Cytokine Correlation Between Sinus Tissue and Nasal Secretions Among Chronic Rhinosinusitis and Controls

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Objectives/Hypothesis: Compare cytokine levels in sinus tissue to sinus secretions from controls and chronic rhinosinusitis patients.

Study Design: In vitro.

Methods: Polyurethane foam sponges were placed into middle meati of patients with chronic rhinosinusitis without nasal polyps (CRSsNP), with polyps (CRSwNP), and controls. Sinus biopsies were then taken from the same location. Protein levels of tumor necrosis factor-alpha (TNF-α), interferon-γ (IFN-γ), and interleukins (IL) 2, 4, 6, 8, 10, and 17A were measured via cytometric bead assay for each sample. Protein values from sinus tissue and secretions were compared with Pearson's correlation between samples as well as one-way ANOVA with posthoc t test between groups.

Results: Samples from 43 patients in total were examined. Mucus was measured from 10 controls, 11 CRSsNP and 10 CRSwNP, and sinus tissue was measured from 10 controls, 15 CRSsNP and 10 CRSwNP. IL-8 and IFN-γ levels were outside of the detectable range of the assay. Levels of secreted IL-2, 4, 6, 10, and 17A correlated with tissue levels (P < 0.05 for all, r > 0.49) while TNF-α did not (P = 0.71). CRSsNP had elevated mucus levels of IL-2, 4, 6, 10, and 17A compared to controls. CRSwNP had elevated mucus levels of IL-4, 6, 10, 17A, and TNF-α compared to controls.

Conclusions: Cytokine levels in sinus secretions correlate with levels in sinus tissue and are elevated in CRS versus control based on Th1/Th2 skewing.

Key Words: Cytokine; Chronic rhinosinusitis; nasal secretion.

Level of Evidence: N/A (in vitro).

INTRODUCTION

Chronic rhinosinusitis (CRS) is characterized by chronic inflammation of the paranasal sinuses. This inflammation involves a complex interplay between the cellular elements of the sinus tissue and the native and recruited immune cells. Central to the inflammatory process are cytokines that regulate various interactions between cells through an autocrine and paracrine fashion. Numerous cytokines have been investigated in CRS with differences based on disease states, severity, and response to therapy.1,2

Measuring cytokine levels from sinus mucosa offers the most direct investigation of inflammation at the site of the disease process. Sinus mucosa biopsy, however, is an invasive procedure associated with patient discomfort that causes tissue injury leading to further inflammation and possible scar formation.3 This sample collection method is reasonable at the time of surgery for patients treated surgically, but is not feasible for those undergoing medical management and cannot provide temporal evaluation of postoperative inflammatory responses and wound healing. This limits the ability of physicians and researchers to monitor changes in cytokine levels over time without additional invasive procedures.

An alternative, less invasive method involves collection of nasal secretions to determine cytokine levels. Nasal secretions are derived from goblet cells, submucous glands, transepithelial ion and water transport, and plasma transudation.4 Several techniques are described for collection of nasal secretions including nose blowing, vacuum collection, absorption, and nasal lavage.5 The assumption is that protein levels in secretions correlate with levels in the underlying tissue. Most studies utilizing secretions focus on allergic rhinitis where the inflammation of interest occurs in the inferior turbinate, but distinct cytokine profiles have also been described for patients with rhinosinusitis compared to controls.6

What is not clear is whether cytokine levels from easily obtainable nasal secretions truly reflect sinus tissue levels in CRS that can only be measured by biopsy. Recently a polyurethane foam sponge collection system
has been developed for obtaining nasal secretions that is well tolerated and with good reproducibility and superior detectability to nasal lavage. In addition, this method allows for sampling of nasal secretions from more precise anatomic locations compared to sinonasal lavage. The goal of the present study is to compare cytokine levels from sinus tissue and sinus secretions among controls, patients with CRS with nasal polyps (CRSwNP), and patients without polyps (CRSsNP) to determine disease state variation and extent of correlation between secretions and sinus tissue.

MATERIALS AND METHODS

Patients

The Institutional Review Board at the Medical University of South Carolina approved the study protocol prior to initiation, and written informed consent was obtained for all patients. Patients were included if they were undergoing sinus surgery for any reason, including repair of cerebrospinal fluid leaks, transphenoidal skull base surgery, or inflammatory sinus disease defined by the criteria outlined by the European position paper on rhinosinusitis 2012. Determination of nasal polyposis was made by endoscopic examination preoperatively to divide CRS patients into CRSsNP and CRSwNP. Patients with allergic fungal rhinosinusitis and Samter’s triad were included in the CRSwNP group based on the presence of nasal polyposis on exam. Patients were excluded if they had taken systemic corticosteroids or immunosuppressants or had any reason, including repair of cerebrospinal fluid leaks, transphenoidal skull base surgery, or inflammatory sinus disease.

