

Original article

Nerve growth factor localization in the nasal mucosa of patients with persistent allergic rhinitis

Background and objectives: Nerve growth factor (NGF) and NGF receptors have been shown to be expressed by structural and infiltrating inflammatory cells in the human allergic bronchial mucosa and conjunctiva. In the nose, a positive immunostaining for NGF was recently reported in biopsies of subjects undergoing surgery for refractory nasal obstruction. This study was aimed at studying by immunohistochemistry NGF expression and localization in the nasal mucosa from subjects with moderate/severe persistent allergic rhinitis and natural allergen exposure.

Methods: Immunostaining for NGF, tryptase and eosinophil cationic protein was performed in human nasal turbinate sections of 25 patients affected by persistent allergic rhinitis and sensitization to *Dermatophagoides pteronyssinus*.

Results: NGF was consistently expressed in the epithelium and in the submucosa of allergic rhinitic subjects, preferentially localized in eosinophils and mast cells. A strong NGF immunostaining was found in mucous cells of the epithelial lining and in the submucosal glands.

Conclusions: As previously shown for allergic asthma and allergic conjunctivitis, NGF is also detectable in the nasal mucosa of patients with persistent allergic rhinitis. The preferential NGF localization in mucous cells of the epithelial lining and in the submucosal glands suggests a possible role for NGF in modulating secretion in allergic rhinitis and possibly other allergic diseases.

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Nerve growth factor (NGF) is the first discovered protein belonging to the neurotrophin family. The name recalls its primary activity: peripheral and central nerve growth, differentiation and survival (1). In addition, NGF is also known to exert a variety of multidirectional effects on nonneural cells as shown by several studies (2, 3).

After the original report of one of us, of increased serum levels of NGF in human allergic diseases and asthma (4), extensive evidence has been accumulated indicating that NGF is involved in the pathophysiology of allergic inflammation (5–7) and neural hyperresponsiveness in the airways (8–10).

In animal models of allergy and asthma NGF positive cells are mainly represented by the mononuclear cells present in the inflammatory infiltrate of the bronchial mucosa and elevated levels of NGF are detected in serum and broncho-alveolar lavage fluids (BALF) in ova-challenged mice (11). More recently, airway epithelial cells have also been shown to represent a major source of neurotrophins (NGF and brain derived neurotrophic factor, BDNF) which, via eosinophil activation, and

chemotaxis enhance the epithelial neurotrophin production and promote eosinophil survival, amplifying allergic inflammation (12). In fact, up-regulation of NGF production by inflammatory mediators during allergic inflammation is a well known contributing factor to the development of neural hyperresponsiveness of the upper and lower airways primarily by affecting peptidergic sensory nerve function (13–15). Moreover, direct evidence was produced showing that inhibition of NGF activation via receptor blockage prevents airway hyperreactivity (AHR) development (16).

In humans, elevated levels of NGF have been demonstrated in serum and in BALF of asthmatic patients (17, 18) and in nasal lavage fluid of rhinitic patients (6) after allergen provocation. Furthermore, in bronchial tissue, pulmonary epithelial cells, fibroblasts and smooth muscle cells synthesize and express NGF in inflammatory conditions (18–20).

Recently, NGF and its receptors were also shown by Wu et al. (21) in biopsies of nasal mucosa from six subjects who underwent surgery including partial turbinectomy for refractory nasal obstruction. However in this study, for identification reasons, no clinical information was available on sample source, although the histamine release from tissues induced by anti-IgE and the presence

Abbreviations: AHR, airway hyperreactivity; BALF, bronchial alveolar lavage fluid; NGF, nerve growth factor; PAR, persistent allergic rhinitis; SP, substance P.

of eosinophils in biopsies was highly suggestive for an allergic origin of the refractory nasal obstruction. Accordingly the authors, in correctly recognizing the limitation of their study, strongly recommended further studies of human diseases with special reference to information on the atopic status, and severity of allergic inflammation and symptoms.

