Nasal mucus proteomic changes reflect altered immune responses and epithelial permeability in patients with allergic rhinitis

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Background: Nasal mucus is the first-line defense barrier against (aero-) allergens. However, its proteome and function have not been clearly investigated.

Objective: The role of nasal mucus in the pathophysiology of allergic rhinitis was investigated by analyzing its proteome in patients with allergic rhinitis (n = 29) and healthy control subjects (n = 29).

Methods: Nasal mucus was collected with a suction device, tryptically digested, and analyzed by using liquid chromatography–tandem mass spectrometry. Proteins were identified by searching the SwissProt database and annotated by collecting gene ontology data from databases and existing literature. Gene enrichment analysis was performed by using Cytoscape/BINGO software tools. Proteins were quantified with spectral counting, and selected proteins were confirmed by means of Western blotting.

Results: In total, 267 proteins were identified, with 20 (7.5%) found exclusively in patients with allergic rhinitis and 25 (9.5%) found exclusively in healthy control subjects. Five proteins were found to be significantly upregulated in patients with allergic rhinitis (apolipoprotein A-2 [APOA2], 9.7-fold; α2-macroglobulin [A2M], 4.5-fold; apolipoprotein A-1 [APOA1], 3.2-fold; α1-antitrypsin [SERPINA1], 2.5-fold; and complement C3 [C3], 2.3-fold) and 5 were found to be downregulated (antileukoproteinase [SLPI], 0.6-fold; WAP 4-disulfide core domain protein [WFDC2], 0.5-fold; haptoglobin [HP], 0.7-fold; IgJ chain [IGJ], 0.7-fold; and Ig hc V-III region BRO, 0.8-fold) compared with levels seen in healthy control subjects.

Conclusion: The allergic rhinitis mucus proteome shows an enhanced immune response in which apolipoproteins might play an important role. Furthermore, an imbalance between cysteine proteases and antiproteases could be seen, which negatively affects epithelial integrity on exposure to pollen protease activity. This reflects the important role of mucus as the first-line defense barrier against allergens. (J Allergy Clin Immunol 2014;133:741-50.)

Key words: *Nasal mucus, proteome, proteomics, allergic rhinitis, mass spectrometry*

Mucus is the first-line defense barrier of the upper respiratory tract, and its proper production and transport maintain a healthy and patent airway and protect the epithelium.[1-4] Mucus mainly consists of polypeptides, cells, and cellular debris,[2,3] but little is known about the distinct proteins that comprise the nasal mucus proteome. Casado et al[5] were the first to publish a proteomics study about nasal mucus in healthy control subjects, identifying 111 different proteins.

According to the Allergic Rhinitis and its Impact on Asthma guidelines, allergic rhinitis is a disorder of the nose induced after allergen exposure by IgE-mediated inflammation of the nasal mucosa, during which rhinorrhea is one of the cardinal symptoms.[6] Thus nasal mucus must somehow be involved in its pathophysiology. As a highly prevalent disease, especially in Western countries, allergic rhinitis is a huge problem for patients and the health care system.[6] The aim of our study was to investigate the nasal mucus proteome in patients with allergic rhinitis compared with healthy control subjects. As a part of this first-line defense barrier against harmful agents, nasal mucus proteins are most likely involved in physiologic and pathologic processes. The major question we asked was whether proteomic changes are involved in allergic rhinitis or whether there are no differences compared with healthy control subjects. On the one hand, we sought to shed light on how immune system responses are represented through the mucus proteome in patients with allergic rhinitis. On the other hand, we hypothesized that the mucus proteome might confirm and extend theories of transepithelial transport of allergens.[7] Regarding the latter point, pollen grains were found to contain proteinases,[8] which can degrade tight junctions,[9] leading to allergen penetration through the epithelial barrier. Thus we were also interested in the presence of innate protease inhibitors and proteases and whether their balance was altered in the mucus of patients with allergic rhinitis compared with that of healthy control subjects.

The objective of this proteomic study was to obtain a large spectrum of proteins present in nasal mucus and identify key proteins, such as apolipoproteins and cysteine protease inhibitors. The function of these proteins could reflect their involvement in immune responses leading to allergic rhinitis, whereas other proteins could reduce the immune response and deactivate harmful pollen content, such as proteases acting as defense mechanisms in healthy control subjects. Targeting these proteins

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Abbreviations used

A2M: α2-Macroglobulin
APOA1: Apolipoprotein A-1
APOA2: Apolipoprotein A-2
APOA4: Apolipoprotein A-4
APOB: Apolipoprotein B-100
BPIFA1: BPI fold–containing family A member 1
BPIFB1: BPI fold–containing family B member 1
C3: Complement C3
C5: Complement C5
GRN: Granulin
HP: Haptoglobin
IGJ: IgJ chain
LC-MS/MS: Liquid chromatography–tandem mass spectrometry
LTF: Lactotransferrin
LYZ: Lysozyme C
RNASE2: Nonsecretory ribonuclease
SC: Spectral count
SERPINA1: α1-Antitrypsin
SLPI: Antileukoproteinase
VTN: Vitronectin
WFDC2: WAP 4-disulfide core domain protein

for interventions might offer new therapeutic strategies on the mucus level because it is the first-line defense barrier of the nasal mucosa.

