

Decreased expression of E-cadherin and ZO-1 in the nasal mucosa of patients with allergic rhinitis: Altered regulation of E-cadherin by IL-4, IL-5, and TNF-alpha

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ABSTRACT

Background: Allergic rhinitis is a chronic nasal inflammatory disease mediated by an immunoglobulin E mediated process to environmental allergens. Although atopy is a potent predisposing risk factor for allergic rhinitis, local tissue susceptibilities are inevitable for disease expression. The nasal epithelium maintains tissue homeostasis by providing a physical barrier controlled by epithelial junctional proteins. However, the expression of epithelial junctional proteins has not been studied in patients with allergic rhinitis. We sought to elucidate the expression and the regulation of epithelial junctional proteins in the nasal epithelium of patients with allergic rhinitis.

Methods: The expression of E-cadherin and zonula occludens (ZO) 1 in epithelium of turbinate was measured by using real-time polymerase chain reaction, Western blot, and immunohistochemical assays, and was compared between control subjects and patients with allergic rhinitis. In addition, the expression levels of E-cadherin and ZO-1 were determined in cultured epithelial cell treated with interleukin (IL) 4, IL-5, tumor necrosis factor (TNF) alpha, and interferon gamma.

Results: The expression and the immunoreactivity of E-cadherin and ZO-1 were decreased in the nasal epithelium of patients with allergic rhinitis. Interestingly, the stimulation of cultured epithelial cells with IL-4, IL-5, and TNF-alpha resulted in downregulation of E-cadherin expression only in cultured epithelial cells of patients with allergic rhinitis, whereas E-cadherin expression in cultured epithelial cells of controls was not affected by stimulation with the same panel of cytokines.

Conclusion: Decreased expression of epithelial junctional proteins was found in patients with allergic rhinitis. The disruption of epithelial integrity by IL-4, IL-5, and TNF-alpha in vitro indicated a possible role for these cytokines in the pathogenesis of patients with allergic rhinitis.

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Airway epithelial cells are an important component of the innate immune system in the airway mucosa.¹ Epithelial cells form a barrier to the external environment that is composed of surface gel layer, sol layer, and apical junctional complexes (AJC) that bind neighboring cells. AJCs consist of the apical tight junction (TJ) and underlying adherens junction (AJ), which establishes cell-cell binding, cell polarity, and the regulation of the paracellular flux of ions or macromolecules.^{2–4} These cell contacts are mediated by various types of junctional proteins, such as cadherins; occludin; claudins; junctional adhesion molecule; members of the immunoglobulin family; and intracellular membrane proteins, including zonula occludens (ZO) 1, catenin, desmoglein, and desmocollin.^{5–9} E-cadherin is one of the best-characterized transmembrane glycoproteins (molecular weight 124 kDa) in AJs and is expressed on many types of epithelial cell surfaces, including the nasal epithelium.^{9,10} In addition, ZO-1 is a cytoplasmic AJC associated protein (molecular weight 225 kDa) in TJs and is thought to make direct contact with occludin, which serves as an anchor for the underlying cytoskeleton via mechanisms under active investigation.^{7,11}

Disruption of AJCs can lead to entrance of pathogens, noxious stimuli, and environmental antigens (e.g., allergens), into the tissue. Altered AJCs

are associated with multiple diseases, including those seen in patients with inflammatory intestinal diseases, e.g., Crohn's disease, and skin diseases, e.g., atopic dermatitis and psoriasis.^{12–14} Recently, the presence of defective epithelial AJCs was identified in the lower airways of patients with asthma^{15–22}; however, the exact mechanisms and consequences of this airway inflammation are not yet clear. Moreover, specific inflammatory cytokines can cause barrier disruption that results in the formation of a positive feedback loop. Therefore, regulation of AJCs is surging as a significant checkpoint in asthma immunology. Moreover, studies reported that allergic inflammation also induces epithelial damage in the upper airway mucosa.^{23,24}

When considering that AJCs are a potent barrier in the upper airway mucosa, altered levels of expression of junctional proteins may play an important role in the pathogenesis of allergic rhinitis. However, the precise participation of junctional proteins in allergic rhinitis has not been sufficiently evaluated. Therefore, the aims of the present study were as follows:

1. To determine the expression levels and distributional patterns of E-cadherin and ZO-1, and to evaluate the possible effects of E-cadherin and ZO-1 in the pathogenesis of allergic rhinitis by comparing expression between normal and allergic nasal mucosa.