We report here the results of an immunohistological study of NGF localization in a large homogenous series of subjects with moderate-severe persistent allergic rhinitis (PAR) and sensitization to *Dermatophagoides pteronyssinus*.

Materials and methods

Subjects

Twenty-five subjects with PAR diagnosed according to ARIA guidelines (22) were included in the study. Mean age of study subjects was between 18 and 65 (13 males and 12 females) and all had symptoms of PAR, in accordance with allergic rhinitis and its impact on asthma (ARIA) guidelines for moderate/severe persistent allergic rhinitis, for at least 1 year and a positive skin test to house-dust mites (*D. pteronyssinus*, Der p) and/or the presence of serum Der p-specific IgE (Cap System®, Phadia). Patients who had taken (within a defined time period before surgery) one of the following drugs were excluded: oral or nasal antihistamines (7 days), astemizole (3 months), nasal cromoglycate (7 days), corticosteroids in any form (4 weeks) and parenterally administered corticosteroids (3 months).

The study was approved by the ethics committee of the Montpellier Hospital and was conducted in accordance with the Declaration of Helsinki and after written and informed consent from the patients.

Biopsies

Nasal biopsies were obtained from the posterior part of the inferior turbinate with a 2 mm Microfrance forceps after local anaesthesia with a xylocaine and epinephrine spray.

Tissue preparation and morphological analysis

Biopsies (at least one sample for each patient) were fixed at -20°C in acetone containing protease inhibitors. Within 24 h the specimens were processed and embedded in the water-soluble resin glycolmethacrylate (GMA, JB4 Kit, Park Science Ltd, Northampton, UK) by the technique of Britten et al. (23). Sections of $2\text{ }\mu\text{m}$ were cut from the resin-embedded specimen and prepared glass slides were observed using a $20\times$ Splan Apo objective on a BHS microscope (Olympus, Tokyo, Japan) equipped with a Sony DXC-950P 3CCD colour video camera (Sony, Tokyo, Japan). This technique allows co-localization studies in the $2\text{ }\mu\text{m}$ subsequent sections without the need of double staining. All computer images were analysed by sis 2.01 Software for image analysis (Soft-imaging Software GmbH, Olympus, Munster, Germany).

Immunostaining

Nerve growth factor. Sections were incubated with polyclonal sheep anti-human NGF- β $1.5\text{ }\mu\text{g/ml}$ (Chemicon International Inc., Temecula, CA, USA) overnight at 4°C and then 1 h at 37°C ,

followed by a 1 h incubation at room temperature (RT) with biotinylated anti-sheep IgG antiserum (Dako, Glostrup, Denmark). After a further 2 h incubation at RT with avidin biotinylated complex-horse radish peroxidase, samples were processed with H_2O_2 /diaminobenzidine for 15 min to develop the reaction. Previously to antibody incubation antigen retrieval (citrate buffer + microwave oven, $3\times 10\text{ min}$), endogenous peroxidase inhibition (H_2O_2 , 3% 10 min) and nonspecific site blocking with foetal bovine serum (FCS, 10% 30 min) was performed. Finally, sections were washed in distilled water and counterstained with Mayer's haematoxylin, briefly dried and mounted in a gelatin-glycerol solution. Negative control slides were prepared by replacing the primary antibody by its control isotype immunoglobulin from the same species (sheep IgG, Dako). Sections of mouse submandibular gland were used as positive NGF controls. Previous experiments were performed with a monoclonal antibody to NGF (Roche Diagnostic, Indianapolis, IN, USA, France) which was withdrawn from the market during our study (24); immunostaining pattern in the nasal mucosa was identical with both monoclonal and polyclonal antibodies, indirectly demonstrating that our positivity for NGF can not be claimed to nonspecific binding and staining of the polyclonal antibody used subsequently.