METHODS

Patients

Fifty-eight subjects (31 male and 27 female subjects) were included in this study. The mean age was 34 years (range, 20-58 years), and there were 29 (50%) patients with allergic rhinitis and 29 (50%) healthy control subjects. Allergy status was verified by using skin prick tests (Allergopharma GmbH & Co KG, Reinbek, Germany) and specific IgE measurement (ImmunoCAP; Thermo Fisher Scientific, Vienna, Austria), respectively (see Table E1 in this article's Online Repository at www.jacionline.org). Patients sensitized to house dust mite or animals only were excluded to avoid bias because of the small sample size. Thus only patients with symptoms during the pollen season were considered for evaluation. Patients with acute sinusitis, chronic sinusitis, or both, as defined by the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines,[10] were excluded from the study. Furthermore, patients with malignant tumors or any other infectious or cardiopulmonary disease or those treated with systemic or topical drugs, such as antihistamines, corticosteroids, antibiotics, antifungal agents, or any other immunomodulatory drugs, in the 4 weeks before the study were excluded. Informed consent was obtained from all subjects (both patients with allergic rhinitis and healthy control subjects) before entering the study. The study was approved by the Institutional Review Board of the Medical University Graz.

Sample collection

A special suction device (Sinus Secretion Collector; Medtronic Xomed, Jacksonville, Fla) was used to collect nasal mucus. Without previous interventions (decongestants and local anesthetics), untreated mucus was obtained under endoscopic control from the nasal cavity and middle meatus, with meticulous care taken not to touch the mucosa. Then mucus was deep frozen at −80°C before processing for liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Proteomic analysis

Sample preparation, LC-MS/MS analysis, and LC-MS/MS data analysis are described in detail in the Methods section in this article's Online Repository at www.jacionline.org and reported according to "minimum information about a proteomic experiment."[11] In brief, equal amounts of protein digests were separated by means of nano-HPLC and measured online by using tandem mass spectrometry. Spectra were matched to the SwissProt human protein database with appropriate software (Spectrum Mill, Proteome Discoverer, and Mascot). Spectral counting of the total peptides identified (ie, number of MS/MS spectra matched to a protein) was used to compare the relative protein abundances of the same protein between groups.[12,13] Identified proteins were annotated by using data from UniProt (www.uniprot.org), the PANTHER classification system (www.pantherdb.org), and DAVID (DAVID Bioinformatics Resources 6.7, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md; http://david.abcc.ncifcrf.gov/). Enrichment analysis was performed with BINGO 2.44[14] in Cytoscape 2.81 software (www.cytoscape.org).[14]

Statistical analysis

For statistical analysis of group differences of spectral counting data, only proteins with a mean spectral count (SC) of 4 or greater in either group were accepted.[12] SC data are presented as means with SDs or SEMs. The Mann-Whitney *U* test was used to identify significantly altered proteins between groups with SPSS 18.0 software (SPSS, Chicago, Ill). A *P* value of less than .05 was considered significant.

For statistical analysis of enrichment data created with BINGO/Cytoscape, hypergeometric tests were performed and corrected with Benjamini & Hochberg false discovery rate correction at a significance level of .05.

Western blot analysis

Twenty micrograms of lysed protein from different nasal mucus samples was separated by 4% to 20% denaturing reducing SDS-PAGE (Bio-Rad Laboratories, Vienna, Austria). Separated proteins were transferred onto nitrocellulose membranes by means of semidry blotting for 1 hour at 180 mA. Total transferred protein was detected with Pierce MemCode (Thermo Fisher Scientific) reversible stain and imaged on a ChemDocXRS (Bio-Rad Laboratories). Membranes were blocked in blocking buffer (5% skim milk in Tris-buffered saline–Tween buffer [20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.1% Tween 20, pH 6.7]) for 1 hour at room temperature and then incubated with primary antibodies overnight at 4°C. Primary antibodies specific to α1-antitrypsin (SERPINA1; product no. 9400; Abcam, Cambridge, United Kingdom), α2-macroglobulin (A2M; product no. 58703, Abcam), haptoglobin (product no. 13429, Abcam), complement C3 (C3; product no. 97462, Abcam), and apolipoprotein A-2 (APOA2; product no. 24241, Abcam) were used for protein detection. After washing with Tris-buffered saline–Tween, the membrane was incubated with secondary antibodies (goat anti-mouse or anti-rabbit IgG–horseradish peroxidase [HRP] conjugates, Abcam) for 1 hour at room temperature. Immunocomplexes were visualized with Pierce ECL chemiluminescent substrate (Thermo Fisher Scientific). Densitometric evaluation was performed with Image Lab 4.1 software (Bio-Rad Laboratories). Volumes of protein bands were determined by using global background subtraction and normalized on total protein detected in the lane by using MemCode. Means and SEMS of 5 patients in each group were calculated.

RESULTS

Using a shotgun proteomics approach, we identified the nasal mucus proteomes of 29 patients with allergic rhinitis and 29 healthy control subjects (see the Methods section and Table E1 in this article's Online Repository for patients' characteristics and method details). The mean mucus protein concentration was 3.34 mg/mL (SD, 4.1 mg/mL) in patients with allergic rhinitis and 2.88 mg/mL (SD, 3.7 mg/mL) in healthy control subjects, which did not reach significance ($P = .57$). In shotgun proteomics the total sample is digested by a protease, typically trypsin, and the resultant peptides are separated by means of liquid chromatography and sequenced by means of tandem mass spectrometry. Proteins are identified by matching experimental and theoretic peptide spectra and statistically validated. The number of