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Epithelial Permeability Alterations in an In Vitro Air-Liquid Interface Model of Allergic Fungal Rhinosinusitis

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Abstract

Background—Chronic rhinosinusitis (CRS) is an inflammatory upper-airway disease with numerous etiologies. Patients with a characteristic subtype of CRS, allergic fungal rhinosinusitis (AFRS), display increased expression of Th2 cytokines and antigen-specific IgE. Various sinonasal inflammatory conditions are associated with alterations in epithelial barrier function. The aim of this study was to compare epithelial permeability and intercellular junctional protein expression amongst cultured primary sinonasal cells from AFRS patients versus non-inflammatory controls.

Methods—Epithelial cells isolated from paranasal sinus mucosa of AFRS and non-inflammatory control patients were grown to confluence on permeable supports and transitioned to air-liquid interface (ALI). Trans-epithelial resistance (TER) was measured with a horizontal Ussing chamber to characterize the functional permeability of each cell type. After TER recordings were complete, a panel of intercellular junctional proteins was assessed by Western blot and immunofluorescence labeling followed by confocal microscopy.

Results—After 12 samples were measured from each group, we observed a 41% mean decrease in TER in AFRS cells ($296 \pm 89 \text{ ohms} \times \text{cm}^2$) compared to control ($503 \pm 134 \text{ ohms} \times \text{cm}^2$, $P=0.006$). TER deficits observed in AFRS were associated with decreased expression of the tight

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Conflict of Interest Disclosure:

Author	Potential Conflicts of Interest
Kyle A. Den Beste, B.S.	None
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junction proteins occludin and Junctional Adhesion Molecule-A (JAM-A), and increased expression of a leaky tight junction protein claudin-2.

Conclusions—Cultured sinonasal epithelium from AFRS patients displayed increased epithelial permeability and altered expression of intercellular junctional proteins. Given that these cells were not incubated with inflammatory cytokines *in vitro*, the cultured AFRS epithelial alterations may represent a retained modification in protein expression from the *in vivo* phenotype.

Keywords

Adherens junction; allergic fungal rhinosinusitis; allergic rhinitis; claudin-2; E-cadherin; epithelial permeability; junction adhesion molecule-A; occludin; sinonasal epithelium; tight junction

Introduction

Chronic rhinosinusitis (CRS) is a common debilitating condition characterized by sinonasal mucosal edema, nasal discharge, and sinus pain of greater than 12 weeks duration. Numerous etiologic disease processes contribute to the development of CRS, and they can be broadly categorized based on whether they produce CRS with a predominantly Th1 or Th2-mediated cytokine response. The Th1 response often results in the production of interferon-gamma and is seen in patients with CRS from infectious agents.¹ Patients with allergic hypersensitivity in the setting of CRS may present with nasal polyposis, significant tissue eosinophilia, and increased Th2 cytokine expression (IL-4, -5, and -13).

The heightened Th2 response is especially prominent in a specific type of CRS known as allergic fungal rhinosinusitis (AFRS). The classic Bent-Kuhn diagnostic criteria for AFRS include type I IgE-mediated hypersensitivity, nasal polyposis, distinctive radiologic findings, eosinophilic mucin without fungal invasion, and a positive fungal stain.² While eosinophils predominate in the inflammatory infiltrate, mast cells, lymphocytes, and plasma cells can also be found in histology specimens.³ The pathophysiology of AFRS continues to be a topic of debate, but has been associated with fungal antigen exposure, IgE upregulation in local tissues, and a Th2 inflammatory cytokine profile.⁴ Though nasal polyposis as a whole is not specifically associated with allergic hypersensitivity, AFRS patients present with the distinct clinical and inflammatory profile described above. As intercellular junctional proteins modulate epithelial permeability and inflammatory cell migration, it is important to understand how the epithelial barrier is disrupted in the context of AFRS in order to appreciate pathologic mechanisms underlying the disease.

