

Surfactant Protein D Expression in Chronic Rhinosinusitis Patients and Immune Responses In Vitro to *Aspergillus* and *Alternaria* in a Nasal Explant Model

Eng Hooi Ooi, MBBS; Peter-John Wormald, MD; A. Simon Carney, FRACS;
Craig Lloyd James, FRCPA; Lor Wai Tan, PhD

Objectives/Hypothesis: Common fungi have been implicated in the pathogenesis of chronic rhinosinusitis (CRS) with eosinophilic mucus (EMCRS). Surfactant protein (SP)-D plays an important role in the immune response to *Aspergillus fumigatus* in the lungs. We sought to determine whether SP-D is expressed in nasal mucosa and investigated the response of SP-D in vitro to fungal allergens. **Study Design and Methods:** 1) Nasal biopsies from 59 CRS and EMCRS patients, stratified into allergic fungal sinusitis (AFS), nonallergic fungal eosinophilic sinusitis (NAFES), and nonallergic nonfungal eosinophilic sinusitis (NANFES) were studied by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), immunostaining and enzyme-linked immunosorbent assay (ELISA). 2) Nasal tissue from three CRS and three NANFES patients was cultured with fungal allergens in a nasal explant in vitro model for 24 hours at increasing concentrations and mRNA SP-D secreted SP-D protein levels in response to the fungi determined by qRT-PCR and ELISA. **Results:** Staining for SP-D was detected in the submucosal glands from the nasal biopsies in all patient groups except for AFS. By ELISA, SP-D was undetectable in AFS and decreased in NAFES, NANFES, and CRS compared with controls. CRS patients in vitro cultured with *Aspergillus* and *Alternaria* allergens in a nasal tissue explant model induced up-regulation of SP-D by qRT-PCR. In con-

trast, NANFES nasal tissue explants cultured with *Aspergillus* allergens induced down-regulation of SP-D. **Conclusions:** We report for the first time the expression of SP-D in both diseased and normal nasal mucosa. SP-D expression in CRS patients is up-regulated by fungal allergens in an in vitro model. These results may provide potential novel therapy for treatment of CRS. **Key Words:** Chronic rhinosinusitis, allergic fungal sinusitis, eosinophilic mucus chronic rhinosinusitis, surfactant protein-D, *Aspergillus fumigatus*, *Alternaria*.

Laryngoscope, 117:51–57, 2007

INTRODUCTION

The innate immune response is crucial for eliminating pathogens. It is triggered initially and thereby affecting subsequent development of the adaptive immune response.¹ Surfactant protein (SP)-D, a member of the collectin family structurally characterized by a collagenous and C-type lectin domain, is secreted by respiratory epithelial cells. It binds to and agglutinates microorganisms and enhances phagocytosis, chemotaxis, and cytokine production.² SP-D plays an important role in immune defenses in the lung. It also appears to play an important role in allergic inflammation, with growing evidence suggesting it potentially down-regulates allergic hypersensitivity and inflammation.^{3,4} In a mouse model of allergic bronchopulmonary aspergillosis (ABPA), the mice were sensitized to and challenged with *Aspergillus fumigatus* followed by intranasal treatment with SP-D and recombinant SP-D.⁴ A shift from a T-helper (Th)2 to a Th1 cytokine response was detected in the splenic supernatants of these mice, in association with decreased *Afu*-specific immunoglobulin (Ig)G and IgE and peripheral blood and pulmonary eosinophilia.⁴ In vivo, wild-type and SP-D null mice were sensitized and challenged with the allergen ovalbumin in a mouse model of allergic inflammation, and the bronchoalveolar lavage fluid (BALF) was analyzed.³ The SP-D null mice had a greater Th2 response, with higher levels of eosinophilia and interleukin (IL)-13 but a reduced

From the Department of Surgery–Otorhinolaryngology Head and Neck Surgery (E.H.O., P.-J.W., S.C., L.W.T.), University of Adelaide and Flinders University, and Adelaide Pathology Partners (C.L.J.), South Australia.

Editor's Note: This Manuscript was accepted for publication August 14, 2006.

This study was supported by grants from the Garnett Passe and Rodney Williams Memorial Foundation, Victoria, Australia.

Send correspondence to Dr. Peter John Wormald, Department of Otorhinolaryngology Head and Neck Surgery, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South, South Australia 5011, Australia. E-mail: peterj.wormald@adelaide.edu.au

DOI: 10.1097/01.mlg.0000243196.75418.6f

Th1 response with lower levels of interferon (IFN)- γ in the BALF compared with wild-type mice.

