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Novel Role Of Surfactant Protein A In Bacterial Sinusitis

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Abstract

Background—Chronic rhinosinusitis (CRS) is a common inflammatory disorder of the upper airway characterized by chronic inflammation and significant sinonasal remodeling. CRS has two major subgroups based on whether polyps are present or absent. In some cases, it is characterized by colonization with opportunistic pathogens such as *Pseudomonas aeruginosa* (PA), *Staphylococcus aureus* and other bacteria. The innate immune system of the sinonasal epithelium is the first line of defense against inhaled pathogens. Surfactant protein A (SP-A) is a member of the collectin family secreted by the airway epithelia that plays a critical role in airway innate immunity, as it can aggregate bacteria. We hypothesized that SP-A plays a role in bacterial CRS.

Methods—Air-liquid-interface (ALI) cultures of nasal epithelial cells were derived from human *ex vivo* healthy and CRS sinus tissues (n=26) and challenged with PA. SP-A levels were measured with Western Blot and qRT-PCR in ALI and sinus tissues.

Results—We determined that SP-A i) mRNA and protein levels are significantly increased in CRS tissues compared to healthy sinuses; ii) although primarily expressed in the lung, it is also synthesized and expressed in sinonasal epithelia; ii) is expressed in the sinuses of an SP-A humanized transgenic mouse but not in SP-A knock-out mouse; iv) mRNA levels are upregulated significantly during PA challenge, but protein levels are downregulated 4h post challenge and upregulated at 12h.

Conclusions—Our data suggest that SP-A is expressed in the sinuses and it plays a role in the sinus innate immune responses during bacterial infections.

Graphical abstract

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Disclosures

Dr. Chang is a consultant for Acclarent Inc., however the work in this publication is unrelated and does not represent a conflict of interest.

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Keywords

CRS; Innate Immunity; *Pseudomonas aeruginosa*; Rhinosinusitis; SP-A

Introduction

Chronic rhinosinusitis (CRS) is a common inflammatory disorder of the sinonasal airway, affecting up to 15% of the population¹ with direct costs exceeding \$3 billion annually². The innate immune system of the sinonasal epithelium is the first line of defense against inhaled bacterial and viral pathogens. When this defense is compromised, pathogens can thrive and initiate a cycle of chronic infection and inflammation of the sinonasal epithelium. This disease state is characterized by proliferation of recalcitrant bacteria such as *Pseudomonas aeruginosa*, and an enhanced inflammatory response resulting in epithelial remodeling from repeated or unresolved sinusitis.

The mechanism behind inter-individual differences in susceptibility and severity of sinus infections and development of inflammatory sinus disease are poorly understood. The initial innate immune host response to a bacterial infection is critical in maintaining a healthy state of the sinonasal mucosa. The sinonasal epithelium (SNE) is the first contact point with inhaled bacteria. Upon activation, the innate immune system releases antimicrobial cytokines and chemokines, proteins and peptides, followed by recruitment of adaptive cell-mediated responses of lymphocytes and phagocytes that target and eliminate the recognized pathogens. Sinus innate immunity plays a critical role against infection but inappropriate control or imbalance of requisite immune responses can lead to acute or chronic sinus inflammation.

Unlike the adaptive immune system that is antigen-specific, the innate immune system recognizes a limited number of highly conserved structures that are present in a variety of pathogens. These structures, often referred to as pathogen associated molecular patterns (PAMPs), can recognize lipopolysaccharides at the outer membrane of gram-negative bacteria, lipoteichoic acid present in gram-positive bacteria, peptidoglycans and mannans that are found in all bacteria, and bacterial DNA. Recognition of extracellular or endosomal PAMPs is mediated by transmembrane proteins known as toll-like receptors (TLRs). The activation of TLRs by different pathogens results in activation of transcription factors leading to the synthesis of a variety of inflammatory cytokines and antimicrobial peptides. In the lower airway, the surfactant proteins A and D (SP-A, SP-D) can also recognize PAMPs, aggregate and opsonize a variety of bacterial, fungal and viral species. Therefore they are considered as one of the key players of the lung innate immune system³. We postulate that they also have similar functions in the sinuses.