Sponges and Placement

After induction of anesthesia but prior to application of any intranasal topical agent or injection, a polyurethane sponge (Fig. 1, Greer Labs, Lenoir, NC) was placed in the middle meatus under endoscopic guidance. Sponges were left in place for 10 minutes, as has been reported previously. Nasal mucus samples collected on sponges were immediately centrifuged at 4°C, 3,000 rpm for 30 minutes to extract the entire sample from the sponge. The mucus was then transferred by pipette to a cryotube and stored at −80°C until use.

Sinus Tissue Preparation

Biopsy specimens were taken from ethmoid sinus tissue immediately adjacent to where the sponge had been placed. These were placed in a cryotube, snap frozen, and stored at −80°C. Tissue was prepared by removing visible bone, then homogenizing in Tissue Protein Extraction Reagent with Halt Protease Inhibitor Cocktail (Thermo Scientific, Waltham, MA) using a Power Gen 125 handheld homogenizer (Thermo Scientific). After settling, samples were centrifuged at 4°C, 1,500 rpm for 8 minutes and supernatants were collected and stored at −80°C until use.

Cytokine Measurement

Due to the limited sample volume and the number of desired cytokine measurements, levels were detected using commercially available Cytometric Bead Array (CBA) systems with enhanced sensitivity (BD Biosciences, San Diego, CA). Kits and reagents were purchased for eight cytokines representing a broad spectrum of mediators, including tumor necrosis factor (TNF), interferon-gamma (IFN-γ), and interleukins-2, 4, 6, 8, 10, and 17A. Undiluted samples of sinus homogenates and nasal mucus were added in duplicate (50 μl/well) to a MultiScreenHTS Assay System bottom-filtered 96-well plate (EMD Millipore, Billerica, MA). The assay was carried out according to manufacturers’ instructions, including aspirating well contents with a MultiScreenHTS vacuum manifold (EMD Millipore) set no higher than 10 cm Hg. The samples were read on a Guava easyCyte SHT flow cytometer (EMD Millipore) and analysis was performed with FCAP Array Software Version 1.0.1 (BD Biosciences, San Jose, CA). Each CBA kit also contained a standard curve for quality control purposes. The FCAP Array Software used the manufacturer’s recommended four-parameter logistic equation to establish the standard curve and allstandardcurvesachievedtherecommendedminimumcurveaccuracy of 98% (data not shown). The software automatically solved for the unknown analyte values based on the standard curve for each cytokine.

Protein Correction

To control for the variable size of the sinus tissue obtained from each patient, the total protein content of each specimen was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific). Samples were measured in duplicate on 96-well plates (Corning) and compared to an albumin standard curve according to manufacturers’ instructions. Following 30 minutes of incubation at 37°C, absorbance was measured on a Synergy H1 Hybrid plate reader (BioTek, Winooski, VT). Mean protein levels for each sample were calculated and cytokine levels measured by CBA were corrected for the total protein content of the sample and expressed as pg of cytokine per mg of total protein.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 5.04 software (GraphPad Software, La Jolla, CA). Samples above or below the assay sensitivity, as determined by the manufacturer, were excluded. Mean values from sample duplicates were calculated and values from the nasal mucus sample were...
compared to the protein-corrected values of the corresponding sinus tissue samples. Pearson correlation was calculated for sample pairs. For disease-specific mucus and tissue analysis, the left and right side samples were averaged to yield one data point per patient as all CRS patients in this cohort had symmetric disease based on radiographic and endoscopic evaluations. Differences in cytokine levels between disease groups was determined by one-way ANOVA followed by posthoc t test analysis. Significance was determined by a P value < 0.05.

RESULTS

Patients

Samples from 43 patients were included. Our initial focus was to examine disease-specific variations in cytokine levels from tissue and mucus samples independently, which included nine patients. Similar diseasespecific variations were observed for most cytokines in both the tissue and mucus samples, prompting a subsequent examination of cytokine correlations in patients with paired mucus and tissue samples among 34 patients. Two patients' mucus samples were visibly contaminated with blood, which prevented filtration through the bottom-filtered vacuum plate and would have resulted in contamination of secreted cytokines with systemic cytokines. Thus, these samples were excluded from analysis. Demographic details for each patient group including the presence of atopy, asthma, and disease severity determined by Lund Mackay (LM) scores are depicted in Table I. There was no difference in patient age between the groups, but there were differences in gender and race. There was a higher prevalence of atopy and asthma among the CRS patients compared to controls, and LM scores were also higher in the CRS cohorts (P < 0.02 for all). LM scores were not significantly different between the right and left side for any group (data not shown).

