

Localization of nerve growth factor and its receptors in the human nasal mucosa

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Background: Nerve growth factor (NGF) is a pluripotent mediator, the levels of which are elevated in nasal lavage fluids of individuals with allergic rhinitis at baseline. Levels of NGF increase after allergen challenge.

Objective: In the current study, we tested the hypotheses that mast cells are the main source of human nasal NGF, and that NGF can potentially affect mucosal elements other than nerves.

Methods: Immunostaining with antibodies against NGF, tryptase, CD3, eosinophil cationic protein, and the high-affinity (tyrosine kinase A) and low-affinity (p75) receptors for NGF was performed by using human nasal turbinate sections.

Results: Double immunofluorescence staining demonstrated NGF in only 2% (median) (1.3, 2.3; 25th, 75th percentiles) of mast cells, 0.2% (0, 0.4) of T cells, but in 62.2% (56.5, 68) of activated eosinophils. With immunohistology, NGF expression was consistently strongest in the submucosal glands and lesser in the epithelial lining. Both high-affinity and low-affinity receptors for NGF were localized not only on nerves, as expected, but also on nasal epithelium, submucosal glands, and some interstitial cells, but not on vascular endothelium.

Conclusion: This study demonstrates that submucosal glands, nasal epithelium, and eosinophils constitute the major sources of NGF in the human nasal mucosa. That NGF receptors are found in cells other than nerves supports the notion that the role of this neurotrophin is broader than simple modulation of the sensorineural system.

Clinical implications: The distribution of NGF and its receptors and its established release during allergic reactions suggest that this factor participates in the pathophysiology of allergic rhinitis. (*J Allergy Clin Immunol* 2006;118:428-33.)

Key words: Nerve growth factor, TrkA, p75, NGF receptors, rhinitis, mast cells, eosinophils, submucosal glands, sensory nerves, epithelium

Allergic rhinitis is characterized by mucosal inflammation and hyperresponsiveness. Nasal hyperresponsiveness is central to the pathophysiology of allergic rhinitis and accounts for symptoms induced by irritants, a very common problem in this disease. Although the pathobiology of inflammation has been elucidated incrementally over the past decades, that of hyperresponsiveness remains enigmatic.

Our previous work has indicated that hyperresponsiveness in allergic rhinitis could be a result of abnormally exaggerated neural reactivity. Sneezing induced by histamine and secretory neural reflexes induced by capsaicin or hyperosmolar saline are markedly exaggerated in individuals with this disease compared with healthy controls.^{1,2}

Nerve growth factor (NGF), which is an essential element in the development of sensory nerve fibers, has been shown to induce biochemical as well as structural changes in the nerves that can lead to hyperresponsiveness.³ Increased airway responsiveness to stimulation with histamine or capsaicin has been demonstrated in transgenic mice that were engineered to overexpress NGF and in guinea pigs that were exogenously given this neurotrophin.^{4,5}

In human beings, we have found that baseline levels of NGF in nasal lavage fluids are significantly higher in individuals with allergic rhinitis compared with healthy controls. Furthermore, such levels increase immediately on nasal provocation with allergen in the subjects with allergic rhinitis.⁶ On the basis of this observation, and given that mast cells can produce NGF,⁷⁻⁹ we hypothesized that mast cells constitute the primary source of nasal NGF after allergen challenge. Therefore, we conducted this study with the primary aim of determining whether human nasal mucosal mast cells carry NGF. To put the mast cell as an NGF source in perspective, we also examined whether other leukocytes, namely eosinophils and lymphocytes, express this neurotrophin, as previously reported.¹⁰⁻¹² Furthermore, we examined whether NGF can be found in structural elements of the nasal mucosa such as the epithelium, the submucosal glands, or blood vessels. A secondary aim was to obtain information on the distribution of the high-affinity and low-affinity NGF receptors tyrosine kinase A (TrkA) and p75, respectively, because it is quite possible that NGF can have effects on various structures, in addition to nerves.¹³⁻¹⁶

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Abbreviations used

NGF: Nerve growth factor
pAb: Polyclonal antibody
TrkA: Tyrosine kinase A

METHODS

Nasal tissue samples were obtained from patients who underwent surgery that included partial turbinectomy for refractory nasal obstruction. Because of sample deidentification, no clinical information was available.