Chronic rhinosinusitis (CRS) is a debilitating condition with chronic inflammation of the nasal mucosa playing a key role in its pathogenesis.⁵ It is commonly associated with the presence of nasal tissue eosinophilia and a Th2 cytokine response with IL-5 producing lymphocytes.⁵ Allergic fungal sinusitis (AFS) is a distinct form of CRS characterized by nasal polyps, thick eosinophilic mucin (EM), and fungal allergy and histologically similar to ABPA.⁵ The fungi commonly implicated in AFS include *Aspergillus* and *Alternaria*. A subgroup of CRS patients clinically similar to AFS with the characteristic presence of EM (EMCRS) has demonstrated distinct fungal-specific humoral immune response.⁶

Recent studies have demonstrated the presence of SP-D in other nonpulmonary tissues, implicating a wider role in innate immunity at mucosal surfaces other than the lung.⁷ The nasal cavity and paranasal sinuses are considered part of the respiratory tract. We surmise that SP-D may be produced by nasal mucosa given its expression at numerous nonpulmonary sites and investigated its innate immune responses in CRS and AFS patients in vivo and in vitro to fungal allergens.

MATERIALS AND METHODS

Patient Classification

This prospective study was conducted on patients undergoing endoscopic sinus surgery for CRS as defined by published criteria.⁸ Normal controls were endoscopic transsphenoidal pituitary surgery patients rigorously screened to exclude any sinusitis. The patient's atopic status was examined by testing for serum-specific IgE using a Pharmacia CAP (Pharmacia Diagnostics, AB, Uppsala, Sweden) system against a mold mix (*Aspergillus*, *Alternaria*, *Cladosporium*, *Penicillium*, *Candida*, and *Helminthosporium*) and other allergens (mite/house dust mite, grass pollen, animal epithelial mix). Specific IgE greater than 0.35 KUA/L was defined as a positive result. A positive skin-prick test to fungal and nonfungal allergen preparations (Hollister-Stier Laboratories, Spokane, WA) was defined as a wheal equal to or greater than 3 mm in diameter.

The characteristic EM collected from the sinuses at surgery was confirmed by histology and patients defined as fungal positive if either fungal stains or cultures from the EM were positive, as previously published.⁹ Patients were then classified on the basis of the histology and culture results as outlined below:

- CRS. No EM present at time of surgery and negative culture and staining for any fungi.
- EMCRS. CRS patients with EM present within the sinuses at surgery. EMCRS patients were further subcategorized according to the presence or absence of fungi in the EM (by histology or culture), and fungal allergy (positive mold-mix serum-specific IgE, or skin prick testing) as defined below:
- AFS. Presence of EM, fungal allergy, positive fungal stain or culture, characteristic sinus computed tomographic findings, and nasal polyps;
- Nonallergic fungal eosinophilic sinusitis (NAFES). Presence of EM, positive fungal stain or culture, negative fungal allergy;
- Nonallergic nonfungal eosinophilic sinusitis (NANFES). Presence of EM, negative fungal stain and culture, negative fungal allergy.

This study was undertaken at The Queen Elizabeth and Memorial Hospitals. The Ethics of Human Research Committee approved the study, and all patients gave written informed consent. Patients were excluded if they had received systemic or topical corticosteroids, antibiotics, or antifungals at least 1 week before surgery or were immunocompromised with tissue invasive fungal rhinosinusitis.

Allergen Challenge with *Aspergillus* and *Alternaria* extracts in a Nasal Tissue Explant In Vitro Model

Nasal tissue biopsies of approximately 3 mm² collected during surgery from three CRS and three NANFES patients were placed in tissue culture plates (Nunc, Roskilde, Denmark) with airway culture medium DMEM/HamsF12 (Sigma, Sydney, Australia) supplemented with 1% fetal calf serum (Gibco, Invitrogen Corporation, NY) in a sterile laminar flow environment. The nasal tissue explants were challenged with 1,250 ng/ μ L, 250 ng/ μ L, 50 ng/ μ L *Aspergillus fumigatus* and *Alternaria tenuis* (final concentrations, Hollister-Stier Laboratories, Spokane, WA) in a final volume of 400 μ L airway culture medium for 24 hours at 37°C and 5% CO₂. Untreated tissue specimens with equivalent volumes of culture medium served as controls. The nasal tissue and culture supernatant were harvested and analyzed for SP-D mRNA and protein levels. CRS patients were compared with NANFES because AFS patients did not demonstrate the presence of SP-D protein in the initial part of our study. Because of the significant quantities of required tissue to conduct the experiments (replicates and repeated experiments for both reverse-transcriptase polymerase chain reaction [RT-PCR] and enzyme-linked immunosorbent assay [ELISA]), we were ethically unable to remove the required amount of tissue from normal patients for an experimental normal control group. The experiments were performed in duplicate, and the experiment was performed at least three times, with the pooled results represented.

RNA Extraction from Nasal Tissue

Nasal biopsies collected at surgery were immediately placed in RNAlater (Ambion, Austin, TX) to preserve RNA integrity and stored at -80°C until analysis. Total RNA was extracted from the surgical biopsies using the RNeasy Mini Kit, and an additional On-Column Dnase Digestion step with the Rnase-Free Dnase Set according to the manufacturer's protocol (Qiagen, Hilden, Germany) was performed. The total RNA concentration was determined by measuring the absorbance at 260 nm using the Bio-Rad SmartSpec 3000 Spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Each sample was measured in duplicate.

