Differential Expression of Surfactant Protein A in the Nasal Mucosa of Patients With Allergy Symptoms

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Objective: To characterize surfactant protein A (SP-A) expression in human nasal tissue and correlate differential expression of SP-A with symptoms suggestive of allergic rhinitis.

Design: Allergic rhinitis symptom data were prospectively collected in the form of the Rhinitis Symptom Utility Index, the Rhinoconjunctivitis Quality of Life Questionnaire, and a Visual Analog Scale. Immunohistochemical staining for SP-A was performed on resected nasal tissue. Quantitative polymerase chain reaction amplification of the SP-A gene referenced to β-actin was performed on complementary DNA samples synthesized from total RNA isolates.

Setting: Academic tertiary referral center, department of otolaryngology laboratories.

Patients: Twenty-five consecutive patients undergoing nasal surgery.

Main Outcome Measures: Immunohistochemical staining of SP-A in human nasal mucosa and submucosa, polymerase chain reaction amplification of SP-A messenger RNA, and rhinitis symptom scores.

Results: Immunostaining localized SP-A to the mucosa and submucosal glands in specimens. Quantitative polymerase chain reaction demonstrated correlation between SP-A messenger RNA concentration and the total Rhinitis Symptom Utility Index score (0.51, P = .009) as well as “sneezing over the previous week” (0.40, P = .049), “runny nose over the previous week” (0.55, P = .005), and “sneezing today” (0.47, P = .02).

Conclusions: To our knowledge, this is the first report of SP-A expression in human nasal tissue. Furthermore, the degree of expression correlated with severity of disease as measured by the Rhinitis Symptom Utility Index in patients with allergic rhinitis symptoms.


SURFACTANT PROTEIN A (SP-A) is a surfactant-related protein thought to have a role beyond phosphatidylcholine cycling, including immune modulation along the respiratory tract.1 A member of the collectin (calcium-dependent lectin) family, SP-A is related to surfactant protein D, mannose-binding protein, and C1q.2-4 Based on differential post-translational modifications, SP-A exists as a 28 to 36 kDa protein, but in vivo it aggregates into octadecameric oligomers with a flower bouquet shape.5

Its bipolar anatomy, with collagenous tails opposing carbohydrate-binding globular heads, underpins its biphasic immune function. For example, SP-A is able to down-regulate local inflammation by binding with its globular heads to signal inhibitory regulatory protein-α on the surface of tissue macrophages. Alternatively, association of its collagenous tail region with the calreticulin/CD 91 complex on tissue macrophages stimulates cytokine production, P38 phosphorylation, and nuclear factor κB activation, promoting local inflammation.6 In such a role, SP-A also acts as an opsonin and stimulates phagocytosis.

Given its varied role in the innate immunity of the respiratory tract, SP-A expression has been characterized in several disease states. Surfactant protein A down-regulation in allergy was demonstrated in a mouse asthma model in which SP-A expression decreased after allergen challenge in sensitized animals.7 This down-regulation phenomenon has also been observed in eustachian tube mucosa after middle ear perfusion of aller-
gen in sensitized rats (A.C., C.T.W., K.F.L., and R.L.L., unpublished data, 2006). Consistent with its biphasic role in inflammation, in the human respiratory tract, SP-A expression may also be elevated in the setting of allergy or infection.⁴⁻¹⁰ Bronchoalveolar lavage specimens from patients with chronic bronchial asthma have demonstrated increased SP-A,¹¹ as have reports of differential SP-A expression in patients with pneumocystis pneumonia. Surfactant protein A expression has also been assessed in the sinonasal mucosa. In a semiquantitative study, Dutton et al¹² found SP-A to be elevated in the mucosa of rabbits with intercurrent sinusitis or antibiotic-treated sinusitis compared with pathogen-free animals.

Given the important role of nasal inflammation in human disease, our study sought to correlate SP-A expression with rhinitis symptomatic traits in the nasal mucosa of human subjects. Using immunohistochemical staining and real-time, quantitative polymerase chain reaction (PCR), we demonstrate differential expression of SP-A that varies with the severity of allergic rhinitis symptoms as measured by the Rhinitis Symptom Utility Index (RSUI).¹⁴

METHODS

A prospective, blinded analysis of SP-A expression in the nasal mucosa of 25 consecutive adult patients (age, ≥18 years) undergoing turbinate (middle or inferior) reduction surgery was performed using immunohistochemical and quantitative PCR technologies. Institutional review board approval was obtained. All participants had preoperative nasal congestion and/or chronic sinusitis listed as a presenting complaint in their initial clinical encounter. Preoperative computed tomographic imaging was obtained for each patient, demonstrating anatomic disease persisting despite appropriate nonsurgical treatment. The indication for inferior turbinectomy was anatomic disease persisting despite appropriate nonsurgical treatment. Middle turbinates were partially resected if involved with obstructive concha bullosa changes or for improved postoperative surveillance and debridement in difficult revision cases. At resection, none of the study patients had gross mucosal disease suggesting atrophy, suppurative, invasive fungal, or neoplastic lesions.