Anti-IgE-induced histamine release assays were performed to verify the presence of functional mast cells in the samples that were used for NGF immunostaining. Double-immunofluorescence techniques were applied to evaluate colocalization of NGF with mast cells, eosinophils, T lymphocytes, or epithelial cells. Single-stain immunohistochemistry studies were separately performed to evaluate the localization of NGF and its high-affinity (TrkA) and low-affinity (p75) receptors in the nasal mucosa. The Johns Hopkins Medicine Institutional Review Board approved this study.

Reagents and antibodies

The following materials were used: for enzymatic dispersion and challenge of nasal tissue cells, collagenase type II, hyaluronidase type I, DNase I, HBSS, HEPES, FCS, penicillin-streptomycin (Sigma Chemical Co, St Louis, Mo), polyclonal goat antihuman IgE, A23187; for immunofluorescence staining, rabbit antihuman NGF polyclonal antibody (pAb) (H-20; Santa Cruz Biotechnology, Santa Cruz, Calif), mouse antihuman CD3 mAb (UCH-T1; Santa Cruz Biotechnology), mouse antihuman mast cell tryptase mAb (G3; Chemicon International Inc, Temecula, Calif), mouse antihuman eosinophilic cationic protein mAb (EG2; Pharmacia & Upjohn, Kalamazoo, Mich), biotinylated goat antirabbit IgG, streptavidin Alexa 594 and goat antimouse Alexa 488 (Molecular Probes, Eugene, Ore); and for immunohistochemical staining, rabbit antihuman NGF pAb (H-20; Santa Cruz Biotechnology), biotinylated goat antirabbit IgG (Molecular Probes), rabbit antihuman TrkA pAb (Chemicon International Inc), mouse antihuman NGF receptor P75 mAb (clone ME20.4; Sigma Chemical Co), and avidin-peroxidase and peroxidase substrate diaminobenzidine kit (Vector Laboratories, Inc, Burlingame, Calif). The polyclonal rabbit antihuman NGF antibody was raised against a peptide mapping at the N-terminus of human NGF. This antibody recognizes the 27-kd NGF precursor and has no cross-reactivity with mature NGF nor with brain-derived neurotrophic factor, neurotrophin 3, or neurotrophin 4.

Preparation of samples

The nasal tissue specimens were divided immediately on retrieval. One portion was fixed in 4% paraformaldehyde for 4 hours and then divided into 2 pieces, 1 for preparation of paraffin-embedded sections and another for preparation of frozen sections. The second portion of the original specimen was placed in HEPES-HBSS for cell dispersion and histamine release assays.

Cell dispersion and histamine release assay

Dispersion of cells from nasal tissue was performed by using modified methods described previously.¹⁷ In brief, samples were minced and subjected to gentle agitation with 2 changes of calcium-free and magnesium-free HEPES-HBSS solution with 1 mg/mL collagenase, 188 U DNase, 10% FCS, 1 mg/mL hyaluronidase, and 1% penicillin-streptomycin at 37°C for 90 minutes each. The

dispersed cells were filtered through Nitex cloth, then resuspended and cultured overnight at 37°C in 95% O₂/5% CO₂ incubator. The cell suspensions were divided into equal aliquots and challenged in duplicate with piperazine-N, N'-bis (2-ethanesulfonic acid)-albumin-glucose-calcium-magnesium buffer, polyclonal goat anti-IgE (0.3 µg/mL, 1 µg/mL, 3 µg/mL), and A23187 (1 µg/mL) for 30 minutes at 37°C. Histamine levels in supernatant samples were measured by using an automated fluorometric method described previously.¹⁸