Epithelial cells separate luminal contents from interstitial tissue by way of a selectively permeable barrier known as the apical-junctional complex (AJC). The AJC consists of the tight junction and adherens junction, which collectively bind cells together and regulate paracellular transport. Tight junctions are located at the most apical region of the lateral epithelial cell membrane and are associated with the actin cytoskeleton. Proteins comprising the tight junction include zonula occludens-1 (ZO-1), occludin, Junctional Adhesion Molecule-A (JAM-A), and the claudins. The claudin family of tight junction transmembrane proteins determine the paracellular pore characteristics and therefore the permeability properties of the epithelium. Over-expression of “tight” claudins (claudin-1, -4, -5, -8, -11, -14, and -19) decreases paracellular permeability, while over-expression of “leaky” claudins (claudin-2, -7, -10, -15, and -16) increases permeability.⁵ Adherens junction proteins such as E-cadherin and members of the catenin family facilitate cell-cell recognition and are located near the medial aspect of the lateral membrane.

Epithelial barrier permeability as it relates to tight junction integrity has been elucidated through numerous studies of infectious, inflammatory, and autoimmune perturbations. A

generalized mechanism that can be applied to most examples involves the following: inflammatory cytokines or luminal antigens promote AJC disassembly and increased epithelial permeability, causing luminal contents to flow into the interstitium, resulting in antigen exposure and inflammation. As an example, paracellular permeability is compromised in the intestinal epithelium of patients with inflammatory bowel disease which have elevated levels of cytokines and chronic active mucosal inflammation.⁶ Interestingly, increased expression of claudin-2 and perturbed expression of other tight junction proteins such as occludin has been observed in intestinal epithelial cells of inflammatory bowel disease patients.^{6,7} *In vitro* studies of lower- airway respiratory epithelium have shown that inhaled allergens such as pollen and house dust mite modify tight junction protein expression, contributing to increased antigen sensitization and an allergic response.^{8,9} Structural and functional tight junction abnormalities have also been implicated in the pathogenesis of chronic bronchitis, asthma, and cystic fibrosis.^{10–12}

Compared to the existing body of literature on lower-airway bronchial epithelium, relatively little is known about upper-airway epithelial barrier function in the context of chronic inflammatory disease. The aim of this study was thus twofold: to characterize *in vitro* epithelial permeability in AFRS, and to investigate whether permeability changes are associated with specific alterations in AJC protein expression. Considering the intrinsic difficulty in collecting functional and biochemical data from a limited supply of human tissue samples, we proposed to culture primary cells from sinonasal mucosal biopsies. In order to minimize confounding factors, we excluded the possibility of characterizing immortalized human cell lines given that they may have characteristics not shared by their primary cell counterparts. In addition, we cultured primary cells at an air-liquid interface (ALI) to approximate the native respiratory epithelial environment.

Materials and Methods

Human tissue collection

This study was performed with approval from the Emory University Institutional Review Board. Following written informed consent, patients undergoing endoscopic sinus surgery as part of their routine clinical care for AFRS or patients undergoing endoscopic transnasal surgical approaches to orbital or skull base pathology (non-inflammatory controls) had sinonasal mucosa biopsied for primary epithelial cell culture. Inclusion criteria for AFRS patients were based upon the 1994 Bent and Kuhn diagnostic characteristics.² As there is some controversy over the diagnostic criteria for AFRS, at least 4 of the 5 Bent and Kuhn AFRS criteria were required for collection of AFRS tissue for these experiments. Control patients had no significant clinical or radiographic evidence of inflammatory rhinosinusitis. We excluded patients from both AFRS and control groups with a diagnosis of cystic fibrosis, immune deficiency, autoimmune conditions, granulomatous disorders, aspirin-exacerbated respiratory conditions. Prisoners, institutionalized individuals, and other vulnerable populations were also excluded. No patients were receiving systemic steroids at treatment doses for inflammatory conditions. One patient in the control group was on hydrocortisone replacement for hypopituitarism.

