

# Nerve Growth Factor and Eosinophils in Inflamed Juvenile Conjunctival Nevus

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**PURPOSE.** To study both systemic and ocular nerve growth factor (NGF) in inflamed juvenile conjunctival nevus (IJCN), a benign, inflammatory juxtalimbal lesion characterized by many intralesional eosinophils, and to investigate the behavior of eosinophils cocultured on lesional and extralesional fibroblasts obtained from IJCN biopsies, in relation to NGF.

**METHODS.** Eight patients with IJCN (7–15 years old) and six with noninflamed conjunctival compound nevus (12–30 years old) participated in the study. Conjunctival biopsy specimens were used for routine histology, immunohistochemistry (NGF, trkA, eosinophil cationic protein, and tryptase determination), in situ hybridization (NGF mRNA), and determination of fibroblast growth. Blood of patients with IJCN was used to measure NGF levels (by ELISA) and to isolate eosinophils (magnet activated cell sorter [MACS]). Eosinophils were seeded on lesional and extralesional fibroblasts and their adherence, survival (by trypan blue staining), and functional activity (by eosinophil peroxidase [EPO] assay) were assessed after 4 days.

**RESULTS.** NGF in the blood of patients with IJCN and eosinophils and mast cells in their conjunctivas, were significantly elevated. NGF protein, NGF mRNA, and trkA were found to be increased in IJCN biopsy specimens compared with noninflamed compound nevi. Some NGF and trkA colocalized with eosinophils and mast cells. Lesional fibroblasts produced high amounts of NGF in comparison with extralesional fibroblasts and significantly enhanced eosinophil adherence, without influencing either their viability or activation. Adherence and EPO release were increased, in both lesional and extralesional fibroblasts.

**CONCLUSIONS.** The triggering factors that lead to the prominent inflammation in IJCN are unknown. The data in the current study, showing the presence of increased NG-trkA, eosinophils, and mast cells in IJCN and the modulation of eosinophil properties by lesional fibroblasts partly through NGF, suggest a

possible association between IJCN and allergic inflammation. Alternatively, this process may represent a direct immune response induced by the nevus itself. (*Invest Ophthalmol Vis Sci.* 2002;43:1850–1856)

**I**nflamed juvenile conjunctival nevus (IJCN) is a benign, juxtalimbal, usually amelanotic conjunctival nevus that appears in children and adolescents and can grow rapidly and cause congestion.<sup>1</sup> In a very recent extensive retrospective study, it was observed that all patients with IJCN had histories and physical findings of systemic allergy and bilateral papillary conjunctivitis. Histologically, IJCN is associated with extensive inflammatory infiltration, including significant numbers of eosinophils and mast cells.<sup>2</sup>

Nerve growth factor (NGF) is a well-known neurotrophic factor with a relevant role in the immune-endocrine-nervous network<sup>3,4</sup> that shows a highly conserved sequence and great homology within different species, including humans.<sup>5</sup> Its biological activity is mediated through specific cell surface receptors, trkA and p75, showing different affinity.<sup>6,7</sup> NGF levels are enhanced in inflamed tissues and in the blood of patients affected by several chronic inflammatory disorders, such as rheumatoid arthritis, scleroderma, and lupus erythematosus.<sup>8</sup> NGF is also increased in the peripheral blood of patients with various diseases associated with allergy, such as asthma, allergic conjunctivitis (rhinoconjunctivitis), and allergic skin diseases (urticaria angioedema), compared with its presence in healthy subjects.<sup>9</sup> In addition, NGF is produced by and can modulate the function of mast cells and eosinophils, considered to be the key effector cells of allergic reactions.<sup>10–12</sup>

NGF and trkA are also present on the ocular surface,<sup>13</sup> and they are increased in vernal keratoconjunctivitis (VKC), a recurrent or chronic inflammatory allergic disease.<sup>13,14</sup>

In view of these data and of our retrospective study on IJCN, to further assess a possible link of IJCN with allergy in the present study we sought to characterize NGF, eosinophils, and mast cells in the conjunctival biopsy specimens of patients who had undergone surgical excision of IJCN. Fibroblasts from IJCN biopsy tissue, known to be modulator cells of functional activities of both eosinophils and mast cells and recently demonstrated to be a source of NGF, at least in the skin and lung,<sup>15,16</sup> were studied in parallel, to determine whether they influence eosinophil properties through NGF.