Quantitative RT-PCR

First strand cDNA synthesis was performed using Omniscript Reverse Transcriptase kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The no-template control had the Omniscript enzyme omitted from the RT mix. The real-time quantitative (q)PCR assay for SP-D was performed using the QuantiTect SYBR Green PCR kit (Qiagen) on a MyIQ Thermocycler (Bio-Rad Laboratories, Hercules, CA). PCR primers were purchased from Geneworks (Adelaide, Australia), and sequences were derived using Primer Express 3.0 to span an intron (NCBI Accession #BC022318): SP-D forward primer 5'TCATGTGTAGCTCAGTGGAGAGTG3' and reverse primer 5'AGGTTCTCCACAGAGCCATTGT3'; HPRT forward primer 5'TGACACTGGCAAAACAATGCA3' and reverse primer 5'GGTCCTTTTCACCAGCAAGCT3'. Two microliters of diluted RT reaction (equivalent to 10 ng total RNA) was used for the PCR assay in a final total volume of 25 μ L with 2 $\times$ QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden, Germany), 10 nmol/L Fluorescein (Bio-Rad Laboratories, Hercules, CA), and 0.3

$\mu\text{mol/L}$ primers. The PCR assay was performed initially at 95°C for 15 minutes. Thirty-five cycles of amplification were then performed at 95°C for 15 seconds and at 62°C for 1 minute. A melting curve analysis of the products was performed at the end of the PCR assay, and single melting peaks were identified for both the target and reference genes. The PCR products were examined by agarose gel electrophoresis (data not shown), and single bands of the predicted size were identified for both SP-D (177 bp) and the reference gene hypoxanthine phosphoribosyl transferase (HPRT) (93 bp), and no bands were detected for the no-template controls. Sequenced PCR products were confirmed by searching against all available sequences in the National Center for Biotechnology Information database using the BLAST program (www.ncbi.nlm.gov/BLAST).

Cycle threshold (C_t) is defined as the PCR cycle at which detected fluorescence first rises significantly above the baseline threshold. The results were analyzed by the comparative C_t ($\Delta\Delta C_t$) method as published by Livak and Schmittgen¹⁰ rather than an absolute mRNA value, and the efficiencies of the primers used in the PCR assay were appropriately validated. This method analyses the relative change in expression of the target gene relative to a control group. The change in C_t (ΔC_t) = C_t (HPRT) – C_t (SP-D). The control was included with the patients in each run. The relative expression was quantified by calculating $2^{-\Delta\Delta C_t}$ to account for the exponential amplification of the PCR. Triplicate PCRs were run on each sample, and the C_t data for each well was averaged before performing the $2^{-\Delta\Delta C_t}$ calculation.¹⁰

Immunohistochemistry for SP-D

Paraffin-embedded tissue samples were sectioned into $5\ \mu\text{m}$ thickness, deparaffinized with xylene, and rehydrated using a series of alcohol dilutions. The antigen retrieval was performed using $100\ \text{mmol/L}$ Citric acid, pH 6.0, and endogenous peroxide activity quenched with 3% hydrogen peroxide for 30 minutes. Sections were blocked with fetal calf serum. The tissue sections were incubated with a 1:1000 SP-D polyclonal primary antibody (rabbit antimouse SP-D antibody known to cross-react with human SP-D, Chemicon, Temecula, CA) at 4°C overnight. Alkaline phosphatase activity was detected using the biotin-free EnVision G 2 kit performed according to the manufacturer's instructions and Levamisole added to the permanent red chromogen substrate to block endogenous alkaline phosphatase activity (Dako, Glostrup, Denmark). Counterstaining was with Mayer's hematoxylin solution. All rinses were performed with Tris buffered saline with Tween. For negative staining control purposes, adjacent tissue sections were processed with nonimmune rabbit serum (Dako, Glostrup, Denmark) as replacement for and at the same concentration as the primary antibody.

Protein Extraction from Nasal Tissue and Bradford Assay

Total protein was extracted from nasal tissue grinding in liquid nitrogen with Cell lytic-M Mammalian Cell Lysis Extraction reagent (Sigma, Sydney, Australia) and Protease Inhibitor Cocktail (Sigma, Sydney, Australia). The total protein concentration was determined by the mini-Bradford assay.

SP-D ELISA

A commercially available sandwich ELISA detecting SP-D protein with a sensitivity range from $0.08\ \text{ng/mL}$ to $200\ \text{ng/mL}$ was performed according to the manufacturer's instructions (Antibody Shop, Gentofte, Denmark). Fifty micrograms of total protein per sample was used for the assay, all assays were performed in duplicate, and the results are given as the concentration of SP-D ng/mL . Airway medium was used as a blank for the assay.

Statistics

Statistical analysis was performed with GraphPad Prism version 4.0 for Windows software (San Diego, CA). Multiple comparisons were performed by analysis of variance (ANOVA), unless it was nonparametric data, and then a Kruskal-Wallis test was used as indicated, followed by appropriate post hoc multiple pair-wise comparisons with a Bonferroni correction. P values less than .05 were considered statistically significant.

RESULTS

Demographic, Clinical, and Laboratory Data

Fifty-nine patients (AFS $n = 7$, NANFES $n = 13$, CRS $n = 26$, and NAFES $n = 13$) with the diagnosis of CRS undergoing ESS and nine patients undergoing endoscopic transsphenoidal pituitary surgery were recruited for this study. A summary of the demographic, clinical, and laboratory details are provided in Table I.