Primary cell culture at the air-liquid interface (ALI)

Sinonasal biopsies were minced into ~2 mm³ pieces and placed in RPMI 1640 media (Invitrogen, Carlsbad, CA) with antibiotic/antimycotic (Invitrogen) at 2X final concentration. Tissue was digested with protease isolated from *Streptococcus griseus* (Sigma-Aldrich, St. Louis, MO), and the reaction was stopped with heat inactivated fetal bovine serum after 2 hours at 37°C. Supernatant was mechanically separated from tissue pieces, centrifuged (5 minutes, 950 rpm or 101 g), and the cell pellet was resuspended in

Bronchial Epithelial Growth Medium (BEGM): Bronchial Epithelial Basal Medium (BEBM) supplemented with EBM SingleQuot additives (Lonza, Walkersville, MD), antibiotic/antimycotic (Invitrogen), and nystatin (Sigma-Aldrich). Fibroblasts were removed from culture by incubating in a collagen-coated P100 tissue culture-treated petri dish at 37°C for 2 hours. Supernatant containing sinonasal epithelial cells was transferred to collagen-coated T75 cell culture flasks (Corning, Corning, NY) and grown in BEGM at 5% CO₂, 95% humidity, 37°C. BEGM media was changed every 48–72 hours.

Once the cell layers reached 85% confluency, they were washed with Hanks Balanced Salt Solution (HBSS) without Mg²⁺ or Ca²⁺ (Sigma-Aldrich) and trypsinized with Trypsin-EDTA (Invitrogen). After 5 minutes the trypsin reaction was stopped with soybean trypsin inhibitor. The cells were centrifuged (5 minutes, 950 rpm or 101 g), resuspended in BEGM, and seeded onto 0.4 µm pore size Snapwell inserts of 12 mm diameter (Corning). All cell layers were passaged only once from the original T75 cell culture flask to Snapwell inserts in order to reduce the possibility of dedifferentiation.

Cells were grown to confluence with BEGM on the apical and basal surfaces and media changed every 48–72 hours. Once confluence was confirmed by light microscopy, media was removed from the apical surface and basal media was changed to air-liquid interface (ALI) media. ALI media consisted of a 50:50 mixture of BEBM and DMEM high glucose (Invitrogen), along with BEBM SingleQuots, antibiotic/antimycotic, retinol, and bovine serum albumin (Sigma-Aldrich). Cell layers were grown to full polarization and differentiation at the ALI, which was confirmed by beating cilia under phase-contrast light microscopy. The cells were allowed to continue to grow and stabilize for at least 7–10 days prior to being used in experiments. Control and AFRS cell layers were paired for experiments based on the time of ciliary differentiation. There was no significant difference in the time from ciliary differentiation to the time of use in experimentation (Wilcoxon matched-pair signed-rank test, $p = \text{NS}$).

Epithelial barrier permeability measurements

Transepithelial electrical resistance (TER) was measured as an indicator of epithelial barrier integrity using a horizontal Ussing chamber (Harvard Apparatus, Holliston, MA). Confluent cell monolayers on snapwell permeable supports were allowed to equilibrate in the chamber for 15 minutes, and resistance values were continuously measured for an additional 15 minutes. Mean values were computed and recorded for each set of cells. Due to differences in *in vitro* cell density and TER variations in primary cultured sinonasal epithelial cells, exclusion criteria for both groups were defined as a TER less than 200 ohms $\times$ cm² or greater than 800 ohms $\times$ cm².

Antibodies and reagents

A panel of tight junction and adherens junction proteins was analyzed with the following antibodies: anti-occludin, anti-claudin-1, and anti-claudin-2 (Invitrogen), anti-E-cadherin and anti-ZO-1 (Sigma-Aldrich). The monoclonal antibody against JAM-A used in this study has been described previously.¹³ Alexa-488 and Alexa-546-conjugated secondary antibodies were obtained from Invitrogen; horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Immunoresearch Laboratories. All other immunofluorescence staining and Western blotting reagents were obtained from Sigma-Aldrich.