2. To elucidate whether the expression of E-cadherin and ZO-1 is altered on stimulation with cytokines relevant to allergic rhinitis.

METHODS

Tissue Preparation

Twenty patients with allergic rhinitis and 20 healthy control subjects were enrolled in the current study. Patients who were undergoing augmentation rhinoplasty were considered healthy controls and were excluded if they had a history of nasal infection or allergy, smoking, or ongoing drug treatment. All the patients with allergic rhinitis had typical symptoms of perennial nasal allergy, and nasal eosinophilia, and a positive skin test or positive radioallergen sorbent

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test to house-dust mites as well as positive reactions on provocation tests. Total serum immunoglobulin E levels were determined by enzyme-linked immunosorbent assay (CAP system, ImmunoCAP 1000, Aloka, Japan). Patients with allergy and with exacerbation of upper respiratory tract infection during the 6 weeks before the study were excluded. Additional exclusion criteria consisted of current smoking status; a medical history of asthma; recurrent infections of the respiratory tract; and use of topical or oral corticosteroids, leukotriene modifiers, and antihistamines during the 8 weeks before the study. The clinical data of patients, including total serum immunoglobulin E levels, are summarized in Table 1.

Twenty samples of healthy nasal mucosa were obtained from the inferior turbinates of 20 healthy control subjects who had normal nasal mucosa and who were admitted for augmentation rhinoplasty. Before obtaining tissue specimens, the study protocol was approved by the institutional review board for human studies at Korea University Hospital, and all the participants provided written informed consent.

All the tissue samples were divided into two portions. The first portion was immersed in a fixative that contained 4% paraformaldehyde for histology, and the second portion was treated with Dispase (1 mg/mL; GenDEPOT, Barker, TX) to harvest epithelial cells. For histologic analysis, paraffinized turbinate blocks were sectioned at a thickness of 4 μ m.

To harvest epithelial cells, the collected turbinates were treated with Dispase for 24 hours at 4°C. Cells were detached from enzyme-treated turbinate and then cultured or used for extraction of RNA and protein. By immunostaining against cytokeratin, detached cells were confirmed as epithelial cells. For extraction of total RNA from epithelial cells, detached epithelial cells were lysed with QIAzol (Qiagen, Hilden, Germany), and the lysates were sequentially treated with chloroform, isopropanol, and ethanol for messenger RNA (mRNA) purification. Complementary DNA (cDNA) was synthesized from RNA by using cDNA synthesis master mix (GenDEPOT). For extraction of protein from epithelial cells, detached epithelial cells were resuspended in 5X sodium dodecyl sulfate sample buffer and heated for 10 minutes at 100°C.

Real-Time Polymerase Chain Reaction

Gene expression in epithelial cells was measured by quantitative real-time polymerase chain reaction. The prepared cDNA was amplified and quantified by using SYBR Green master mix (Qiagen) with the following primers: ZO-1, forward 5'-ACA GTG CCT AAA GCT ATT CCT GTG A-3' and reverse 5'-TCG GGA ATG GCT CCT TGA G-3'; E-cadherin, forward 5'-TGC TCT TGC TGT TTC TTC GG-3' and reverse 5'-TGC CCC ATT CGT TCA AGT AG-3'; and glyceraldehyde 3-phosphate dehydrogenase, forward 5'-GAG TCA ACG GAT TTG GTC GT-3' and reverse 5'-TTG ATT TTG GAG GGA TCT CG-3'. Polymerase chain reactions were performed by using a real-time thermal cycler system (TP850) (Takara, Shiga, Japan) with 40 cycles of a two-step reaction that consisted of denaturation at 95°C for 15 seconds followed by annealing-extension at 60°C for 45 seconds. Data were analyzed by using the Δ Ct method.

Immunohistochemistry

To evaluate the distribution of cell-to-cell adhesion molecules, turbinate sections were immunostained for E-cadherin (SantaCruz Biotechnology, Dallas, TX) and ZO-1 (Life Technologies, Waltham, MA). The sections were examined with an Olympus BX51 microscope (Tokyo, Japan). Pictures were captured by using an Olympus DP72 with DP2-BSW software.