Patients who were not undergoing tissue resective surgery and those unwilling or unable to supply information on their rhinitis symptoms or their histories were excluded from the study. Allergy testing (skin testing or radioallergosorbent test assay) was not mandated prior to entry into the study, and only 6 patients had allergy testing data in their medical records. These results were excluded from analysis. Thus, the clinical instruments employed in this study were not designed to assess the presence of symptoms commonly attributed to nasal allergy, but they are not a substitute for objective testing. In addition, institutional review board approval for this study did not allow us to control for simultaneous medication usage (topical or systemic).

SYMPTOM SCORES AND TISSUE COLLECTION

To assess allergy symptoms as they were being experienced on the day of tissue resection, 5 symptoms (stuffy or blocked nose; runny nose; sneezing; itchy, watery eyes; and itchy nose or throat) were scored in a self-administered fashion. Patients placed a vertical mark on a 45-mm line, providing a Visual Analog Scale (VAS) of their symptoms. Allergic symptoms over the past 1 week were measured via a modified Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ),¹³ a 28-item, self-administered assessment, and symptoms over the past 2 weeks were assessed by the 5-item RSUI.¹⁴

In the operating room and under sterile conditions, resected specimens were dissected free of gross bone, partitioned, and snap-frozen in liquid nitrogen and stored in labeled nuclelease-free containers at −70°C.

IMMUNOHISTOCHEMICAL ANALYSIS

After thawing to −20°C and overnight decalcification, followed by fixation in 4% paraformaldehyde, 5-µm sections of paraffin-embedded human turbinate samples were placed on positively charged slides and allowed to dry overnight. The paraffin was removed from the slides, and heat-induced epitope retrieval was performed with EDTA buffer (Richard-Allan Scientific, Kalamazoo, Mich) for 20 minutes. Endogenous peroxidase activity was squelched with 0.03% hydrogen peroxide and rinsed. The sections were treated with casein for 20 minutes to block nonspecific binding of the primary antibody. Rabbit anti-human SP-A (Santa Cruz Biotechnology Inc, Santa Cruz, Calif) diluted in antibody diluent (Dako Corp, Carpinteria, Calif) by 1:200 was applied to the tissues and stored in a humidity chamber overnight at 4°C. Sections without primary antibody served as negative controls. We produced localized, visible staining (DakoCytomation Envision+ System, DAB/Peroxidase; DakoCytomation, Carpinteria, Calif) after secondary antibody treatment with polyclonal anti-rabbit IgG and horseradish peroxidase. The slides were finally counterstained with Mayer hematoxylin.

RNA PURIFICATION AND COMPLEMENTARY DNA CONSTRUCTION

Total RNA purification was performed on the 25 specimens (using an Aurum Total RNA Mini Prep-Kit; Bio-Rad, Hercules, Calif), including a DNase 1 digest, according to the manufacturer’s instructions. Reverse transcriptase–PCR was performed on a portion of the purified RNA for each specimen to create complementary DNA (cDNA) libraries using the iScript cDNA Synthesis Kit (Bio-Rad) under the following conditions in a conventional thermocycler: 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C.

