IT area. Although our statistical analysis indicated close correlations between nasal FeNO levels and nasal NO levels in the IT area, some of the subjects had shown relatively higher nasal NO values in the IT area. The tendency was more pronounced in the moderate/severe AR patients. The reasons for this phenomenon are unclear, but several previous studies indicate the same tendency.[4,6] The exhalation process in the moderate/severe AR patients with hypertrophic inferior turbinates may be influenced by modification of the nasal airflow in narrowing pathways.

As for the difference in nasal NO between the right and left nasal cavities, Alexanderson *et al.* reported that in 331 normal and symptomatic subjects, the mean difference in nasal NO between the nostrils was 14 ppb and nearly 95% of the subjects had a difference of <45 ppb.[26] They also found that atopy was significantly associated with a high difference of nasal NO levels between the nostrils. These results are consistent with those of the present study. We also found that 95.6% (44/46) of the normal subjects, 93.3% (14/15) of the mild AR patients, and 68% (17/25) of the moderate/severe AR patients had a difference of <45 ppb in the IT area. These findings indicate that large differences in nasal NO between the cavities may predict the presence of not only atopic diathesis but also an ongoing inflammatory allergic reaction in the inferior turbinate. Another supplementary finding of this study is that the difference in nasal NO levels between the IT area and the MM area can also be used as a marker for AR diagnosis. The MM/IT ratio was significantly lower in the AR group, with the differences being more significant than the FeNO measurement. However, limited data are available for the interpretation of the MM/IT ratio in the present study, because we performed the nasal endoscopy only to evaluate the patency of the middle meatus. Further studies are necessary for the use of this parameter to evaluate various nasal diseases (including AR) in relation to inter-individual differences in anatomical characteristics of the nose.

Nasal NO levels in the MM area have been postulated to depend mainly on the amount of NO produced by the ciliated epithelium of the paranasal sinuses and the size of the paranasal sinus ostia.[29] In the present study, we found that most participants showed higher NO levels in the MM area than in the IT area (94.8% of the normal subjects and 74.7% of the AR patients). By emphasizing the MM area in this study, we assumed that the maxillary and anterior ethmoid sinuses are the dominant source of nasal NO detected in this area. Arnal *et al.* also found that symptomatic AR patients exhibited an acute increase in nasal NO after the administration of a topical vasoconstrictor, suggesting that acute changes in the widening of the ostio-meatal complex may affect the physiological mixing of paranasal sinus NO with that of the nasal cavity.[18] However, since standardized measurements of sinus NO have not yet been established, further studies are required to assess the relative importance of the volume-surface area of the individual sinus in relation to the NO transport to the nasal cavity, where it is commonly measured.

The increased NO in allergic rhinitis has several pathophysiologic consequences, including vasodilation, modification of sensory nerve endings, and accumulation of activated eosinophils. Nitrotyrosine is a stable downstream product of multiple pathways formed in the presence of excess NO production and oxidative stress by the modification of tyrosine residues.[30,31] In patients with severe asthma, the degree of airway eosinophilia can be predicted by a combination of FeNO levels, iNOS expression, and NT production.[32,33] The intranasal administration of eotaxin to AR patients also induced a significant increase in nasal NO with accompanying local eosinophil recruitment.[34] In the present study, the production of both ECP and NT was significantly up-regulated in the AR patients, associated with elevated nasal NO in the IT

area. These results are in accord with those of these previous studies, where the NO concentration was thought to reflect eosinophilic inflammation and autotoxic NO metabolisms.

The limitations of this study include its cross-sectional study design, without ability to see the effects of therapeutic modalities for each patient. Further studies are required to reinforce the usefulness of nasal NO measurement as an objective method for assessing the outcome of various anti-allergic therapies.

The on-line method for nasal FeNO and nasal NO measurements is highly reproducible and easy to perform for both the subject and the clinician. Increased NO levels near the IT surface can be sensitive markers for the diagnosis of allergic rhinitis, with the significance being more prominent than nasal FeNO. Relatively high nasal NO levels in the MM area also indicate the role of paranasal sinuses acting as a physiological NO reservoir in humans. We suggest that such advances in the standardized measurement techniques and established guidelines for standard values will exploit nasal NO for the diagnosis, treatment, and monitoring of relevant upper airway disorders.

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RESPIRATORY RESEARCH

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Pseudomonas aeruginosa elastase causes transient disruption of tight junctions and downregulation of PAR-2 in human nasal epithelial cells

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Abstract

Background: *Pseudomonas aeruginosa* causes chronic respiratory disease, and the elastase enzyme that it produces increases the permeability of airway epithelial cells owing to the disruption of tight junctions. *P. aeruginosa* is also implicated in prolonged chronic rhinosinusitis. However, the effects of *P. aeruginosa* elastase (PE) against the barrier formed by human nasal epithelial cells (HNECs) remain unknown.

Methods: To investigate the mechanisms involved in the disruption of tight junctions by PE in HNECs, primary cultures of HNECs transfected with human telomerase reverse transcriptase (hTERT-HNECs) were used. The hTERT-HNECs were pretreated with inhibitors of various signal transduction pathways, PKC, MAPK, p38MAPK, PI3K, JNK, NF-κB, EGF receptor, proteasome, COX1 and COX2 before treatment with PE. Some cells were pretreated with siRNA and agonist of protease activated receptor-2 (PAR-2) before treatment with PE. Expression and structures of tight junctions were determined by Western blotting, real-time PCR, immunostaining and freeze-fracture. Transepithelial electrical resistance (TER) was examined as the epithelial barrier function.

Results: PE treatment transiently disrupted the epithelial barrier and downregulated the transmembrane proteins claudin-1 and -4, occludin, and tricellulin, but not the scaffold PDZ-expression proteins ZO-1 and -2 and adherens junction proteins E-cadherin and β-catenin. The transient downregulation of tight junction proteins was controlled via distinct signal transduction pathways such as the PKC, MAPK, PI3K, p38 MAPK, JNK, COX-1 and -2, and NF-κB pathways. Furthermore, treatment with PE transiently decreased PAR-2 expression, which also regulated the expression of the tight junction proteins. Treatment with a PAR-2 agonist prevented the downregulation of the tight junction proteins after PE treatment in HNECs.

Conclusions: PE transiently disrupts tight junctions in HNECs and downregulates PAR-2. The transient disruption of tight junctions by PE might occur repeatedly during chronic rhinosinusitis.

Keywords: *Pseudomonas aeruginosa* elastase, Tight junctions, Barrier function, Human nasal epithelial cells, Signal transduction, PAR-2

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Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a virulent Gram-negative bacterium that causes aggressive infections in patients compromised by pre-existing respiratory disease such as cystic fibrosis and diffuse panbronchiolitis [1,2]. *P. aeruginosa* is also associated with prolonged chronic rhinosinusitis (CRS) [3].

P. aeruginosa secretes several virulence factors such as exotoxin A, exoenzyme S, pyocyanin, and elastase, which play an important role in pathogenesis [4,5]. *P. aeruginosa* elastase (PE) increases paracellular permeability in lung epithelial cells via mechanisms involving tight junction disruption and cytoskeletal reorganization [6]. PE affects epithelial cells via multiple mediators of signaling including activation of PKC, EGFR, ERK1/2, NF-κB, urokinase/uPAR, and protease activated receptor-2 (PAR-2) [1,2,7-11]. PKC signaling is involved in PE-induced epithelial barrier disruption via tight junction translocation and cytoskeletal reorganization in the human bronchial adenocarcinoma cell line Calu-3 [2].

PE disables PAR-2 in respiratory epithelial cells [1]. Protease-activated receptors (PARs) are G protein-coupled receptors with seven transmembrane domains, which are cleaved at an activation site within the N-terminal exodomain by a variety of proteases [1]. Four PARs (PAR-1, -2, -3, and -4) have been identified and are widely expressed by cells in blood vessels, connective tissue, leukocytes, epithelium, and many airway cells [12]. PAR-2 is expressed in airway epithelium, and its activation initiates multiple effects including enhanced airway inflammation and reactivity [13]. Upregulation of PAR-2 is observed in the respiratory epithelium of patients with asthma and chronic rhinosinusitis [14,15]. PAR-2 activation also affects the airway epithelial barrier [16]. However, details of the mechanistic effects of PE against the epithelial barrier via PAR-2 remain unknown.

