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IL-4 and IL-13 Compromise the Sinonasal Epithelial Barrier and Perturb Intercellular Junction Protein Expression

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Abstract

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Introduction—Altered expression of epithelial intercellular junction proteins has been observed in sinonasal biopsies from nasal polyps and epithelial layers cultured from nasal polyp patients. These alterations comprise a “leaky” epithelial barrier phenotype. We hypothesize that Th2 cytokines IL-4 and IL-13 modulate epithelial junction proteins thereby contributing to the leaky epithelial barrier.

Methods—Differentiated primary sinonasal epithelial layers cultured at the air-liquid interface were exposed to IL-4, IL-13, and controls for 24 hours at 37°C. Epithelial resistance measurements were taken every 4 hours during cytokine exposure. Western blot and immunofluorescence staining/confocal microscopy were used to assess changes in a panel of tight and adherens junction proteins. Western blot densitometry was quantified with image analysis.

Results—IL-4 and IL-13 exposure resulted in a mean decrease in transepithelial resistance at 24 hours to 51.6% (n=6) and 68.6% (n=8) of baseline, respectively. Tight junction protein JAM-A expression decreased 42.2% with IL-4 exposure (n=9) and 37.5% with IL-13 exposure (n=9). Adherens junction protein E-cadherin expression decreased 35.3% with IL-4 exposure (n=9) and 32.9% with IL-13 exposure (n=9). Tight junction protein claudin-2 showed more variability but had a trend toward higher expression with Th2 cytokine exposure. There were no appreciable changes in claudin-1, occludin, or ZO-1 with IL-4 or IL-13 exposure.

Conclusion—Sinonasal epithelial exposure to Th2 cytokines IL-4 and IL-13 results in alterations in intercellular junction proteins, reflecting increased epithelial permeability. Such changes may explain some of the phenotypic manifestations of Th2-mediated sinonasal disease, such as edema, nasal discharge, and environmental reactivity.

Keywords

Epithelial cell; cytokine; inflammation; interleukin 4; IL-4; interleukin 13; IL-13; Th2 inflammation; rhinosinusitis; allergic fungal sinusitis

INTRODUCTION

Chronic rhinosinusitis (CRS) has a prevalence of 10.9% in Europe and 12.6% in the United States.^{1–3} Nasal polyposis occurs in approximately 20% of CRS patients is one of the most frequent indications for endoscopic sinus surgery.^{4,5} Nasal polyposis is a phenotypic manifestation that may accompany various disease etiologies, such as cystic fibrosis, aspirin exacerbated respiratory disease (AERD), allergic fungal rhinosinusitis (AFRS), and others. The inflammatory pattern of nasal polyposis relates to the underlying disease entity. For example, nasal polyps from atopic individuals demonstrate a T-helper 2 (Th2) skewed profile, including interleukin (IL)-4, IL-5, IL-6, IL-25, IL-33, eotaxin-3, and abundant eosinophils.^{6–8} In contrast, nasal polyps from those without atopy may show a Th1 skewed profile and higher production of interferon (IFN)- γ .⁸ Nasal polyps from cystic fibrosis patients exhibit neutrophilic inflammation, whereas AFRS nasal polyps have copious eosinophils and increased IL-5.^{9–12}

The epithelial barrier is increasingly recognized as a modulator and target of inflammatory processes. Located in the apical pole of the lateral membrane of polarized epithelial cells, the apical-junctional complex (AJC) is a selectively permeable barrier comprised of the tight

and adherens junctions, which regulates paracellular transport and cell-cell adhesion. Proteins in the apical tight junction include claudin proteins, zonula occludens-1 (ZO-1), occludin, and junctional adhesion molecule-A (JAM-A). Paracellular permeability in epithelial tight junctions is dependent on protein composition. Increased presence of pore-forming claudins (claudin-2, -10, -15, and -16) confers a more “leaky” barrier, while increased “tight” claudins (claudin-1, -4, -5, -8, -11, -14, and -19) decrease paracellular permeability.¹³ The adherens junction, subjacent to the tight junction, contains E-cadherin and members of the catenin protein family. The adherens junction facilitates cell-cell recognition and adhesion.