Western Blotting

Protein expression of cell-to-cell adhesion molecule in epithelial cells was detected by immunoblotting. Cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with

Table 1 Clinical characteristics of patients

Characteristics	Healthy Nasal Mucosa (n = 20)	Allergic Rhinitis (n = 20)
No. women/men	9/21	8/22
Age, y, mean $\pm$ SD	34.2 $\pm$ 7.3	35.4 $\pm$ 7.7
Rhinitis duration, y, mean $\pm$ SD		12.5 $\pm$ 2.1
Serum total immunoglobulin E, IU/mL, mean $\pm$ SD	32.4 $\pm$ 12.3	523.2 $\pm$ 205.2

SD = standard deviation.

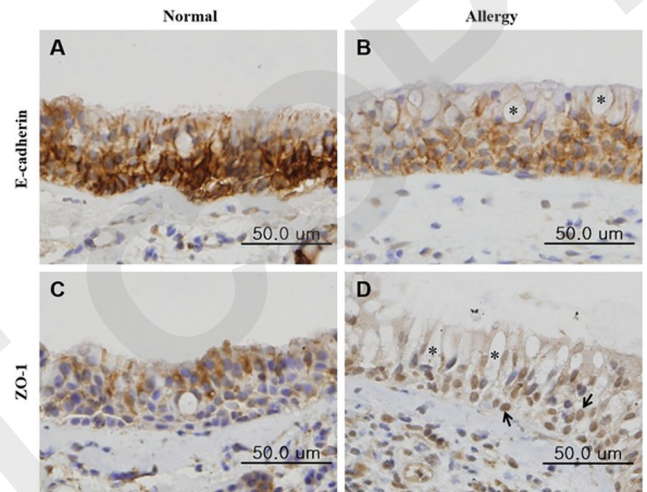


Figure 1. Immunohistochemical analysis of the cell-to-cell adhesion molecules E-cadherin and zonula occludens 1 (ZO-1) in normal and allergic turbinates. Normal turbinates (A and C) and turbinates from patients with allergic rhinitis (B and D) were stained with an anti-E-cadherin antibody (A and B) or anti-ZO-1 antibody (C and D). *Goblet cells. Arrows indicate nuclei stained with the anti-ZO-1 antibody. Bar = 50 μ m.

E-cadherin antibody for target molecule or β -actin antibody (SantaCruz Biotechnology) for reference. After color development, proteins were visualized with a ChemiDoc imaging system (Bio-Rad, Hercules, CA).

Epithelial Cell Culture

To investigate the responsiveness of epithelial cells from patients with allergy to cytokines, epithelial cells were resuspended in bronchial epithelial cell basal medium (Lonza, Basel, Switzerland) and cultured in 6-well plates. On reaching 80% confluency, the cells were subcultured in 12-well plates. On reaching confluency, the subcultured epithelial cells were treated with either 100 ng/mL of the T-helper (Th) type 1 cytokines tumor necrosis factor (TNF) α and interferon (IFN) γ , or the Th2 cytokines interleukin (IL)-4 and IL-5 (PeproTech, Rocky Hill, NJ). After overnight incubation, RNA and protein were extracted from control and cytokine-treated cells as described above.

Statistical Analysis

Statistical analyses were carried out by using SPSS (Armonk, NY). Data in graphs are expressed as the mean $\pm$ standard error of at least three independent determinations. Differences between normal and allergic samples were determined by using the Student's *t*-test, and differences among cytokine-treated groups were calculated by one-way analysis of variance. Differences with *p* values of < 0.05 were considered significant.