Immunofluorescence labeling and confocal microscopy

Immunofluorescence labeling and confocal laser microscopy was used to examine tight and adherens junction protein expression and localization. Samples were fixed and permeabilized in 100% ethanol for 20 minutes at –20°C. All subsequent steps were

performed at room temperature. Samples were washed with HBSS with Mg^{2+} and Ca^{2+} (HBSS+) and blocked in HBSS+ with 3% bovine serum albumin for 1 hour. Primary antibodies were diluted in blocking buffer and incubated with samples for 1 hour. Primary antibody concentrations included the following: anti-claudin-1 (1:250), anti-occludin (1:500), anti-JAM-A (1:100), and anti-E-cadherin (1:100). Samples were washed in HBSS+ and incubated with Alexa Fluor secondary antibodies diluted 1:500 in blocking buffer for 1 hour. After a final wash, samples were mounted on slides with p-phenylenediamine anti-quench reagent and sealed.

Stained cell layers were examined using a Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging Inc., Thornwood, NY) coupled to a Zeiss 100M Axiovert with a 63× Pan-Apochromat oil lens. Fluorescent dyes were imaged sequentially to eliminate cross talk between channels, and images were processed with Zeiss LSM5 image browser software. Images shown are representative of multiple images taken per slide.

Cell lysis and Western immunoblotting

Cells were washed twice with HBSS+ to remove media and scraped into RIPA buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, pH 7.4) with a protease inhibitor cocktail (Sigma-Aldrich). To ensure complete cell lysis, samples were sonicated on ice and rotated for 30 minutes at 4°C. Samples were centrifuged (1,000 × g, 5 minutes then 4500 × g, 10 minutes) to remove nuclear debris, and supernatants were normalized for protein concentration using a bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA). Lysates were boiled in SDS sample buffer containing 10% 2-mercaptoethanol for 10 minutes, and equal concentrations of protein were loaded onto SDS-polyacrylamide gels for Western blot analysis.

Statistics

TER data from AFRS and control samples was analyzed for significance by a Wilcoxon matched-pair signed-rank test with significance set at $P < 0.05$. Data shown are representative of 12 separate recordings from culture-age matched pairs of AFRS and control cell layer inserts.

Results

Transepithelial electrical resistance (TER) was measured as an indicator of epithelial barrier integrity. The resistance values of twelve individual populations of cells from each experimental group were measured continuously over a period of 30 minutes with a horizontal Ussing chamber. Cultured sinonasal AFRS cell layer samples displayed a significant decrease in TER, and thus an increase in epithelial permeability, when compared to non-inflammatory control cell samples ($P = 0.006$, $n = 12$). Figure 1A shows the mean TER value from each of the twelve sets of AFRS and control cells which were age-matched by length of time in ALI culture and the presence of beating cilia. As shown in Figure 1B, the overall mean AFRS TER calculated from all twelve sets was 296 ± 89 ohms $\times$ cm², a decrease of 41% from the control value of 503 ± 134 ohms $\times$ cm².

After TER recordings were completed, cells were processed for Western blotting or immunofluorescence labeling/confocal microscopy to analyze a panel of AJC proteins. Figure 2A shows the relative expression levels of tight junction proteins from 4 representative sets of non-inflammatory control and AFRS cells, and figure 2B displays the corresponding densitometric immunoblot analysis normalized to actin levels and expressed as a percentage of the total signal intensity. Due to the small sample size in the Western

immunoblot experiment, we chose not to perform statistical analysis of densitometric values. Protein expression values are slightly different in each set due to the inherent variability in primary cell culture, but general trends can be appreciated. Decreased expression of the tight junction-associated proteins JAM-A and occludin were seen in AFRS, as well as increased expression of a pore-forming, “leaky” tight junction protein claudin-2 when compared to control (Figure 2A). Densitometry confirmed a decrease of 50% in JAM-A and occludin, and an increase of 90% in claudin-2 when comparing AFRS to control (Figure 2B). There were no changes in ZO-1 or claudin-1 expression (data not shown). We also observed a large decrease in the expression of the adherens junction protein E-cadherin in AFRS cells (75% decrease compared to control). (Figure 2A).

Immunofluorescence labeling and confocal microscopy results coincided with the trends observed by Western blot analysis of protein expression. JAM-A, occludin, and E-cadherin protein expression was decreased throughout AFRS cell culture layers compared to control (Figure 3). Additionally, claudin-2 localized to an intracellular pool in control cell monolayers and to cell-cell junctions in AFRS. Collectively, these data indicate altered composition of the epithelial tight and adherens junctions in the setting of AFRS.