The epithelial barrier is sensitive to inflammatory cytokines and surface antigens. In a simplified explanation, inflammatory mediators or surface antigens alter distribution of AJC proteins, thereby compromising the epithelial barrier function. Multiple chronic inflammatory disease states exhibit epithelial permeability and AJC defects. Asthma, chronic bronchitis, and cystic fibrosis lung disease are associated with abnormalities of tight junction structure and function.^{14–16} Inhaled environmental antigens alter tight junction protein expression *in vitro* in lower-airway respiratory epithelium, potentially causing increased antigen sensitization and allergic response.^{17,18} In inflammatory bowel disease, changes in paracellular permeability and alterations in levels of occludin and claudin-2 occur in intestinal epithelium with cytokine exposure and chronic mucosal inflammation.^{19,20} Our prior work demonstrates decreased epithelial expression of tight junction proteins claudin-1 and occludin and desmosomal proteins DSG-2 and DSG-3 in patients with various etiologies nasal polyposis.^{21,22} We have also shown that sinonasal epithelial cultures from AFRS patients have decreased transepithelial resistance (TER), decreased expression of occludin and JAM-A, and increased expression of claudin-2.²³

In this study, we examined the profile of specific tight and adherens junction proteins in a characteristic Th2-mediated atopic nasal polyposis phenotype, AFRS. We also evaluated the influence of specific Th2 cytokines found in nasal polyp disease IL-4^{11,24,25}, IL-5^{11,26–28}, and IL-13^{11,28} on sinonasal epithelial resistance and AJC protein expression *in vitro*. We hypothesized that AFRS polyps would demonstrate alterations in junctional protein expression consistent with a “leaky” epithelial barrier, and further that Th2 cytokine exposure would decrease transepithelial electrical resistance (TER) in sinonasal epithelial cell layers *in vitro* and contribute to altered tight and adherens junction protein expression, consistent with an increased permeability phenotype.

METHODS

Patient characteristics and tissue collection

Control participants were undergoing endoscopic transnasal skull base surgery and were without significant clinical or radiographic evidence of CRS. Control participants were free of active allergy symptoms at the time of tissue collection, although a history of mild seasonal allergic rhinitis did not require exclusion. AFRS participants were undergoing endoscopic sinus surgery as part of the routine care of their disease. Patients in the AFRS group fulfilled at least 4 of 5 of the 1994 Bent and Kuhn criteria.²⁹ Exclusion criteria were:

cystic fibrosis, immune deficiency, autoimmune conditions affecting the sinonasal cavities, granulomatous disorders, AERD, and oral steroid use 7 days preoperatively.

Tissue for immunofluorescence or protein extraction was taken from the ethmoid or sphenoid sinuses in control patients, nasal polyps in AFRS patients, and inferior turbinates (qualitative internal comparison) in both groups. Control sinus tissue for cell culture was biopsied from the ethmoid or sphenoid cavities. No cell culture specimens were taken from the nasal cavity or turbinates. Cell culture was performed only from non-inflammatory control patients so that the effects of Th2 cytokine exposure could be isolated without undue influence of source patient inflammatory disease.²³ Emory University Institutional Review Board granted study approval. All patients gave written informed consent.

Primary sinonasal air-liquid interface (ALI) culture

Cell culture techniques have been described previously.²³ In brief, sinus tissue was placed in RPMI 1640 media (Invitrogen, Carlsbad, CA) with antibiotic/antimycotic (Invitrogen, Carlsbad, CA) and digested with *Streptococcus griseus* protease (Sigma-Aldrich, St. Louis, MO). Large tissue pieces were removed, supernatant was centrifuged (5 minutes, 101g), 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, Carlsbad, CA), and nystatin (Sigma-Aldrich, St. Louis, MO). Fibroblasts were removed by incubating in a tissue culture-treated petri dish at 37°C for 2 hours. Epithelial cell rich supernatant was transferred to collagen-coated T75 culture flasks (Corning, Corning, NY) and grown in BEGM at 5% CO₂, 95% humidity, 37°C. BEGM media was changed every 48–72 hours.

At approximately 85% confluence, cells were released with trypsin-EDTA (Invitrogen, Carlsbad, CA), centrifuged (5 minutes, 101g), resuspended in BEGM, seeded onto collagen-coated Transwell inserts of 6.5 or 24 mm diameter (Corning, Corning, NY), and maintained with BEGM on the apical and basal surfaces. At confirmation of confluence by light microscopy, apical media was removed and cells were fed from the basal chamber only with air-liquid interface (ALI) media, consisting of a 50:50 mixture of BEBM and DMEM high glucose (Invitrogen, Carlsbad, CA), along with BEBM SingleQuots, antibiotic/antimycotic, retinol, and bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Polarization and differentiation of epithelial cell layers was confirmed by visualization of beating cilia under phase-contrast light microscopy.