Surfactant protein A (SP-A) is a member of the collectin family that is secreted by the proximal and distal airway epithelia. SP-A plays a critical role in airway innate immunity, and can directly eliminate bacteria as it binds to their cell wall, inhibits bacterial growth, and mediates enhanced opsonization by other effector cells, such as macrophages. Recently, SP-A mRNA was detected in *ex vivo* sinus tissues of those with and without CRS^{4, 5}, with the

SP-A gene being significantly upregulated in CRS specimens⁶. However, it is unclear how SP-A responds to bacterial challenges or its role in the inflammatory cascade of CRS.

In the present study, we hypothesized that SP-A is expressed in the sinonasal epithelium and that it plays a role in the innate immune responses of bacterial sinusitis. We determined that SP-A is synthesized and expressed in the nasal epithelia and that its expression levels are altered in response to *P. aeruginosa* challenge.

Materials and Methods

Human Subjects and Sample Collection

Study participants were patients who were undergoing endoscopic sinus surgery at the Banner University Medical Center, Tucson, Arizona⁷. This study was evaluated and approved by the institutional review board at The University of Arizona (IRB 1502660530). Each subject was fully informed and provided written consent before participating. CRS was diagnosed by a rhinologist according to the guidelines set forth by the American Academy of Otolaryngology² and the European position paper on rhinosinusitis and nasal polyps⁸. All individuals had symptoms for longer than 12 weeks, with nasal endoscopy and findings of mucosal thickening on computed tomography of the sinuses. Healthy controls were individuals undergoing surgery for benign pituitary disorders who had an absence of symptoms or history of chronic rhinosinusitis and a negative CT scan for disease. Demographic information is presented in Table 1.

All tissue specimens were collected from the uncinate process of the middle meatus or the anterior surface of the middle turbinate due to ease of access and the representative nature of this area. Samples were immediately placed in sterile collection tubes, labeled with de-identified study identification numbers, and snap-frozen in CoolBox M30 System (BioCision LLC, San Rafael, CA). Biospecimens were stored at -80°C before RNA and protein extraction.

Cultures of human nasal epithelial cells at the air liquid interface and *P. aeruginosa* challenge

Culture of SNECs at air liquid interface (ALI) was performed as described previously⁹. Briefly, middle meatus tissues were dissociated in minimum essential medium (MEM) media containing pronase and DNase I for 48h at 4°C ; cells were spun down and plated in primary 10-cm plates (Fisher Scientific, Waltham, MA) for 4 h 37°C in 5% CO_2 to remove fibroblasts. Non-adherent cells were plated into 10-cm that have been coated with collagen and cultured in BEGM media (Lonza, New Jersey, NJ) until they reached 80% confluence. Cells were trypsinized, counted and seeded onto culture inserts containing BEGM; upon reaching 90% confluence, apical media was removed and cells were cultured at ALI for three weeks, mimicking the physiological conditions. Elimination of fibroblasts was confirmed by absence of vimentin in the ALI (Supplementary File 1). Mature differentiated nasal epithelial cells were infected apically with 1.0×10^6 CFU/ml of *P. aeruginosa* strain K (PAK)¹⁰ in 50 μl of DBPS, or vehicle treatment for 4 h, as described previously^{11, 12}. Cells

were washed with 50 µl of DBPS and lysed at 4 and 12h post challenge for protein and RNA analysis as described below.

Animals

We used a total of ten C57BL/6 mice, five SP-A (–/–) knockouts and five humanized transgenic (hTG) knock-ins SP-A2 (+/–)¹³. Mice were euthanized by CO₂ asphyxiation and whole nasal meatuses were harvested and snap frozen with liquid nitrogen followed by Bessman Tissue Pulverization. All procedures involving animals used protocols that were approved by the Institutional Animal Care and Use Committee at the University of Arizona, College of Medicine. All mice were maintained in facilities under pathogen-free conditions or in barrier containment facilities.