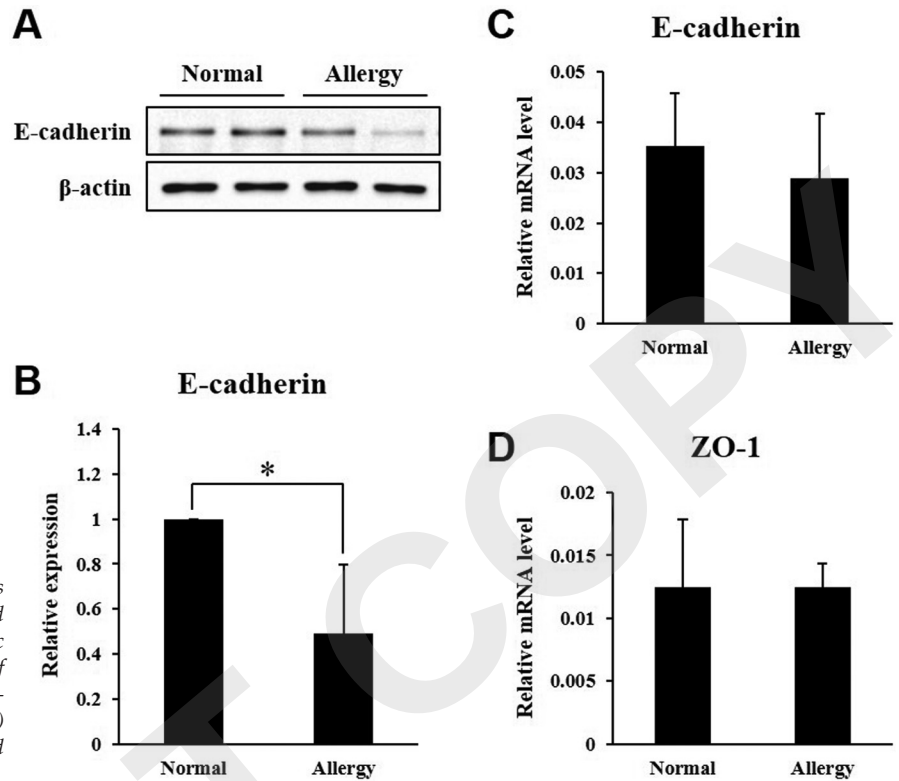


Figure 2. Translational and transcriptional analysis of the cell-to-cell adhesion molecules E-cadherin and zonula occludens 1 (ZO-1) in normal and allergic turbinates. (A) Representative immunoblot images of E-cadherin expression in normal and allergic turbinates with (B) graphic and statistical analysis. (C) Relative expression of E-cadherin messenger RNA and (D) ZO-1 messenger RNA. * $p < 0.05$.

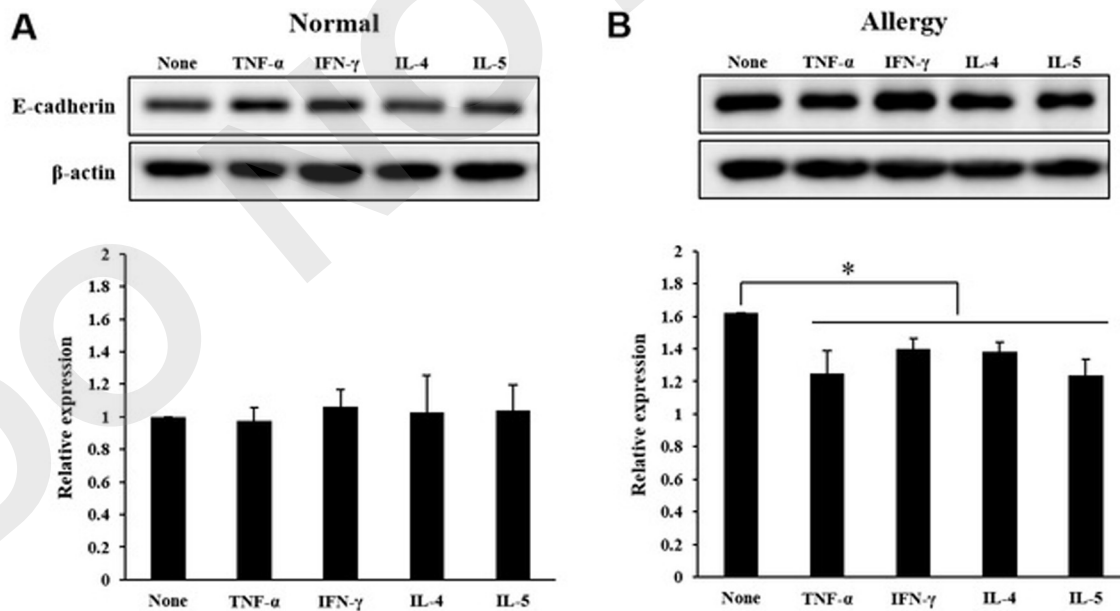


Figure 3. Immunoblotting analysis of E-cadherin in epithelial cells treated with T-helper (Th) cell type 1 and Th2 cytokines. Epithelial cells from (A) normal turbinate and (B) allergic turbinates were treated with Th1 cytokines tumor necrosis factor (TNF) α and interferon (IFN) γ or the Th2 cytokines interleukin (IL) 4 and IL-5 as indicated. Relative expression of E-cadherin is shown in the ratio of cytokine-treated cells to nontreated cells (None). (A and B) Representative immunoblot images with graphic and statistical analysis. * $p < 0.05$.