Discussion

Epithelial barrier function is a key homeostatic control mechanism for regulating paracellular fluid transport, immune sensitization, and disease progression in numerous organ systems. We sought to characterize epithelial permeability and AJC protein expression in cultured primary sinonasal epithelial cells isolated from patients with AFRS, a distinct subtype of CRS characterized by Th2 cytokine overexpression and the development of exuberant nasal polyposis.

Transepithelial electrical resistance was measured as an indicator of paracellular permeability, with decreases in TER correlating with increased permeability. Traditional TER recordings are performed manually with hand-held electrodes placed into the apical and basolateral chambers of permeable cell supports. This method has been validated as a standard technique for assessing epithelial barrier integrity, though repeated measurements can vary significantly depending on electrode placement within the chambers. In the present study, cells were grown to confluence on permeable supports and sealed within horizontal Ussing chambers for continuous TER measurement at one-second intervals over a period of 30 minutes. By recording the TER from each experimental group simultaneously with a common set of electrodes, a large number of highly objective data points were generated. After calculating the mean TER values from 12 cell layer inserts each group, we observed a significant resistance drop in AFRS cell layers compared to non-inflammatory controls.

Increased epithelial permeability can be caused by gross mechanical defects in the epithelial barrier and by changes in AJC permeability mediated by altered protein expression. Though inflammatory cytokines can induce apoptosis and subsequent epithelial barrier degradation, their effects on paracellular permeability have been shown to primarily involve AJC protein internalization.¹⁴ Microscopic evaluation of our cell layers did not show any transient denuded zones in the epithelium, and therefore the possibility of ulceration or erosion facilitating the observed phenotype was ruled-out. Additionally, a lesion-mediated mechanism would have had extreme effects on TER recordings, reducing transepithelial resistance to nearly zero, which was not observed in any of the sample sets. Biochemical data generated by Western blot and immunofluorescence microscopy confirmed that the increased epithelial permeability seen in the AFRS samples was due to decreased expression of the tight junction-associated proteins occludin and JAM-A, and increased expression of claudin-2, a “leaky” pore-forming member of the claudin family.

The observed decreases in occludin and JAM-A expression may be caused by residue modifications preventing interactions with scaffolding proteins at the apical surface such as ZO-1, or by increased protein turnover. Farshori et al. demonstrated that occludin phosphorylation at C-terminal residues increases protein internalization to a cytosolic pool, disrupting tight junction structure and function.¹⁵ Immunofluorescence microscopy images shown in Figure 3 indicate a decrease in occludin and JAM-A expression at the plasma membrane in AFRS cell layers compared to controls. The data do not indicate an appreciable increase in cytosolic protein levels in the setting of AFRS. Though this mechanism cannot be completely ruled-out without further biochemical analysis, we must consider the possibility that occludin and JAM-A protein degradation is increased at the expense of synthesis and membrane integration.

It is well known that tight junction proteins are continually recycled during normal junction maintenance and homeostasis. It is, however, unclear how “tight” claudins are exchanged for pore-forming, “leaky” claudins in the context of inflammation. The mechanism is proposed to involve changes in the type of claudin expressed as well as the density of pore-forming claudins integrated into the membrane.¹⁶ For example, variations in cell line-specific paracellular permeability have been attributed to claudin protein composition and the number of intercellular pores.¹⁷ Claudin-2 has been identified among the pore-forming claudins as a specific paracellular channel for small cations, and overexpression has resulted in decreased TER and tight junction strand irregularities.^{18,19}

Given the observed increases in claudin-2 expression along with decreases in JAM-A and occludin, an overarching mechanism involving protein alteration at the transcriptional level would be a powerful explanation for tight junction remodeling in AFRS. One form of transcriptional modification that has been associated with Th2 cytokine-induced epithelial cell dysfunction is epigenetic DNA methylation and histone acetylation.^{20,21} Following an environmental stimulus, transcription factors bind to DNA and increase the acetylation of core histones, leading to chromatin decondensation, and subsequent transcription. An equally possible explanation involves translational or post-translational modification that increases internalization or delays recycling. We can only speculate on possible mediators until future studies examining mRNA transcripts are performed. The transcription factor NFkB is a likely candidate, as it has been shown to disrupt TER and occludin and claudin expression in the setting of pro-inflammatory cytokine treatment.²²

