Upregulation of Elafin and Cystatin C in the Ethmoid Sinus Mucosa of Patients With Chronic Sinusitis

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Objectives: To investigate the expression levels and distribution patterns of elafin and cystatin C in normal and inflammatory human sinus mucosa and to evaluate their roles in chronic sinusitis.

Design: A controlled, prospective study.

Setting: A tertiary academic institution.

Patients: Normal sinus mucosa was obtained from the ethmoid sinus during surgery in 30 patients with blow-out fractures. Inflammatory sinus mucosa was obtained from 30 patients undergoing endoscopic sinus surgery for chronic polypoid sinusitis.

Interventions: Reverse transcription–polymerase chain reaction, immunohistochemical analysis, and Western blotting.

Main Outcome Measures: Expression levels and distribution patterns of elafin and cystatin C in normal and inflammatory mucosa.

Results: Expression of elafin and cystatin C messenger RNAs and proteins analyzed by means of reverse transcription–polymerase chain reaction and Western blot was detected in all normal and inflammatory sinus mucosa tested. Their expression levels were increased in inflammatory vs normal mucosa. Elafin in normal and inflammatory sinus mucosa was distinctly expressed in goblet cells, which are increased in inflammatory sinus mucosa. Elafin in submucosal glands was usually weak in staining intensity, except for a few scattered submucosal glands showing moderate intensity in inflammatory sinus mucosa. Cystatin C was also localized in goblet cells and submucosal glands in normal and inflammatory mucosa. Staining intensity was increased more in inflammatory vs normal sinus mucosa.

Conclusion: Elafin and cystatin C may play an important role in the protection of normal sinus mucosa and further in regulation of the inflammatory condition in chronic sinusitis.


Asal mucosal surfaces are under the constant threat of pathogenic attack. Like other mucosal surfaces, a major function of nasal mucosa is to present a barrier to the penetration of pathogens and other potentially injurious agents from the environment into the body. These mucosal defense mechanisms of nasal mucosa depend on respiratory epithelium covered by a superficial layer of mucus and a deeper serious mucociliary layer. It does this by mucociliary clearance, allowing the physical removal of inhaled pathogens. In addition, nasal airway secretions and their constituent proteins contribute to defend nasal epithelia and mucosal surfaces against microorganisms. Human nasal secretions contain a variety of well-characterized antimicrobial products. Defensins and cathelicidins are the most well characterized of the antimicrobial peptides found in nasal mucosa. However, there are many materials with antimicrobial functions that have not been evaluated regarding their presence or expression in normal and inflammatory sinus mucosa.

Recently, various antimicrobial substances, including elafin and cystatin C, were reported to be present or expressed in various organs, including skin. Elafin, also known as skin-derivated antileukoproteinase or elastase-specific inhibitor, is a serine proteinase inhibitor that is reported to play a regulatory role in inflammation. Cystatin C, an endogenous cysteine proteinase inhibitor, is a nonglycosylated low-molecular secretory protein produced by nucleated cells and has been found in a variety of human tissues. It has been reported that cystatin C is associated with the regulation of inflammation. In this respect, these molecules, better known for antimicrobial peptides, can also be con-
considered as functional antimicrobial substances in the nasal mucosa. However, little attention has been given to the expression of these protease inhibitors in human sinonasal mucosa.

Therefore, the present study investigates the expression levels and distribution patterns of elafin and cystatin C in normal and inflammatory human sinus mucosa to evaluate whether these materials may play a role in chronic sinusitis.

### METHODS

#### SAMPLE PREPARATIONS

Normal ethmoid sinus mucosa samples (n = 30) were obtained from the ethmoid sinus during endoscopic reduction in 30 patients (23 men and 7 women; age range, 20–45 years) with blowout fractures. These patients had no history of nasal infections, allergy, smoking, or ongoing drug treatment. During surgery, normal-appearing sinus mucosa that was not injured by the fracture was removed and was used as a control. Inflammatory ethmoid sinus mucosa samples (n = 30) were obtained from 30 patients (22 men and 8 women; age range, 22–47 years) undergoing endoscopic sinus surgery for chronic sinusitis with nasal polyposis. None of these patients had a history of nasal allergy, asthma, aspirin sensitivity, or ongoing drug treatment. Before the tissue specimens were obtained, the protocols and the informed consent form were approved by the institutional review board for humans at the Korea University College of Medicine.

Tissue samples (20 each of normal and inflammatory sinus mucosa) were cut into 2 portions; one portion was dissected, frozen in liquid nitrogen, and stored at −80°C for subsequent RNA isolation, and the other portion was used for protein isolation. For immunohistochemical analysis, the remaining samples (10 each of normal and inflammatory sinus mucosa) were fixed overnight in a freshly prepared fixative containing 4% paraformaldehyde in a phosphate-buffered saline solution (pH 7.4) and were then dehydrated in a graded series of ethanol.