Airway epithelium of human nasal mucosa acts as a physical barrier that protects against inhaled substances and pathogens because of its tight junctions, the most apical intercellular junctions [17-19]. Tight junctions are formed by not only the integral membrane proteins claudins, occludin, tricellulin, and junctional adhesion molecules (JAMs), but also by many peripheral membrane proteins, including the scaffold PDZ-expression proteins zonula occludens (ZO) and non-PDZ-expressing proteins [20-23]. We previously reported that, in HNECs *in vivo*, the tight junction molecules occludin, tricellulin, JAM-A, claudin-1, -4, -7, -8, -12, -13, and -14, and ZO-1 and -2 were detected together with well-developed tight junction strands [17,24,25]. The tight junctions and the well-developed barrier function in primary *in vitro* cultures of HNECs transfected with human telomerase reverse transcriptase (hTERT-HNECs) were very similar to those observed in HNECs *in vivo* [24-27]. Furthermore, in the *in vitro* HNECs, tight junction molecules and barrier function are upregulated by various stimuli via distinct signal transduction pathways [25].

In the present study, to investigate the effects of elastase on the tight junction barrier of HNECs, hTERT-HNECs were treated with PE. Treatment with PE transiently disrupted the epithelial barrier and downregulated the transmembrane proteins claudin-1 and -4, occludin, and tricellulin but not the scaffold PDZ-expression proteins ZO-1 and -2 and adherens junction proteins E-cadherin and β-catenin. Downregulation of tight junction proteins because of PE treatment was mediated via distinct signal transduction pathways. Furthermore, treatment with PE transiently decreased PAR-2 expression, which partially regulated the expression of the tight junction proteins. A PAR-2 agonist prevented the downregulation of tight junction proteins after PE treatment in HNECs.

Materials and methods

Reagents

A pan-PKC inhibitor (GF109203X), MEK1/2 inhibitor (U0126), p38 MAPK inhibitor (SB203580), and PI3K inhibitor (LY294002) were purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). JNK inhibitor (SP600125) and NF-κB inhibitor (IMD-0354) were purchased from Sigma-Aldrich (St. Louis, MO). Epidermal growth factor (EGF) receptor inhibitor (AG1478) was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). Proteasome inhibitor (MG132), the COX1 inhibitor (FR122047), and COX2 inhibitor were purchased from Calbiochem Novabiochem Corporation (San Diego, CA). *Pseudomonas aeruginosa* elastase and neutrophil elastase were purchased from Elastin Products Company, Inc. (Owensville, USA). Protease activated receptor 2 (PAR-2) agonist (*SLIGKV*-NH2) was purchased from R&D Systems, Inc. (Minneapolis, MN). Alexa 488 (green)- and Alexa 594 (red)-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Invitrogen. HRP-conjugated polyclonal goat anti-rabbit immunoglobulins were purchased from Dako A/S (Glostrup, Denmark). The ECL Western blotting 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 and the KKR Sapporo Medical Center Tonan Hospital. Informed consent was obtained from all patients and this study was approved by the ethical 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 human nasal epithelial cells were as reported previously [26]. 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 [26]. The cells were plated on 35-mm or 60-mm culture dishes (Corning Glass Works, Corning, NY, USA), which were coated with rat tail collagen (500 μg of dried tendon/ml 0.1% acetic acid). The cells were cultured in serum-free bronchial epithelial cell basal medium (BEBM, Lonza Walkersville, Inc.; Walkersville, MD, USA) 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 humidifier, 5% CO2:95% air incubator at 37°C. This experiment used cells in the second and third passage. The hTERT-HNECs were treated with 0.1 U (a unit of 3.83 μg/ml) *Pseudomonas aeruginosa* elastase (PE) or 0.01 U (a unit of 1.25 μg/ml) neutrophil elastase (NE). Some cells were pretreated with or without inhibitors of pan-PKC, MEK1/2, p38MAPK, PI3K, JNK, NF-κB, EGF receptor, proteasome, COX1, COX2 and PAR-2 agonist 30 min before treatment with 0.1 U PE. The concentrations of the various inhibitors were used following our previous reports [28,29].

Transfection with small interfering RNA (siRNA)

siRNA duplex oligonucleotides against human PAR 2 (sc-36188) were synthesized by Santa Cruz Biotechnology, inc. (Santa Cruz, CA). The hTERT-transfected HNECs at 24 h after plating were transfected with 100 nM siRNA of PAR-2 using Lipofectamine™ RNAiMAX Reagent (Invitrogen). Some cells were treated with 0.1 U PE after transfection with 100 nM siRNS of PAR-2 for 48 h.

RNA isolation, RT-PCR, and real-time RT-PCR analysis

Total RNA was extracted and purified from hTERT-transfected HNECs using TRIzol reagent (Invitrogen). One microgram of total RNA was reverse transcribed into cDNA using a mixture of oligo(dT) and Superscript II RTase using 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 MgCl2, 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. The final elongation step was 7 min at 72°C. Nine microliters of the 20 μl total PCR reaction was analyzed by gel electrophoresis with 2% agarose after staining with ethidium bromide. 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, occludin, tricellulin, claudin-1, -4, -7, PAR-1, and -2 are indicated in Table 1.

Real-time PCR detection was performed using a TaqMan Gene Expression Assay kit with a StepOnePlus™ real-time PCR system (Applied Biosystems; Foster City, CA, USA). The amount of 18S ribosomal RNA (rRNA) (Hs99999901) in each sample was used to standardize the quantity of the following mRNAs: tricellulin (Hs00930631), claudin-1 (Hs00221623), claudin-4 (Hs00533616), claudin-7 (Hs00154575), occludin (Hs00170162).

The relative mRNA expression levels between the control and treated samples were calculated by the difference of the threshold cycle (comparative C_T [$\Delta\Delta C_T$] method) and presented as the average of triplicate experiments with a 95% confidence interval.

Western blot analysis

The hTERT-transfected HNECs were scraped from a 60 mm dish containing 300 μl of buffer (1 mM NaHCO3 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, USA). Aliquots of 15 μl of protein/lane

Table 1 Primers for RT-PCR

Gene	Forward primer	Reverse primer	Product size (bp)
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452
PAR-1	GGATATTTGACCAGCTCCTGG	AGATGGCCAGACAAGTGAAGG	400
PAR-2	CTGCATCTGTCCTCACTGGAA	ATTGCCAGGGAGATGCCAATG	400
Claudin-1	GCTGCTGGGTTTCATCCTG	CACATAGTCTTTCCCACTAGAAG	619
Claudin-4	AGCCTTCCAGGTCCTCAACT	AGCAGCGAGTAGAAG	249
Claudin-7	AGGCATAATTTTCATCGTGG	GAGTTGGACTTAGGGTAAGAGCG	252
Occludin	TCAGGGAATATCCACCTATCACTTCAG	CATCAGCAGCAGCCATGTACTCTTCAC	189
Tricellulin	AGGCAGCTCGGAGACATAGA	TCACAGGGTATTTTGCCACA	200

for each sample were separated by electrophoresis in 5–20% SDS polyacrylamide gels (Daiichi Pure Chemicals Co.; Tokyo, 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 anti-claudin-1, -4, and -7 anti-occludin, anti-tricellulin, anti-ZO-1, and -2, anti-β-catenin, anti-E-cadherin, anti-actin and anti-PAR-2 antibodies (Table 2) 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 blotting system.

Immunocytochemical staining

The hTERT-transfected 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 anti-occludin and anti-claudin-1 antibodies (Table 2) 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).

Table 2 Antibodies

Antibody	Type	Dilution		Company
		IS	WB	
claudin-1	pAb	1:100	1:1000	Zymed Laboratories (San Francisco, CA)
claudin-4	pAb		1:1000	Zymed Laboratories (San Francisco, CA)
claudin-7	pAb		1:1000	Zymed Laboratories (San Francisco, CA)
occludin	pAb	1:100	1:1000	Zymed Laboratories (San Francisco, CA)
tricellulin	pAb		1:1000	Zymed Laboratories (San Francisco, CA)
ZO-1	pAb		1:1000	Zymed Laboratories (San Francisco, CA)
ZO-2	pAb		1:1000	Zymed Laboratories (San Francisco, CA)
actin	pAb		1:1000	Sigma-Aldrich (St. Louis, MO)
E-cadherin	mAb (36)		1:2000	BD Biosciences (San Jose, CA)
β-catenin	pAb		1:1000	Zymed Laboratories (San Francisco, CA)

pAb; rabbit polyclonal antibody, mAb; mouse monoclonal antibody, IS; immunostaining, WB; Western blotting.

Freeze-fracture analysis

For the freeze-fracture technique, the cells were immersed in 40% glycerin solution after fixation in 2.5% glutaraldehyde/0.1 M phosphate-buffered saline (PBS). The specimens were fractured at −150°C to −160°C in a JFD-7000 freeze-fracture device (JEOL, Ltd., Tokyo, Japan) and replicated by deposition of platinum/carbon from an electron beam gun positioned at a 45° angle followed by carbon applied from overhead. Replicas were examined at 100kV with a JEM transmission electron microscope (JEOL Ltd., Tokyo, Japan).