Mast cells and eosinophils. Specific staining for mast cells and eosinophils was also performed on the sections immediately following those on which immunohistochemistry for NGF was performed. Primary antibodies included mouse monoclonal anti-human tryptase (anti AA1 IgG $2\text{ }\mu\text{g/ml}$, Dako) for mast cells and mouse monoclonal anti-human eosinophil cationic protein (ECP) (anti EG2 IgG $0.5\text{ }\mu\text{g/ml}$, Pharmacia) for eosinophils.

Results

Samples from all patients showed the typical histological findings of allergic rhinitis with subepithelial increased representation of inflammatory cells including mast cells, lymphocytes and eosinophils. The degree of inflammation appeared to vary among patients and in the same patient among different sections and microscopic fields, although it was not possible to express this variability in quantitative terms.

NGF was heavily expressed in the nasal mucosa of all subjects with moderate/severe persistent allergic rhinitis. Immunostaining was prevalent in mononuclear cells of the submucosa, preferentially localized in eosinophils (Fig. 1), and mast cells (Fig. 2) and in the cytoplasm of secreting cells in glandular structures (Fig. 3). The epithelial cell layer showed a positivity for NGF (Fig. 4) in the cytoplasm of mucous cells and in limited cases in the cells of the basal layer. Occasionally, a positive staining for NGF was detectable in stromal cells with a clear morphological aspect of fibroblasts.

Discussion

Our study shows that NGF is highly and consistently expressed in biopsies from patients with moderate/severe persistent allergic rhinitis sensitized to *D. pteronyssinus*.

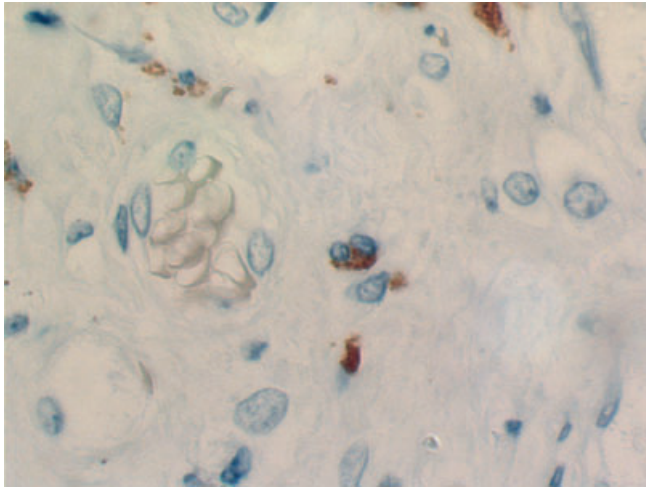


Figure 1. Sub-mucosa (lamina propria): eosinophils. Immunohistochemistry for nerve growth factor in the submucosa of the inferior turbinate nasal biopsies. Sheep anti-human NGF- β antibody (Chemicon International Inc.) shows localization in eosinophils. Sections of 2 μ m were observed using a 25 $\times$ Splan Apo objective on a BHS microscope.

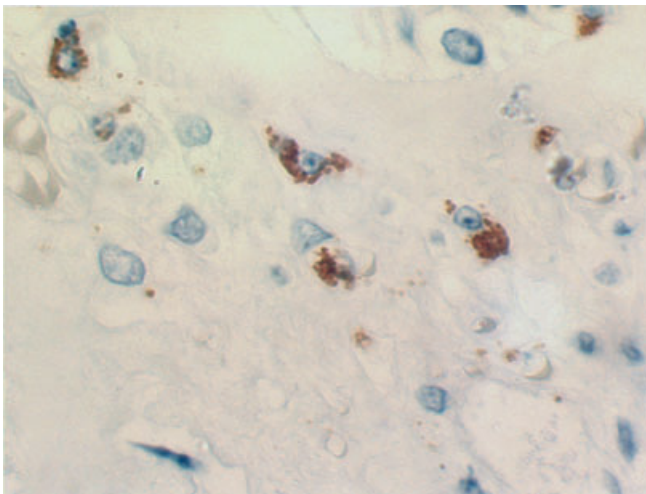


Figure 2. Sub-mucosa (lamina propria): mast cells. Immunohistochemistry for nerve growth factor in the sub-mucosa of posterior turbinate nasal biopsies. Sheep anti-human NGF- β (Chemicon International Inc.) antibody shows localization in sub-mucosa cell layer. Sections of 2 μ m were observed using a 25 $\times$ Splan Apo objective on a BHS microscope.