Human SP-D Expression in EMCRS and CRS Patients

SP-D transcript was detected in human nasal mucosa for the first time by qRT-PCR. The fold change of SP-D mRNA relative to the control group was calculated using the comparative C_t method. There was variability in the SP-D mRNA expression levels between patients in each group, as shown in Figure 1. However, there were no statistically significant differences between the different groups (Kruskal-Wallis test, $P = .9$).

Using a commercial ELISA kit quantifying SP-D protein, we detected reduced SP-D protein levels in CRS (median, $1.9\ \text{ng/mL}$) and NANFES (median, $1.9\ \text{ng/mL}$) patients relative to the control (median, $4\ \text{ng/mL}$), but this was not statistically significant (Kruskal-Wallis test, $P = .064$) (Fig. 2). Interestingly, all the AFS patients and the majority of NAFES (7/8) patients did not have detectable levels of SP-D protein within the sensitivity range of the ELISA.

Immunohistochemical Localization of SP-D Expression

SP-D protein expression was detected in nasal tissue biopsies by immunohistochemistry. There was strong positive staining in the lumen of the ducts and ductal epithelial cells of the submucosal glands, as shown in Figure 3A. This indicates that SP-D is secreted by glandular epithelium. Faint staining for SP-D was also detected in the nasal epithelial cell layer (Fig. 3C). However, SP-D staining was not uniformly present in all submucosal glands (Fig. 3B). Furthermore, SP-D staining was detected in the nonsinusitis controls, CRS, and NANFES patients and was not detected in the AFS patients, in support of our ELISA results. As a negative staining control, sections were incubated with nonimmune rabbit serum instead of SP-D antibody, resulting in loss of staining (Fig. 3D).

Alternaria and Aspergillus Allergens Up-Regulate SP-D Expression in a Nasal Explant In Vitro Model in a Dose-Response Effect

CRS patients. Challenging the nasal tissue in our explant in vitro model induced a significant dose response increase in SP-D mRNA expression in CRS patients, as

TABLE I.
Demographic, Clinical, and Laboratory Data for Patients and Controls.

	CRS (n = 26)	AFS (n = 7)	NAFES (n = 13)	NANFES (n = 13)	Controls (n = 9)
Median age (range)	42 (27–80)	33 (21–48)	46 (18–74)	46 (24–78)	53 (35–64)
Male/female	15/11	5/2	6/7	9/4	5/4
Median Lund-Mackay CT score (range)	10 (2–22)	19 (2–21)	12.5 (3–22)	21 (10–24)	0
Fungal allergy (%)	3/24 (13)	7/7 (100)	0	0	0
Other nonfungal allergy (%)	12/24 (50)	5/7 (71)	1/15 (6)	5/12 (42)	1/9 (11)
Microbiology*	Coagulase negative Staph. (n = 1), S. aureus (n = 3)	Aspergillus sp. (n = 3), Alternaria sp. (n = 3), Drechslera, Phoma glomerata, Ulocladium sp.	Aspergillus sp. (n = 5), Alternaria sp. (n = 2), Penicillium, Paecilomyces, Arthrographis sp.	S. aureus (n = 3)	Not applicable
Histology N = Fungus ⁺	0	3	6	0	Not applicable

*Some patients cultured more than one organism.

CRS = chronic rhinosinusitis; AFS = allergic fungal sinusitis; NAFES = nonallergic fungal eosinophilic rhinosinusitis; NANFES = nonallergic nonfungal eosinophilic rhinosinusitis; CT = computed tomography.

shown in Figure 4. Both *Aspergillus* and *Alternaria* at 1,250 ng/ μ L induced increases in SP-D mRNA approximately fourfold greater than 250 ng/ μ L or 50 ng/ μ L of fungal allergens. These results demonstrate that SP-D expression at the mRNA level in CRS patients is up-regulated in response to fungal allergens. Next, we were able to demonstrate that SP-D protein is secreted by nasal tissue and detected by ELISA in our explant in vitro model, as shown in Figure 5A. However, there was no statistically significant correlation with the mRNA expression and no statistically significant differences between challenge with and without fungal allergens (one-way ANOVA, $P = .9$; SP-D protein levels were below the detectable limits of the assay for the control tissue homogenates). We also observed variability in SP-D mRNA and protein levels, even though all tissue samples were harvested and processed in the same manner.

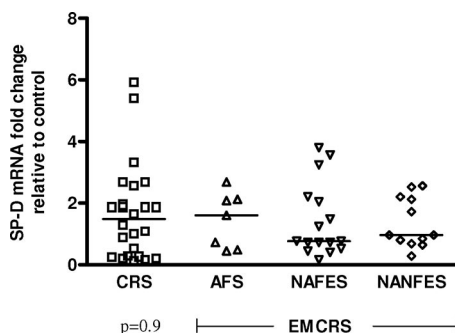


Fig. 1. Expression of surfactant protein (SP)-D mRNA in nasal biopsies from allergic fungal rhinosinusitis (AFS), nonallergic fungal rhinosinusitis (NAFES), nonallergic nonfungal eosinophilic sinusitis (NANFES), and chronic rhinosinusitis (CRS) patients. Data analyzed by quantitative reverse-transcription polymerase chain reaction and SP-D mRNA levels are normalized to hypoxanthine phosphoribosyl transferase (HPRT) and expressed as fold-change in SP-D mRNA levels relative to controls (pituitary patients). Data are represented as scatter dot plot with the line at the median.