Continuous online measurements of transepithelial electrical resistance (TER)

Cells were cultured to confluence on the inner chambers of 12-mm Transwell 0.4-μm pore-size filters (Corning Life Science). Transepithelial electrical resistance (TER) was monitored using a cellZscope (nanoAnalytics, Germany), a computer controlled automated multi-well device (12 wells). The values are expressed in standard units of ohms per square centimeter and presented as the mean ± SD of triplicate experiments.

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. Results are given as means ± SEM. Differences between groups were tested by ANOVA followed by a *post-hoc* test and an unpaired two-tailed Student's *t* test and considered to be significant when $p < 0.05$.

Results

P. aeruginosa elastase (PE) transiently reduces the expression of transmembrane proteins in the tight junctions in HNECs

To investigate whether *Pseudomonas aeruginosa* elastase (PE) affects the protein and mRNAs expression of tight junction and adherens junction molecules in HNECs, hTERT-HNECs were treated with 0.1 U PE for 30 min, 1 h, 2 h, and 4 h. Western blots showed that claudin-1, -4, and tricellulin protein levels decreased at 30 min but were restored at 2 h, whereas occludin protein was transiently reduced at 1 h (Figure 1). No changes in claudin-7, ZO-1, ZO-2, E-cadherin, and β-catenin protein levels were observed post-treatment (Figure 1). The mRNA levels of claudin-1, -4, occludin, and tricellulin decreased at 30 min and were restored at 2 h, whereas claudin-7 mRNA level was slightly reduced from 30 min until 2 h (Figure 2).

Furthermore, we investigated the effects of neutrophil elastase (NE) on the expression of tight junction and adherens junction molecules in HNECs, to compare the effects of PE. When hTERT-HNECs were treated with

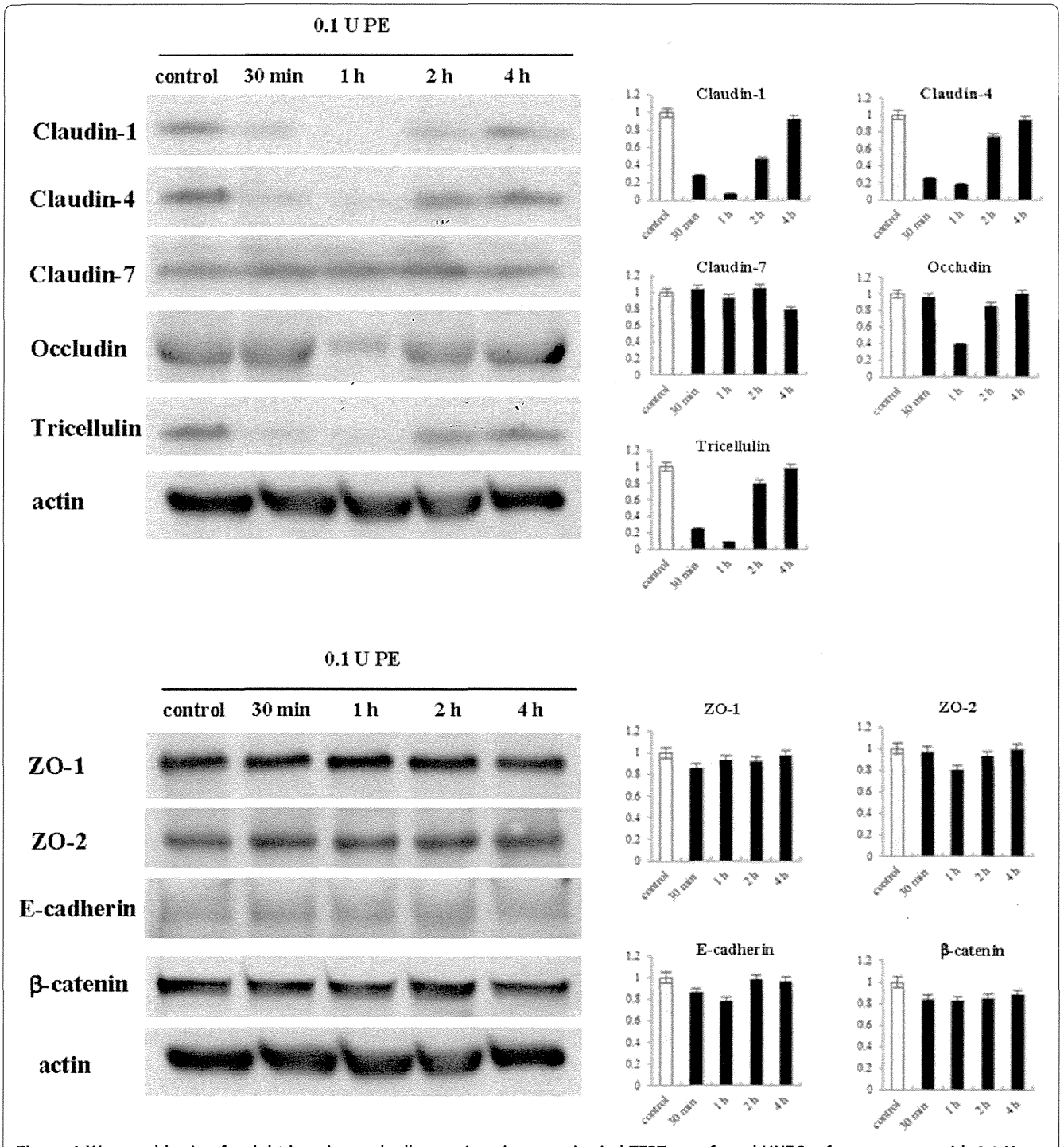

Figure 1 Western blotting for tight junction and adherens junction proteins in hTERT-transfected HNECs after treatment with 0.1 U *Pseudomonas aeruginosa* elastase. The corresponding expression levels are shown as bar graphs. PE: *Pseudomonas aeruginosa* elastase.

0.01 U NE for 30 min, 1 h, 2 h, and 4 h, claudin-1, occludin, and tricellulin protein levels were transiently reduced 30 min post-treatment with NE, while no changes in claudin-4, -7, ZO-1, ZO-2, E-cadherin and β-catenin protein levels were observed (Additional file 1). NE did not affect mRNA levels of claudin-1, occludin, and tricellulin (Additional file 1).

PE affects the distribution of transmembrane tight junction proteins and the formation of tight junction strands in HNECs

We investigated changes in the distribution of tight junction proteins in hTERT-HNECs 1 h, 2 h, and 4 h post-treatment with 0.1 U PE. In the control cells (0 h), strong immunoreactivity of occludin and claudin-1 was

