Expression of Nerve Growth Factor Receptors on the Ocular Surface in Healthy Subjects and during Manifestation of Inflammatory Diseases

Alessandro Lambiase, Paolo Rama, Stefano Bonini, Sergie Bonini, and Luigi Aloe

PURPOSE. Recent studies have suggested the involvement of nerve growth factor (NGF) in the conjunctival inflammatory process and in corneal epithelium proliferation and differentiation. To verify the hypothesis that NGF could locally modulate the inflammatory and reparative processes, the authors evaluated the expression of NGF high-affinity receptor on the ocular surface in normal and pathologic conditions.

METHODS. Ten conjunctival biopsies (obtained from three healthy subjects, five patients affected by vernal keratoconjunctivitis (VKC), and two patients with cicatricial pemphigoid (CP)) and five corneal specimens obtained from the Eye Bank of Veneto (Italy) were evaluated. All specimens were processed to identify the NGF high-affinity receptor (TrkA).

RESULTS. All tissues expressed immunoreactivity for NGF receptors. In conjunctival specimens of healthy subjects, basal epithelial cells strongly expressed immunoreactivity and, in the stroma, rare cells were immunopositive for TrkA. No significant difference in immunoreactivity was observed in the conjunctival epithelium between healthy subjects and patients with inflammatory conjunctival diseases, whereas there were more immunopositive cells observed in the conjunctival stroma of VKC and CP patients than in the controls. The immunoreactivity in the cornea was confined to basal epithelial cells and endothelium.

CONCLUSIONS. The NGF receptor is present on the human ocular surface. The authors' data support the possibility that NGF modulates ocular inflammation and corneal epithelial proliferation and differentiation through its receptors. (Invest Ophthalmol Vis Sci. 1998;39:1272-1275)
### Table 1. Characteristics of Patients and Criteria Used for Diagnosis

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<th>Vernal Keratoconjunctivitis</th>
<th>Age (yr)</th>
<th>Previous and Associated Atopic Diseases</th>
<th>Clinical Signs</th>
<th>Total IgE (KU/l)</th>
<th>RAST-Positive*</th>
<th>ECP (mg/l)</th>
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Ig, immunoglobulins; BMZ, basement membrane zone; ECP, eosinophil cationic protein.
*Scrum measurement of specific IgE include rye grass (Gram.), Dermatophagoides pteronyssinus (Opt.), Parietaria officinalis (Par. off.), Olea europea milk, and egg proteins. We considered patients RAST positive if they showed a class 1 positivity for at least one of the allergens tested.

### MATERIALS AND METHODS

#### Subjects and Procedure

Ten conjunctival biopsies obtained from five patients (3 males, 2 females; age range, 8–23 years) with VKC, two men with cicatricial pemphigoid (CP) (aged 55 and 64 years), and three healthy controls (2 males, 1 female; aged 14, 16, and 19 years) were evaluated for the presence of NGF receptors. Five corneas not suitable for corneal transplantation (3 men, 2 women; age range, 70–85 years) obtained from the Eye Bank of Veneto (Italy) were also included in the study. The diagnoses of VKC and cicatricial pemphigoid were made on the basis of history and clinical examination and confirmed by histologic evaluation (Table 1).^{5,7}

All patients were untreated for at least 1 week before the study was performed.

The tenets of the Declaration of Helsinki were followed, informed consent was obtained from the patients or the relatives, and institutional human experimentation committee approval was granted.

#### Histologic Evaluation

Biopsy specimens were obtained from the tarsal conjunctiva of patients with VKC and from the bulbar conjunctiva of patients with CP and of the healthy subjects. Conjunctival specimens were taken from each patient after anesthesia with subconjunctival 2% mepivacaine hydrochloride (Polocaine; Astra Pharmaceutical Products, Westboro, MA Cm-Ti zymography). Specimens were fixed in a 0.1 M sodium phosphate buffer (phosphate-buffered saline, pH 7.4) containing 4% paraformaldehyde. The specimens were stored in 20% sucrose, 0.1 M phosphate-buffered saline (pH 7.4) and sectioned at 6 μm with a freezing microtome. The slides were then stained with hematoxylin–eosin or acidic toluidine blue and evaluated by light microscopy. In patients with VKC, lymphocytes, eosinophils, and mast cells were counted in three fields (×40) for each of three randomly selected sections. Data are presented as mean ± SE. The same procedure was used to process corneal specimens.