NANFES Patients. SP-D mRNA expression in NANFES patients was induced by *Alternaria* allergens at only the highest concentration of 1,250 ng/ μ L *Alternaria*, which induced a median twofold increase greater than that of the controls or *Aspergillus* challenged tissue explants, as shown in Figure 6 (one-way ANOVA, $P = .0084$). In contrast, SP-D mRNA levels were down-regulated with *Aspergillus* allergens (fold-change <1). There was a reduced amount of SP-D protein produced and secreted by NANFES patients detected in our explant in vitro model in response to both fungal allergens compared with the controls (no fungal allergens, tissue homogenates median 2 ng/mL, conditioned media 5 ng/mL; one-way ANOVA, $P = .0012$), as shown in Figure 5B. From the qRT-PCR results, we would not expect any increase in SP-D levels with *Aspergillus* except perhaps for the highest concentration of *Alternaria*, and we were surprised to find a significantly less amount of SP-D protein

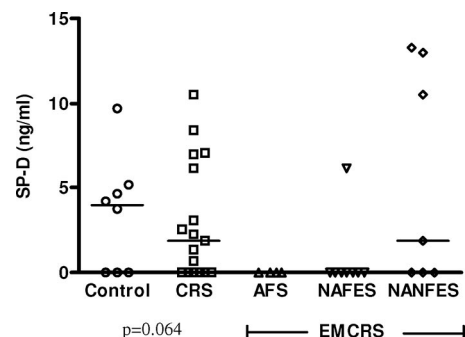


Fig. 2. Enzyme-linked immunosorbent assay measuring surfactant protein (SP)-D levels in tissue homogenates of nasal biopsies from allergic fungal rhinosinusitis (AFS), nonallergic fungal rhinosinusitis (NAFES), nonallergic nonfungal eosinophilic sinusitis (NANFES), chronic rhinosinusitis (CRS) patients, and normal controls (pituitary patients). Data are represented as scatter dot plot with the line at the median and values represented as ng/mL SP-D protein.

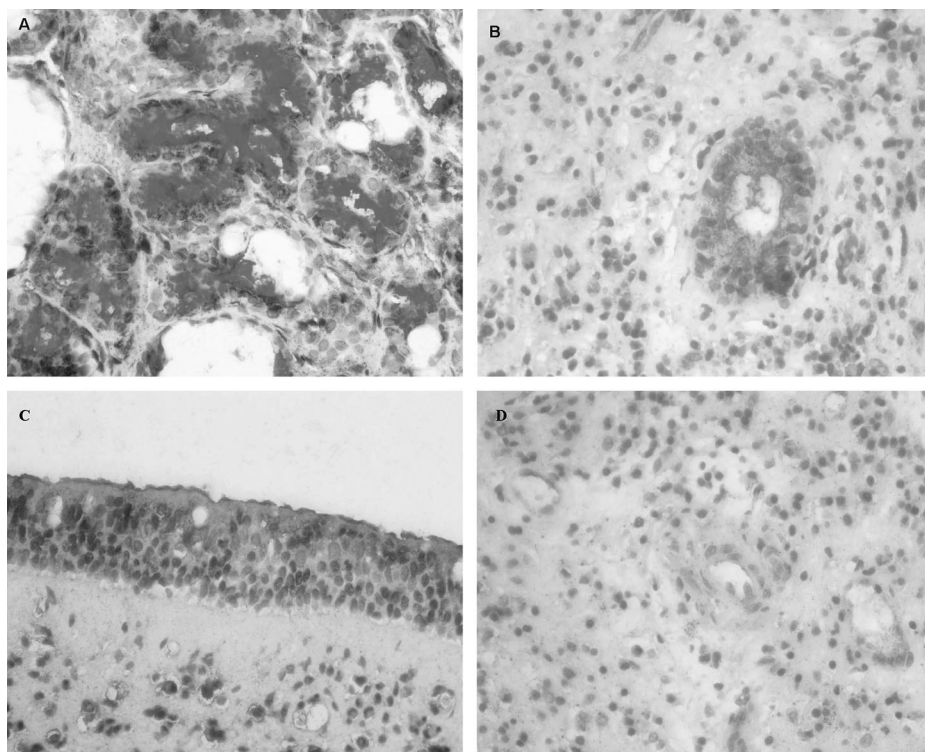


Fig. 3. Immunohistochemical staining for surfactant protein (SP)-D in nasal tissue biopsies. Binding of primary antibody is detected by alkaline phosphatase, which yields dark red color reaction. Staining is detected in duct epithelial cells and lumen of submucosal glands of human nasal mucosa (A). Some glands exhibited weak staining for SP-D (B). Faint staining is detected in nasal epithelial cells (C). Nasal tissue stained with nonimmune rabbit serum instead of primary antibody results in loss of staining (D) (magnification, $\times 40$).

detected for both *Aspergillus* and *Alternaria* compared with the untreated tissue specimens.