RESULTS

Cell Adherent Molecules Were Reduced in the Epithelial Cells of Allergic Turbinates

We initially hypothesized that cell-to-cell interactions between epithelial cells may be disturbed in patients with allergy. To evaluate

this hypothesis, we investigated the expression of the cell-to-cell adhesion molecules E-cadherin and ZO-1 in turbinate epithelium by immunostaining. E-cadherin was localized at the basolateral side, whereas ZO-1 was located much more toward the apical side. The expression of E-cadherin at the AJ of normal turbinate epithelium was more prominent than that of allergic turbinate epithelium, which

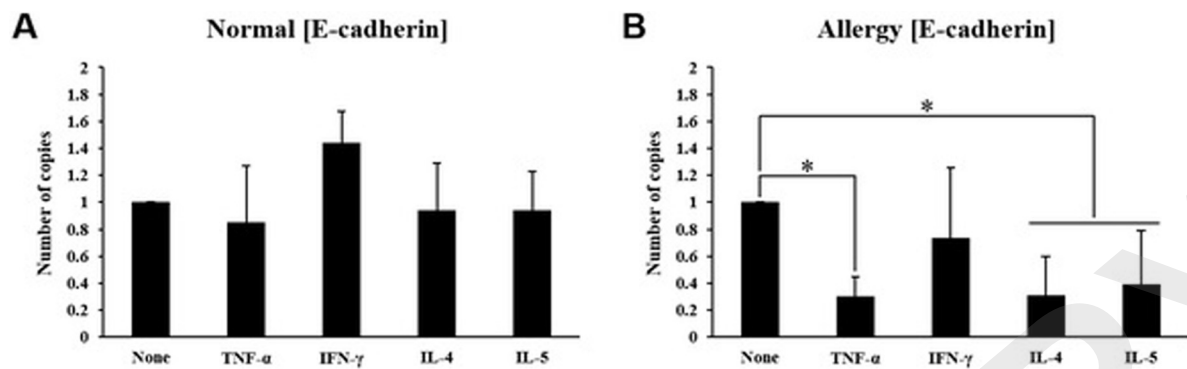


Figure 4. Transcriptional analysis of E-cadherin expression in epithelial cells treated with T-helper (Th) type 1 and Th2 cytokines. Epithelial cells from (A) normal turbinate and (B) allergic turbinates were treated with the Th1 cytokines tumor necrosis factor (TNF) α and interferon (IFN) γ or the Th2 cytokines interleukin (IL) 4 and IL-5 as indicated. * $p < 0.05$.

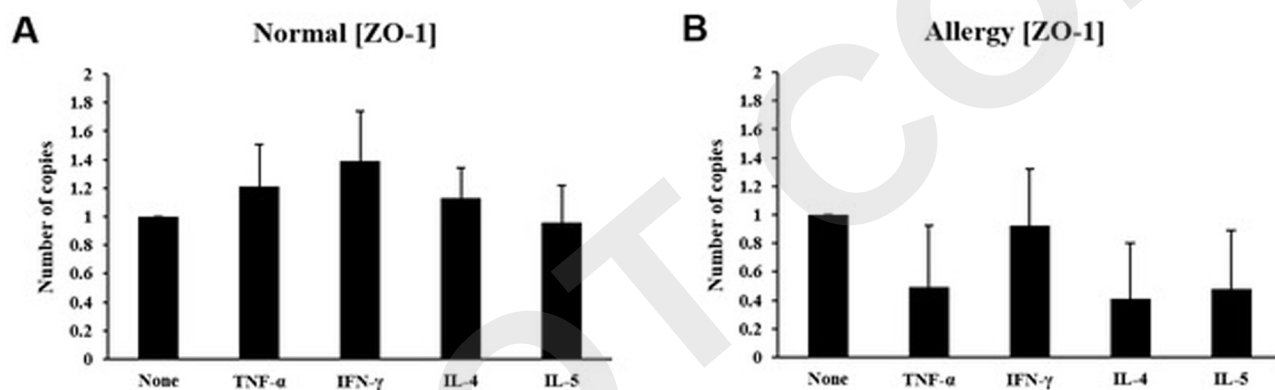


Figure 5. Transcriptional analysis of zonula occludens 1 (ZO-1) expression in epithelial cells treated with T-helper (Th) Th1 and Th2 cytokines. Epithelial cells from (A) normal turbinate and (B) allergic turbinates were treated with the Th1 cytokines tumor necrosis factor (TNF) α and interferon (IFN) γ or the Th2 cytokines interleukin (IL) 4 and IL-5 as indicated.