Cytokine Correlation

Of the eight cytokines investigated, the values for two cytokines fell outside of the detection range of the assay. IFN-γ levels were below the detectable range, while IL-8 levels were above the upper limit of the assay. Figure 2 demonstrates cytokine levels for each disease state, with mucus levels presented in the left column measured in pg/ml and tissue levels in the right column measured in pg/mg protein. Overall, patients with CRS had higher cytokine levels in both tissue and mucus than control patients. Specifically, CRSsNP mucus had higher levels of IL-2, IL-4, IL-6, IL-10, and IL-17A compared to controls, while CRSwNP mucus levels were higher than controls for IL-4, IL-6, IL-10, IL-17A, and TNF-α. There were significant correlations between paired sinus tissue and mucus levels for all measurable cytokines except TNF-α (Fig. 3). There was a moderately strong correlation between sinus and mucus cytokines for IL-4, IL-6, IL-10, and IL-17A (r = 0.573, 0.566, 0.491, 0.598, respectively) with a strong correlation for IL-2 (r = 0.762). Cytokine levels were not different between right and left sides for any group or cytokine measured (data not shown).

DISCUSSION

Categorizing and understanding the role of inflammatory biomarkers in CRS is an area of intense scientific interest. A recent study among CRSwNP patients evaluated nearly 100 specific molecules for this subcategory alone. The mechanisms responsible for the chronic inflammation in CRS are complex and incompletely understood. Further elucidation of the detailed interactions involved will improve our understanding of these complex mechanisms, but has yet to provide many clinical advances in the care of patients with CRS because most of the studies are limited to in vitro investigations of samples acquired surgically. Availability of a reliable, noninvasive method of assessing levels of inflammatory markers would allow researchers and clinicians to explore these factors in nonsurgical patients and monitor changes at multiple points throughout treatment.

Specimen Collection Technique and Cytokine Correlation

The goals of this study were to determine how cytokines in nasal mucus differ by disease type and to examine if mucus cytokine values correlate with values measured in sinus tissue. Of the many collection techniques available for obtaining nasal secretions, there is evidence that passive absorption via capillary action is superior. Nasal blowing results in inconsistent results, while vacuum suctioning or microsuctioning tubes cause mucosal trauma with variable results. Nasal lavage requires the instillation of a fixed amount of solution

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**TABLE I.**

Demographic and Disease Characteristics of Control and CRS Patients.

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>Control</th>
<th>CRSsNP</th>
<th>CRSwNP</th>
<th>P Value</th>
<th>Test Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Mean ± SD)</td>
<td>14</td>
<td>15</td>
<td>12</td>
<td>0.817</td>
<td>ANOVA</td>
</tr>
<tr>
<td>Gender (% female)</td>
<td>50.0%</td>
<td>73.3%</td>
<td>25.0%</td>
<td>0.044</td>
<td>Chi-square</td>
</tr>
<tr>
<td>Race (% Caucasian)</td>
<td>35.7%</td>
<td>86.7%</td>
<td>50.0%</td>
<td>0.016</td>
<td>Chi-square</td>
</tr>
<tr>
<td>Atopy</td>
<td>0.0%</td>
<td>33.3%</td>
<td>75.0%</td>
<td>0.0004</td>
<td>Chi-square</td>
</tr>
<tr>
<td>Asthma</td>
<td>7.1%</td>
<td>26.7%</td>
<td>60.0%</td>
<td>0.018</td>
<td>Chi-square</td>
</tr>
<tr>
<td>Lund-Mackay score (Mean ± SD)</td>
<td>0.5 ± 0.5</td>
<td>7.8 ± 3.9</td>
<td>14.9 ± 6.2</td>
<td>&lt;0.0001</td>
<td>ANOVA</td>
</tr>
</tbody>
</table>
Fig. 2. Cytokine levels displayed by disease groups among mucous samples (left column) and tissue samples (right column): (A) IL-2, (B) IL-4, (C) IL-6, (D) IL-10, (E) IL-17A, and (F) TNF-α. Values are expressed as means with standard deviations. Mucous levels are expressed in pg/ml while tissue samples are expressed in pg/mg of total protein to correct for total protein content of each sample. Differences between groups were measured with ANOVA and posthoc t test. The symbol * denotes $P < 0.05$ between indicated CRS group versus controls while # denotes $P < 0.05$ between CRSsNP and CRSwNP.
(usually isotonic saline), resulting in a variable collection volume with unpredictable dilution effects. Nasal secretions have generally been studied in allergic rhinitis, with limited data available for CRS. The only direct connection between nasal secretions and corresponding sinus tissue levels has been shown for matrix metalloproteinase-9 by enzyme-linked immunosorbent assay from ethmoid sinus biopsies and secretions. The authors found a significant correlation between tissue and secretion levels among the 30 patients with CRS studied but did not use tissue sample weight or protein content to standardize sample size, thus introducing a potentially confounding factor.