DISCUSSION

CRS with nasal polyps and EM is associated with eosinophilia, a Th2 inflammatory response, and elevated serum IgG3 levels.^{5,6} However, the expression of SP-D, an important innate immune modulator, has not been previ-

ously investigated in CRS. We report here for the first time the presence of SP-D expression in human nasal mucosa. Recent studies have demonstrated the presence of SP-D in other tissue sites by RT-PCR and immunohistochemistry.^{7,11} Expression of SP-D mRNA was highest in the lung but was also detected in the kidney, trachea, brain, testis, pancreas, salivary gland, heart, prostate, small intestine, and placenta. Low levels of expression were detected in the uterus, stomach, mammary glands, spleen, adrenal gland, and liver.¹¹

By immunohistochemistry, we detected strong SP-D staining in the duct epithelial cells and lumen of the submucosal glands in the CRS and NANFES patients. Immunohistochemical staining of normal human tissues for SP-D revealed that it was strongly expressed in alveolar type II epithelial cells, Clara cells, on and within alveolar macrophages, in epithelial cells and ducts of numerous glandular tissue, and intrahepatic bile ducts.^{7,11} Our finding is consistent with the wide distribution of SP-D at mucosal surfaces exposed to pathogens. However, we were unable to detect staining for SP-D in the AFS patients, indicating possible reduction in SP-D synthesis or intracellular reserves.

The SP-D protein levels were below the detection limit of the assay in the AFS patients in support of our immunohistochemistry results, and we observed a trend for decreased SP-D levels in CRS and NANFES patients compared with controls. A possible explanation for this observation is that absence of significant SP-D gene up-regulation and depletion of SP-D protein reserves during the progression from acute to CRS may result in failure to clear the pathogen from the sinuses and increase the chances of re-infection or recolonization post-

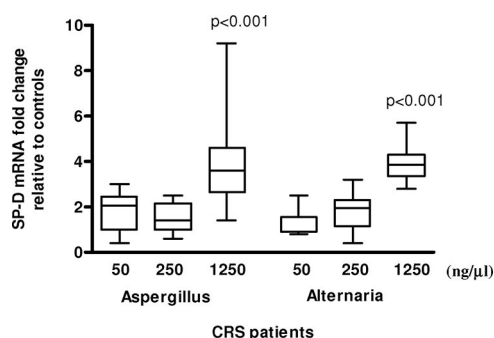


Fig. 4. Expression of surfactant protein (SP)-D mRNA by quantitative reverse-transcription polymerase chain reaction in human nasal tissue from chronic rhinosinusitis (CRS) patients challenged with *Aspergillus fumigatus* and *Alternaria tenuis*. Levels are normalized to hypoxanthine phosphoribosyl transferase (HPRT). Data are expressed as fold-change in mRNA levels of SP-D relative to controls (no fungal allergens). Horizontal bars and boxes indicate median and 25th to 75th percentiles. Error bars indicate range. Both *Aspergillus* and *Alternaria* showed dose-dependent increases in nasal tissue SP-D mRNA expression (one-way analysis of variance, $P < .0001$). 1,250 ng/μL *Aspergillus* compared with 250 ng/μL, 50 ng/μL *Aspergillus* and 250 ng/μL, 50 ng/μL *Alternaria* ($P < .001$); 1,250 ng/μL *Alternaria* compared with 250 ng/μL, 50 ng/μL *Aspergillus* and 250 ng/μL, 50 ng/μL *Alternaria* ($P < .001$) by Bonferroni's multiple comparison test.