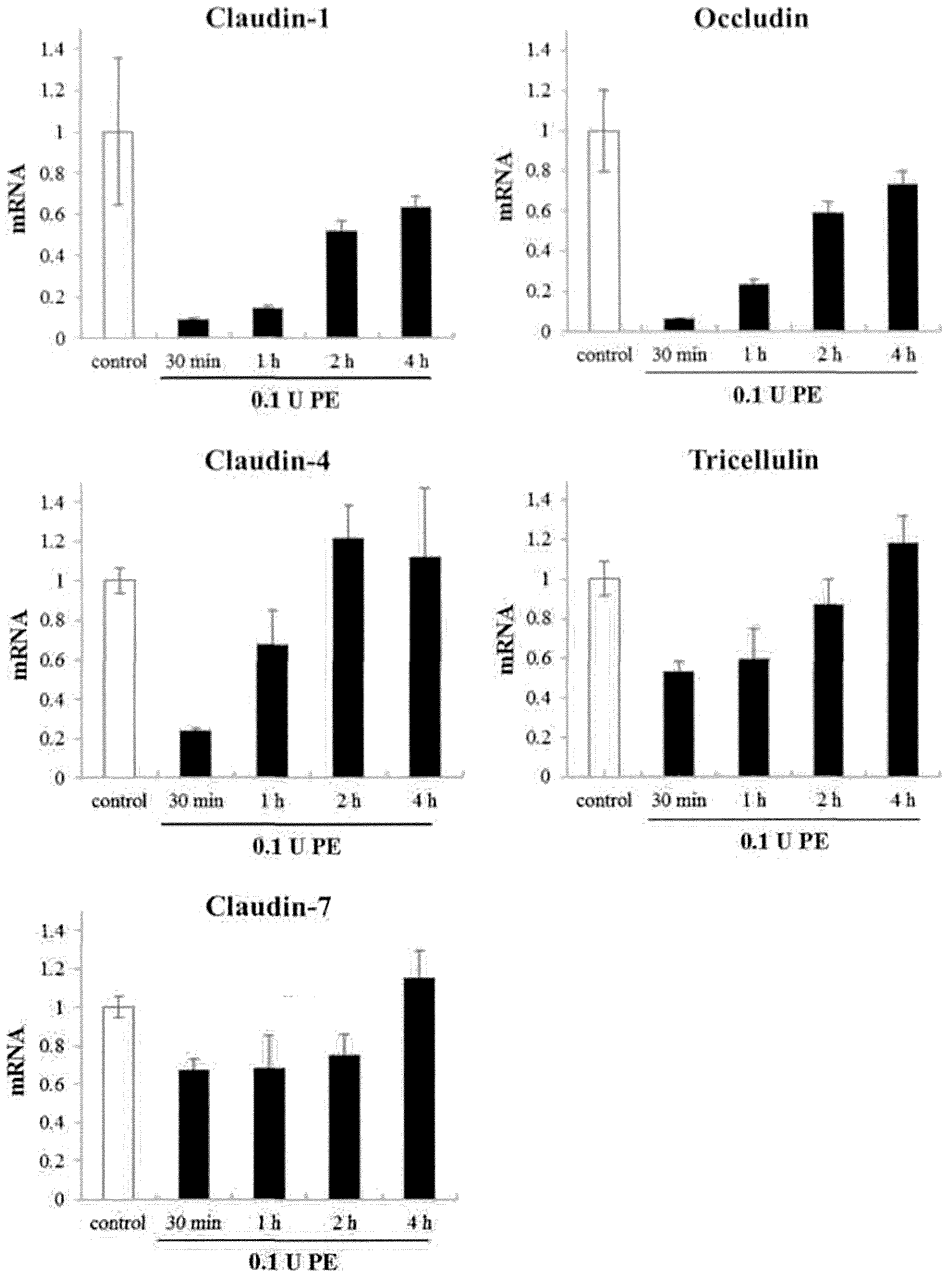

Figure 2 Real-time PCR analysis of claudin-1, -4, and -7, occludin, and tricellulin mRNA in hTERT-transfected HNECs after treatment with 0.1 U *Pseudomonas aeruginosa* elastase. PE: *Pseudomonas aeruginosa* elastase.

observed at the membranes (Figure 3A). The immunoreactivities of occludin and claudin-1, in part, disappeared at the borders of some cells 1 h after treatment with PE and were then recovered 2 h post-treatment (Figure 3A).

Furthermore, we performed freeze-fracture analysis to investigate changes in the tight junction strands in hTERT-HNECs 1 h after treatment with 0.1 U of PE. In the control cells, a network composed of several continuous tight junction strands was observed (Figure 3B). The cells treated with PE exhibited a reduced number of tight junction strands, which were partially disrupted (Figure 3B).

PE transiently reduces the tight junction barrier function of HNECs

To investigate the effects of PE on the tight junction barrier function of HNECs, hTERT-HNECs were treated with 0.1 U PE and then examined with continuous online measurements of transepithelial electric resistance (TER) by using a cellZscope. As shown in Figure 4, the TER was continuously decreased from 20 min to 120 min

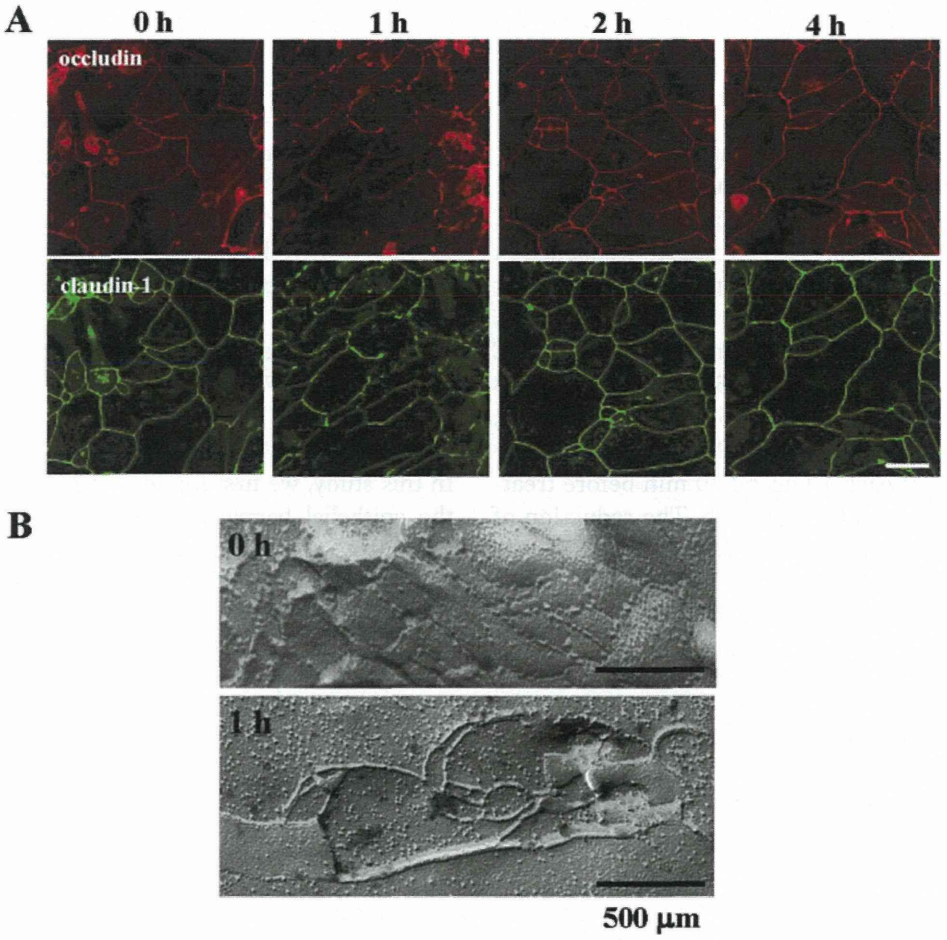

Figure 3 Immunocytochemical and transmission electron microscopic findings. (A) Immunocytochemical staining of occludin and claudin-1 and **(B)** Freeze-Fracture replicas in hTERT-transfected HNECs after treatment with 0.1 U *Pseudomonas aeruginosa* elastase. Bars: A=20 μm, B=500 μm.

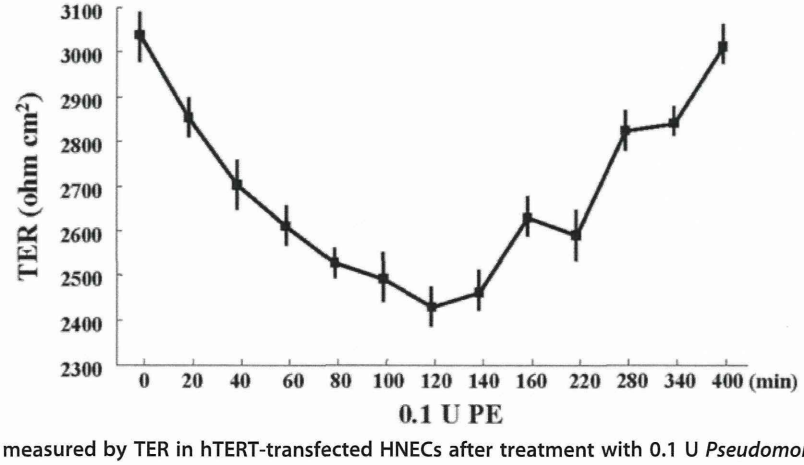

Figure 4 Barrier function measured by TER in hTERT-transfected HNECs after treatment with 0.1 U *Pseudomonas aeruginosa* elastase. PE: *Pseudomonas aeruginosa* elastase.

after PE-treatment, and continuously increased from 140 min to 400 min, recovering to the level prior to PE-treatment (Figure 4).

The reduction of transmembrane tight junction proteins by PE is regulated via distinct signaling pathway

It is reported that PE disrupts tight junctions via a PKC pathway (2). To investigate which signal transduction pathways affected the reduction of transmembrane tight junction proteins in HNECs after treatment with PE, hTERT-HNECs were pretreated with inhibitors of pan-PKC (GF109203X), MEK1/2 (U0126), PI3K (LY294002), p38 MAPK (SB203580), JNK (SP600125), EGFR (AG1478), COX1 (FR122047), COX2, NF-κB (IMD-0354), and Proteasome (MG132) at each 10 μg/ml 30 min before treatment with 0.1 U PE for 30 min or 1 h. The reduction of claudin-1 and occludin at 1 h after treatment with PE was prevented by GF109203X, U0126, LY294002, SP600125, inhibitors of COX1 and COX2, IMD-0354 and MG132 (Figure 5). The reduction of tricellulin at 1 h after treatment with PE was prevented by GF109203X, U0126, LY294002, and IMD-0354 (Figure 5). GF109203X, U0126, LY294002, SB203580, SP600125, and inhibitors of COX1 and COX2 inhibited claudin-4 reduction 30 min post PE treatment (Figure 5). No change of all tight junction proteins was observed at the concentrations of the various inhibitors without PE (Additional file 2).