Th2 cytokine exposure

Confluent, polarized, differentiated, ciliated primary sinonasal epithelial cell cultures were exposed to chosen Th2 cytokines during transepithelial resistance measurements and for 24 hours prior to assessment of junctional protein changes. Cytokines were added to ALI media in the basal Transwell chamber at the following final concentrations: recombinant human IL-4 (high concentration 50 ng/ml, low concentration 10 ng/ml; R&D Systems, Minneapolis, MN)^{30,31}, recombinant human IL-5 (high concentration 200 ng/ml, low concentration 40 ng/ml; R&D Systems, Minneapolis, MN)³², recombinant human IL-13 (high concentration 50 ng/ml, low concentration 10 ng/ml; R&D Systems, Minneapolis,

MN)³⁰, IFN- γ (100 IU/ml, Genentech, San Francisco, CA) and recombinant human TNF- α (500 ng/ml, BioVision, Mountain View, CA) combination, serving as a positive control.

Sinonasal epithelial resistance measurements

At baseline and every 4 hours following cytokine exposure, TER measurements were taken in 6.5 mm diameter sinonasal epithelial cell culture Transwells with the EVOM² Epithelial Voltmeter (World Precision Instruments, Sarasota, FL). The EVOM² probe was cleaned in 70% ethanol for 15 minutes, air dried for 15 minutes, and equilibrated in ALI media for 10 minutes prior to use. ALI media was placed on the apical surface of the Transwell inserts for 15 minutes for equilibration as well. TER measurements were taken in triplicate and averaged. Resistance was calculated according to the EVOM² package insert, as $R_{\text{total}} - R_{\text{blank}} = R_{\text{tissue}}$, where R_{total} is the resistance reading from the EVOM² output, R_{blank} is the resistance measurement of an empty Transwell insert, and R_{tissue} is the true resistance of the epithelial layer. By convention, tissue resistance measurements were converted to unit area resistance using the formula $[R_{\text{tissue}} (3.14) (\text{diameter}^2)]/4 = \text{resistance in ohms}\cdot\text{cm}^2$. Resistance measurements over time were tabulated as a fraction of the baseline unit area resistance for each individual well.

Antibodies and reagents

Tight and adherens junction proteins evaluated in this study were: claudins -1 and -2, JAM-A, occludin, ZO-1, and E-cadherin. The chosen proteins were a result of a preliminary mRNA array identifying transcripts for various AJC component proteins, as well as our prior experiments and literature reports. Antibodies used were: anti-claudin-1, anti-claudin-2, anti-ZO-1, anti-occludin, Alexa-488 and Alexa-546 conjugated secondary antibodies (Invitrogen, Carlsbad, CA); anti-E-cadherin (Sigma-Aldrich, St. Louis, MO); anti JAM-A (Western blot; BD Biosciences, San Jose, CA); and horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The monoclonal antibody against JAM-A used in immunofluorescent labeling and confocal microscopy in these experiments has been described previously.³³ Unless stated, all other immunofluorescence staining and Western blotting reagents were obtained from Sigma-Aldrich.

Immunofluorescence labeling and confocal microscopy

Tight and adherens junction protein expression and localization was assessed via immunofluorescence labeling and confocal laser microscopy. Surgical tissue biopsies were snap frozen in Tissue Tek OCT (Sakura, Torrance, CA) and maintained at -80°C . Six μm sections were cut, placed onto positively charged slides, and fixed in absolute ethanol at -20°C for 20 minutes. All remaining steps were performed at room temperature. Samples were washed with Hank's Balanced Salt Solution with Mg^{2+} and Ca^{2+} (HBSS+) and blocked in 5% normal goat serum. Samples were then incubated with primary antibodies for 1 hour (diluted in blocking buffer), washed in HBSS+, incubated with Alexa-Fluor secondary antibodies for 1 hour (1:500 in blocking buffer), again washed in HBSS+, and incubated with To-Pro 3-iodide nuclear stain for 5 minutes (1:1000 in blocking buffer; Invitrogen, Carlsbad, CA), followed by a final HBSS+ wash. Primary antibody concentrations were: claudin-1 (1:250), claudin-2 (1:250), occludin (1:500), JAM-A (1:100),

ZO-1 (1:100), and E-cadherin (1:100). P-phenylenediamine anti-quench reagent was added, and slides were sealed.