Isolation of mRNA and qRT-PCR

Total RNA from the sinus tissues or SNECs was isolated using RNeasy Mini and Micro Kit, respectively, (Qiagen, Valencia, CA) following vendor's protocol. cDNA was generated with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and then used as a template for real-time qPCR with TaqMan Gene expression assays for SFTP-A2 (SP-A2) and GAPDH (housekeeping gene) (Thermo Scientific). The variability across specimens was assessed by standard deviation and the p-values were calculated based on a Student's t-test. P-values of < 0.05 were considered statistically significant.

Protein Extraction and Western Blot Analysis

Proteins were extracted from either tissue or cultured nasal epithelial cells with 200 µl of RIPA buffer (Thermo Fisher Scientific, Waltham, MA) according to vendor's protocol. Protein concentration was determined by BCA assays (Pierce, Waltham, MA). Proteins extracts (20 µg) were reduced with 20mM of dithiothreitol (DTT) in SDS loading buffer, denatured at 95°C for 5 min and fractionated in SDS-PAGE, transferred to nitrocellulose membrane, blocked with 5% BSA in PBS 0.05% Tween 20. Blots were probed with specific primary polyclonal rabbit antibody anti-SP-A2 (Aviva, San Diego, CA) (1:1000 dilution), followed by a secondary goat anti-rabbit IgG HRP conjugate (Bio-Rad, Hercules, CA) (1:2000 dilution) and ECL Plus chemiluminescence substrate (Pierce). For loading control, we used a specific monoclonal rat anti-β-actin AC-15 HRP conjugated antibody (Novus, Littleton, CO). Densitometry was performed with ImageJ software.

Immunohistochemistry

Paraffin-embedded nasal tissue sections slides were deparaffinized in xylene and ethanol, according to standard procedures. Antigen retrieval was carried out in 0.05% trypsin (5 min), after which slides were incubated in 3% H₂O₂ for 10 min to block peroxidase activity. Sections were blocked using the M.O.M. Immunodetection Kit (Vector Laboratories), according to the manufacturer's instructions. Primary specific rabbit anti-SP-A (AB3420-I, Millipore, Billerica, MA) was added at 1:1000 dilution for 18h, followed by a secondary goat anti-rabbit IgG HRP conjugate (Bio-Rad). For staining control, nasal tissues were pre-incubated with molar excess of purified human SP-A (100 µg) followed by primary anti-SP-A and same secondary antibody. Antibody reactivity was visualized using DAB substrate

(Vector Laboratories) and counterstained with hematoxylin. Imaging was performed with inverted Leica DMI6000 fully motorized microscope (Leica, Buffalo Grove, IL) at the University of Arizona Cancer Center Imaging Core Facility.

Results

SP-A is increased in CRS sinus tissues

SP-A mRNA has been detected previously and was shown to be increased in nasal tissues without polyps from CRS patients as compared to healthy controls⁶. We confirmed that SP-A mRNA levels were significantly increased in our CRS patients as compared to healthy subjects by 2.5- fold (Figure 1, Panel A). In addition, we determined the expression of SP-A protein by western blot analysis (Figure 1, Panels B and C) and report that in tissues from CRS patients there is a significant 3.0- fold increase of the levels of SP-A protein as compared to the healthy controls. In our cohort, we did not see any significant differences in the expression levels of SP-A between subjects with or without nasal polyps.

SP-A is synthesized and expressed in the sinonasal epithelium

To investigate whether SP-A is expressed in the sinus tissues we employed the hTG SP-A2 (+/–) and the SP-A KO (–/–) mouse models. We found that human SP-A protein is detected with western blot in the nasal tissues of the hTG and is absent at the KO (Figure 2). By immunohistochemistry, we detected SP-A in the sinonasal epithelium of human sinus nasal tissues along the entire apical surface of the ciliated epithelia and in the submucosal glands (Figure 3). These findings support the concept that SP-A, apart from its role in the lung, it is also expressed in the nasal cavity.