### Immunohistochemistry for Nerve Growth Factor High-Affinity Receptor

Immunoperoxidase and immunofluorescence localization of p140trkA was performed using a specific rabbit polyclonal antibody (0.3 μg/ml; Santa Cruz Biotechnologies, Santa Cruz, CA). The slides were mounted with Kaiser's glycerol and evaluated by light microscopy. The specimens were then stained with hematoxylin–eosin or acidic toluidine blue and evaluated by light microscopy. In patients with VKC, lymphocytes, eosinophils, and mast cells were counted in three fields (×40) for each of three randomly selected sections. Data are presented as mean ± SE. The same procedure was used to process corneal specimens.

![Figure 1. Immunohistochemistry for nerve growth factor high-affinity receptor shows that basal epithelial cells are immunopositive in conjunctiva specimens of healthy subjects. Original magnification, ×40.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933206/ on 06/24/2017)
FIGURE 2. Immunohistochemistry for nerve growth factor high-affinity receptor shows that basal cells in the epithelium and several cells in the stroma are immunopositive in conjunctival specimens of patients with cicatricial pemphigoid. Original magnification, ×40.

CA). Phenotypic analysis was performed by indirect immunofluorescence and immunoperoxidase using a previously described procedure. To detect nonspecific binding of antibodies, goat or rabbit purified immunoglobulins were used as control. Cells positive for TrkA were counted in three fields (×40) for each of three randomly selected sections. Data are presented as mean ± SE.

To verify the expression of TrkA on eosinophils and T-lymphocytes we performed a double immunofluorescence technique in two patients with VKC, using TrkA antibody with anti-eosinophil cationic protein (ECP; clone EG2, Pharmacia, Sweden) or anti-CD4-fluorescein-conjugated antibody (Sigma Chemical, St. Louis, MO). Briefly, for immunofluorescent staining, the slides were incubated for 15 minutes in 0.1 M phosphate-buffered saline (pH 7.4) containing 0.1% Triton X-100 and over night at 4°C with monoclonal antibody anti-ECP diluted 1:200 or with anti-CD4-fluorescein-conjugated antibody diluted 1:100. Excess antiserum was removed, and the slides processed with anti-ECP were incubated for 1 hour at room temperature with fluorescein-conjugated sheep anti-mouse Ig, F(ab')2 fragment (Boehringer, Mannheim, Germany) that was diluted 1:10 with the same buffer. All slides were then incubated with anti-TrkA antibody following the procedure previously described. A similar procedure was followed for the second immunofluorescence staining with rhodamine-conjugated anti-rabbit Ig, F(ab')2 (Cappel, Malvern, PA) diluted 1:20 with phosphate-buffered saline.

RESULTS

In conjunctival specimens of healthy subjects a strong immunoreactivity was observed in the basal cells of the epithelium, whereas rare cells were immunopositive for TrkA in the stroma (Fig. 1). A similar immunoreactivity pattern for TrkA was found in the conjunctival epithelium of patients with CP and VKC. On the contrary, a marked increase of immunopositive cells was seen in the conjunctival stroma of VKC and CP patients (CP = 25 ± 5 cells/field; VKC = 80 ± 15 cells/field) when compared with healthy controls (3 ± 2 cells/field) (Fig. 2).

The histologic analysis of the patients with CP showed the presence of marked inflammatory infiltrate with plasma cells, lymphocytes, histiocytes, mast cells, and rare eosinophils in the stroma. In patients affected by VKC the stromal infiltrate was mainly represented by lymphocytes (75.3 ± 11.4 cells/field), eosinophils (28.0 ± 9.2 cells/field), and mast cells (20 ± 3.4 cells/field). Colocalization for TrkA and ECP or CD4 showed that approximately 80% of activated eosinophils (ECP positive) and 80% of the T-helper lymphocytes (CD4 positive) expressed the high-affinity NGF receptor.

All of the corneal tissues expressed immunoreactivity for NGF receptor as well. The basal cells of the corneal epithelium and the endothelium expressed immunoreactivity for TrkA, whereas no immunoreactivity was detected in the stroma (Fig. 3).