The current study found significant correlations between tissue and sinus secretion cytokine levels for five of the six cytokines within the detectable ranges of the assay. Furthermore, tissue samples were standardized by total protein content to avoid confounding based on varying tissue density from individual patients. Benefits of this technique include ease of sample collection and processing, small sample volume (50 μl) needed for analysis, and the ability to measure multiple factors simultaneously. Drawbacks include the need for specialized laboratory equipment and analysis software, and the need to use the same sample dilution factor for all measured cytokines. We analyzed undiluted samples, which resulted in IFN-γ values below the detectable range (despite the enhanced sensitivity kits used) and IL-8 values above the upper limit of the assay. Sample dilution would have reduced IL-8 values into the measurable range but would have diluted most of the remaining factors below the detectable limit. Another limitation is that up to 15% of samples for a given cytokine generate low event errors, resulting in no data for that particular analyte. This occurred with all cytokines but most frequently with IL-2, IL-4, and IL-17A. Additionally, we discovered that samples visibly contaminated by blood could not be filtered through the vacuum-assisted filtration system used and had to be omitted. We omitted bloody samples both for technical reasons and because secretions contaminated with blood would be expected to have cytokine levels approximating those in circulation rather than local sinus tissue.

**Cytokine Comparisons Between Groups**

Most of the cytokines measured demonstrated increased levels in CRS patients compared to controls. Riechelmann measured 17 biomarkers from nasal secretions among patients with CRSwNP, CRSsNP, acute rhinosinusitis, and controls to determine profiles for each disease. The authors demonstrated higher levels of nearly all cytokines in sinusitis versus controls with CRSwNP possessing a Th2-skewed profile, while CRSsNP was Th1-dominant. In the current study, cytokines involved in a broad range of immunologic...
responses were investigated, including Th1 (IFN-γ and IL-2), Th2 (IL-4), proinflammatory (TNF-α, IL-6, and IL-8), and regulatory (IL-10 and IL-17A).

IL-2 is primarily produced by local T cells and dendritic cells and is important in T-cell differentiation and activation. IL-2 production has been implicated in Th1 disease processes, and our data demonstrate elevated levels among the Th1-skewed CRSsNP population. IFN-γ is a major Th1 cytokine secreted by CD8+ lymphocytes, natural killer cells, B cells, and other antigen-presenting cells. Levels of IFN-γ are increased in CRSsNP and decreased in CRSwNP patients. Unfortunately, the levels of IFN-γ from our samples were below the detectable range for assay used, but we would expect to see elevated levels in CRS compared to controls with the highest levels among the CRSsNP subgroup, as demonstrated by others. It is possible that IFN-γ production occurs primarily in parenchymal cells such that tissue levels are increased without an increase in levels secreted into the sinus cavity.

IL-6 is a proinflammatory cytokine produced by several cells, including epithelial cells, T and B lymphocytes, macrophages, eosinophils, mast cells, and fibroblasts, that plays a role in lymphocyte and mast cell maturation, IgE synthesis, and fibrosis. IL-6 affects B- and T-cell regulation through the activation of Th17 cells and the production of IL-17. Increased levels of IL-6 have been observed in both subtypes of CRS compared to controls, and we observed a similar pattern with elevated levels in CRSwNP and CRSsNP compared to controls.

TNF-α is another proinflammatory cytokine produced primarily by mast cells, neutrophils, macrophages, monocytes, and eosinophils, with the ability to augment both Th1 and Th2 responses. Evidence suggests that eosinophils are the primary source of TNF-α among CRSwNP patients. Studies have shown elevated TNF-α among both subtypes of CRS compared to controls; however, our data demonstrate elevated mucus levels in the CRSwNP subgroup only and no difference in tissue levels for any group or correlation between the mucus and tissue levels. One possible explanation for this may be that TNF-α is constitutively produced but released only under select conditions. Therefore, cells that have been lysed may yield differing results from those obtained from secreted cytokines in mucus under more physiologically relevant conditions. Alternatively, since TNF-α is made by a wide range of cell types, there may be different sources of TNF-α between subsets of CRS, thus resulting in poor correlations.