Immunofluorescence staining of sinonasal epithelial cell culture samples was undertaken according to the steps above, except as detailed here. Transwell inserts were washed with HBSS+, fixed in absolute ethanol (or a 50:50 mixture of methanol and acetone for claudin staining) for 20 minutes at -20°C and blocked with 3% bovine serum albumin. Transwell filters were cut and placed onto slides for mounting and confocal microscope visualization. Primary antibody concentrations were adjusted to allow appropriate confocal visualization of junctional proteins in cultured sinonasal epithelial layers.

Slides were examined with a Zeiss LSM510 laser scanning confocal microscope (Zeiss Microimaging Inc., Thornwood, NY) coupled to a Zeiss 100M Axiovert with a 40X or 63X Pan-Apochromat oil lens. Fluorescent dyes were imaged sequentially to eliminate cross talk between channels. Images were processed with Zeiss LSM5 image browser software. For quantitative pixel analysis of protein staining on sinonasal epithelial biopsy sections, the epithelial area was outlined on the Image J image analysis program. All epithelium on a given slide was outlined and analyzed. Pixel intensity was noted for the outlined region and then divided by the outlined area (Figure 1). Pixel intensity per area difference was compared statistically amongst cytokine exposure groups for each protein.

Protein isolation and Western blotting

Sinonasal biopsy specimens were snap frozen and stored in cryovials at -80°C for protein extraction. Samples were thawed and lysed with 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, St. Louis, MO). Tissue was homogenized on ice and placed on a rotator at 4°C for 1 hour. Tissue pieces and nuclei were centrifuged at 12,000g for 15 minutes at 4°C . The supernatant was again centrifuged at the same settings and time. The final supernatant was then quantified for protein concentration by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA).

Following 24-hour cytokine incubation, sinonasal epithelial cell culture cells were washed with HBSS+ and scraped into RIPA buffer with protease inhibitors. Samples were sonicated on ice and incubated for 10 minutes at 4°C . Nuclear debris was removed from samples by centrifugation (1,000g for 5 minutes, then 4,500g for 10 minutes), and sample protein concentrations were normalized by bicinchoninic acid assay.

Samples were boiled in SDS sample buffer with 10% 2-mercaptoethanol for 10 minutes, run on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes for Western blotting. Protein loading control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). To ensure protein alterations were not the result of cell death, apoptosis marker poly-ADP ribose polymerase (PARP) cleaved product level was assessed by Western blot.

Relative quantification of protein densitometry for cytokine exposure experiments was performed with the Image J program. Each protein was normalized to the GAPDH loading

control for that experiment. Protein levels were collated across triplicate measurements for each of 3 experimental runs to provide representative protein densities.

Statistical analysis

Statistical calculations were performed with IBM SPSS version 19.0 (Chicago, IL). Pixel intensity on sinus biopsy specimens was performed with Mann-Whitney U pairwise comparisons between disease groups (control sinus v. AFRS sinus). Statistical significance was set at $p < 0.05$. The Western blot experiments on sinonasal biopsy specimens were performed as a confirmatory method to validate the results of the initial immunofluorescence analysis. Statistical analysis was not performed on the biopsy specimen Western blot data.

Descriptive statistics are provided for *in vitro* Western blot densitometry experiments. Due to the repeated measures design, involving 3 sets of experiments each performed in triplicate, significance testing was deemed inappropriate for this analysis.

RESULTS

Tight junction and adherens junction protein expression sinonasal biopsy specimens

In order to determine the staining pattern for chosen sinonasal epithelial tight and adherens junction proteins, as well as any significant difference in these proteins by disease process (control v. AFRS), pixel density per epithelial area analysis was undertaken. Each protein was stained by immunofluorescence labeling of 9 control sinus and 9 AFRS sinus tissue sections. Inferior turbinate tissue served as a qualitative internal comparison in these experiments, as inferior turbinate tissue does not traditionally form polyps.

Immunofluorescence staining of sinonasal epithelial biopsies resulted in stain largely concentrated along the apical surface and lateral cell membranes in the expected region of the AJC. Pixel density analysis revealed a significant increase in claudin-2 in AFRS sinus versus control sinus tissue ($p = 0.015$). These results indicate that AFRS sinus tissue has a tendency toward a more leaky epithelial barrier versus non-inflamed control sinus tissue. These results are supported by Western blotting of claudin-2 in representative tissue samples. (Table 1, Figure 2). No significant differences in sinus tissue pixel analysis were seen between AFRS and control sinus tissue for JAM-A, E-cadherin, occludin, ZO-1, or claudin-1.