indicated that cell-to-cell contact may be disturbed in allergic rhinitis (Fig. 1, A and B). In addition, staining of ZO-1 at the TJ was more prominent in normal turbinate epithelium (Fig. 1, C and D). However, the nuclei of epithelial cells from allergic rhinitis were much more prominently stained with the ZO-1 antibody compared with normal turbinate epithelium (Fig. 1 D arrows), which indicates that ZO-1 is localized to the nucleus during remodeling as a result of decreased cell-to-cell contact in allergic rhinitis epithelium. On histologic examination, goblet cells were more prominent in allergic turbinate epithelium than in normal epithelium (Fig. 1, B and D asterisk), which accounted for mucus overproduction and airway hyperresponsiveness in allergic rhinitis.

To confirm the decrease in cell-to-cell interactions in allergic rhinitis, we next examined the protein and mRNA levels of E-cadherin and ZO-1. Consistent with immunohistochemical results, immunoblotting showed that expression of E-cadherin protein was decreased in allergic epithelial cell extracts (Fig. 2, A and B). However, the mRNA levels of E-cadherin between normal and allergic epithelial cell extracts were not significantly different according to real-time polymerase chain reaction, although a tendency for reduced mRNA expression under allergic conditions was noted (Fig. 2 C). Similar to E-cadherin, the mRNA level of ZO-1 in allergic epithelial cell extracts was not different from that of normal epithelial cells (Fig. 2 D).

Epithelial Cells of Allergic Turbinate Were More Vulnerable to Cytokines

To observe the characteristics of the allergic epithelial cells, enzymically detached epithelial cells from patients with allergic rhinitis

were treated with several cytokines and compared with normal epithelial cells. When epithelial cells were treated with the Th1 cytokines TNF- α and IFN- γ or the Th2 cytokines IL-4 and IL-5, protein expression of E-cadherin in normal epithelial cells was not different among any of the treatment groups, whereas, in allergic epithelial cells, the expression levels of E-cadherin in the cytokine treatment groups were significantly different from the control group (Fig. 3, A and B), which indicated a vulnerability of allergic epithelial cells against both Th1 and Th2 cytokines. Unexpectedly, however, there was no difference between Th1 and Th2 cytokines (Fig. 3 B), despite only Th2 cytokines being widely associated with allergic responses. Contrary to tissue staining results, the expression of E-cadherin in control cells from patients with allergy was 1.6 times higher than the expression of normal control cells (Fig. 3 A none versus B none), which indicated that the properties of epithelial cells may have been altered under *in vitro* culture conditions due to a loss of allergic stimulation.

We next evaluated the transcriptional expression of E-cadherin in epithelial cells from patients with allergy after treatment with Th2 cytokines and found that E-cadherin mRNA expression was significantly reduced compared with control cells (Fig. 4 B). However, in Th1 cytokine cells, TNF- α reduced the expression of E-cadherin, whereas IFN- γ had no effect (Fig. 4 B). Given the similarity of E-cadherin expression among the treatment groups compared with normal epithelial cells (Fig. 4 A), this result indicated the vulnerability of allergic epithelial cells against Th1 and Th2 cytokines, and also indicated that Th2 cytokines were more effective than Th1 cytokines at eliciting this response.

In contrast to E-cadherin, the mRNA expression of the TJ protein ZO-1 in normal cells was not different between control and treatment groups (Fig. 5 A). Also, with respect to epithelial cells from patients with allergy, the expression of ZO-1 exhibited a similar pattern as E-cadherin, although there was no significant difference between control and treatment groups (Fig. 5 B).

DISCUSSION

Defects in the airway epithelial barrier allow access to the subepithelial tissue, which results in an inflammatory response in the airway mucosa. In addition, decreased expression of E-cadherin and ZO-1 in the bronchial biopsy specimen has been observed in patients with asthma.^{18,22} Likewise, in the nasal mucosa from patients with chronic rhinosinusitis with nasal polyp, expression of ZO-1 and occludin is decreased.²⁵ Thus, our findings were consistent with those of previous studies that showed that junctional proteins are disrupted during airway inflammation.