## METHODS

### Patients

Eight patients with symptomatic IJCN (7 boys, 1 girl; 7–15 years old) participated in the study. Six specimens of noninflamed conjunctival compound nevi were used as the control (6 males; 12 to 30 years old). Informed consent was given by the parents of patients with IJCN, in accordance with the Helsinki Declaration. Compound nevi were taken from the archives of the Ophthalmic Pathology Laboratory at the Hadassah-Hebrew University Hospital and were exempt from the Helsinki Declaration and the approval of the Institutional Committee. None of the patients was treated by systemic or topical drugs during

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**TABLE 1.** NGF Levels, Total IgE, and Percentage of Eosinophils in Peripheral Blood of Patients with IJCN

| Patient | Gender | Age (y) | NGF (pg/mL) | Eosinophils (%) | Total IgE [kU/L] |
|---------|--------|---------|-------------|-----------------|------------------|
| 1       | M      | 9       | 540.00      | 5.3             | 48               |
| 2       | M      | 15      | 198.69      | 2.7             | 334              |
| 3       | M      | 15      | 110.22      | 7.0             | 11               |
| 4       | M      | 7       | 88.39       | 6.4             | 116              |
|         |        |         | 1596.00*    | 25.0*           |                  |
| 5       | M      | 12      | 175.38      | 14.00           | 249              |
| 6       | F      | 11      | 77.83       | 4.7             | ND               |
|         |        |         | 561.00*     | 19.0*           |                  |
| 7       | M      | 9       | 106.36      | 6.3             | 24               |
| 8       | M      | 7       | 499.30      | 13.7            | 203              |

ND, not determined.

\* NGF levels obtained when blood was withdrawn during allergy season.

the 3 months preceding surgery. The clinical diagnosis of IJCN was based on the presence of prominent congested conjunctival, mostly amelanotic, lesions, usually close to the limbus, with small cystic structures in most of them. All the patients with IJCN had conjunctival papillary reaction. Histologically, the nevi were infiltrated and surrounded by prominent inflammatory cells and contained cystic and solid epithelial islands.<sup>2</sup> Characteristics of the patients taking part in this study are summarized in Table 1. Peripheral blood from patients with IJCN was collected at the time of surgery and from two patients again on a routine check-up at the clinic after surgery, during the high allergic season. Total IgE was measured in the serum by paper-radioimmunosorbent assays, and the percentage of circulating eosinophils was measured in the blood by routine automatic counting.

### Conjunctival Fibroblast Growth

The conjunctival biopsy tissue from IJCN lesions and from extralesional areas from the same subject were placed immediately after surgery as explants in 24 multiwell plates (Nunc, Roskilde, Denmark) in 1 mL Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Biological Industries, Beit Haemek, Israel) at 37°C in 5% CO<sub>2</sub>, according to a standard protocol.<sup>16</sup> The fibroblasts that grew from the biopsy specimens were subcultured by treatment with 0.1% trypsin and 0.02% EDTA in Hanks' balanced salt solution without Ca<sup>2+</sup> and Mg<sup>2+</sup> (Sigma Chemical Co., St. Louis, MO). The fibroblasts were used between the third and fifth passages.

### NGF Determination in the Blood and in the Supernatant of Conjunctival Fibroblasts

NGF was measured in the serum of patients with IJCN by a modified, highly sensitive, two-site immunoenzymatic assay (ELISA), according to a protocol previously reported.<sup>17,18</sup> This assay specifically recognizes human NGF, but not brain derived neurotrophic factor, by the use of anti-NGF antibodies (clone 27/21, Chemicon International, Inc., Temecula, CA). Its lower limit of sensitivity is 0.5 pg/mL. Confluent fibroblast monolayers in 24 multiwell plates were incubated with fresh medium containing 2.5% FCS for 4 days and the supernatants withdrawn for determination of NGF. The yield of exogenous NGF was calculated by subtracting the amount of endogenous NGF from the level of endogenous plus exogenous NGF. The biological activity of NGF was estimated *in vitro* using the PC12 bioassay.<sup>3</sup> Data are represented as mean picograms per milliliter (±SEM), and all the assays were performed in triplicate for the indicated number of times.