Transepithelial electrical resistance (TER) in sinonasal epithelial culture following Th2 cytokine exposure

To further evaluate epithelial permeability, we sought to test the *in vitro* effects of specific Th2 cytokines IL-4, IL-5, and IL-13 that have been observed in the mucosa of patients with nasal polyposis and atopy. Therefore, TER measurements were obtained with Th2 cytokine exposure. Mean ($\pm$ standard error) baseline TER measurement across all culture wells prior to cytokine exposure was 500.47 ± 46.40 ohms-cm². No wells were used with baseline TER less than 250 ohms-cm². Control wells (no cytokine exposure, $n = 5$) showed a mild decrease in TER over the 24-hour cytokine exposure time course with 24-hour mean TER at

81.2±11.5% of baseline values. This TER decrease in control wells was likely due to manipulation of the ALI cell layer every 4 hours by placement of apical media for TER measurement and subsequent removal of the apical media for continued incubation in the interim. However, this protocol was deemed necessary as leaving the apical media in place for the full 24 hours resulted in poor cell morphology in prior trials.

At 24 hours of cytokine exposure, the positive control IFN γ -TNF α exposure demonstrated mean TER at 64.1±10.6% of baseline values (n=6). (Figure 3a) IL-4 exposure had the most profound effect on TER of all Th2 cytokines tested, with the 50 ng/ml high concentration exhibiting mean TER at 24 hours of 51.6±7.2% of baseline values (n=6) and the 10 ng/ml low concentration demonstrating mean 24-hour TER of 57.2±11.9% of baseline values (n=5). (Figure 3b) Less consistent TER results were seen for IL-5. The 200 ng/ml high concentration exposure of IL-5 resulted in 24-hour mean TER of 80.5±10.6% of baseline values (n=5), and the 40 ng/ml low concentration exposure showed mean TER at 24 hours of 68.5±11.5% of baseline values (n=5). (Figure 3c) Finally, IL-13 50 ng/ml high concentration exposure demonstrated 24-hour mean TER at 68.6±5.8% of baseline values (n=8) and the 10 ng/ml low concentration exhibited 24-hour mean TER of 58.6±4.3% of baseline values (n=5). (Figure 3d) These results indicate that exposure to Th2 cytokine for 24 hours, especially IL-4, decreases TER in sinus epithelium.

The effect of IL-4 exposure on sinonasal epithelial tight and adherens junction protein expression *in vitro* was further tested in subsequent experiments via Western blot and immunofluorescence labeling/confocal microscopy. Along with IL-4 exposure, IFN γ -TNF α control and IL-13 (shared receptor complex subunits with IL-4 receptor) were also tested for effects on tight and adherens junction protein expression.^{34,35} IL-5 was not further tested for effects on tight and adherens junction protein expression *in vitro* as the TER results for this cytokine were inconsistent and not concentration dependent. In addition, availability of tissue resources limited the number of cytokines and replicates that could be employed in additional experiments.

Tight and adherens junction protein expression in sinonasal epithelial culture following Th2 cytokine exposure

The effect of IL-4 (50 ng/ml) and IL-13 (50 ng/ml) exposure on tight and adherens junction protein expression in sinonasal epithelial cell culture was performed to investigate if changes in these proteins could account for the increased epithelial permeability. Following 24-hour cytokine exposure, tight and adherens junction protein expression was assessed via Western blot analysis and associated densitometry measurements. Densitometry results presented are the combination of 3 separate experiments, each performed in triplicate. Each individual protein densitometry reading was normalized to the GAPDH loading control for that sample. Values are presented as mean ± standard error.

Tight junction protein JAM-A decreased 42.2±16.7% with IL-4 exposure (n=9) and 37.5±12.3% with IL-13 exposure (n=9). Adherens junction protein E-cadherin decreased 35.3±9.0% with IL-4 exposure (n=9) and 32.9±11.5% with IL-13 exposure (n=9). In keeping with a more permeable epithelial barrier phenotype, “leaky” tight junction protein claudin-2 increased 27.0±27.9% with IL-4 exposure and 53.2±41.6% with IL-13 exposure.