PE reduces PAR-2 expression in HNECs

PE disables PAR-2 in airway epithelial cells A549 and 16 HBE cells (1). To investigate whether PE affects PAR-2 expression in HNECs, mRNA and protein in hTERT-HNECs 30 min, 1 h, 2 h, and 4 h after treatment with 0.1 U PE were examined by RT-PCR and Western blotting. PAR-2 but not PAR-1 mRNA was markedly decreased at 30 min and was restored at 2 h (Figure 6A). PAR-2 protein was transiently reduced 1 h after treatment (Figure 6B).

Knockdown of PAR-2 downregulates transmembrane tight junction proteins in HNECs with or without treatment with PE

We investigated whether PAR-2 expression affects transmembrane tight junction proteins in HNECs with or without treatment with PE. Occludin and claudin-1 mRNA and protein levels in hTERT-HNECs without PE-treatment were reduced by the knockdown of PAR-2 using siRNA (Figure 6C and D). The occludin and claudin-1 protein levels in hTERT-HNECs 1 h post treatment with 0.1 U PE decreased after the knockdown of PAR-2 (Figure 6E).

The downregulation of transmembrane tight junction proteins by PE treatment is prevented by PAR-2 agonist

We investigated whether PAR-2 agonist prevents the reduction of transmembrane tight junction proteins caused by PE treatment in HNECs. When hTERT-HNECs were pretreated with 10–200 μM PAR-2 agonist 30 min before treatment with 0.1 U PE for 1 h, disruption of occludin and claudin-1 at the membranes after PE treatment was prevented in cells that were treated with PAR-2 agonist concentrations of 100 μM or more (Figure 7A). The downregulation of occludin and claudin-1 mRNAs by PE was prevented by treatment with 100 μM of PAR-2 agonist (Figure 7B).

Discussion

In this study, we first found that PE transiently disrupted the epithelial barrier of HNECs by the downregulation of transmembrane tight junction proteins via distinct signal transduction pathways. Furthermore, PE decreased PAR-2 expression, which plays a crucial role in the maintenance of tight junction proteins in HNECs.

PE increases paracellular permeability in lung epithelial cells by causing tight junction disruption and cytoskeletal reorganization [6]. Furthermore, PE decreases epithelial barrier function in a time-dependent manner in the human bronchial adenocarcinoma cell line Calu-3 by reduced localization of occludin and ZO-1 in the membrane fraction [2]. In these airway epithelial cells, the barrier function is not recovered after treatment with PE. In the present study, treatment with PE transiently decreased the epithelial barrier of HNECs together with downregulation of the transmembrane proteins claudin-1 and -4, occludin, and tricellulin but not the scaffold PDZ-expression proteins ZO-1 and -2 and adherens junction proteins E-cadherin and β-catenin. Furthermore, reduced localization of occludin and claudin-1 and the disruption of tight junction structure were observed following PE treatment. Nevertheless the expression of claudin-1 and occludin by treatment with PE was markedly reduced at the level of mRNA and protein compared to the control, the immunostaining of these two proteins did not represent the dramatic reduction. In the present study using HNECs, it is possible that PE may strongly affect the synthesis of the tight junction proteins rather than the localization, although the detailed mechanisms are unclear. Treatment with NE also transiently decreased claudin-1, occludin, and tricellulin protein levels in HNECs. The sensitivity to PE in HNECs and other airway epithelial cells is different. PE, as a thermolysin-like metalloproteinase [30], may more strongly degrade the extracellular loops of transmembrane proteins in HNECs than other airway epithelial cells.

The tight junction proteins are regulated by various cytokines and growth factors via distinct signal transduction

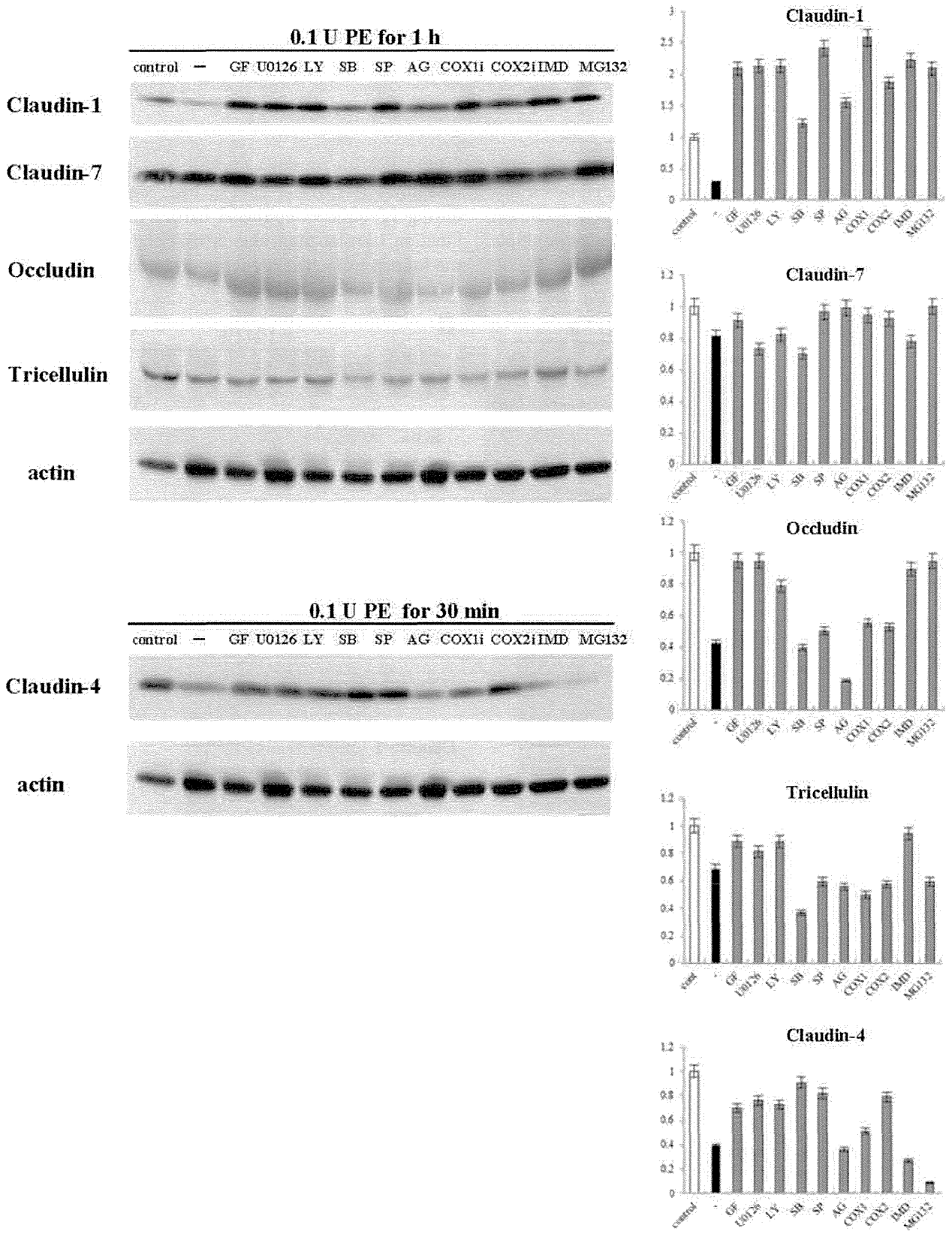

Figure 5 (See legend on next page.)

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Figure 5 Western blotting analysis. Western blotting for tight junction proteins in hTERT-transfected HNECs pretreatment with pan-PKC inhibitor (GF109203X), MEK1/2 inhibitor (U0126), PI3K inhibitor (LY294002), p38 MAPK inhibitor (SB203580), JNK inhibitor (SP600125), epidermal growth factor (EGF) receptor inhibitor (AG1478), COX1 inhibitor (FR122047), and COX2 inhibitor, NF-κB inhibitor (IMD-0354), and Proteasome inhibitor (MG132) before treatment with 0.1 U *Pseudomonas aeruginosa* elastase for 30 min or 1 h. The corresponding expression levels are shown as bar graphs. PE: *Pseudomonas aeruginosa* elastase.

pathways [23,31]. In HNECs *in vitro*, tight junction proteins and the barrier function are also regulated by various stimuli via distinct signal transduction pathways [25]. On the other hand, PE affects the epithelial cells via multiple mediators of signaling, including activation of PKC, EGFR, Erk1/2, NF-κB, urokinase/uPAR and protease activated receptor-2 (PAR-2) [1,2,7-11]. PKC signaling is involved during PE-induced epithelial barrier disruption via tight junction translocation and cytoskeletal reorganization in the human bronchial adenocarcinoma cell line Calu-3 [2].