### Histology, Immunohistochemistry, and In Situ Hybridization

Biopsy specimens were fixed in formalin, embedded in paraffin, and consecutive 5-µm-thin sections were dewaxed after a standard proto-

col. Slides were stained either with hematoxylin and eosin (H&E) or with acidic toluidine blue (pH 0.5). Eosinophils in H&E-stained slides and mast cells in toluidine blue-stained slides were counted in five separate high-power fields (×40) for each of three randomly selected sections. Parallel sections were processed for immunohistochemistry by the avidin-biotin-peroxidase method, according to the procedure suggested by the kit's manufacturer (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA). The following specific antibodies were used: purified goat anti-mouse NGF antibody, which recognizes human NGF (10 µg/mL)<sup>19</sup>; rabbit anti-human trkA antibody (2 µg/mL; Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes human trkA without cross-reacting with other trk receptors<sup>6,20</sup>; mouse monoclonal anti-human tryptase antibody (AA1; 1/200, R&D Systems, Minneapolis, MN)<sup>14</sup>; and mouse anti-human eosinophil cationic protein antibodies (anti-ECP, EG<sub>2</sub> clone, 1/50; Pharmacia Upjohn, Uppsala, Sweden).<sup>14</sup> Pretreatment to block endogenous peroxidase activity (0.3% H<sub>2</sub>O<sub>2</sub> and 10% methanol in PBS) and preincubation to block aspecific signals (2% BSA and 10% normal serum solution) were performed before incubating the sections with the specific antibodies. To detect nonspecific binding, control immunostaining was performed in parallel. Staining specificity for NGF was assessed by preabsorbing the anti-NGF antibodies with a 500-fold excess of purified NGF protein. To detect nonspecific binding of trkA, control immunocytochemistry was performed by substituting the primary antibody with control rabbit IgG (Vector Laboratories). The NGF-positive cells were counted and data presented as the mean ± SEM.

For experiments with double staining, the sections were pretreated with the blocking avidin-biotin solution (Vector Laboratories) before incubation with the specific secondary antibody. Aminoethylcarbazol (Sigma Chemical Co.) was used as the first chromogen and developed according to the protocol suggested by the manufacturer. To detect nonspecific binding of trkA, AA1 and EG<sub>2</sub> control immunohistochemistry was performed by substituting the primary antibody with control rabbit IgG (Vector Laboratories).

*In situ* hybridization for NGF mRNA was performed as previously described.<sup>21</sup> Briefly, 3'-end biotin-labeled oligonucleotide complementary to bases 703 to 742 of the human NGF mRNA sequence, was used at a final concentration of 30 to 40 ng/mL. The avidin-biotin complex technique was used to reveal the immunoreaction of sections hybridized with both antisense (specific signal) and sense (aspecific signal) biotin-labeled oligonucleotides.

### Isolation and Purification of Human Peripheral Blood Eosinophils

Eosinophils were purified from peripheral blood of patients with IJCN.<sup>22</sup> Briefly, venous blood (30–75 mL) was collected in heparinized syringes and left to sediment in 6% dextran (Sigma Chemical Co.). Leukocytes were removed by centrifugation at 700g in density gradient (Ficoll-Paque, density 1.077; Sigma Chemical Co.) for 25 minutes at 4°C, whereas the neutrophils and lymphocytes in the granulocyte-enriched pellet were tagged with micromagnetic beads bound to anti-CD16 and to anti-CD3 antibodies respectively (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The tagged cells were eliminated by passing them through a magnetic field (magnet activated cell sorter [MACS]). Eosinophils were collected at a purity of 99% or more, assessed by Kimura staining, and at a viability of 99% or more, assessed by trypan blue staining.

### Coculture of Eosinophils with Conjunctival Fibroblasts

Eosinophils (0.5 × 10<sup>5</sup>/mL) were seeded on confluent fibroblast monolayers from IJCN lesional and extralesional conjunctival biopsy tissue in 96-well plates, either in 2.5% FCS medium alone or in the presence of either NGF (50 ng/mL) or neutralizing anti-NGF antibodies (10 µg/mL).

NGF was purified from mouse submaxillary gland, according to the method of Bocchini and Angeletti.<sup>23</sup> The specific antibodies against ultrapure 2.5S NGF were raised in a goat and purified by column

chromatography.<sup>24</sup> The neutralizing anti-NGF antibodies were added to the fibroblast monolayers 2 hours before or immediately after the eosinophils were seeded. Cocultures were incubated for 4 days at 37°C in 5% CO<sub>2</sub> and 95% humidity. Media were changed after 2 days and replaced with fresh media containing the supplements.