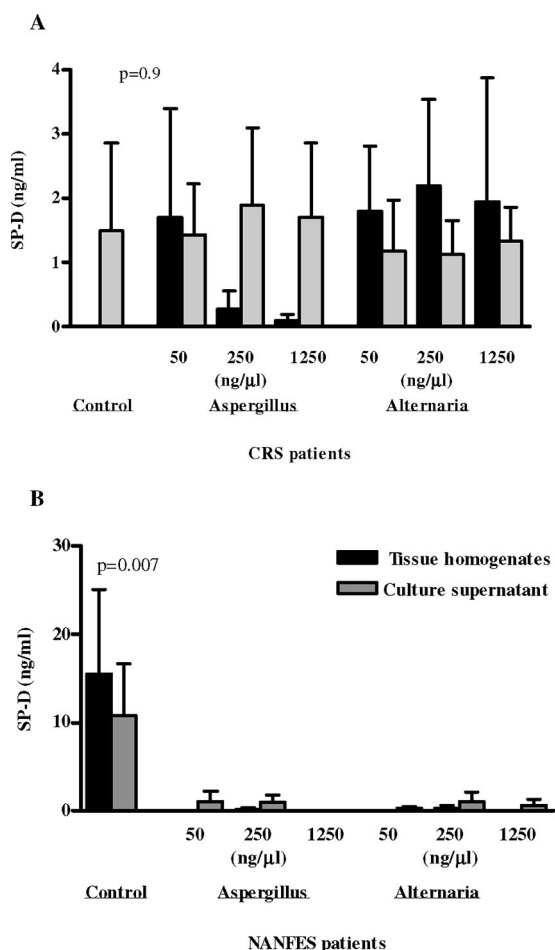


Fig. 5. Expression of tissue and secreted surfactant protein (SP)-D protein levels measured by enzyme-linked immunosorbent assay in chronic rhinosinusitis (CRS) (A) and nonallergic fungal eosinophilic sinusitis (NANFES) (B) patients in response to fungal allergen challenge in nasal explant in vitro model compared with controls (no fungus). Controls (B) compared with tissue homogenates challenged with 1,250 ng/μL, 250 ng/μL, and 50 ng/μL *Aspergillus* and *Alternaria* in NANFES patients by Bonferroni's multiple comparison test ($P < .05$). Data are represented as mean $\pm$ standard error of mean.

operatively. Grubor et al.,¹² using an ovine model of chronic pneumonia, demonstrated decreased SP-D expression as measured by real-time RT-PCR and immunohistochemistry. Healthy lambs were infected with either saline or *Mannheimia hemolytica* (a Gram-negative bacteria that colonizes the mucosa of the upper respiratory tract and nasopharynx of sheep) in their lungs to cause pneumonia, the lambs were killed, and the lungs harvested at 1 day (acute), 15 days (subacute), and 45 days (chronic) postinfection.¹² SP-D mRNA expression increased initially after infection as measured by real-time RT-PCR, but at 45 days, the SP-D mRNA levels were not significantly different from the saline inoculated control animals. There was an associated decrease in intensity of SP-D protein staining and number of SP-D positive cells in the lungs of the infected lambs compared with the saline treated lambs, with increased inflammation on histology associated with decreased amount of staining, possibly caused by exhaustion of SP-D protein reserves.

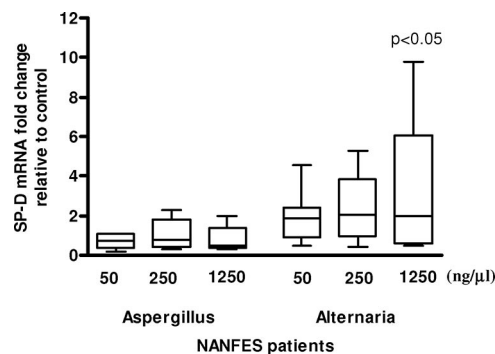


Fig. 6. Expression of surfactant protein (SP)-D mRNA by quantitative reverse-transcription polymerase chain reaction in human nasal tissue from nonallergic fungal eosinophilic sinusitis (NANFES) patients challenged with *Aspergillus fumigatus* and *Alternaria tenuis*. Levels are normalized to hypoxanthine phosphoribosyl transferase (HPRT). Data are expressed as fold-change in mRNA levels of SP-D relative to controls (no fungal allergens). Horizontal bars and boxes indicate median and 25th to 75th percentiles. Error bars indicate range. 1,250 ng/μL *Alternaria* compared with 1,250 ng/μL and 250 ng/μL *Aspergillus* by Bonferroni's multiple comparison test ($P < .05$).

SP-D also plays a role in regulating inflammation and protecting against pulmonary hypersensitivity disorders, as demonstrated by several human and mice studies. Human SP-D has been shown in vitro to bind to 3-week culture filtrate allergens and the purified glycosylated allergens gp45 and gp55 of *Aspergillus fumigatus*. It also inhibits binding of allergen-specific IgE from Aspergillosis patients to the allergens and inhibits the *A. fumigatus* allergen-induced histamine release from sensitized basophils of ABPA patients.¹³ Intranasal treatment with SP-D (1 μg/mouse) and recombinant SP-D (1 μg/mouse) in a mice model of ABPA decreased blood eosinophilia, pulmonary infiltration, and levels of allergen-specific IgG and IgE.⁴ The levels of IL-2, IL-4, and IL-5 decreased, whereas IFN-γ increased in the splenic supernatants of the treated ABPA mice, indicating a marked shift from a Th2 to a Th1 cytokine response.⁴ The effects of SP-D may be potentially beneficial in CRS patients with a Th2 cytokine response.