Double-immunofluorescence staining

Cryostat sections of 10- to 12-µm thickness mounted on lysine precoated slides and dried were washed, blocked in goat serum solution for 1 hour, and then incubated with primary antibody for 16 to 18 hours at 4°C. The primary antibodies included antihuman mast cell tryptase (1:100), antihuman eosinophil cationic protein (EG2, 1:150), anti-CD3 (1:100), antiepithelial surface antigen (E-29, 1:100), and antihuman NGF (1:100). After rinsing, sections were incubated with secondary antibodies including goat antimouse Alexa 488 (1:100) for tryptase, EG2, E-29, and CD3. For NGF, the secondary antibody was a biotinylated goat antirabbit IgG (1:100), which was incubated with the tissue sample for 1 hour at room temperature. Then, the sections were incubated with streptavidin Alexa 594 (1:100) for 1 hour at room temperature. After washing with PBS, the slides were cover-slipped with antifade glycerol. Negative control slides were prepared (1) by omission of the primary antibody and (2) by use of rabbit or mouse nonimmune IgG. Positive control samples were prepared by using submandibular salivary glands dissected from male mice.

For quantification, nasal submucosal areas excluding glands below the basement membrane were viewed with an Olympus BX60 microscope (Olympus Optical Co, Hamburg, Germany) and appropriate filter sets by 2 blinded investigators. Positive cells for NGF, CD3, tryptase, and EG2 and double-positive cells for NGF/CD3, NGF/tryptase, and NGF/EG2 were counted under ×400 magnification. Results were expressed as the number of positive cells or the number of double positive cells per square millimeter.

Immunohistochemical staining for NGF, TrkA, or p75

Briefly, 5-µm paraffin-embedded sections were cut and mounted on slides, then rehydrated, washed, blocked in goat serum solution for 1 hour, and incubated with antihuman NGF pAb for 16 to 18 hours at 4°C. The sections were then incubated with biotinylated goat antirabbit IgG for 1 hour, with avidin-peroxidase for 2 hours, and then reacted with diaminobenzidine/H₂O₂. After counterstaining with hematoxylin, the sections were dehydrated, then cover-slipped with permount. Negative control slides were prepared by isotype-matched nonimmune IgG. The same method was applied for immunostaining with antihuman TrkA or antihuman p75 antibodies. Mouse submandibular gland sections were used as a positive control for immunostaining with antihuman NGF.

Statistical analysis

Data are presented as medians with 25th and 75th percentiles. The percentages of tryptase⁺, CD3⁺, and EG2⁺ cells that were immunoreactive for NGF were calculated and compared by using the Kruskal-Wallis test. A *P* value less than .05 was considered statistically significant.

RESULTS

Nasal tissue samples from 6 individuals were used for double-immunofluorescence studies after exhibiting

TABLE I. Immunoreactive cells in human nasal mucosa (median numbers [25th, 75th percentiles] of cells per mm²)

Tryptase⁺ NGF⁺	Total tryptase⁺	%*
1 (0.8, 1)	47 (42.8, 55.3)	2 (1.3, 2.3)
CD3⁺ NGF⁺	Total CD3⁺	%*
1 (0.3, 1.8)	398.5 (392.5, 411.5)	0.2 (0, 0.4)
EG2⁺ NGF⁺	Total EG2⁺	%*
15.5 (13, 21.3)	25 (21, 34.3)	62.2 (56.2, 68)

% Indicates the percentage of tryptase⁺, CD3⁺, or EG2⁺ cells that also stained positive for NGF.

*Kruskal-Wallis test comparing the above percentages: $P = .001$.

histamine release induced by anti-IgE, which indicates the presence of functional mast cells (data not shown). The percentages of tryptase⁺, CD3⁺, and EG2⁺ cells that were also immunoreactive for NGF were 2% (1.3, 2.3), 0.2% (0, 0.4) and 62.2% (56.2, 68), respectively (median [25th, 75th percentile]; $P = .001$; Table I; Fig 1). Among the interstitial cells that were NGF⁺, the vast majority (76%) were EG2⁺ cells, presumably eosinophils, and only 18% were tryptase⁺, presumably mast cells.