#### TOTAL RNA ISOLATION, REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION, AND SEMIQUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

The total RNA (1 µg) from each sample was reverse transcribed in 20 µL of a reaction mixture containing 2.5 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Grand Island, New York) and 50 pmol of random hexanucleotides at 42°C for 60 minutes. RNA integrity and the success of the reverse transcription (RT) reaction were monitored by means of polymerase chain reaction (PCR) amplification of glyceraldehyde-3-phosphate dehydrogenase transcripts. Negative controls consisted of omission of the reverse transcriptase enzyme from the complementary DNA (cDNA) synthesis for each specimen. The primer sequences of the genes used in this study are demonstrated in the Table. Amplified PCR products were resolved in 2% agarose gel, stained with ethidium bromide, and photographed under UV light. The identity of each PCR product was confirmed by means of sequencing and was found to be identical with the messenger RNA (mRNA) sequence of each gene, as deposited in the National Center for Biotechnology Information database.

Semiquantitative PCR was performed on the samples to assess the differential expression of elafin and cystatin C mRNA in normal and inflammatory sinus mucosa. The optimum number of PCR cycles for each cDNA species was determined by plotting the PCR product yield of different cycles on a semilogarithmic graph, and the cycle number that represented the exponential amplification was chosen for the final amplification.

For quantification of the PCR products of all the samples, the expected bands were analyzed by means of densitometry, and these data are expressed as the ratio between each cDNA and the corresponding glyceraldehyde-3-phosphate dehydrogenase cDNA. Data are presented as mean (SD). The statistical significance of differences between the groups was evaluated using the Mann-Whitney test, and the level of statistical significance was set at P < .05.

#### IMMUNOHISTOCHEMICAL AND WESTERN BLOT ANALYSES

Immunohistochemical staining was performed using a peroxidase-labeled streptavidin-biotin technique to evaluate the expression and distribution of elafin and cystatin C in normal and inflammatory sinus mucosa. Briefly, tissue sections were deparaffinized, rehydrated, and treated with 3% hydrogen peroxide in methanol for 15 minutes to quench endogenous peroxidase activity. After washing in a 10mM phosphate-buffered saline solution (pH 7.4), the sections were incubated overnight at room temperature with a 1:100 dilution of goat anti–elafin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, California) or rabbit anti–cystatin C polyclonal antibody (BioChain Institute Inc, Hayward, California). The color was developed using 3, 3′-diaminobenzidine.

For Western blot analysis, equal amounts of total protein were separated on 12% sodium dodecyl sulfate–polyacrylamide gel and were transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, Massachusetts). The blots were then blocked for 1 hour at room temperature with Tris-buffered saline containing 1% skim milk and thereafter were incubated with each antibody in Tris-buffered saline overnight at room temperature. Antibody reactions were detected by using a Western blotting detection kit (ECL; Amersham Bioscience, Piscataway, New Jersey), followed by detection of chemiluminescence on radiographs. As an internal control, β-actin expression was analyzed in parallel blots using the β-actin antibody (Sigma-Aldrich Corp, St Louis, Missouri). The intensity of the detected bands was quantified using Scion Image Beta 4.0.2 (Scion Corp, Frederick, Maryland). The relative intensity of each protein signal was obtained by dividing the intensity of each protein signal by the β-actin signal. The statistical significance of differences was evaluated using Mann-Whitney tests, and the level of statistical significance was set at P < .05.
RESULTS

The RT-PCR assays were performed to detect the expression of elafin and cystatin C mRNA in normal and inflammatory sinus mucosa. The RT-PCR experiments using each gene-specific primer showed the presence of elafin and cystatin C mRNA and revealed that cDNA fragments of the expected length were amplified in all normal and inflammatory sinus mucosa tested in the present study. The relative abundance of these genes was significantly increased in inflammatory vs normal sinus mucosa (Figure 1). As a control for RNA integrity and the RT-PCR procedure, all the RNA samples were analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are given as means; error bars, SD.

The tissue distribution of elafin and cystatin C protein was analyzed using immunohistochemical staining. In immunohistochemical analysis, a homogenous pattern was observed on all samples of normal and inflammatory sinus mucosa examined in the present study. In normal and inflammatory sinus mucosa, elafin was exclusively localized in goblet cells and in submucosal glands. The immunostaining of elafin was strongly positive in goblet cells, which were markedly increased in inflammatory sinus mucosa, suggesting the increased expression levels of elafin. Immunostaining of submucosal glands for elafin was usually weak in staining intensity, except for a few scattered submucosal glands showing moderate intensity in inflammatory sinus mucosa (Figure 2A and B). Cystatin C was also expressed in normal and inflammatory sinus mucosa, where goblet cells and submucosal glands showed positive staining. In normal sinus mucosa, goblet cells and submucosal glands showed light immunostaining intensity for cystatin C, with moderate or intense staining intensity in inflammatory sinus mucosa (Figure 2C and D).

Western blot analysis detected elafin and cystatin C in normal and inflammatory sinus mucosa. Expression levels of elafin and cystatin C were increased in inflammatory vs normal sinus mucosa (P < .05) (Figure 3).