SP-A is regulated after bacterial challenge

Given the fact that SP-A has innate immune functions in the lower lung, we investigated its role in the upper airway. SNECs that were isolated from healthy subjects were challenged with *Pseudomonas aeruginosa* strain K (PAK) for 4h and we monitored the response of SP-A immediately after PAK challenge at 4h and 12h post challenge. Interestingly, we found that SP-A mRNA levels increased significantly by 2- fold immediately after PAK challenge and by 3- fold 12h post challenge (Figure 4, Panel A). We also monitored the expression of SP-A protein levels over time and found that after PAK challenge, SP-A levels were initially decreased at the 4h time point whereas at 12h levels they were increased (Figure 4, Panel B). Data were obtained from three different subjects (n=3). These findings suggest that SP-A is synthesized in the pseudostratified nasal epithelium and expression is altered during bacterial challenge.

Discussion

The sinonasal epithelium not only acts as a physical barrier between the environmental insults and the respiratory system, but it also has immunological functions against bacteria. Therefore, this barrier is also the sentinel, frontline defense mechanism of the respiratory system. Upon sinus bacterial infections a cascade of adaptive and innate immune responses are initiated leading to production of antimicrobial cytokines and chemokines such as

interferons¹⁴, defensins^{15, 16}, and lactoferrin¹⁷. These confer to rapid and effective clearance of most pathogens, although sometimes pathogens manage to evade the defense mechanisms. In the latter case, microbes can replicate in the sinonasal epithelial cells (SNECs), produce cytotoxic chemokines that disrupt the epithelial repair mechanisms, thereby enhancing paracellular permeability. Furthermore, these cytotoxins can interfere with the nasal mucosal microflora, paving the way for secondary bacterial infections that can easily migrate to the lower bronchial epithelium, and ultimately to the lungs.

Surfactant proteins A (SP-A) and SP-D are critical in the innate immune response. These belong to the secreted pattern recognition receptors and function as opsonins for phagocytosis¹⁸. SP-A and SP-D help modulate the immune system^{19, 20}, its inflammatory response²¹, and trigger the clearance of apoptotic cells by alveolar macrophages (AMs). SP-A and SP-D can promote clearance and elimination of pathogens directly by binding to them or indirectly by stimulating phagocytosis mediated by other phagocytic mediators, such as the AMs and lymphocytes.

In primates and humans there are two distinct SP-A genes (SFTP-A1 and SFTP-A2) that encode two different proteins SP-A1 and SP-A2 that have biochemical and functional differences. Unlike primates, rodents have only one gene. It is believed that the relative amount of the two genes (SP-A1 and SP-A2) rather than the total SP-A plays a role in lung disease susceptibility. Even though they can maintain normal lung function, the SP-A knock out mice are more vulnerable to bacterial and viral infections, and the effect of oxidative stress (ozone exposure)^{22–24}. These findings implicate SP-A in regulating inflammation and limiting epithelial damage in response to environmental insults (bacteria, viruses, ozone). SP-A can bind both Gram-negative (recognize their membrane lipopolysaccharides) and Gram-positive bacteria (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Staphylococcus aureus*), as well as fungi (recognize the mannans) such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Candida albicans*, *Pneumocystis jiroveci*^{11, 25}. Also, SP-A can recognize, bind and aggregate viruses such as influenza A and RSV^{23, 26} that are implicated in the nasal and respiratory diseases.

SP-A mRNA has been detected and shown to increase in CRS patients' nasal tissues without polys comparing to healthy controls⁶. This report is consistent with our present study where we looked not only at mRNA, but also at protein levels of SP-A. In our cohort of patients, we found that in CRS nasal tissues SP-A was upregulated significantly by 2.5- fold at mRNA level, and by 3- fold at protein level, compared to healthy nasal tissue specimens (Figure 1, Panels A, B, and C). These collective data suggest the potential use of SP-A levels as a biomarker of sinusitis. We also confirmed the expression of SP-A at the sinus mucosa utilizing a humanized transgenic mouse model that expresses human SP-A instead of the mouse protein (Figure 2). This additional observation shows that SP-A is expressed in the nasal cavity.