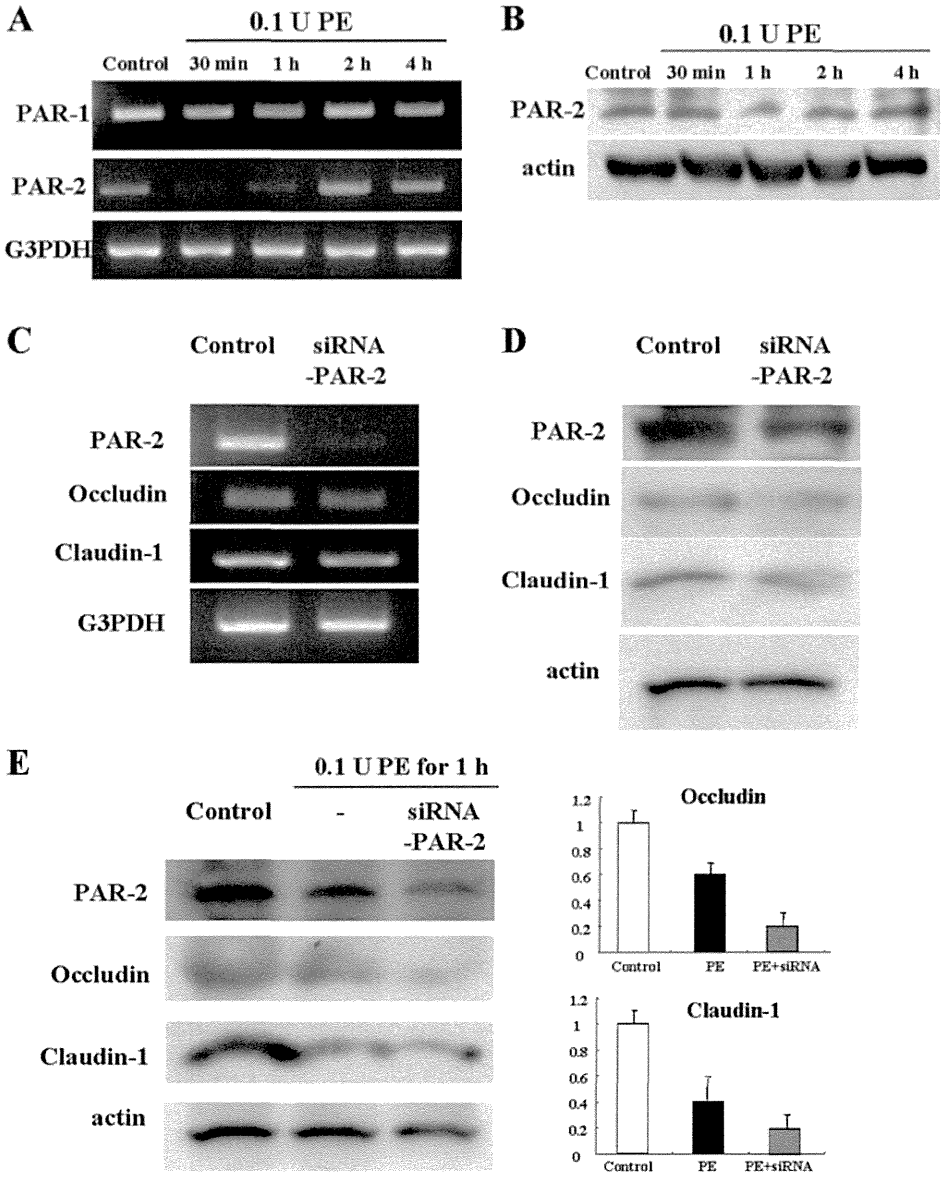

Figure 6 RT-PCR and Western blotting analyses. (A) RT-PCR for PAR-1 and -2 and **(B)** Western blotting for PAR-2 in hTERT-transfected HNECs after treatment with 0.1 U *Pseudomonas aeruginosa* elastase. **(C)** RT-PCR and **(D)** Western blotting for PAR-2, occludin, and claudin-1 in hTERT-transfected HNECs after treatment with siRNA of PAR-2 for 48 h. **(E)** Western blotting for PAR-2, occludin, and claudin-1 in hTERT-transfected HNECs pretreated with PAR-2 siRNA before treatment with 0.1 U *Pseudomonas aeruginosa* elastase for 1 h. The corresponding expression levels are shown as bar graphs. PE: *Pseudomonas aeruginosa* elastase.

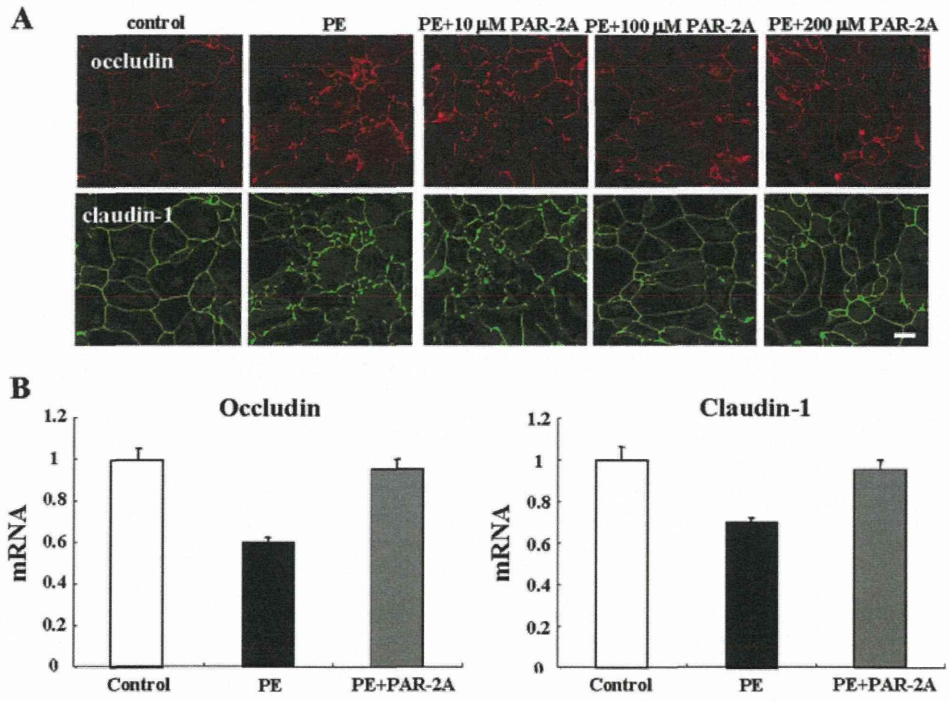

Figure 7 Immunocytochemistrical finding and Real-time PCR analysis. (A) Immunocytochemistrical staining for occludin and claudin-1 and occludin in hTERT-transfected HNECs pretreated with 10–200 μM PAR-2 agonist before treatment with 0.1 U *Pseudomonas aeruginosa* elastase for 1 h. Bar: 10 μm. **(B)** Real-time PCR for occludin and claudin-1 mRNAs in hTERT-transfected HNECs pretreated with 100 μM PAR-2 agonist before treatment with 0.1 U *Pseudomonas aeruginosa* elastase for 1 h. PE: *Pseudomonas aeruginosa* elastase. PAR-2A: PAR-2 agonist.

In the present study of HNECs, the transient downregulation of the transmembrane tight junction proteins by treatment with PE was controlled via distinct signal transduction pathways such as PKC, MEK1/2, PI3K, p38 MAPK, JNK, COX-1, -2 and NF-κB. Furthermore, there are, in part, the different signal pathways among downregulation of the tight junction proteins by PE. Treatment with PE transiently downregulated mRNAs of the tight junction molecules in HNECs. These data suggest that PE rapidly induces the activation of multiple signaling mediators in HNECs and indirectly affects the synthesis of transmembrane tight junction proteins via distinct signaling pathways.