QUANTITATIVE PCR

Intron-exon spanning SP-A primers were used in the quantitative PCR amplification reactions with the following design: 5′-AAGCCACACTCCAGCATTTAGA-3′ and 5′-GCCCATGCTGGGAGAACCT-3′ (Integrated DNA Technology Inc, Coralville, Iowa). Commercially available β-actin primers for the amplification of the housekeeping gene were used: 5′-TCTAGAGTGTGACGTTGACATCCGT-3′ and 5′-GTCTAGACGATTTCGTTTACGATC-3′ (Promega, Madison, Wis). In a quantitative PCR detection system (My IQ Thermal Cycler; Bio-Rad), 96-well reaction plates were used to perform real-time, quantitative PCR with the sample wells containing the following: 25-µL 2X Supermix with SYBRgreen (a fluorescent double-stranded DNA-binding reporter molecule), 1 µL each of SP-A primer (100 ng/µL) or β-actin primers (100 pmol/µL) for control wells, a 1-µL sample complementary DNA, and 22 µL of nuclease-free water. After an initial activation step at 95°C for 3 minutes, amplification was performed across 45 cycles with the following parameters: denaturation for 30 seconds at 95°C, annealing for 20 seconds at 58°C, and elongation for 30 seconds at 72°C. Digital capture of SYBRgreen fluorescence was performed.
during the annealing phase for threshold cycle (C_T) assessment. The C_T values were automatically stored in an output file, and these were analyzed to determine relative starting messenger RNA (mRNA) concentrations according to the 2^-ΔΔC_T method using a proprietary macro (Bio-Rad) with Excel 2003 XT (Microsoft Corp, Redmond, Wash).

A melt-curve analysis confirmed specimen uniformity and estimated product size in each sample well in the 96-well plate. The following protocol was programmed into the My iQ Thermal Cycler (Bio-Rad) to produce the melt curves for each sample well after amplification using SYBRgreen as the reporter: 1 minute at 95°C, 1 minute at 55°C, and 10 seconds at 55°C for 80 cycles.

Finally, to verify PCR product sequence, random samples were prepared and sequenced by the Vanderbilt University (Nashville, Tenn) DNA sequencing core laboratory. Data were compared with the human genome via BLAST downloadable software (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9606).

All data compiled in this study were analyzed statistically using SigmaStat software (version 2.03; SPSS Inc, Chicago, Ill).

**RESULTS**

Concerning descriptive data, 25 consecutive patients underwent nasal turbinate reduction surgery from December 2004 to March 2005, and, after consenting to be in the study, provided information regarding their allergy symptoms and had the salient portions of their medical records reviewed. The mean age of the group was 42 years, with a 1:1.8 male-female distribution. At the time of tissue collection, 2 patients were receiving oral corticosteroids; 10, nasal corticosteroids; 11, oral or nasal antihistamines; 5, oral leukotriene inhibitors; and 4, oral antibiotics. The mean (SD) symptom scores and maximum scores possible for the VAS, RQLQ, and RSUI are given in **Table 1**.

The SP-A antibody immunohistochemical studies showed variable staining in the mucosa and submucosal glands in each of the samples (**Figure 1**). Negative control slides demonstrated no staining (**Figure 1**). A blinded analysis rating the intensity of staining as 0 (no appreciable staining), 1, 2, or 3 (intense staining) failed to correlate with quantitative mRNA expression (−0.087, P = .79 [Pearson product moment correlation]).

Following total RNA purification and complementary DNA synthesis via reverse transcriptase-PCR for each of the samples, quantitative PCR was performed to estimate the relative starting concentrations of SP-A. Samples 9 to 11, 15, 17, and 28 had a relative-fold expression of SP-A mRNA greater than 50 000. Modest expression was seen in samples 2, 5, 7, 12, 16, 20, and 24. The remaining samples had a modicum of SP-A mRNA expression at the time of tissue resection (**Figure 2**).

To confirm that the amplicons from the real-time quantitative PCR were indeed the SP-A gene sequence, a melt-curve analysis and DNA sequencing were employed. The

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<thead>
<tr>
<th>Instrument</th>
<th>Mean Score (SD)</th>
<th>Maximum Score Possible</th>
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<tbody>
<tr>
<td>VAS</td>
<td>160 (106)</td>
<td>500</td>
</tr>
<tr>
<td>RQLQ</td>
<td>127 (56)</td>
<td>168</td>
</tr>
<tr>
<td>RSUI</td>
<td>12 (5)</td>
<td>30</td>
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Abbreviations: RQLQ, Rhinoconjunctivitis Quality of Life Questionnaire; RSUI, Rhinitis Symptom Utility Index; and VAS, Visual Analog Scale.
melt-curve step demonstrated 2 peaks: one at 86.8°C, consistent with β-actin amplicon, and the other at 89.5°C, denoting SP-A (Figure 3). Amplicons chosen at random for DNA sequencing were found to have 100% homologous correspondence with the human SP-A1 gene.