### Assessment of Adherence, Viability, and Functional Activity of Eosinophils in the Cocultures

The adherence of the seeded eosinophils to the fibroblast monolayers was assessed after the culture media were aspirated, and 2 mL fresh media were added twice, with gentle shaking, and the media were aspirated again to remove all the nonadherent eosinophils. Adherent eosinophils were thereafter counted in five randomly selected fields, with a  $\times 40$  phase objective. The percentage of adherence per group was calculated as follows: % adherence = (number of adhered cells at day 4/number of cells seeded on day 0)  $\times$  100. Cell viability was assessed by the trypan blue exclusion test.<sup>11</sup>

Functional activity of the eosinophils was determined by incubating eosinophil fibroblast cocultures with lipopolysaccharide (10  $\mu$ g/mL, LPS; R&D) for 20 minutes at 37°C. Eosinophil activation was determined by analyzing eosinophil peroxidase (EPO) released in the supernatant, using an enzymatic colorimetric assay.<sup>11</sup>

### Statistical Analysis

Nonparametric statistical analysis (*t*-test or Tukey-Kramer post hoc for multiple comparisons) was performed to compare NGF and eosinophil percentages in the peripheral blood of patients with IJCN or NGF and the number of eosinophils and mast cells in the conjunctival biopsy specimens of patients with IJCN and compound nevus. Parametric ANOVA, followed by Tukey-Kramer post hoc, was used to analyze the coculture data.  $P \leq 0.05$  was considered statistically significant.

## RESULTS

### NGF, Total IgE, and Percentage of Eosinophils in Peripheral Blood

The blood of the eight patients with IJCN was assessed for NGF, total IgE levels, and percentage of eosinophils (Table 1). The amount of NGF in the peripheral blood of the patients ranged from 77.83 to 561.00 pg/mL. In patients 4 and 6, whose blood was also collected during the high allergic season, we observed a further increase in circulating NGF levels. The levels of NGF were found to be high. Total IgE levels were also found to be increased in 4 of 8 of the patients, ranging from 100 to 334 kU/L. The percentage of circulating eosinophils was also elevated in patients with IJCN, ranging from 2.7% to 14.0%, with values reaching 19.0% and 25.0% (patients 4 and 6, respectively) during the high allergic season.

### Presence of Eosinophils and Mast Cells, NGF, and trkA in IJCN Conjunctival Biopsy Specimens

Examination of IJCN biopsy specimens stained with H&E revealed the presence of infiltrating lymphocytes and an increase in the number of eosinophils (not shown). Toluidine blue-stained tissue showed an increased number of mast cells, some of which looked partially degranulated (not shown). Both eosinophils and mast cells were found inside and surrounding the nevus. On quantification, the number of eosinophils, counted in five separate high-power fields ( $\times 40$ ) for each of three randomly selected IJCN sections was  $6.51 \pm 1.16$  compared with  $1.33 \pm 0.81$  in compound nevus biopsy specimens ( $P < 0.05$ , Tukey-Kramer post hoc). The number of mast cells in IJCN biopsy specimens was  $11.43 \pm 9.64$  compared with  $2.84 \pm 5.91$  in compound nevus tissue samples ( $P < 0.05$ , Tukey-Kramer post hoc).

The in situ hybridization analysis for NGF mRNA displayed the presence of an increased number of NGF mRNA-positive cells in IJCN sections, compared with the number of positive cells found in compound nevus sections (Figs. 1A, 1B, respectively,  $\times 20$ ). By immunohistochemical analysis, it was found that, similar to the mRNA of NGF, the expression of NGF protein was also higher in IJCN than in the compound nevus (Figs. 1C, 1D, respectively,  $\times 10$ ). In fact, the quantification of NGF-positive cells showed that the number in IJCN sections was  $9.11 \pm 3.23$  compared with  $1.78 \pm 0.31$  in compound nevus ( $P < 0.001$ , Tukey-Kramer post hoc). Immunohistochemistry for trkA revealed a slightly increased number of trkA-positive cells in IJCN biopsy specimens, compared with those in compound nevus ( $13.82 \pm 2.16$  vs.  $7.12 \pm 1.83$  cells per field;  $P = 0.0332$ ; Figs. 1E, 1F, respectively,  $\times 20$ ). By means of the use of double immunohistochemistry (Fig. 2), it was found that some NGF colocalized with both EG<sub>2</sub>-positive cells (Fig. 2A,  $\times 40$ ) and AA1-positive cells (Fig. 2B,  $\times 40$ ), confirming that eosinophils and mast cells represent a source of NGF in the IJCN conjunctiva.