In the present study, E-cadherin was studied as a representative cell-to-cell molecule that might be regulated in an allergic state. E-cadherin is normally synthesized as a 135-kDa inactive precursor protein but is ultimately incorporated into the cell membrane as a 124-kDa active epithelial specific glycoprotein.²⁶ In AJs, E-cadherin interacts with other E-cadherin molecules on adjacent cells to form E-cadherin homodimers, which are critical for the paracellular barrier in the airway epithelia.²⁷ Interestingly, we found that protein expression of E-cadherin in tissue was inconsistent with mRNA expression, which may indicate that this inconsistency might be counterbalanced by transcriptional regulation of E-cadherin mRNA in an allergic state.

With respect to turbinates of patients with allergic rhinitis, we found that ZO-1 was present mainly in the nuclei of epithelial cells rather than TJs according to immunohistochemistry. Normally, ZO-1 is detected in the upper layers of the epithelium of human nasal mucosa, where it participates in the first physical barrier of the body as a peripheral membrane protein in TJs by associating with polymerization of claudin, an integral membrane protein.²⁸ However, according to *in vitro* experiments, ZO-1 is localized in the nucleus in immature cell-to-cell contacts or contacts undergoing remodeling after injury.²⁹ This finding indicated that nuclear localization of ZO-1 in the turbinates of human patients with allergic rhinitis reflects dissociation of cell-to-cell contact between epithelial cells. Furthermore, because transfected ZO-1 binds to the cadherin-catenin complex and actin filaments in exogenous E-cadherin expressing cells,³⁰ the nuclear localization of ZO-1 in this research might have affected, or been affected by, the distribution of E-cadherin in epithelial cells of allergic rhinitis turbinates.

It is currently known that intestinal permeability in patients with inflammatory bowel disease is caused by cytokine-induced barrier disruption in the absence of apoptosis,^{31,32} and it is becoming clear that similar responses are likely involved in the airway. Inflammatory cytokines, including IL-4, IL-13, TNF- α , and IFN- γ have been shown to be involved in airway epithelial barrier functions through diverse mechanisms,^{33–36} although their roles in regulating airway epithelial junctional proteins remain controversial. In primary cultured bronchial epithelial cells from healthy controls and patients with cystic fibrosis, TNF- α and IFN- γ synergistically reduce the expression of ZO-1 and junctional adhesion molecule.³³ IL-4 does not induce any changes in the expression of epithelial junctional proteins, such as E-cadherin, ZO-1, occludin, claudin-1, and claudin-3 in 16HBE14o- human bronchial epithelial cells.³⁴ However, IL-4, TNF- α , and IFN- γ all significantly downregulate ZO-1 and occludin in Calu-3 airway epithelial cells.¹⁷ Soyka *et al.*²⁵ demonstrated that there are no changes in the mRNA expression of junctional proteins such as ZO-1, occludin, and claudin-1 in primary human nasal epithelial cells after stimulation with IL-4 and IL-17, although IFN- γ -stimulated cultures from patients with chronic rhinosinusitis with nasal polyposis seem to have higher mRNA expression of junctional proteins than those of control subjects.²⁵ In the present study, there was no change

in the mRNA and protein expression of ZO-1 and E-cadherin after stimulation of normal cells with IL-4, 5, TNF- α , and IFN- γ . Indeed, only in the nasal mucosa from patients with allergic rhinitis did cytokines decrease expression of E-cadherin at the mRNA and protein level. Therefore, our data may indicate that epithelial cells in allergic nasal mucosa are more vulnerable to stimulation by cytokines compared with cells of normal mucosa. However, additional studies will be required to evaluate the specific effects of cytokines in the nasal mucosa of patients with allergic rhinitis.

CONCLUSION

To our knowledge, this is the first report that explored the association between expression of junctional proteins and human allergic rhinitis. Our results indicated that junctional proteins may be involved in the pathogenesis of allergic rhinitis and could be modulated differentially by cytokines in the nasal mucosa of patients with allergic rhinitis compared with healthy individuals. Further studies are needed to evaluate the functional role of these findings in relationship to *in vivo* manifestation of increased allergen exposure, tissue edema, and nasal discharge.

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