With immunohistochemistry, all samples showed strong NGF positivity at the submucosal glands and, to a lesser extent, the nasal epithelium (Fig 2). As expected, we demonstrated similarly strong NGF immunoreactivity in mouse submandibular glands (data not shown).

Adequate tissue samples from 3 of the individuals were available for evaluation of the high (TrkA) and low (p75) receptors for NGF. In all of these samples, immunoreactivity of both TrkA and p75 was conspicuously present in the nasal epithelium. TrkA was notable at the superficial epithelial layer, whereas p75 was localized in basal epithelial cells. Positive staining for TrkA, which was stronger than for p75, was also found in the submucosal glands. Both receptors were prominently evident on nerves and some interstitial cells, but not on vascular endothelium (Figs 3 and 4).

DISCUSSION

Our interest in NGF stems from the fact that this biologic product has enough characteristics to be considered a prime culprit leading to neural hyperresponsiveness in rhinitis,³ a phenomenon that has been very well documented and may play a central role in the clinical manifestations of nasal disease.^{1,2,19}

In this article, we present the first report on NGF and NGF receptor localization in the human nasal mucosa. We tested the hypothesis that the mast cell is a primary source of NGF, but found that only a small percentage of these cells are NGF⁺ on immunofluorescence. In contrast, a larger percentage of eosinophils were positive with NGF antibodies. Importantly, NGF immunoreactivity was very prominent in submucosal glands and in the epithelium. An additional observation was that high-affinity and low-affinity NGF receptors were expressed not only on

mucosal nerves but also on epithelial and glandular cells. Finally, we report a clear differentiation in the expression of NGF receptors: the high-affinity receptor is seen primarily in superficial epithelial cells, whereas the low-affinity receptor is localized in basal cells.

The first aim of our study was to test the hypothesis that nasal mast cells contain NGF. The hypothesis was based on our previous observation that large quantities of NGF are acutely released in nasal secretions on allergen (and not on histamine) stimulation⁶ and on previous reports that mast cells can produce and secrete NGF.⁷⁻⁹ The presence of functional mast cells in the tissue samples was ascertained by demonstrating anti-IgE induced release of histamine. It is possible that basophils contributed to the histamine released by the tissue, but it is generally accepted that, in the absence of an acute allergic reaction (allergen challenge), the primary source of histamine in nasal tissue is the mast cell. Contrary to our hypothesis, only a small fraction of nasal mast cells were found to contain NGF. Likewise, T lymphocytes in the human nasal mucosa rarely contained this neurotrophin. The latter result is in contrast with *in vitro* findings in animals and human beings, suggesting that T lymphocytes constitutively synthesize and secrete NGF.^{11,20,21} On the other hand, almost 2/3 of activated eosinophils exhibited NGF immunoreactivity. This result is compatible with the findings of Solomon et al,¹² who demonstrated the presence of NGF mRNA and protein in isolated human peripheral blood eosinophils. Their findings were recently confirmed by Kobayashi et al,¹⁰ who noted that such cells constitutively synthesize and secrete biologically active NGF and that nasal lavage levels of NGF correlated with levels of other known eosinophil products.

In further assessing sources of NGF, we examined the structural elements of the nasal mucosa. Multiple studies have established that NGF is produced by mouse submandibular salivary glands, and we used these glands successfully as a positive control for our immunohistochemistry studies. De Vicente et al²² previously demonstrated NGF in this murine tissue, but they did not detect it in human submandibular glands. We were thus surprised to discover the consistently striking presence of NGF in human nasal submucosal glands. Epithelial staining, on the other hand, was an expected finding because it has been previously reported in the human lower airway.^{23,24}

The presence of NGF in the glandular apparatus raises the possibility that the large increase in nasal fluid NGF levels we have previously observed after allergen challenge⁶ is a result of glandular activation, a prominent element of the nasal allergic reaction. If so, it is perplexing why, in our hands, nasal provocation with histamine, which does induce glandular secretions,²⁵ failed to result in increased NGF levels.⁶ A possible explanation for this paradox is that the release of NGF by glands is stimulus-dependent and that an allergic mediator other than histamine may be responsible for this phenomenon.