### NGF Production by IJCN Fibroblasts

High amounts of NGF were found in the 4-day conditioned media of lesional fibroblasts in comparison with extralesional fibroblasts ( $257.57 \pm 41.12$  pg/mL vs.  $35.83 \pm 10.38$  pg/mL;  $P = 0.0022$ , Tukey-Kramer post hoc). Of particular note, after three passages in culture, a further increase in NGF was found both in lesional and extralesional fibroblasts ( $597.01 \pm 242.52$  pg/mL vs.  $368.43 \pm 224.5$  pg/mL;  $P = 0.09$ ). Conjunctival fibroblast supernatant induced differentiation in PC12 cells into neuronlike cells after 4 days of incubation under standard conditions, in the presence or absence of neutralizing anti-pan-TGF- $\beta$  antibodies (not shown).

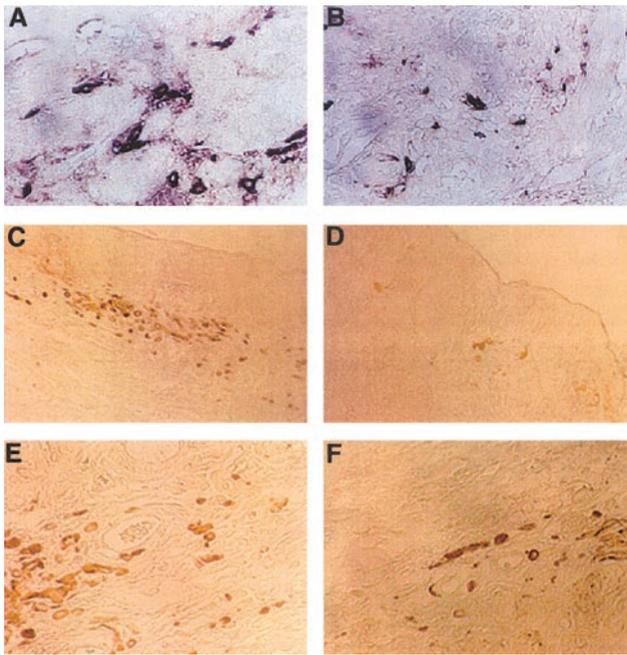
### Adherence, Viability, and Functionality of IJCN Eosinophils in Coculture with IJCN Fibroblasts

IJCN peripheral blood eosinophils were cocultured with lesional and extralesional fibroblasts, and their adherence to the fibroblast monolayers was evaluated at day 4. Sixty-eight percent  $\pm$  6.39% of eosinophils were found to adhere to lesional fibroblast monolayers, whereas only  $34.50\% \pm 6.38\%$  adhered to extralesional fibroblasts. This difference in adhesion was statistically significant ( $P < 0.01$ , ANOVA followed by Tukey-Kramer post hoc, Fig. 3A).

Addition of neutralizing anti-NGF antibodies reduced, although not significantly, the eosinophils' adherence to lesional fibroblasts (from  $68.00\% \pm 6.39\%$  to  $47.50\% \pm 5.56\%$ ;  $P > 0.05$ ) and increased adherence to extralesional fibroblasts (from  $34.50\% \pm 6.38\%$  to  $46.00\% \pm 5.70\%$ ;  $P < 0.05$ ; Fig. 3A). The addition of NGF to the cocultures increased eosinophil adherence to both lesional (from  $68.00\% \pm 6.39\%$  to  $88.00\% \pm 6.00\%$ ;  $P > 0.05$ ) and extralesional (from  $34.50\% \pm 6.38\%$  to  $73.00\% \pm 3.00\%$ ;  $P < 0.01$ ) fibroblasts.

The survival of eosinophils cultured on lesional and extralesional fibroblast monolayers was similar ( $29.00\% \pm 0.71\%$  in lesional and  $20.60\% \pm 0.64\%$  in extralesional, Fig. 3B). The addition of neutralizing anti-NGF antibodies reduced, although not significantly, the viability of the eosinophils (to  $12.00\% \pm 1.60\%$  in lesional and  $16.00\% \pm 0.86\%$  in extralesional fibroblasts). When NGF was added, the number of viable eosinophils increased in both cases, although not significantly (to  $36.00\% \pm 1.19\%$ , in lesional fibroblasts and to  $40.00\% \pm 0.64\%$  in extralesional fibroblasts).

