Expression of Neutrophil Gelatinase–Associated Lipocalin in Nasal Polyps

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Objective: To investigate the expression and localization of neutrophil gelatinase–associated lipocalin (NGAL), an antimicrobial peptide, in the normal nasal mucosa and human nasal polyps. Neutrophil gelatinase–associated lipocalin has been identified as a key element in the innate host defense system. However, scant knowledge exists about the expression of NGAL in the human sinonasal tract.

Design: Prospective study.

Setting: Academic medical center.

Patients: Normal inferior turbinate mucosa was obtained from 10 patients who were undergoing augmentation rhinoplasty. The nasal polyps were obtained from 10 patients who were undergoing endoscopic sinus surgery for chronic rhinosinusitis with polyps.

Interventions: We performed semiquantitative reverse transcription–polymerase chain reaction, immunohistochemical staining, and Western blot analysis.

Main Outcome Measures: We analyzed the expression of the NGAL messenger RNA (mRNA) and localization of the NGAL protein.

Results: The NGAL mRNA and NGAL protein were highly expressed in the nasal polyps. The ratio of NGAL mRNA to glyceraldehyde-3-phosphate dehydrogenase mRNA in the nasal polyps was greater compared with that in the normal turbinate mucosa (P = .002). The NGAL protein was observed in the epithelium, the infiltrating inflammatory cells, and the submucosal gland of the nasal polyps, but it was very rarely detected in the normal nasal mucosa.

Conclusion: Expression of NGAL is upregulated in nasal polyps, and additional work is needed to reveal the possible role of NGAL in the defense systems of the nasal mucosa and the process of polyp formation.


The nasal mucosa is a potential site for the deposition of toxic or infectious pathogens. The normal local host defense systems effectively eliminate these harmful pathogens. Human nasal secretions contain a number of well-characterized antimicrobial molecules. If the defense mechanisms fail, then microorganisms can adhere to epithelial cells and begin to colonize.

Neutrophil gelatinase–associated lipocalin (NGAL) is a 25-kDa protein that was initially purified from human neutrophil granules during a search for specific markers of neutrophils. The protein belongs to the lipocalin superfamily, and the members of this superfamily are characterized by their ability to bind small lipophilic molecules. Several recent reports have shown that NGAL acts as a potent bacteriostatic agent because of its ability to capture and deplete siderophores. Siderophores are small iron-binding molecules that are synthesized and used by bacteria for the uptake of the essential nutrient iron. Unlike other antimicrobial proteins such as lactoferrin and transferrin, NGAL does not bind iron directly but rather binds it indirectly via the bacterial siderophore; thus, NGAL constitutes a critical component of the innate immunity against bacterial infection.

In addition to its well-known antimicrobial activity, NGAL appears to have other complex functions. In response to renal tubular injury, humans and rodents experience a rapid increase of NGAL expression that has been detected in the urine and serum; therefore, this protein is used as an early marker of renal failure. Increases in the serum NGAL level have been reported even during the acute-phase response in those systemic diseases that are without overt bacterial in-
However, although multiple stimuli capable of inducing NGAL expression have been identified, the functional role of NGAL is not well understood.

The specific expression of the messenger RNA (mRNA) of the NGAL gene (GenBank NM_005564) has been seen in the bone marrow, uterus, prostate, salivary gland, stomach, appendix, colon, trachea, and lung. However, little is known about its expression in the human sinonasal tract. Therefore, this study aimed to investigate the differential expression and localization of NGAL in the normal human nasal mucosa and nasal polyps.

**METHODS**

**TISSUE COLLECTION**

The nasal polyp tissues were obtained during endoscopic sinus surgery from patients with chronic rhinosinusitis and polyps (5 men and 5 women; mean age, 46.4 years). Normal inferior turbinate tissues were obtained from 10 cases of augmentation rhinoplasty (5 men and 5 women; mean age, 32.0 years). Patients with allergic rhinitis were excluded from this study. All patients stopped taking oral and topical corticosteroids and antibiotics for at least 4 weeks before surgery.

The study protocols were approved by the institutional review board for human studies at the Yeungnam University Medical Center.

**TISSUE PREPARATION**

All of the tissue samples were divided into 2 portions. The first portion was immediately frozen in liquid nitrogen and stored at −70°C for subsequent RNA studies and Western blot analysis. The other portion was immersed overnight in a freshly prepared fixative containing 4% paraformaldehyde in phosphate-buffered saline (PBS) solution (pH, 7.41) for immunohistochemistry.

**REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION ANALYSIS OF THE NGAL GENE**

The total RNAs from the nasal polyp and the inferior turbinate specimens were extracted using a commercially available reagent (TRIzol; Gibco BRL, Gaithersburg, Maryland) according to the manufacturer's instructions. Fifteen micrograms of the total cellular RNA underwent reverse transcription (RT) into complementary DNA (cDNA) at 37°C for 70 minutes in 60 µL of a volume reaction mixture that contained 150 U of reverse transcriptase (Superscript-II; Invitrogen, Carlsbad, California); 10 µL each of 10mM deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxyctydine triphosphate; and 100 µg/mL of oligo-dT primer (Amersham Co, Buckinghamshire, England). The reactions were stopped by heat inactivation at 85°C for 10 minutes. Two microliters of each cDNA sample from the RT was amplified by means of polymerase chain reaction (PCR) in a volume of 50 µL that contained 0.5 U of Taq DNA polymerase; 2 µL of 50mM magnesium chloride; 1 µL each of 10mM deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyctydine triphosphate, and deoxyguanosine triphosphate; and 500 ng/µL of forward and reverse primers. The PCR products were separated on a 4% agarose gel, stained with ethidium bromide, and visualized by means of UV fluorescence. Semiquantitative analysis of the RT-PCR product was performed on the scanned gel images, and the intensity of the PCR product was measured using commercially available imaging software (Scion software; Scion Corporation, Frederick, Maryland). We determined the relative intensity of the individual bands on a gel image as the ratio of the intensities of NGAL mRNA to those of GAPDH mRNA. Peripheral blood leukocytes were used for a positive control sample. As a negative control, the RT of the NGAL primer was omitted from the RT-PCR procedure.

**IMMUNOLOCALIZATION OF THE NGAL PROTEIN**

For immunohistochemical analysis, the obtained samples were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin wax. We cut 4-µm sections and mounted them on silane-coated glass slides. To minimize any variations during processing of the tissue, we mounted pairs of sequential sections on each slide, one for NGAL and the other for a negative control. We deparaffinized the sections with xylene and rehydrated them serially with 100%, 90%, 80%, and 70% alcohol solutions. We deparaffinized the sections with xylene and rehydrated them with PBS. Endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol at room temperature for 30 minutes, and then the slides were rinsed in PBS. The slides were then blocked with normal blocking serum diluted at 1:50 in 0.05% polysorbate 20 (Tween 20; Merck Research Laboratories, West Point, Pennsylvania) in PBS for 20 minutes. Monoclonal antibody to human NGAL (Santa Cruz Biotechnology, Santa Cruz, California) diluted at 1:200 was added for 1 hour at room temperature. After rinsing with PBS, biotinylated secondary antibody was added for 1 hour at room temperature. After rinsing, the immunoreactive NGAL was visualized with a peroxidase kit (Vectastain Elite ABC kit; Vector Laboratories, Inc, Burlingame, California) while using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Corp, St Louis, Missouri) for color development. The tissue sections were counterstained with Mayer hematoxylin-eosin. For the negative controls, nonimmune serum was used instead of the primary antibody or PBS.

**WESTERN BLOT ANALYSIS OF THE NGAL PROTEIN**

The tissues were homogenized, and the cells were lysed in protein extract buffer (30-mmol/L TRIS hydrochloride [pH 8.0], 10-mmol/L EDTA, 1% Triton X-100, 100-mmol/L sodium chloride, and 1-mmol/L phenylmethylsulfonyl fluoride) and then stored at −70°C. The proteins were then separated by means of 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane. The membranes were then blocked for 30 minutes in TRIS-buffered saline–polysorbate 20 buffer (20-mmol/L TRIS hydrochloride, 50-mmol/L NaCl, 0.05% polysorbate 20). The proteins were transferred to a nitrocellulose membrane. The membranes were blotted for 1 hour at room temperature. After rinsing with PBS, biotinylated secondary antibody was added for 1 hour at room temperature. After rinsing, the immunoreactive NGAL was visualized with a peroxidase kit (Vector Laboratories, Inc, Burlingame, California) while using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Corp, St Louis, Missouri) for color development. The tissue sections were counterstained with Mayer hematoxylin-eosin. For the negative controls, nonimmune serum was used instead of the primary antibody or PBS.
The expression of neutrophil gelatinase–associated lipocalin (NGAL) messenger RNA in the human normal nasal mucosa (control) and the nasal polyps by means of reverse transcription–polymerase chain reaction. bp indicates base pairs; GAPDH, gluceraldehyde-3-phosphate dehydrogenase; +, positive control; and −, negative control.

STATISTICAL ANALYSIS

We performed statistical analysis using commercially available software (SPSS, version 10.0; SPSS Inc, Chicago, Illinois). We calculated the mean for each of the obtained quantitative values. Comparisons were made using the nonparametric Mann-Whitney test. For all tests, we considered a P value of less than .05 statistically significant.

RESULTS

RT-PCR ANALYSIS OF NGAL GENE EXPRESSION

On the RT-PCR study, NGAL mRNA was scantly expressed in the normal nasal mucosa. However, the nasal polyps showed strong expression of NGAL mRNA (Figure 1). A similar-sized product was expressed in the peripheral blood leukocytes as the positive control. The RT-PCR negative control showed no NGAL mRNA expression. As a control for the RNA integrity and the RT-PCR procedure, the same RNA samples were also analyzed for their GAPDH expression. The GAPDH primer produced a PCR product (145 bp) from each of the tissue samples. All of the samples were normalized with respect to GAPDH. Semiquantitative analysis of the PCR samples showed a statistically significant difference in the NGAL:GAPDH mRNA ratios between the nasal polyps and the normal nasal mucosa (P = .002) (Figure 2).