One caveat with imaging is that cellular staining does not provide definitive evidence for synthesis or secretion. It is possible, for example, that some of these cells take

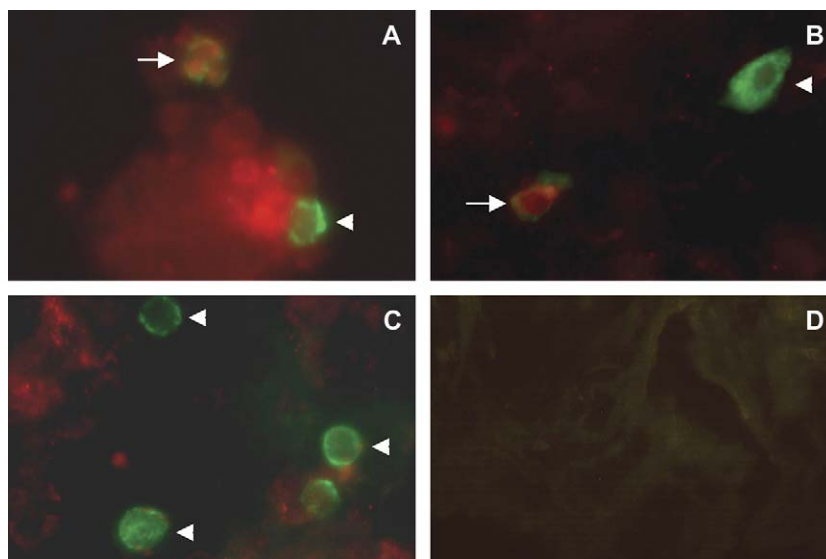


FIG 1. NGF colocalization with EG2 (eosinophils), CD3 (T lymphocytes), and tryptase (mast cells) by double immunofluorescence staining in human nasal mucosal tissue (representative samples out of 6 subjects). NGF-Alexa 594 (red); EG2, CD3, and tryptase-Alexa 488 (green); double-positive cell (orange). **A**, EG2⁺/NGF⁺ nasal interstitial cell (arrow, orange), EG2⁺/NGF⁻ interstitial cell (arrowhead, green). **B**, Tryptase⁺/NGF⁺ interstitial cell (arrow, orange), tryptase⁺/NGF⁻ interstitial cell (arrowhead, green). **C**, CD3⁺/NGF⁻ cells (arrowhead, green). **D**, Negative control (stained with rabbit and mouse nonimmune IgG). Original magnification $\times 400$.