When activated for 20 minutes by an optimal concentration of LPS, the eosinophils cocultured for 4 days on either lesional or extralesional fibroblasts released EPO in a similar fashion ( $0.33 \pm 0.02$  OD vs.  $0.31 \pm 0.02$  OD respectively; Fig. 3C). A



**FIGURE 1.** NGF mRNA, NGF protein, and trkA immunoreactivity in IJCN conjunctival biopsy specimens in comparison with compound nevus conjunctival biopsy specimens. NGF mRNA-positive cells were increased in IJCN conjunctival sections (A) compared with compound nevus sections (B). No positive signal was detected in both sections hybridized with sense-specific oligonucleotides. NGF protein-positive cells were increased in IJCN (C) compared with compound nevus (D). Increased trkA positivity also was observed in IJCN (E) compared with compound nevus (F). No positivity was observed in control sections. Magnification: (A, B, E, F)  $\times 20$ ; (C, D)  $\times 10$ .

slight decrease in EPO release by the eosinophils was detected after the addition of neutralizing anti-NGF antibodies to lesional (to  $0.21 \pm 0.03$  OD;  $P < 0.05$ ) and to extralesional fibroblasts (to  $0.25 \pm 0.02$  OD;  $P > 0.05$ ). However, EPO release was significantly increased when NGF was added to the lesional fibroblasts (to  $0.55 \pm 0.06$  OD;  $P < 0.05$ ) and to the extralesional ones (to  $0.49 \pm 0.03$  OD;  $P < 0.05$ ).

## DISCUSSION

In this study we report that patients with IJCN had increased levels of IgE, eosinophils, and NGF in the peripheral blood. Moreover, NGF mRNA and NGF protein and trkA receptor-expressing cells, as well as eosinophils and mast cells, were increased in IJCN conjunctiva. We have also shown that conjunctival fibroblasts from lesional areas produced higher amounts of NGF, compared with those from extralesional areas, and influenced eosinophil functional properties in coculture.

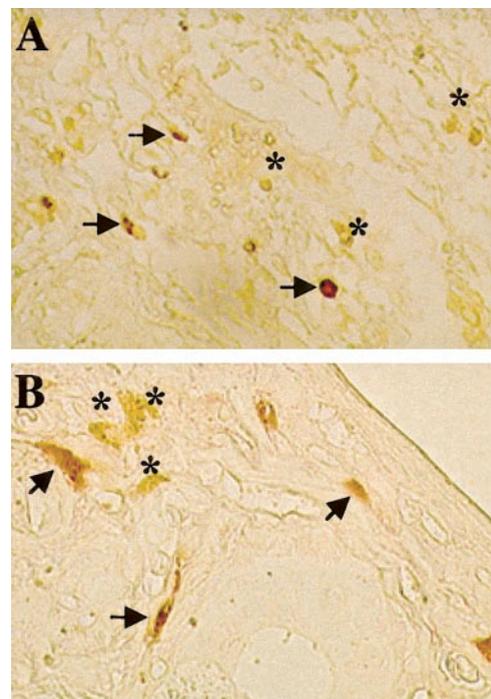
NGF is a neurotrophic factor with a wide spectrum of action on cells belonging to nervous, endocrine, and immune systems.<sup>3,4</sup> NGF is produced by and can modulate mast cell, T-helper cell-0 and Th-2 lymphocytes, eosinophils, and monocytes-macrophages.<sup>5,10,11,25,26</sup> The circulating NGF levels, as well as the trkA expression, are affected in some ocular diseases, such as VKC and ocular cicatricial pemphigoid (OCP).<sup>13,14</sup> In VKC, characterized by activation of conjunctival mast cells associated with a recruitment of eosinophils in the conjunctiva, the increase in plasma levels of NGF and NGF receptors in the conjunctiva are particularly evident.<sup>14</sup> Another interesting observation concerning NGF and the ocular surface relates to the profound epithelial

healing effects of locally applied NGF on otherwise untreatable corneal ulcers.<sup>27</sup>