PE disables PAR-2 in A549 airway epithelial cells and in 16 HBE cells [1]. The activation of PAR-2 initiates multiple effects including enhanced airway inflammation and reactivity [13]. PAR-2 also affects the airway epithelial barrier [16]. In the present study, PE transiently reduced PAR-2 at mRNA and protein level in HNECs. Knockdown of PAR-2 using siRNA resulted in the downregulation of occludin and claudin-1 at the mRNA and protein levels. Furthermore, the knockdown of PAR-2 greatly enhanced the downregulation of occludin and claudin-1 by treatment with PE. It is thought that PAR-2 may play a crucial role in maintenance of tight junctions in HNECs. These data indicate that PE affects expression of tight junction proteins via PAR-2 in HNECs. We investigated whether PAR-2 agonist prevents the reduction of transmembrane tight junction proteins by treatment with PE in HNECs. Treatment with more than 100 μM PAR-2 agonist could prevent delocalization of occludin and claudin-1 and downregulation of the mRNAs. However, in the present study, the knockdown of PAR-2 with siRNA did not affect the barrier function in the control HNECs (data not shown). Furthermore, when we measured TER in HNECs pretreated with a PAR-2 agonist before treatment with PE, a PAR-2 agonist did not protect the disruption of barrier function by PE (data not shown). These suggest that PAR-2 in part regulates the expression of tight junction proteins but not the barrier function in HNECs.

In conclusion, PE transiently disrupts tight junctions in HNECs through multiple effects: direct degradation, distinct signal transduction, and downregulation of PAR-2. *P. aeruginosa* is related to prolonged CRS [3]. The transient disruption of tight junctions may be repeatedly caused during CRS by PE and induce secondary infection by bacteria. PAR-2 agonists might be useful for the prevention and treatment of CRS.

Additional files

Additional file 1: (A) Western blotting for tight junction and adherens junction proteins in hTERT-transfected HNECs after treatment with 0.01 U neutrophil elastase. (B) RT-PCR for mRNAs of

tight junction molecules in hTERT-transfected HNECs after treatment with 0.01 U neutrophil elastase. NE: neutrophil elastase.

Additional file 2: Western blotting for tight junction proteins in hTERT-transfected HNECs treatment with pan-PKC inhibitor (GF109203X), MEK1/2 inhibitor (U0126), PI3K inhibitor (LY294002), p38 MAPK inhibitor (SB203580), JNK inhibitor (SP600125), epidermal growth factor (EGF) receptor inhibitor (AG1478), COX1 inhibitor (FR122047), and COX2 inhibitor, NF-κB inhibitor (IMD-0354), and Proteasome inhibitor (MG132) without *Pseudomonas aeruginosa* elastase.

Competing interests
The authors declare that they have no competing interest.

Authors' contributions
KN and TK carried out the genetic cell biological studies and drafted the manuscript. KO, RM and SH participated in cell culture. KT participated in the design of the study and performed the statistical analysis. TH and SN conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Local expression of interleukin-17a is correlated with nasal eosinophilia and clinical severity in allergic rhinitis

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ABSTRACT

Interleukin (IL)-17A is a major cytokine produced by Th17 cells, which are associated with chronic inflammations. The local expression of IL-17A in allergic rhinitis (AR) remains to be characterized. We sought to determine the role of IL-17A expression in human inferior turbinate mucosa in the pathophysiology of AR. Inferior turbinate mucosa was sampled from medical treatment–resistant, surgery-required patients with perennial AR (PAR, n = 21), nonallergic rhinitis with eosinophilia syndrome (NARES, n = 7), and nonallergic hypertrophic rhinitis (HR, n = 13). IL-17A expression was determined with immunohistochemical staining. The mean number of IL-17A$^+$ cells and eosinophils per field were counted. Total serum immunoglobulin E (IgE) levels, blood eosinophil count, and forced expiratory volume in 1 second (FEV_1)/forced vital capacity (FVC) ratio were also examined in each patient. IL-17A was primarily expressed in infiltrating inflammatory cells. The number of IL-17A$^+$ cells in nasal mucosa was significantly higher in the PAR group compared with HR ($p = 0.002$) and NARES ($p = 0.021$) groups. There was a significant and positive correlation between the number of IL-17A$^+$ cells and total nasal symptom score (rho = 0.403; $p = 0.011$), especially sneezing score (rho = 0.471; $p = 0.003$). The number of IL-17A$^+$ cells was significantly and positively correlated with the degree of eosinophil infiltration (rho = 0.623; $p < 0.001$), but not with total serum IgE levels (rho = 0.284; $p = 0.098$), blood eosinophil counts (rho = 0.302; $p = 0.056$), or FEV_1/FVC ratio (rho = 0.092; $p = 0.569$). The present study provides evidence that IL-17A expression in the nasal mucosa is associated with the pathophysiology of AR, including disease severity and nasal eosinophilia.

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Allergic rhinitis (AR) is the inflammation of nasal mucosa, and allergen-specific CD4$^+$ Th2 cells, which produce interleukin (IL)-4, IL-5, IL-13, and IL-31, are believed to play a central role in its pathogenesis.[1,2] Other CD4$^+$ T-cell subsets, including Th1, Tr1, and Treg cells, can regulate Th2 responses and inflammation of AR.[3–5] Recently, Th17 cells were characterized as a distinct lineage of CD4$^+$ T cells and were found to be associated with autoimmune diseases and involved in the protection against microbial infections and chronic inflammation.[6,7]

IL-17A is a proinflammatory cytokine synthesized by Th17 cells.[6] IL-17A acts on a broad range of respiratory cells to induce the expression of cytokines, chemokines, matrix metalloproteinase proteins, and mucus proteins.[8,9] For example, we have recently reported that IL-17A expression is associated with eosinophilic inflammation in chronic rhinosinusitis (CRS) both *in vivo* and *ex vivo*.[10]

Research on the role of IL-17A in the pathogenesis of AR has accumulated.[11–19] Ciprandi *et al.* showed that serum concentrations of IL-17A are significantly elevated in patients with AR compared with healthy controls, and a significant positive relationship between serum IL-17A levels and symptom severity was observed.[11,12] They also found that peripheral blood mononuclear cells from AR patients have higher frequencies of IL-17A producing T cells and CD161$^+$ circulating T cells compared with those from normal subjects.[13,16] Neiminen *et al.* indicated that specific allergen-induced IL-17A mRNA expression in peripheral blood mononuclear cells of pediatric patients with AR was significantly and positively correlated with the symptom–medication score.[14] Additionally, Xu *et al.* showed that IL-17A levels in nasal lavages of patients with AR were significantly higher compared with those of controls. They further indicated that IL-17A enhanced CCL-20 and IL-8 expression in human nasal epithelial cells.[15] More recently, Baumann *et al.* found a significant increase of IL-17A in the nasal lavages of patients with seasonal AR after nasal allergen challenge.[20] On the other hand,

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Table 1 **Subject characteristics**

	HR	NARES	PAR
No. of patients	13	7	21
Sex (male/female)	11/2	6/1	16/5
Age (yr)	37 (24–57)	44 (21–58)	31 (20–57)
CAP titer to *Dermatophagoides farinae* (UA/mL)	0 (0–0)	0 (0–0)	42.2 (0.55 to >100)
Total IgE (IU/mL)	77 (5–182)	149 (36–280)	1139 (24–9831)
Blood eosinophil (μL^{-1})	171 (68–610)	156 (34–305)	409 (37–1754)
FEV_1/FVC ratio	82 (70–100)	82 (72–92)	86 (67–100)
Patients with asthma	0	0	3

HR = hypertrophic rhinitis; NARES = nonallergic rhinitis with eosinophilic syndrome; PAR = perennial allergic rhinitis; IgE = immunoglobulin E; FEV_1 = forced expiratory volume 1 s; FVC = forced vital capacity.

Groger *et al.* showed that elevated levels of IL-17A in nasal secretions were found in patients with nonallergic rhinitis with eosinophilia syndrome (NARES) compared with healthy controls as well as AR.[17] Mouse models have shown that IL-17A contributes to the development and regulation of AR.[18,19,21] However, local IL-17A expression in AR remains to be characterized.

In the present study, we sought to determine the expression of IL-17A in human inferior turbinate mucosa and compared the expression between patients with AR and nonallergic rhinitis. Furthermore, we analyzed the correlations between IL-17A expression in nasal mucosa and various pathophysiological parameters. We believe that the results presented here may provide insight into the role of IL-17A and Th17 in the pathophysiology of AR.