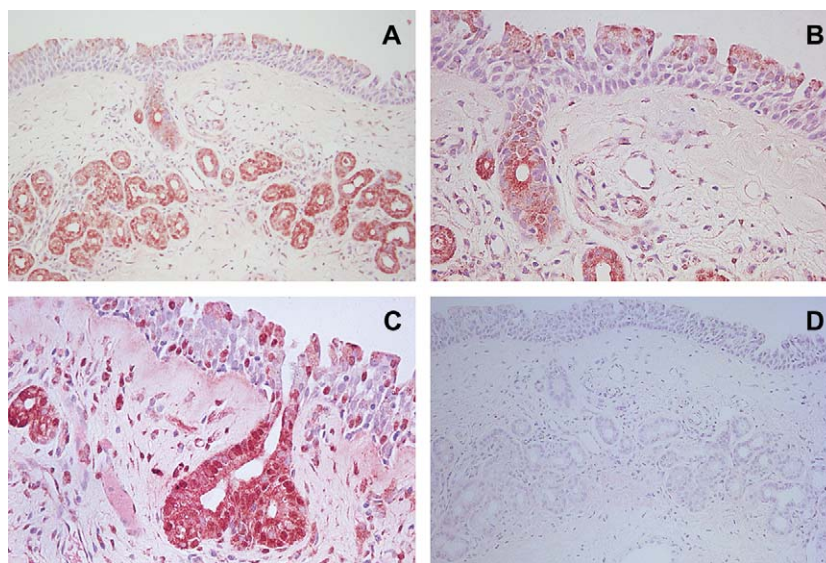


FIG 2. NGF immunohistochemical staining in the human nasal mucosa (diaminobenzidine, brown; representative sample out of 3 subjects). **A**, NGF staining is observed on submucosal glands and the epithelial layer ($\times 200$). **B**, Higher magnification view of **A** concentrating on the epithelium ($\times 400$). **C**, High magnification view of a submucosal gland ($\times 400$). **D**, Negative control (stained with isotype matched nonimmune IgG; $\times 200$).

up NGF from the environment but do not produce it. Functional studies are required to address this issue in depth. Furthermore, the state of cellular activation may influence the NGF content of various inflammatory or structural cells. The lack of clinical information from the patients who donated the tissue does not allow us to comment further on this matter, and this is certainly a

limitation of this study. For example, atopic status or the degree of mucosal inflammation may influence the expression of NGF as demonstrated by our work in the human nose⁶ and in lower airway studies involving subjects with asthma.^{23,24} It is important, therefore, that dynamic studies examining NGF cell content at various stages of activation in human tissue are conducted in the future.

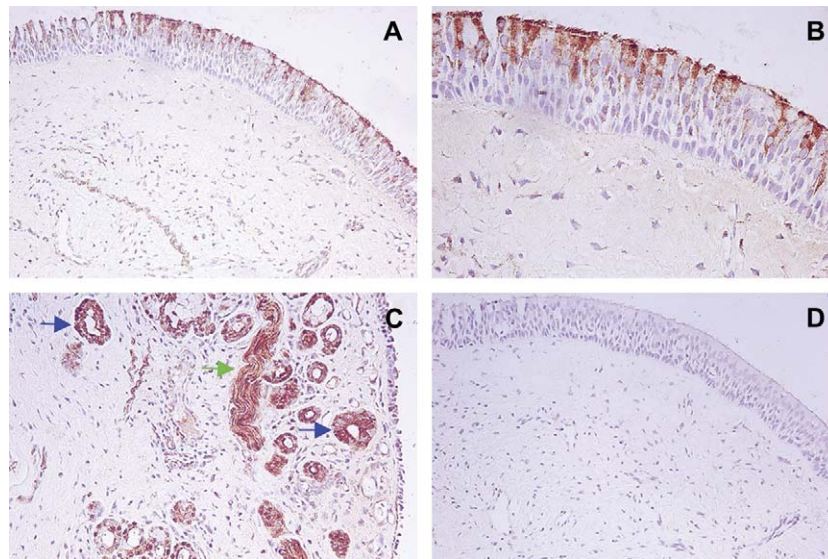


FIG 3. High-affinity NGF receptor (TrkA) immunohistochemical staining in the human nasal mucosa (diaminobenzidine, brown; representative sample out of 3 subjects). **A**, TrkA staining is observed on the superficial epithelial layer ($\times 200$). **B**, Higher magnification view of **A** ($\times 400$). **C**, TrkA staining is also observed on nerves (green arrow) and on submucosal glands (blue arrows; $\times 200$). **D**, Negative control (stained with isotype matched nonimmune IgG; $\times 200$).