IL-4 is a major Th2 cytokine produced by eosinophils, basophils, mast cells, and other Th2 cells. IL-4 plays a role in Th2 differentiation of naive T-helper cells, immunoglobulin class shifting to IgE production, and recruitment of eosinophils. Levels of IL-4 are elevated among both CRSwNP and CRSsNP compared to controls, and we found a similar pattern in our patients.

IL-10 is an antiinflammatory cytokine produced by a wide range of immunologically active cells, especially Th2 and T regulatory (Treg) cells. IL-10 production has been implicated in Th2-skewed disease states. Our results demonstrate increased levels of IL-10 among both CRS subtypes compared to control with the highest levels in the Th2-skewed CRSwNP population.

IL-17A is a regulatory cytokine produced primarily by Tregs and Th17 cells. IL-17A is associated with T-cell priming, fibroblast stimulation and production of proinflammatory cytokines, and autoimmune processes. IL-17A has been shown to attract neutrophil migration and predict the severity of disease in asthma, but it has also been associated with eosinophilic inflammation in nasal polyps with higher IL-17A levels in polyp patients compared to controls. We found elevated IL-17A among both CRSsNP and CRSwNP compared to controls, without a difference between subgroups.

The cytokines included in this study were selected to encompass a range of inflammatory patterns with factors known to play a role in CRS with commercially available CBA kits; yet these cytokines represent only a fraction of the multiple factors important in CRS. Future studies are needed to investigate additional cytokines important in Th1/Th2 skewing, tissue fibrosis, regulation and termination of the inflammatory cascade, and cellular interaction and cross talk. Moreover, classification of inflammatory responses into Th1 and Th2 pathways is an oversimplification, with additional pathways such as IL-17 and Tregs playing an important role. Our CRSwNP patients demonstrated elevated Th2 cytokines (IL-4) in addition to elevated IL-17A, IL-6, and IL-10, suggesting a mixed pattern of inflammation. Inclusion of other important Th2 cytokines such as IL-5 and IL-13 may have provided additional insights. The distinction between CRSsNP and CRSwNP into two dichotomous classes is also an oversimplification. Patterns of cytokine expression have been shown to vary between these two groups, but results are not always consistent and subtypes of each group are also recognized based on the presence of eosinophilia, atopy, and aspirin sensitivity.

Our study included patients from these subgroups, including allergic fungal rhinosinusitis and Samter’s triad representing all comers who presented for evaluation. Therefore, cytokine variation with disease type should be interpreted with caution in a purely homogeneous patient population, but this does suggest that cytokine correlation between mucus and tissue occurs among multiple subgroups of patients.

Future Directions

Absorbent sponges were placed under endoscopic guidance while patients were under general anesthesia and left in place for 10 minutes prior to topical preparation of the nasal cavity. This method was chosen to provide the highest yield for potential correlation between the secretions collected by the sponge and cytokine levels in neighboring sinus tissue. The size of the sponge relative to the middle meatus made endoscopic placement difficult at times, especially if there was significant polyp disease; however, sponge placement was successful in nearly all patients using a freer to gently compress
the sponge and insert it into the middle meatus. Care must be taken with this maneuver to avoid mucosal trauma and contamination of the sponge with blood, which precludes sample processing, as occurred in two patients in this study. Based on these results that suggest a correlation exists, we hope to modify the technique to make it better suited for use in a clinical setting. Questions to be addressed include: 1) does topicalization of the nose with anesthetics and/or vasoconstrictors affect cytokine correlation; 2) does the sponge need to contact the sinus mucosa directly or do secretions collected from the nasal cavity (e.g., head of the middle turbinate) correlate with sinus tissue values; 3) do cytokine levels correlate to clinical symptoms or disease severity indices; and 4) can cytokines measured from secreted mucus be used to track clinical responses to medical and/or surgical treatment.

CONCLUSION

Cytokine measurements from sinus secretions correlate with levels in sinus mucosa when levels fall within the detectable range of the assay. This technique may provide a useful method for clinicians and researchers to detect and monitor cytokite levels of individual patients. Further efforts are needed to address the clinical questions listed above to help establish a reliable correlation between inflammatory profiles and clinical outcomes.

Acknowledgement

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