However, the Western blots for claudin-2 were somewhat less reliable than those for other tight and adherens junction proteins. The pooled densitometry results for claudin-2 blots were from a total of 5 samples rather than 9, and the data variability for claudin-2 is substantially more than for the other proteins tested. Therefore, the claudin-2 results should be interpreted in light of these issues. There were no notable changes in claudin-1 (n=9), occludin (n=8), or ZO-1 (n=9) with IL-4 or IL-13 exposure. (Figure 4a, b) Based upon the levels of PARP cleaved product (no difference across exposures), the tight and adherens junction protein changes with cytokine exposure were not the results of cell death. Immunofluorescence staining and confocal microscopy images supported these findings, with decreases in JAM-A and E-cadherin following IL-4 and IL-13 exposure. (Figure 4c) The control images for JAM-A and E-cadherin both exhibited intense, continuous staining along the cell borders. In contrast, the IL-4 and IL-13 exposed cell layers demonstrated decreased staining intensity and disrupted continuity along the cell membrane for JAM-A and E-cadherin. There were no changes in occludin, ZO-1, or claudin-1 staining across cytokine exposure groups. Claudin-2 staining, as demonstrated in Figure 4d, was much less intense overall and somewhat variable. However, there were areas of apparent concentration in claudin-2 along the cell-cell interfaces with IL-4 and IL-13 exposure.

DISCUSSION

The experimental results presented here support the concept that AFRS polyp epithelium is comprised of a more “leaky” barrier, with evidence of increased claudin-2, compared to control sinus tissue. Further, *in vitro* exposure of cultured sinus epithelium to Th2 cytokines IL-4 and IL-13 results in lower TER and associated decreased expression of AJC proteins JAM-A and E-cadherin, along with increased expression of claudin-2. Taken together, these findings support the role of Th2 cytokines in perpetuation of increased epithelial permeability in AFRS, a characteristic subset of polypoid disease in CRS classically associated with atopy.

Epithelial barrier compromise allows access to the subepithelial tissue, resulting in an inflammatory response in some individuals. Decreased tight junction claudin-1 and occludin in bronchial epithelial cells has been shown with house dust mite antigen Der p1 exposure.¹⁷ Der p1, a cysteine protease, also cleaves ZO-1 and occludin in respiratory epithelial cells.³⁶ Further, our group has shown decreases in claudin-1 and JAM-A upon exposure to recombinant Der p1 in preliminary sinonasal epithelial culture experiments.³⁷ These results suggest that certain antigens may directly alter the respiratory epithelial barrier by disrupting the AJC.

The respiratory epithelium also exhibits changes as a result of exposure to inflammatory mediators. Ahdieh *et al.* demonstrated decreased TER and decreased ZO-1 and occludin expression in IL-4 and IL-13 treated human lung epithelial cell lines.³⁰ Soyka *et al.* noted decreased trans-tissue resistance in CRS with nasal polyp (CRSwNP) biopsy specimens, decreased TER in CRSwNP *in vitro* cell layers, and decreased ZO-1 and occludin expression in CRSwNP sinonasal epithelial biopsy and culture specimens versus controls.³⁸ Soyka *et al.* also report decreased TER and tight junction disruption in sinonasal epithelial cell culture layers stimulated with IL-4 and IFN γ .³⁸ Previous work from our group has

demonstrated decreased TER, decreased occludin and JAM-A expression, and increased claudin-2 expression in sinonasal epithelial cultures from AFRS patients.²³

The results of the current study show some similarities to the previous literature, as well as some differences. First, in CRSwNP biopsy specimens, Soyka *et al.*³⁸ noted decreased ZO-1 and occludin protein and decreased claudin-4 and occludin mRNA. We have previously demonstrated decreases in claudin-1 and occludin in nasal polyp biopsies from a group of patients with heterogeneous nasal polyp etiology.²¹ While the specific tight junction protein changes across studies are different (claudin-2 increased in AFRS polyps [present study] and ZO-1, occludin, claudin-1, and claudin-4 decreased in CRSwNP [previously reported]), all of these patterns would be indicative of an increase in epithelial permeability *in vivo*. The increased claudin-2 in AFRS polyp biopsies identified in the present study is potentially different from previous findings due to the specificity of the AFRS patient population compared to heterogeneous groups of nasal polyp patients in the studies by Soyka *et al.*³⁸ and Rogers *et al.*²¹ Additional study of AJC protein changes specific to other etiologies of nasal polyposis (i.e. cystic fibrosis, aspirin exacerbated respiratory disease) may yield different results. Further, the patient groups are small in all of these studies, and the results should be interpreted accordingly.

Next, considering epithelial barrier and AJC protein changes *in vitro* with cytokine exposure, similar to Soyka *et al.*³⁸, we noted decreased TER in sinonasal epithelial cultures exposed to IL-4. We also noted decreased TER in cultures exposed to IL-13, which has common receptor subunits with IL-4. Whereas Soyka *et al.*³⁸ describe disruption of tight junction strands following IL-4 and IFN γ exposure, we specifically demonstrated decreases in JAM-A and E-cadherin expression with IL-4 and IL-13 stimulation. We also noted a trend toward increased claudin-2 expression in sinonasal epithelial cultures stimulated by IL-4 and IL-13, although this finding was more variable (indicated by larger standard error measurements in claudin-2 experiments [see Results section]).