Both SP-A and SP-D have been shown to be immunolocalized in the submucosal glands of both healthy controls and CRS patients⁴. Consistent with this finding, we found expression of SP-A not only in the submucosal glands but also at ciliated lining of the columnar nasal epithelial cells (Figure 3). The consensus is that surfactant (proteins and lipids) is

predominantly produced in the lungs by the alveolar type II pneumocytes and it has been shown to play two roles. While the protein portion of surfactant has innate immune functions against inhaled pathogens, the lipid fraction lowers the surface tension at the alveoli during the respiration process^{19, 20}. It has been also proved that the bronchial epithelial cells (Clara cells) produce surfactant proteins and play a major immune role in the bronchial mucosa²⁷. One possibility is that the surfactant present in the sinonasal cavity could be initially produced in the lower airway. However, in the present study when we isolated nasal epithelial cells from human tissue subjects, cultured them, expanded them, and brought them to air-liquid interface, we managed to produce a pseudostratified sinonasal epithelium and we found the presence of SP-A at both mRNA and protein levels (Figure 4, Panels A and B, mock treatment). This finding demonstrates that SP-A is synthesized and secreted by the sinonasal epithelium itself. Further experimentation is required to find which of the epithelial cells is producing SP-A. Based on immunohistochemistry of paraffin embedded nasal tissues it is very likely that the columnar ciliated epithelial cells are producing SP-A (Figure 3). Furthermore, although SP-A and SP-D have been found by others in the nasal tissue by real time quantitative polymerase reaction (RT-qPCR), enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry²⁸, we are the first group to report the presence of SP-A both at mRNA and protein levels at the pseudostratified sinonasal epithelium.

Since SP-A is expressed in the SNE, we surmised that it plays an antimicrobial role. We challenged SNECs with PAK and we found that SP-A mRNA levels were significantly upregulated by 2- fold at 4h and by 3- fold at 12h post challenge (Figure 4, Panel A). Interestingly, the SP-A protein levels did not follow the same pattern. Although SP-A protein levels were initially decreased at 4h post challenge, they were upregulated at 12h post challenge (Figure 4, Panel B). We postulate that soon after PAK challenge, at 4h, a signal transduction cascade (yet to be determined) takes place, and initiates the transcriptional activation of SP-A gene (2- fold mRNA upregulation); the secreted SP-A is binding to PAK to enhance its aggregation. This probable scenario explains the reduced SP-A protein levels at 4h in the lysates. At 12h post challenge while SP-A transcript is still upregulated (by 3- fold), the SP-A protein levels are also increased. Additionally, *P. aeruginosa* has been shown to colonize and infect the sinuses of CF patients, and to degrade surfactant components, including SP-A²⁹. That may explain the observed protein reduction at 4h post PAK challenge. We postulate that although there is upregulation of SP-A gene expression levels (mRNA) and subsequent protein production, SP-A protein is rapidly degraded in the presence of PAK. These observations support the concept that SP-A is synthesized in the nasal epithelium and that SP-A plays a key role in the sinus innate immunity mechanisms.

In other studies, SP-D was found to be abundantly expressed in the submucosal glands of CRS patients but was undetectable in allergic fungal sinusitis patients²⁸. CRS patients' nasal tissue explant models after challenged with fungal allergens resulted in downregulation of SP-D levels as assessed by immunohistochemistry. Authors assumed that the downregulation of SP-D may result in lack of clearance of the pathogens from the sinuses and this probably contributes to the chronic phenotype of the sinusitis⁵. Interestingly, we are seeing similar findings with *P. aeruginosa* infections (Figure 4, Panel B) in our nasal tissue

explant model but the specific role of SP-A in the sinus innate immunity remains to be determined.

In conclusion, although SP-A originates from the lung and the submucosal glands, our findings demonstrate that SP-A not only is constitutively produced by the sinonasal epithelia, but it is increased in CRS patients, and its expression levels are induced during PAK challenge. These data indicate that SP-A may play a role in the local defense mechanisms of the sinus innate immunity responses during bacterial infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALI	Air-liquid-interface
AMs	alveolar macrophages
CRS	Chronic rhinosinusitis
AR	allergic rhinitis
CF	cystic fibrosis
COPD	chronic obstructive pulmonary disease
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
hTG	humanized transgenic
IRB	independent review committee
MEM	minimum essential medium
PAMPs	pathogen associated molecular patterns
PA	<i>Pseudomonas aeruginosa</i>
PAK	<i>Pseudomonas aeruginosa</i> strain K
RT-qPCR	real time quantitative polymerase reaction
SNE	sinonasal epithelium

SFTP-A1	Surfactant Protein A1 gene
SFTP-A2	Surfactant Protein A2 gene
SNECs	sinonasal epithelial cells
SP-A	Surfactant protein A
SP-D	Surfactant protein D
SP	surfactant proteins
TLRs	Toll-like receptors

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Figure 1.