IMMUNOLOCALIZATION OF THE NGAL PROTEIN

The tissue distribution of the NGAL protein was analyzed by means of immunohistochemical staining. The positive immunostained cells appeared brown. In the nasal polyps, the NGAL protein immunoreactivity was strongly expressed in the epithelial cells, the infiltrating inflammatory cells, and the submucosal gland (Figure 3). The NGAL protein was very rarely present in the normal nasal mucosa. There was no specific localization noted with the negative control, confirming the specificity of the NGAL antibody.

WESTERN BLOT ANALYSIS OF THE NGAL PROTEIN

Using a specific antibody to human NGAL, we detected a protein band of 25 kDa that corresponded to NGAL on the Western blot analysis results. The NGAL protein was very rarely detected in the normal nasal mucosa, whereas it was strongly detected in the nasal polyps (Figure 4).

Neutrophil gelatinase–associated lipocalin has broad antibacterial activity against a number of gram-negative and gram-positive bacteria, as well as against fungi and the enveloped viruses. The synergistic effects combined with other antimicrobial substances such as lysozyme, lactoferrin, cathelicidin, and defensin make this peptide more potent against infectious pathogens. In the present study, we investigated whether NGAL was expressed in the normal human nasal mucosa and nasal polyps. There was a significant increase in the NGAL mRNA expression in the nasal polyps compared with normal nasal mucosa; thus, this finding suggests that NGAL was upregulated in the nasal polyp. Moreover, NGAL was detected in the ductal epithelium of the submucosal glands of the nasal polyp, which is representative of nasal inflammatory disease. In a study by Cowland and Borregaard, high steady-state levels of NGAL expression were found in the uterus, prostate, salivary gland, stomach, appendix, colon, trachea, and lung. These locations constitute tissues that are often exposed to microorganisms or the glands that secrete to such tissues. Nelson et al reported that NGAL was observed in the nasal secretions from healthy human nasal mucosa. These reports and our results imply
that NGAL may play a certain role for the nasal host defenses in the nasal secretions; this role may be similar to that of the other antimicrobial substances.

An infection and its subsequent inflammatory reaction in the nasal mucosa enhance mucosal vascular permeability, producing edematous stroma. This process may play an important role in the development of nasal polyps.\(^{13,14}\) Although the precise mechanism of polyp formation is incompletely understood, the significant proliferation and thickening of mucosal epithelial cells with glandular hyperplasia are histologic characteristics of polyps.\(^{13} \) These features are caused by cellular interactions that are implemented by chronic inflammatory cells. The molecules or signals involved in these processes include interleukins, neuropeptides, cytokines, and growth factors, and most of these can be produced by inflammatory and structural cells.\(^{15}\) In addition to its antimicrobial characterics, NGAL acts as a growth and differentiation factor in various cell types. In cultured renal collecting duct cells, NGAL seems to play a regulatory role in epithelial morphogenesis by promoting the organization of cells into tubular structures.\(^{16}\) In addition, NGAL converts 4T1-ras–transformed mesenchymal tumor cells to an epithelial phenotype.\(^{17}\) In the present study, results of immunohistochemical staining also found that the NGAL protein was localized in epithelial cells and in the infiltrating inflammatory cells of the polyps. These findings point to the possible role of NGAL in nasal polyp formation, and this should be more clearly determined in future studies.

In conclusion, our results showed that a strong expression of NGAL was found in the nasal polyps. Thus, we can postulate that NGAL is upregulated in nasal polyps and may play a role in the defense of the nasal mucosa. Further studies are needed regarding the mechanism of NGAL upregulation in the nasal polyp and its possible role in nasal polyp formation.

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Figure 3. Immunohistochemical staining for neutrophil gelatinase–associated lipocalin (NGAL). In the normal nasal mucosa (control), NGAL stains very weakly in the epithelium (A) and the submucosal glands (C). On the other hand, NGAL is highly expressed mainly in the epithelial cells (B), the infiltrating inflammatory cells (B), and the ductal epithelium of the submucosal glands (D) in the nasal polyp (original magnification ×200).

Figure 4. Western blot analysis of neutrophil gelatinase–associated lipocalin (NGAL) protein in the nasal polyps and the normal nasal mucosa (control). The number indicates the size of the immunodetected proteins.

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Author Contributions: Drs Woo, Min, Bai, Song, Lee, and Kim had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Woo, Min, Lee, and Kim. Acquisition of data: Woo, Min, Song, Lee, and Kim. Analysis and interpretation of data: Woo, Min, Bai, and Kang. Drafting of the manuscript: Woo, Min, and Kim. Critical revision of the manuscript for important intellectual content: Woo, Min, Bai, Song, Kang, Lee, and Kim. Statistical analysis: Song. Study supervision: Lee and Kim.

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