In a recent paper by Saatian *et al.*³⁹ it was shown that IL-4 and IL-13 exposure reduced TER, increased FITC-dextran flux, and disrupted cell-cell contacts involving ZO-1, occludin, E-cadherin, β -catenin, and claudin-4. Claudin-2. was reported not to play a role in this process. The Saatian *et al.*³⁹ paper has a number of important differences versus our study. Saatian *et al.*³⁹ used a human bronchial epithelial line rather than primary sinonasal epithelial cells, performed experiments in submerged (not ALI) culture, and exposed cell layers to cytokines on the apical and basolateral surfaces. Nonetheless, this study highlights an interesting point about claudin-2. We previously showed that claudin-2 is increased in AFRS sinonasal epithelial cultures and associated with decreased TER.²³ Others have identified claudin-2 in human adenoid epithelium grown *in vitro* but not from *in vivo* biopsy samples,⁴⁰ whereas some indicate that claudin-2 is not present in sinonasal epithelium or does not have a substantial role in sinonasal AJC function.⁴¹ Based upon our results, it is possible that claudin-2 is present at low or variable levels in AFRS sinonasal tissue at baseline and higher levels *in vitro* or with Th2 cytokine exposure. While we have identified claudin-2 by Western blot and immunofluorescence, our experiments are preliminary, and this question is yet to be fully resolved.

The true physiology of AFRS is unknown. Nonetheless, taking into account the studies related to the sinonasal epithelial barrier and AFRS, we hypothesize that the initiation of epithelial barrier disruption is related to external antigen contact and disruption of AJC protein complexes, as well as the influence of Th2 cytokines. Dependent upon which areas of epithelial cells are being disrupted (i.e. those in contact with antigen versus those remote from direct antigen but still in the vicinity of Th2 cytokine exposure), Th2 cytokine exposure likely has the ability to influence and perpetuate increased epithelial barrier permeability in AFRS, leading to egress of fluid and inflammatory mediators to the external environment. These processes may be pathologic or physiologic, with probable variation amongst individuals.

The limitations of any study must be considered. The first limitation of this study is the use of immunofluorescence pixel density analysis for AJC protein quantification in biopsy samples. Immunofluorescence staining has inherent variability. In order to control this variability as much as possible, an equal number of control and AFRS samples were stained daily, staining protocols were followed precisely from day to day, and all confocal microscopy images were taken at the same settings for each protein stained. The increased claudin-2 results by immunofluorescence pixel intensity analysis were confirmed with Western blot. The second limitation is the use of primary sinonasal epithelial cell culture for *in vitro* TER and AJC protein expression experiments with Th2 cytokine exposure. While using primary culture more closely mimics the *in vivo* state versus cell lines, there is also inherent variability in working with primary cell culture. Therefore, TER experiments were performed with at least 5 samples per exposure group, and Western blot experiments were performed in triplicate and repeated 3 times (9 sets total). A third limitation is that TER measurements do not directly reflect macromolecular transepithelial permeability. FITC dextran flux experiments were considered as well. However, leaving apical media on the primary sinonasal epithelial ALI cultures for 12–24 hours, as indicated for FITC dextran experiments, resulted in undesirable changes in the cell morphology. Therefore, we complemented our TER results with investigations of AJC protein changes via immunofluorescence and Western blots. Finally, sample sizes are relatively small, which may have an impact upon detecting significant differences in protein analysis of sinonasal biopsy specimens. Nonetheless, these preliminary results are promising and warrant further confirmation and investigation.

These studies demonstrate that a leaky sinonasal epithelial barrier phenotype is present in AFRS and with Th2 cytokine exposure, but a precise mechanism by which this occurs is not yet clear. Whether these changes occur as a result of changes in protein expression, fluctuations in cell membrane turnover, modifications of protein folding, or an alternative mechanism have not been elucidated. These questions support the need for ongoing investigations in this area.

CONCLUSION

In these studies, an epithelial barrier with characteristics of increased permeability is demonstrated in nasal polyp biopsies from AFRS, a disease entity classically demonstrating a robust allergic phenotype and local expression of Th2 cytokines. By exposing sinonasal

epithelial layers to Th2 cytokines *in vitro*, we show a modest decrease in TER as a marker of increased epithelial permeability. We also demonstrate decreased expression of JAM-A and E-cadherin, following IL-4 and IL-13 exposure *in vitro*, providing a likely mechanism for the epithelial permeability changes. Taken together, these preliminary studies indicate that exposure of sinonasal epithelial cells to Th2 cytokines *in vivo* contributes to a leaky epithelial barrier in nasal polyp tissue. These findings may relate to *in vivo* manifestations of increased allergen exposure, tissue edema, and nasal discharge.