Our data indicate that NGF expression is related to allergic inflammation since preferentially localized in inflammatory cells, which are well known to be absent in the mucosa of normal subjects. This finding extends to the nasal mucosa what previously found in the bronchi (18–20) and in the conjunctiva (25), and confirms, on quite a large number of allergic patients, the preliminary observation of Wu et al. in the nasal mucosa of subjects with refractory nasal obstruction. Unfortunately no

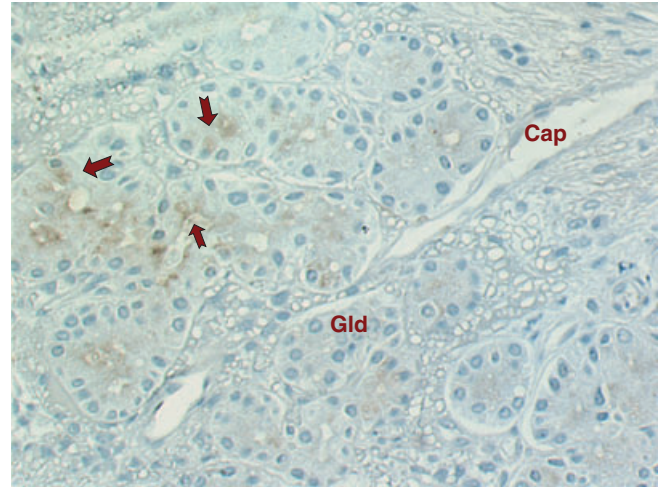


Figure 3. Sub-mucosa (lamina propria): glands. Glandular structure in the nasal mucosa of the inferior turbinate. The arrows points to glandular cells with positive NGF staining. Gld, glandular structure; Cap, blood capillary.

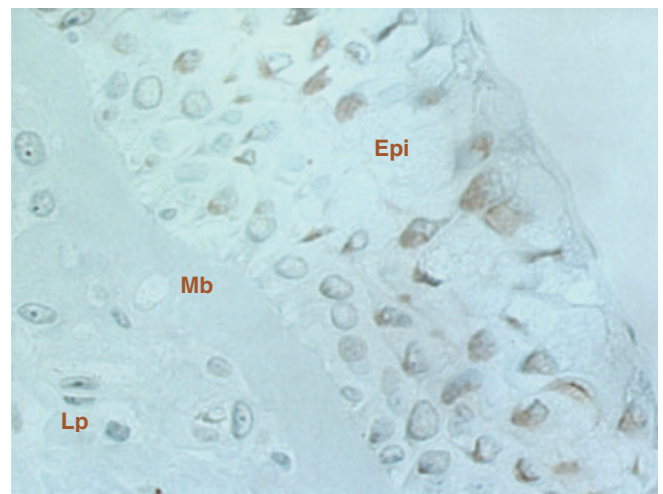


Figure 4. Epithelium. Positive staining for NGF is observed within the epithelial layer of the nasal mucosa. Sections of 2 μ m were observed using a 40 $\times$ Splan Apo objective on a BHS microscope. Epi, epithelium; Bm, basal membrane; Lp, lamina propria.

clinical correlation between morphological and clinical data could be made.

The evaluation of subsequent 2 μ m tissue sections with antibodies against NGF, tryptase and eosinophils cationic protein allowed us to show a co-localization of this neurotrophin in the inflammatory cells infiltrating the submucosa (mast cell and eosinophils mainly). NGF was also localized in structural cells (epithelial cells, mucous cells and fibroblasts). The prevalent localization of NGF in

the inflammatory infiltrate and in the remodelling elements justifies the quantitative differences in NGF expression in the mucosal tissues of allergic subjects *vs* normal subjects, as previously reported for the bronchial and conjunctival mucosa and as we could confirm (data not shown).