The raw data from the clinical instruments, medical record review, and quantitative PCR were correlated. The VAS scores positively correlated with RSUI scores (0.58, \( P \) = .003), and RSUI scores positively correlated with raw data from the RQLQ (0.56, \( P \) = .004). No significant correlation was found between the VAS and the RQLQ (0.27, \( P \) = .20) (Pearson product moment correlation). To determine the effects of medical allergy therapy with corticosteroids, antihistamines, leukotriene inhibitors, and antibiotics, we performed a multivariate linear regression analysis and found no statistically significant impact of these therapies on the clinical instrument scores or quantitative PCR data. An isolated analysis of the individual therapies’ effects on mRNA expression again found no statistically significant effects (Table 2).

Pearson product moment correlation demonstrated that several symptoms from the VAS and RQLQ were predicted by high SP-A mRNA expression: “sneezing over the previous week” (RQLQ) (0.40, \( P \) = .049), “runny nose

Figure 2. Relative-fold expression of surfactant protein A (SP-A) messenger RNA reported by quantitative polymerase chain reaction for 25 consecutive patients undergoing nasal surgery.

Figure 3. Melt curve for quantitative polymerase chain reaction assay of surfactant protein A messenger RNA (89.5°C peak) expression relative to the expression of β-actin (86.8°C peak).
over the previous week” (RQLQ) (0.55, \(P = .005\)), and “sneezing today” (VAS) (0.47, \(P = .02\)). The remainder of the individual symptoms assessed by the VAS and RQLQ instruments did not correlate significantly with quantitative PCR. However, total RSUI scores were predictive of high SP-A mRNA expression in a positive and statistically significant correlation (0.51, \(P = .009\)).

Using immunohistochemical staining and quantitative PCR, this study characterizes the differential expression of SP-A in the nasal tissue of human subjects with symptoms suggestive of allergic rhinitis. To our knowledge, this is the first demonstration of SP-A in human nasal mucosa by immunohistochemical analysis (Figure 1). Furthermore, SP-A expression was found to be directly related to the clinical realm with a higher total RSUI score predictive of elevated SP-A mRNA expression (0.51, \(P = .009\)).

By design, our pilot study had a small sample size. Negative controls consisted of patients with fewer symptoms (Table 1) rather than true negative controls consisting of healthy individuals without rhinitis complaints. Also, all samples were resected during the winter months, before the release of most environmental pollens. However, performing the study in the winter months does not reduce exposure to perennial allergens. Despite these limitations, in this initial study, we have demonstrated SP-A expression in human nasal mucosa and correlated it to rhinitis symptoms.

The literature is discordant regarding the expression of SP-A in allergic states. Surfactant protein A has been shown to specifically bind pollens, and patients with pollen allergy have been reported to have an increased proportion of smaller oligomeric forms of SP-A compared with controls. In a small study of bronchoalveolar lavage fluids in patients with bronchial asthma, Cheng et al determined that SP-A was diminished in the eustachian tube mucosa in allergic rats (A.C., C.T.W., K.F.L., and R.L.L., unpublished data, 2006). These variable reports of SP-A expression in disease reflect the bivalency of SP-A, the small sample numbers in each study, and variability in the assays used to detect and quantify SP-A.

Our study of SP-A expression in the nasal mucosa of human subjects with symptoms suggestive of allergic rhinitis agrees with previous studies relating chronic allergic inflammation to higher SP-A expression. Variability in SP-A expression was noted on immunostaining (Figure 1) and confirmed by quantitative PCR (Figure 2). Although the institutional review board confines placed on this study did not allow us to control for simultaneous medication usage, our data suggest that SP-A expression was not significantly suppressed by medical therapy because no statistical effects of antihistamine, antibiotic, corticosteroid, or leukotriene inhibitor treatment were observed on the symptom scores or SP-A mRNA expression levels (Table 2). Future work is needed with improved controls or pertinent in vitro or ex vivo data to confirm that these drugs do not exert a significant effect on tissue expression of SP-A.

Specifically, patients who continued to express significant allergic symptoms despite medical treatment and who were requiring surgery to treat their nasal disease had more SP-A mRNA in their tissues at the time of resection. The melt-curve analysis (Figure 3) and DNA sequencing of randomly selected amplicons support these claims. Separation of the 2 amplified genes is demonstrated thermally. The third, low peak at 81.5°C represents 2 wells assessing SP-A primer dimer formation (Figure 3).