MATERIALS AND METHODS

Patients

Twenty-one Japanese patients with perennial AR (PAR), 7 patients with NARES, and 13 patients with nonallergic hypertrophic rhinitis (HR) were enrolled in the study. All of them presented with persistent nasal obstruction, were resistant to medical treatment, and underwent endoscopic nasal surgery (inferior turbinectomy with or without septoplasty). PAR and NARES were defined based on the Practical Guideline for Management of Allergic Rhinitis in Japan.[22] Patients having CRS were excluded. All of the PAR patients were sensitized with *Dermatophagoides farinae*, as confirmed by the presence of specific immunoglobulin E (IgE) antibodies (range, 0.55 to >100 UA/mL; mean, 42.22 ± 40.54 UA/mL), which were detected *via* an ImmunoCAP kit (Phadia AB, Uppsala, Sweden). Conversely, HR patients did not show nasal eosinophilia or sensitization to airborne allergens related to the symptoms. Three patients with PAR were asthmatic, and no patients had aspirin sensitivity. None of the participants received systemic steroid treatment for a period of at least 8 weeks before surgery, and none received pharmacotherapy for rhinitis, such as intranasal steroids, for a period of at least 3 weeks before surgery. Patients treated with allergen-specific immunotherapy were excluded. Before surgery, we examined the total serum IgE levels, blood eosinophil count, and forced expiratory volume in 1 second/forced vital capacity ratio in each patient. The severity of nasal symptoms was graded according to the criteria outlined by Okuda *et al.*, in which three nasal symptoms (*i.e.*, sneezing, rhinorrhea, and nasal congestion) were rated on a 4-point scale from 0 to 3 (0 = no symptoms, 1 = minimal, well-tolerated symptoms, 2 = bothersome but tolerated symptoms, 3 = severe and hard to tolerate symptoms).[23] The clinical characteristics of the patients are presented in Table 1. All patients provided informed consent before their participation, and the study was approved by the Human Research Committee of the Okayama University Graduate School of Medicine and Dentistry.

Immunohistochemistry

During surgery, the mucosa of the inferior turbinate was sampled from all of the patients. We performed immunohistochemical staining for IL-17A, according to a previously described protocol.[22] Briefly, 4-μm sections were collected from paraffin-embedded tissue blocks, deparaffinized, and rehydrated. The sections were incubated with trypsin for antigen retrieval and primary antibody, including 1:50 diluted rabbit anti-human IL-17A polyclonal antibody (H-132; Santa Cruz Biotechnology, Santa Cruz, CA) or control serum (Universal Negative Control; Dako Japan, Tokyo, Japan), at 4°C overnight. A Histofine MAX-PO(R) (Nichirei Bioscience, Tokyo, Japan) with diaminobenzidine substrate was used according to the manufacturer's instructions. The sections were then nuclear stained with hematoxylin and examined under a light microscope.

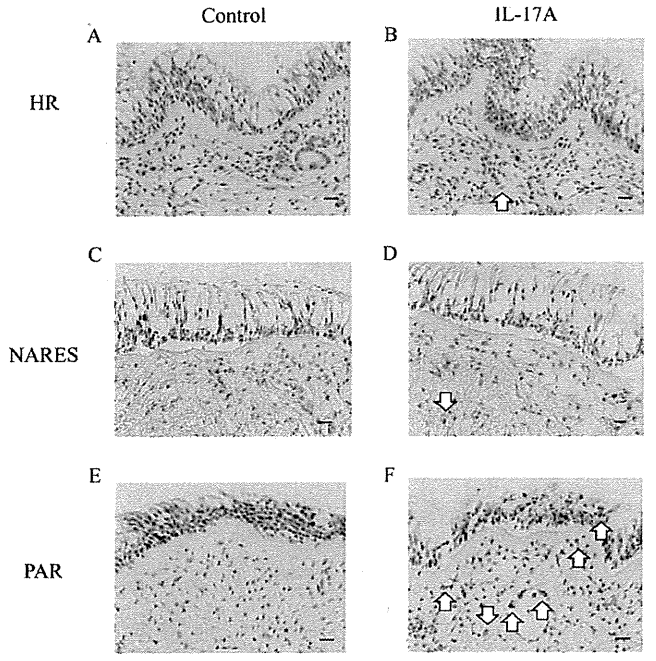

Figure 1. Immunohistochemical staining of interleukin (IL)-17A in nasal mucosa from a patient with (A and B) nonallergic hypertrophic rhinitis (HR), (C and D) nonallergic rhinitis with eosinophilia syndrome (NARES), and (E and F) perennial allergic rhinitis (PAR). Sections were reacted with rabbit polyclonal antibody against (B, D, and F) IL-17A (A, C, and E) or control after which they were stained using a Histofine MAX-PO (Nichirei Bioscience, Tokyo, Japan) with a diaminobenzidine substrate. Arrows indicate IL-17$^+$ cells (scale bar = 20 μm).

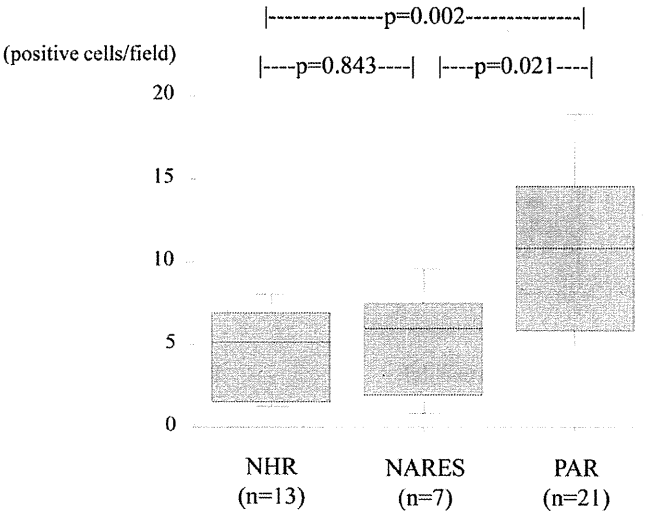

Figure 2. Comparison of numbers of interleukin (IL)-17A$^+$ cells in the nasal mucosa among patients with nonallergic hypertrophic rhinitis (HR), nonallergic rhinitis with eosinophilia syndrome (NARES), and perennial allergic rhinitis (PAR). The rectangle includes the range from the 25th to the 75th percentiles, the horizontal line indicates the median, and the vertical line indicates the range from the 10th to 90th percentiles. The p values were determined by the Mann-Whitney U test.

Positive-stained cells were counted in five fields at high power (10 × 40), where the highest cellular infiltration was observed. The mean number of positive cells was then determined. Additionally, sections were stained with hematoxylin and eosin, and the number of eosinophils that had infiltrated into the nasal mucosa was counted in the same manner.

Statistical Analysis

Values are presented as median values. A nonparametric Mann-Whitney *U* test was used for comparing data between groups. A correlation analysis was performed using a nonparametric Spearman's correlation coefficient by rank. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using StatView software (Version 4.5; Abacus Concepts, Berkeley, CA).

RESULTS

Local Expression of IL-17A in Nasal Mucosa

We immunohistochemically examined the expression and distribution of IL-17A in the nasal mucosa of inferior turbinate. IL-17A protein was primarily expressed in infiltrating inflammatory cells, but not epithelial cells, vascular endothelial cells, glands, or fibroblasts. The expression levels of IL-17A were determined in every group, with a greater expression observed in the PAR group versus the HR and NARES groups (Fig. 1). The number of IL-17A$^+$ cells in the nasal mucosa was significantly higher in the PAR group compared with HR ($p = 0.002$) and NARES ($p = 0.021$) groups. Conversely, the number was similar between the HR and NARES group ($p = 0.843$; Fig. 2).

Pathophysiological Significance of IL-17A Expression in Nasal Mucosa

A significant and positive correlation was seen between the number of IL-17A$^+$ cells in the nasal mucosa and total nasal symptom score, which was determined from the sum of sneezing, rhinorrhea, and congestion scores ($\rho = 0.403$; $p = 0.011$; Fig. 3 *A*). In detail, the sneezing score *per se* was significantly and positively correlated with the number of IL-17A$^+$ cells ($\rho = 0.471$; $p = 0.003$; Fig. 3 *B*), whereas the rhinorrhea ($\rho = 0.291$; $p = 0.066$; Fig. 3 *C*) and congestion ($\rho = 0.206$; $p = 0.192$; Fig. 3 *D*) were not.

The number of IL-17A$^+$ cells did not correlate with total serum IgE levels ($\rho = 0.284$; $p = 0.098$; Fig. 3 *E*), blood eosinophil counts ($\rho = 0.302$, p = .056, Fig. 3 *F*), or forced expiratory volume in 1 second/forced vital capacity ratio ($\rho = 0.092$; $p = 0.569$; Fig. 3 *G*). However, the degree of eosinophil infiltration into the nasal mucosa was significantly and positively