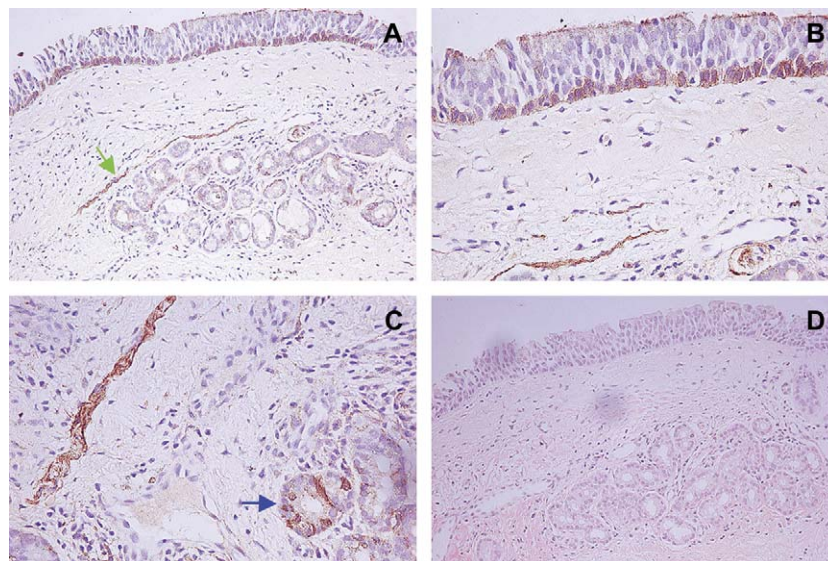


FIG 4. Low-affinity NGF receptor (p75) immunohistochemical staining (diaminobenzidine, brown) in the human nasal mucosa (representative sample out of 3 subjects). **A**, p75 staining is observed on the basal epithelial layer and on nerves (green arrow; $\times 200$). **B**, Higher magnification view of **A** ($\times 400$). **C**, p75 staining is also observed on submucosal glands (blue arrow), but staining of glands with p75 is less prominent than that for TrkA (Fig 3; $\times 400$). **D**, Negative control (stained with isotype matched nonimmune IgG; $\times 200$).

Another possible limitation of this study is that the antibody used to identify NGF expression with either immunofluorescence or immunochemistry recognizes the NGF precursor, a 27-kd molecule, and not mature NGF, a 13-kd molecule. Although mature NGF has stronger biologic activity, at least as a neurotrophin,²⁶ its presence in various tissues is scarce compared with the precursor

molecule.^{27,28} It is thus quite possible that the NGF precursor is biologically more relevant. On the other hand, the pattern of NGF expression we present in this report might have been different if an antibody against mature NGF had been used. Notably, in the published work in which NGF has been identified in human lower airway tissue, the antibody used was identical to ours.^{23,24}

Nerve growth factor is known to have activity on several leukocytes and is considered by many investigators to be not just a neurotrophin and neurokinin²⁹ but a cytokine as well.³⁰ Interestingly, it has been suggested that NGF plays a role in tissue healing,^{15,16,31} which raises the hypothesis that it may also affect structural tissue components. In this context, we investigated the localization of NGF receptors *in vivo*. As expected, we found strong immunoreactivity of both NGF high-affinity tyrosine kinase receptor (TrkA) and low-affinity receptor (p75) along the sensory nerves in the human nasal mucosa. However, we also localized both receptors in the epithelium, submucosal glands, and interstitial cells. Shibayama and Koizumi³² similarly examined the immunolocalization of TrkA in nonneuronal human tissue, but not the nasal mucosa, and found strong expression on the epithelium and glands of the gastrointestinal tract. The role of NGF on glands and epithelium is unknown, but the abundance of receptors and of NGF itself, as well as our current state of knowledge, raise the possibility of autocrine and paracrine effects, potentially including functional modulation and growth. Insights into the role of NGF may also be obtained by understanding the reasons why the high-affinity receptors were localized in cells of the superficial epithelial area, whereas low-affinity receptors were found primarily in basal cells.

In summary, our findings indicate that submucosal glands and eosinophils may be the most important sources of NGF in the human nasal airway. In addition, the fact that NGF receptors are found in structural cells other than nerves supports the notion that the role of this neurotrophin is much broader than the modulation of the sensorineural system. NGF research in the human airway mucosa is thus becoming a progressively more exciting field.

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