We investigated the effects of fungal allergens *Aspergillus* and *Alternaria* on SP-D expression in a nasal explant in vitro system. This explant system allows us to investigate the responses of whole nasal tissue to fungal allergens. Nasal tissue explants have been used previously to investigate the role of superantigens on glucocorticoid receptor expression¹⁴ and the role of macrolides and corticosteroids on cytokine responses in vitro.¹⁵ However, the SP-D mRNA and protein responses to fungal allergens in a nasal explant model have not been previously described. Our observations suggest that there is low SP-D expression in nasal tissue in the CRS and NANFES patients. We demonstrated that fungal allergens induce SP-D mRNA expression by nasal tissue in our explant model. *Alternaria* increased SP-D mRNA expression in both CRS and NANFES patients, whereas *Aspergillus* increased SP-D mRNA expression in CRS but not in NANFES patients. In support of our findings, Haczku et al.¹⁶ have reported a lack of SP-D gene up-regulation to *As-*

pergillus in vivo in a mouse model of airway inflammation. We also demonstrated that SP-D is detected in nasal tissue homogenates and secreted in response to fungal allergens in CRS patients. However, in NANFES patients, only the highest dose of *Alternaria* used *in vitro* induced up-regulation of SP-D. We were also intrigued to find that the levels of SP-D were significantly reduced in NANFES patients challenged with higher doses of fungal allergens relative to the controls. The relative deficiency in SP-D levels of cystic fibrosis (CF) patients compared with normal controls or those with acute lung infection is implicated as a cause in the increased frequency of bacterial colonization in CF children.¹⁷ Local treatment with SP-D has been proposed to be useful in CF by helping to combat infections and modulating inflammatory responses to fungal infection.¹⁸ In the future, treatment with intranasal SP-D in EMCRS patients may also modulate the inflammatory response to fungal allergens.

CONCLUSIONS

In conclusion, we demonstrated the presence of SP-D mRNA and protein in nasal mucosa from normal and CRS patients for the first time using real-time RT-PCR and immunohistochemistry. SP-D expression in CRS patients is up-regulated by fungal allergens in a nasal explant *in vitro* model. Our results suggest that SP-D is a novel innate defense peptide in human nasal mucosa and may be important in fungal allergen induced inflammation.

Acknowledgments

The authors thank Drs. Guy Rees, Nick Vrodos, Steve Santerenos, and Steve Floreani for providing some of the patients for this study. The authors also thank Lianne Hawke and Drs. Smita Nagaonkar, Deepti Singhal, and Graham Van Renen for technical assistance.

BIBLIOGRAPHY

1. Medzhitov R, Janeway C Jr. Innate immunity. *N Engl J Med* 2000;343:338–344.
2. Wright JR. Host defense functions of pulmonary surfactant. *Biol Neonate* 2004;85:326–332.
3. Schaub B, Westlake RM, He H et al. Surfactant protein D deficiency influences allergic immune responses. *Clin Exp Allergy* 2004;34:1819–1826.
4. Madan T, Kishore U, Singh M et al. Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. *J Clin Invest* 2001;107:467–475.
5. Hamilos DL. Chronic sinusitis. *J Allergy Clin Immunol* 2000;106:213–227.
6. Pant H, Kette FE, Smith WB et al. Fungal-specific humoral response in eosinophilic mucus chronic rhinosinusitis. *Laryngoscope* 2005;115:601–606.
7. Stahlman MT, Gray ME, Hull WM, Whitsett JA. Immunolocalization of surfactant protein-D (SP-D) in human fetal, newborn, and adult tissues. *J Histochem Cytochem* 2002;50:651–660.
8. Meltzer EO, Hamilos DL, Hadley JA et al. Rhinosinusitis: establishing definitions for clinical research and patient care. *J Allergy Clin Immunol* 2004;114:155–212.
9. Collins M, Nair S, Smith W et al. Role of local immunoglobulin E production in the pathophysiology of noninvasive fungal sinusitis. *Laryngoscope* 2004;114:1242–1246.
10. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 2001;25:402–408.
11. Madsen J, Kliem A, Tornøe I et al. Localization of lung surfactant protein D on mucosal surfaces in human tissues. *J Immunol* 2000;164:5866–5870.
12. Grubor B, Gallup JM, Ramirez-Romero R et al. Surfactant protein D expression in normal and pneumonic ovine lung. *Vet Immunol Immunopathol* 2004;101:235–242.
13. Madan T, Kishore U, Shah A et al. Lung surfactant proteins A and D can inhibit specific IgE binding to the allergens of *Aspergillus fumigatus* and block allergen-induced histamine release from human basophils. *Clin Exp Immunol* 1997;110:241–249.
14. Fakhri S, Tulic M, Christodoulopoulos P et al. Microbial superantigens induce glucocorticoid receptor beta and steroid resistance in a nasal explant model. *Laryngoscope* 2004;114:887–892.
15. Wallwork B, Coman W, Feron F et al. Clarithromycin and prednisolone inhibit cytokine production in chronic rhinosinusitis. *Laryngoscope* 2002;112:1827–1830.
16. Haczku A, Atochina EN, Tomer Y et al. *Aspergillus fumigatus*-induced allergic airway inflammation alters surfactant homeostasis and lung function in BALB/c mice. *Am J Respir Cell Mol Biol* 2001;25:45–50.
17. Postle AD, Mander A, Reid KB et al. Deficient hydrophilic lung surfactant proteins A and D with normal surfactant phospholipid molecular species in cystic fibrosis. *Am J Respir Cell Mol Biol* 1999;20:90–98.
18. Clark H, Reid K. The potential of recombinant surfactant protein D therapy to reduce inflammation in neonatal chronic lung disease, cystic fibrosis, and emphysema. *Arch Dis Child* 2003;88:981–984.