COMMENT

In this study, we used RT-PCR, immunohistochemical analysis, and Western blotting to study and compare the expression levels and distribution patterns of elafin and cystatin C in normal and inflammatory sinus mucosa. These data demonstrate that elafin and cystatin C mRNAs and proteins were expressed in normal and inflammatory sinus mucosa. These substances were also increased in their expression levels in inflammatory vs normal sinus mucosa.
Figure 3. The expression levels of elafin and cystatin C were more increased in inflammatory sinus mucosa than in normal sinus mucosa. A, Western blot analysis of elafin and cystatin C in normal (NE) and inflammatory (IE) ethmoid sinus mucosa. B, Bands were quantified by means of densitometric scanning, and the relative amount of each gene was calculated by dividing by the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Data are given as means; error bars indicate SD.

Therefore, based on the anti-inflammatory function of these proteins, these results suggest that these proteins may play an important role in the protection of normal sinus mucosa and further in the prevention of aggravation of the inflammatory condition in chronic sinusitis.

Proteases are one group of innate immunity effectors responsible for tissue damage. These enzymes are produced by a variety of phagocytic inflammatory cells, including the neutrophil, to degrade ingested pathogens and permit cell motility through the extracellular matrix. In response to these enzymes, the host secretes antiprotease molecules, which serve to neutralize any excess protease load and to protect host tissues. In purulent nasal secretions, strong protease activity was also measured, and a protease inhibitor was isolated from nasal secretions of patients with chronic sinusitis. These results demonstrate that specimens with high protease activity were thought to be the granulocyte proteases, whereas the protease inhibitor was similar to antileukoprotease and suggested that antiprotease molecules may also play a role in the depression of protease activities in nasal purulent secretion. It has been generally known that antiprotease molecules are produced either locally at mucosal sites or systemically by the liver. Although most serine protease inhibitors are found in the circulatory system, a few of this class are locally active at the tissue level. One of these inhibitors, termed skin-derived antileukoprotease, otherwise known as elafin, was originally isolated from psoriatic scales and was subsequently identified in the trachea and breast. Subsequently, elafin was found to be expressed in multiple epi-

thelia and in bronchoalveolar lavage fluid from healthy individuals and was increased in patients with inflammatory lung disease. In the present study, elafin was exclusively expressed in the goblet cells of normal and inflammatory sinus mucosa, and its expression was increased in inflammatory vs normal sinus mucosa. Its localization in sinus mucosa is in accord with the results of a study showing that goblet cells of the trachea and large intestine are the major sites of elafin synthesis. Goblet cells are the primary source of secretory cells of the sinonasal mucosa and, hence, may secrete elafin in addition to other antimicrobial proteins that were detected in human nasal secretion. This location of elafin seems to be ideal for effective inactivation of destructive bacterial proteases. In this way, elafin may be a candidate for antibacterial activity in nasal secretion and may contribute to the mucosal defense of the sinonasal mucosa with other antimicrobial peptides reported previously.

Cystatin C is a potent, reversible, competitive inhibitor of cystein proteases and is detected in many biological fluids, with the highest concentrations being found in seminal plasma, cerebrospinal fluid, and synovial fluid; it is also found in nasal secretions. Numerous studies have indicated an important pathophysiologic function of cystatin C, with a variation in local concentrations demonstrated in processes such as inflammatory diseases of the skin, gingiva, and lung. To our knowledge, cystatin C has not been studied immunohistochemically or quantitatively in human nasal mucosa. To our knowledge, this is the first study to investigate the expression levels and distribution patterns of cystatin C in normal and inflammatory sinus mucosa. In the present study, cystatin C was expressed and upregulated in chronic inflammatory vs normal sinus mucosa, as confirmed by the results of RT-PCR and Western blotting. It is also localized in goblet cells and submucosal glands in normal and inflammatory sinus mucosa. These results suggest that cystatin C may be excreted from goblet cells and submucosal glands of nasal mucosa on the exposure to microorganisms inhaled into the nasal cavity, playing an important role in the regulation of normal physiologic proteolysis and in inflammatory diseases. Therefore, the synergistic effects of cystatin C with other antimicrobial substances, such as lysozyme, lactoferrin, and defensins, may be effective in making these factors more potent against infectious pathogens. Moreover, it can be considered that alteration in the activity or distribution of cystatin C has some association with the affinity of pathogens to sinonasal mucosa. This suggestion is supported by the facts showing that clinically, a patient’s altered cystatin C levels in body fluid and serum are monitored or are used to predict the progression of diseases. On the other hand, it has been known that the balance between cystatin C and the family C1 cathepsin is of major importance in the regulation of proteolytic activity under normal physiologic conditions but also in pathologic degradation of protein in inflammatory diseases. Further study is necessitated to show whether the balance between cystatin and cathepsin can affect the pathogenesis of chronic sinusitis.

In conclusion, this study established that elafin and cystatin C are expressed in normal sinus mucosa and are upregulated in inflammatory sinus mucosa, suggesting
that elafin and cystatin C are constitutively expressed in normal sinus mucosa and may have key roles in the protection of normal sinonasal mucosa against inhaled toxic, viral, and bacterial products and in the regulation of inflammatory processes in chronic sinusitis. We did not reach any conclusions regarding the regulation of elafin and cystatin C in normal and inflammatory sinus mucosa. This remains an area for further investigation.

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REFERENCES