Effect of CRS in the SP-A levels. **Panel A:** There is a significant 3- fold increase in the relative SP-A mRNA levels of CRS patients compared to healthy subjects as assessed by qRT-PCR. **Panel B:** Expression levels of SP-A protein levels in CRS and healthy nasal tissues were assessed by Western Blot. Bands were detected with specific polyclonal rabbit anti-SP-A, secondary goat anti-rabbit IgG HRP conjugate, followed by chemiluminescence (see material and methods); β -actin was used a loading control and was detected by monoclonal rat anti- β -actin HRP conjugated antibody. **Panel C:** Quantification of bands intensity was performed with ImageJ software. We found a significant 3- fold increase in the SP-A protein levels of CRS patients compared to healthy subjects. Data were generated with n=12 CRS patients and n=5 healthy subjects and 3 replicates/subject. *, p=0.0171; **, p=0.0026.

Figure 2.

Human SP-A is expressed in the sinonasal tissues of humanized transgenic SP-A2 (+/-) mice but not in SP-A KO (-/-) mice. Figure shows representative western blot. Data generated from n=5 hTG SP-A2 mice and n=5 SP-A KO mice. Antibody detecting specifically human SP-A was used (see material and methods).

Figure 3.

Immunohistochemistry of paraffin-embedded nasal tissues. SP-A is detected at the columnar nasal epithelial cells and the submucosal glands (brown stained, denoted with arrow).

Nuclear counterstaining was performed with hematoxylin (blue stained). Specific primary rabbit-anti-SP-A antibody was used detected SP-A, followed by a secondary goat-anti-rabbit IgG HRP conjugated antibody. To confirm the specificity of the primary antibody incubation of nasal tissues with molar excess of human SP-A and anti-SP-A antibody was performed, followed by the same secondary antibody (staining control). Figure shows representative data from 4 human subjects.

Figure 4.

Effect of *P. aeruginosa* strain K (PAK) challenge in SP-A levels of sinonasal epithelium. Epithelial cells were cultured in air-liquid interface and upon maturity they were challenged apically with PAK for 4h and cells harvested immediately after challenge at 4h and at 12h post infection. **Panel A:** SP-A mRNA levels increased significantly by 2- fold at 4h and by 3- fold at 12h as assessed by qRT-PCR. GAPDH was used as a normalizing housekeeping gene. **Panel B:** SP-A protein levels decrease at 4h immediately after *P. aeruginosa* challenge of the sinonasal epithelium, followed by an increase at 12h post challenge as assessed by western blot analysis. β-actin was used as loading control. Data were generated by n=3 independent experiments (three different subjects). *, p=0.0533; **, p=0.0164.

Table 1

Human Subjects Cohort study to investigate the association between chronic sinusitis and expression levels of SP-A

	Sex	Age	Race	CRS	Polyps	# of past surgeries	Asthma	AR	COPD
Average in healthy n=7	42.9% M	49.7 (±21.9)	57.1% Cauc.	0%	0%	0%	0%	28.6%	0%
Average in CRS n=19	63.2% M	42.7 (±19.7)	68.4% Cauc.	100%	47.4%	31.6% multiple surg.	57.9%	52.6%	0%
Average in cohort n=26	57.7% M	44.6 (±20.1)	65.4% Cauc.	73.1%	34.6%	22.2% multiple surg.	42.3%	46.2%	0%

CRS, chronic rhinosinusitis; AR, allergic rhinitis; COPD, chronic obstructive pulmonary disease; PA, *Pseudomonas aeruginosa*; %, percentage, M, males.