DISCUSSION

We have demonstrated the presence of the high-affinity NGF receptors on the human ocular surface. The biologic effects of NGF are mediated by two classes of receptors: p75, a 75-kDa glycoprotein that belongs to a superfamily of cytokine receptors, which includes tumor necrosis factor receptor, Fas, CD27, CD40, and CD30; and a transmembrane tyrosine kinase of 140 kDa (TrkA), phosphorylated on tyrosine after binding to its ligand. Considerable evidence is now available showing that stimulation of TrkA is necessary and sufficient to elicit a full biologic response. In contrast, other reports have highlighted the crucial role of the association of TrkA and p75 in regulating NGF biologic activities. A general agreement seems to confirm that a TrkA expression is necessary for NGF signal transduction and NGF

FIGURE 3. Corneal tissues from donors show immunoreactivity for TrkA in the basal epithelium (A) and in the endothelium (B). Original magnification, ×40.
biologic actions. The trophic effect of NGF on specific neurons in the peripheral and central nervous systems and its proliferative action on keratocytes are well known. In vitro study has shown that NGF and other growth factors modulate the proliferation and differentiation of rabbit corneal epithelial cells. The presence of NGF receptors on human conjunctival and corneal epithelium suggests that this neurotrophin plays a role in the modulation of one or more proliferative mechanisms of the epithelium, such as during corneal injury. It has been demonstrated that corneal epithelial injury induces the expression of promoter genes (such as c-fos and c-jun) in the trigeminal nucleus, which through the superior cervical ganglion stimulate cells of the lacrimal glands to produce several growth factors, including tumor necrosis factor-α. Growth factors are able to modulate the proliferation and differentiation of the epithelial cells, as has been demonstrated in vitro and in vivo studies for a variety of growth factors, such as epidermal growth factor, fibroblast growth factor, and NGF. Because it has been recently reported that murine corneal sensory innervation is NGF dependent and that murine strain knockout for NGF high-affinity receptor (TrkA) gene displays impairment of corneal sensitivity, the possibility exists that NGF is released by the sensitive nerve endings to directly modulate the proliferation of epithelial cells.

The significance of NGF receptor expression by corneal endothelium is at present unclear. We have previously reported an increase of NGF levels in the rabbit aqeous humor during experimental high intraocular pressure. It can be hypothesized that NGF could be involved in the trophism of endothelial cells in the basal condition and during intraocular injury to provide trophic support.

The increased immunopositive cells observed for TrkA in the conjunctival stroma during manifestation of inflammatory diseases is consistent with previous reports that show the involvement of NGF in the pathogenesis of human allergic and autoimmune diseases. NGF receptors have been demonstrated on different human tissues, not only of the central and peripheral nervous systems, but also on immune cells, including human monocytes, lymphocytes, and T-helper clones. Indeed, there is evidence suggesting that NGF acts on the immune cells by modulating the inflammatory reaction. NGF is involved in the induction of mast cell and basophil degranulation, suppression of leukotriene C4 formation by eosinophils, proliferation of human peripheral blood lymphocytes, and stimulation of the expression of IL-2 receptors on these cells. An increase of NGF levels in the blood or tissues of patients affected by different diseases (all characterized by an activation of the immune cells) has been reported, suggesting an involvement of this molecule in the immune processes. Moreover, an increased amount of NGF has been found in tissues of patients with systemic sclerosis and in the synovial fluid of patients with chronic autoimmune arthritis. High circulating NGF levels were measured in murine schistosomiasis and in patients with systemic lupus erythematosus, allergic diseases, asthma. We have reported increased circulating NGF levels in vernal keratoconjunctivitis and a direct correlation between NGF levels and number of mast cells infiltrating the conjunctiva. All these observations raise the question as to whether NGF could locally influence the inflammatory process. Our results, showing an increase of cells expressing NGF receptor during the conjunctival inflammatory process, strongly support this hypothesis. Moreover, present data confirm the expression of NGF receptor on eosinophils and T-helper lymphocytes in two patients affected by VKC and suggest a functional role for NGF receptors on eosinophils and human T-lymphocytes to modulate the inflammatory reactions in human conjunctivitis.

In conclusion, the presence of high-affinity NGF receptors on the ocular surface during basal and inflammatory conditions highlights the role of NGF in the mechanisms of inflammatory reaction and in the reparative process.

References


4. Aloe L, Bracci-Laudiero L, Bonini S, Manni L. The expanding role of nerve growth factor: from neurotrophic to immunologic dis-