We found that symptoms tended to correlate locally with SP-A expression as ocular manifestations of allergy, itchy nose or throat, and tiredness and fatigue, and that the emotional consequences of allergy did not correlate with SP-A expression. The molecular basis for the local effects of SP-A in allergy are well described. Depending on its oligomeric orientation with respect to tissue macrophages, SP-A either down-regulates (via signal inhibitory regulatory protein-\(\alpha\)) or up-regulates (via calreticulin/C/91) inflammation. Surfactant protein A has been shown to directly enhance the production of tumor necrosis factor \(\alpha\), interleukin 1\(\alpha\), interleukin 1\(\beta\),

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<tr>
<th>Instrument</th>
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<th>Leukotriene Inhibitors</th>
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**Table 2. \(t\) Test Comparison of the Effects of Medical Allergy Therapy on Symptom Scores and Quantitative Polymerase Chain Reaction (PCR) Results for 25 Patients**

Abbreviations: RQLQ, Rhinoconjunctivitis Quality of Life Questionnaire; RSUI, Rhinitis Symptom Utility Index; VAS, Visual Analog Scale.
interleukin 6, and interferon-γ, whereas levels of tumor necrosis factor α and macrophage inflammatory protein 2 are increased in SP-A–deficient mice challenged with lipopolysaccharide antigen, bacterial pathogens, or respiratory syncytial virus.20,22

The question that remains to be definitively answered is, when SP-A is found to be elevated in allergic states, as in our study, does this represent a pathologic elevation inciting autoimmunity, or is SP-A responsively elevated to quiet inflammation and competitively inhibit IgE binding? Again, in the pulmonary literature, one study evaluating SP-A knockout rats found mixed results. Compared with wild-type mice, hyperesoinophilia and inflammation characterized the SP-A knockout mice at baseline, prior to sensitization of both groups to Aspergillus fumigatus antigen. This suggested that SP-A was functioning as an anti-inflammatory. However, post-sensitization, SP-A knockout mice deteriorated further with exogenous administration of SP-A whereas wild types given SP-A improved.23 Absent from their analysis was an assessment of native SP-A expression in the control animals prior to and following sensitization and SP-A therapy. Therefore, the temporal modulation of SP-A during inflammation also remains unclear.

Perhaps most interesting is the later work by Wang et al.,24 which assesses the anti-inflammatory effects of exogenous SP-A in vitro in children with asthma. They found SP-A to inhibit histamine release in the early phase of allergen provocation and suppress lymphocyte proliferation in the late phase of bronchial inflammation in controls and those with stable asthma. The third study group, those in the throws of an acute asthma attack, showed only mild inflammatory suppression with SP-A treatment. Wang et al.25 hypothesize that when T cells have already been activated during an asthmatic attack, the inhibitory effect of SP-A is quenched because the surface molecules of activated lymphocytes are overexpressed.

Despite the controversies in the literature, our translational data on the relative expression of SP-A in the nasal mucosa of patients with allergy symptoms suggest some immediate applications. First, these data represent another key step in the understanding of the molecular basis of nasal immunomodulation. Second, in terms of a possible future clinical application, PCR quantification of SP-A in patient samples could be used as a diagnostic marker when the etiology of rhinitis symptoms is unclear (analogous to hemoglobin A1c testing in patients with diabetes mellitus) if further studies incorporating definitive allergy testing (skin testing or radioallergosorbent test) corroborate our results. Besides prospectively mandating allergy testing as an inclusion criterion into such a study, additional studies of SP-A variation in patients who are or who are not receiving medical therapy should be conducted. Finally, as suggested through wild-type mice responses to exogenous SP-A administration in knockout mice and in vitro human studies, there may be a role for topical SP-A therapy in human allergic rhinitis disease.

In this prospective study, we demonstrate SP-A in human nasal tissue for the first time using immunohistochemical staining. In addition, this study correlates the severity of allergic rhinitis symptoms with the degree of SP-A expression using quantitative PCR. Previous work has implicated SP-A as an important molecule in local inflammatory responses elsewhere in the respiratory tract. Linking SP-A expression to the severity of nasal symptoms (sneezing and runny nose) suggests that SP-A may be an important molecule in local nasal inflammation as well, but additional work is necessary to determine whether SP-A elevation is a reaction to local allergy or a mediator of it.

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Author Contributions: Drs Wootten, Chen, and Lane had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Wootten, Labadie, Chen, and Lane. Acquisition of data: Wootten and Lane. Analysis and interpretation of data: Wootten, Labadie, and Lane. Drafting of the manuscript: Wootten. Critical revision of the manuscript for important intellectual content: Wootten, Labadie, Chen, and Lane. Statistical analysis: Wootten. Obtained funding: Wootten, Labadie, and Chen. Administrative, technical, and material support: Wootten and Lane. Study supervision: Wootten, Labadie, and Lane.

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