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Figure 1.

(a) Example black and white image of claudin-1 immunofluorescence staining on control sinus tissue epithelial biopsy. (b) Epithelial area outlined. Pixel analysis was performed in ImageJ analysis program, with pixel intensity per epithelial area compared amongst samples for each protein of interest.

Figure 2.

(a) Graph of pooled pixel intensity per unit area analysis on sinus tissue biopsies. N=9 per group. AFRS = allergic fungal rhinosinusitis. * denotes significant difference ($p=0.015$) between control and AFRS sinus tissue groups. Error bars = standard error. (b) Example immunofluorescence photographs of claudin-2 staining [green], which exhibited significantly higher pixel intensity per area in AFRS sinus than in control sinus. Especially note increased staining along the apical surface. For qualitative comparison, an example

immunofluorescence photo set of claudin 2 inferior turbinate staining and JAM-A staining in sinus and inferior turbinate tissues [red] are also included. There was no significant difference in pixel intensity per area across disease groups for JAM-A. (c) Sinonasal tissue Western blot demonstrating increased expression of claudin-2 in AFRS sinus biopsies versus controls and AFRS turbinate biopsies (n=3 tissue samples per group).

Figure 3.

Mean transepithelial electrical resistance (TER) time course graphs demonstrating 24-hour change in sinonasal epithelial resistance with cytokine exposure. (a) Non-inflammatory control versus IFN γ -TNF α positive control, (b) Control versus IL-4 high concentration [50 ng/ml] and IL-4 low concentration [10 ng/ml], (c) Control versus IL-5 high concentration [200 ng/ml] and IL-5 low concentration [40 ng/ml], (d) Control versus IL-13 high concentration [50 ng/ml] and IL-13 low concentration [10 ng/ml]. Error bars = standard error.

Protein Densitometry Analysis Following Cytokine Exposure

Figure 4.

(a) Graph of pooled Western blot densitometry values across *in vitro* sinonasal epithelial cytokine exposure experiments. Pooled densitometry values for JAM-A and E-cadherin reveal a decrease following IL-4 and IL-13 exposure (*), whereas densitometry values for “leaky” tight junction protein claudin-2 have some variability but display a general increase with Th2 cytokine exposure (**). (b) Example Western blots revealing decreased JAM-A and E-cadherin expression and increased claudin-2 expression following IL-4 and IL-13 exposure, with no change demonstrated for claudin-1, occludin, ZO-1, or PARP cleaved

product. (c) Example immunofluorescence photographs for control, along with IL-4 and IL-13 exposed sinonasal epithelial cultures. JAM-A [green] and E-cadherin [red] staining is reduced following IL-4 and IL-13 exposure, whereas occludin [green] and ZO-1 [green] staining does not change. Photographs taken at 40x magnification; nuclei stained with To-pro 3-iodide [blue]; scale bars 100 μ m. (d) Example immunofluorescence photographs of claudin-1 [green] and claudin-2 [green] with ZO-1 counterstain [red]. There is no visible change in claudin-1. Similar to Western blot, claudin-2 staining was somewhat variable, but a more focused pool of claudin-2 can be seen at some cell borders with IL-4 and IL-13 exposure. Photographs taken at 63x magnification; nuclei stained with To-pro 3-iodide [blue]; scale bars 50 μ m.

TABLE 1

Pixel analysis of tight and adherens junction proteins in control and AFRS sinus biopsy specimens

PROTEIN	Mean±SEM Pixel per area	Control sinus v. AFRS sinus p-value
JAM-A		
Control sinus	15.92±1.95	0.314
AFRS sinus	15.57±2.38	
E-cadherin		
Control sinus	15.49±2.21	0.401
AFRS sinus	17.92±2.80	
Occludin		
Control sinus	19.46±2.20	0.359
AFRS sinus	20.92±2.59	
ZO-1		
Control sinus	6.69±1.18	0.188
AFRS sinus	8.70±0.91	
Claudin-1		
Control sinus	25.95±4.63	0.310
AFRS sinus	32.52±6.03	
Claudin-2		
Control sinus	13.63±1.01	0.015*
AFRS sinus	21.21±2.45	