Immunohistochemical studies allow just speculations on whether the above cells actively synthesize and represent a source of NGF or a target for it. However, several *in vitro* functional studies provide convincing evidence that both structural and inflammatory cells actively synthesize and respond to NGF, possibly through autocrine and intercellular signalling mechanisms (11, 26, 27).

The functional role of NGF and its receptors in allergic diseases is still object of investigation (26, 27). Our observation in biopsies of subjects with persistent allergic rhinitis as well as other studies in tissues and secretions of other allergic diseases certainly document that NGF is consistently present in allergic inflammation, independently from the target organ considered. Since, in animal models, NGF reproduces signs and symptoms of allergic diseases (10) and NGF antagonism with anti-NGF antibodies has a inhibiting effect (10, 16, 28–30), it seems that many of the postulates for supporting an active role for NGF in modulating allergic inflammation are in fact answered.

The most interesting finding in our study was an intense immunostaining for NGF in the mucous cells of the epithelial lining and at the level of submucosal glands. Similar findings are also reported by Wu et al. in their study (21).

In rats, glands have been shown as the most important source of NGF, and the submandibular glands are usually used as a positive control for NGF in immunohistochemistry for their intense staining. Allergic diseases of the airways are characterized by excess mucous secretion, airway obstruction, inflammation, increased sensitivity to insulting constricting stimuli and airway remodelling.

Substance P (SP), a tachykinin whose release is controlled by NGF has been implicated in animal models as inflammatory mediator of mucous secretion (31) and microvascular leakage (32). Moreover, SP along with neurokinin A, another member of the family, leads to hypersensitivity due to offending stimuli (allergens, hypertonic saline solution, capsaicin) and other features of allergic neurogenic inflammation (33). In animals, substance P level is increased in mouse models of allergic airway inflammation and its release influences tear secretion and goblet cell function, in rats (31); moreover, in

humans, increased levels of SP are found, in the lung of asthmatics after allergen provocation (34). Accordingly, in man, we found that in vernal conjunctivitis – an allergic disease with excessive mucous production – the increased NGF content in plasma is a significantly related to SP levels (35). Finally, NGF in inflamed tissues has a major regulatory role in the content and transport of SP in sensory nerves (36). On the basis of the above data we therefore speculate that NGF produced and released in epithelial and glandular structures of the airways may play a critical role, possibly via tachykinin release, in mucous hypersecretion, a typical sign of allergic inflammation of the airways occurring in both asthma and rhinitis.

Moreover, mucus production is a major component of tissue remodelling in the lower airways (37, 38). Preliminary studies of our group indicate that transgenic mice overexpressing NGF in Clara cells show goblet cell hyperplasia and muc 5AC overexpression (39). Similarly, NGF *in vitro* induces muc 5AC overexpression in epithelial cell lines (40). Since it is well documented that NGF has a very potent effect on fibroblasts activation both *in vivo* (41) and *in vitro* (42, 43), the hypothesis that NGF plays a relevant role in tissue remodelling associated with allergic inflammation (44) finds a strong support by our findings.

In fact, although in the allergic nose remodelling processes are definitely less relevant than in the lower airways, in our study NGF was also found to be localized in sub-mucosal fibroblasts (45).

In conclusion, our original report NGF expression in subjects with persistent moderate/severe allergic rhinitis is consistent with observations previously reported in other allergic diseases such as allergic asthma and allergic conjunctivitis, thus suggesting a more general role for NGF in allergic inflammation. The morphological finding of NGF expression in secretory elements, should prompt further functional studies on the effects of this neurotrophin on modulation of mucus secretion and tissue remodelling in allergic diseases.

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