Multiple studies have demonstrated epithelial barrier defects as a consequence of upregulated Th1 cytokine expression in intestinal and respiratory mucosa.^{7,22–25} Previous work conducted in our laboratory has shown that TNF- α and IFN- γ decrease TER and epithelial intercellular junctional protein expression in sinonasal epithelium.^{26,27} Th2 cytokines are traditionally recognized for promoting IgE production and an eosinophilic response in atopic patients. Indeed, sinus epithelial tissue specimens from AFRS patients display significantly more total IgE as well as antigen-specific IgE compared with non-inflammatory controls.²⁸ Recent literature has indicated that Th2 cytokines may also mediate mucosal reactions and modify tight junction protein expression in disease states. IL-13 has been shown to affect cell migration and wound healing after intestinal ulceration in inflammatory bowel disease.²⁹ In addition, IL-13 has been implicated in barrier dysfunction by increasing expression of claudin-2 in T84 model intestinal epithelial cells.³⁰ *In vitro* data supports the role of IL-4 in increasing permeability in airway and intestinal epithelial cells, causing decreased expression of ZO-1 and occludin, and increased expression of claudin-2.^{31,32}

An important point to consider is that the primary cells cultured in this study were disassociated from the inflammatory milieu of the nasal cavity and underlying endothelium

for 6–8 weeks before experiments were conducted. Though epithelial cells secrete cytokines, their relative contribution would be expected to be significantly lower than that of dedicated immune cells, which were largely selected-out during specimen processing and would not have persisted in ALI culture. The observed phenotype may thus involve epithelial perpetuation of Th2 cytokine release, or a sustained intracellular response to the cytokine exposure that occurred *in vivo*. Future studies measuring cytokine production from AFRS and non-inflammatory control sinonasal ALI-cultured cells may be able to further characterize the disease mechanism. We propose that an initial insult to the epithelium and fungal allergen exposure upregulate Th2 cytokine expression, resulting in modulation of specific tight junction-associated proteins, decreased barrier function, and a cycle of increased allergen exposure and inflammation.

Conclusions

Primary sinonasal cells isolated from patients with AFRS displayed increased epithelial permeability and altered expression of tight junction-associated proteins. These results indicate a possible mechanism for the phenotype observed in AFRS, wherein patients are pre-disposed to chronic mucosal edema and inflammation. Given that these cells were not incubated with inflammatory cytokines *in vitro*, it is postulated that the AFRS phenotype represents a retained modification in protein expression from the *in vivo* phenotype.

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Figure 1.

A. TER measurements from 12 sets of cultured AFRS and non-inflammatory control primary sinonasal epithelial cells. Cells were paired based on monolayer maturity, which was judged by length of time from development of beating cilia. **B.** Overall mean TER values calculated from all cell sets. Mean AFRS TER was $296 \pm 89 \text{ ohms} \times \text{cm}^2$, a decrease of 41% from the control value of $503 \pm 134 \text{ ohms} \times \text{cm}^2$ (* $P < 0.01$). Data are graphed as mean $\pm$ SE ($n=12$).

Figure 2.

A. Western immunoblots for tight and adherens junction-associated proteins in AFRS and non-inflammatory control primary sinonasal epithelial cells. JAM-A, occludin, and E-cadherin are decreased in AFRS compared to control. Claudin-2, a “leaky,” pore-forming claudin is increased in AFRS. **B.** Densitometric analysis of Western immunoblots. Data were normalized to actin levels and are presented as mean signal intensity $\pm$ SE as a percentage of the total signal ($n=4$).

Figure 3.

Representative immunofluorescence staining in AFRS and non-inflammatory control cultured sinonasal epithelial cells. JAM-A, occludin, and E-cadherin signal intensity at cell-cell junctions is diminished in AFRS cell layers compared to controls. Claudin-2 localizes to a nonfunctional cytosolic pool in control cell layers, and to cell-cell junctions in AFRS.