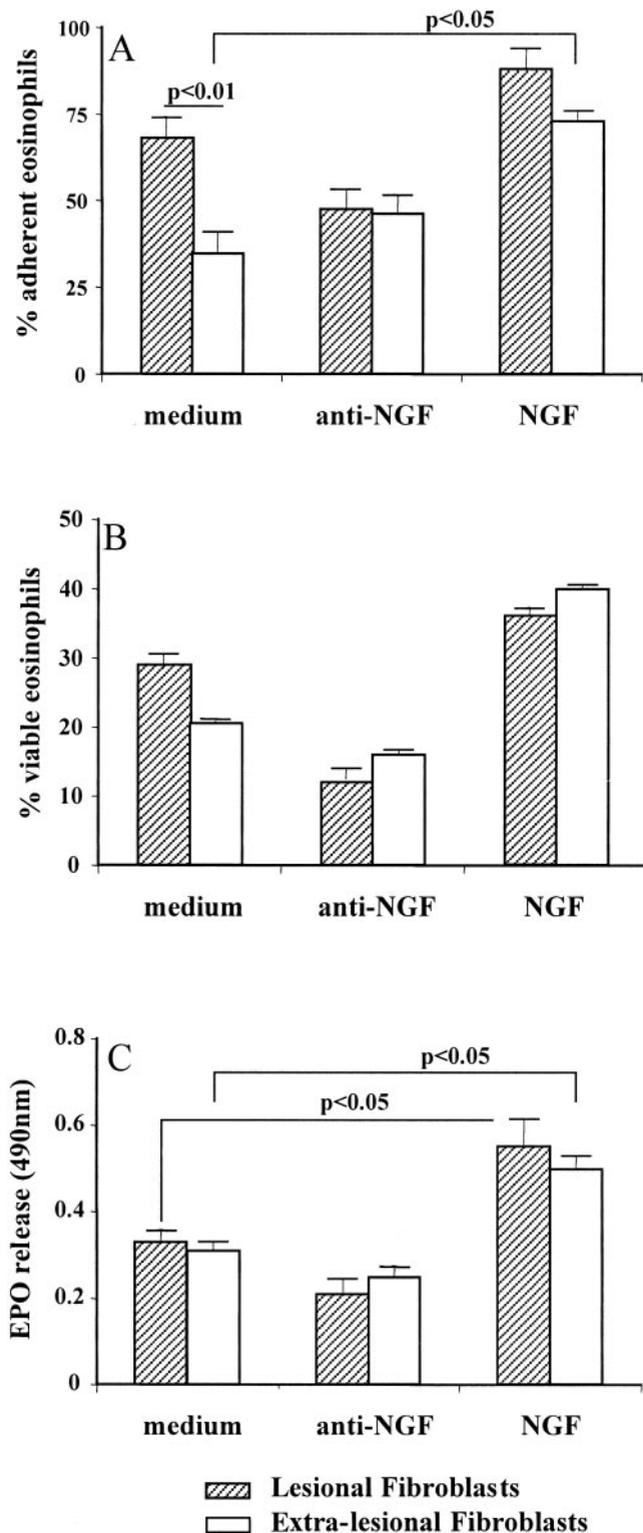
We wanted to investigate whether NGF is involved in IJCN, an ocular lesion characterized by benign, mostly amelanotic lesions localized in the juxtalimbal conjunctiva that may grow rapidly and cause congestion.<sup>1,2</sup> Only lesions with significant inflammatory component were diagnosed as IJCN. We found this unique entity to have features suggesting allergy, including history of systemic allergy and bilateral papillary conjunctivitis. In addition, we found high numbers of eosinophils and mast cells infiltrating the conjunctiva, as reported in a previous extensive retrospective study.<sup>2</sup> In contrast, compound nevus is observed in older patients and is not associated with inflammatory and allergic conditions.<sup>28</sup>

High levels of serum NGF (ranging from 77.83 to 561.00 pg/mL) were found in patients with IJCN in comparison with normal healthy subjects ( $3.8 \pm 1.7$  pg/mL, reference levels previously reported by others<sup>9</sup>), and this systemic increase did not correlate with either peripheral eosinophilia or total IgE levels observed in the patients.<sup>9</sup> We find it interesting that in VKC no correlation between NGF plasma levels and IgE was observed, supporting our data on IJCN. In contrast, an inverse relation was found between the NGF levels and the amount of circulating eosinophils and the ECP serum levels.<sup>14</sup>

Histologic analysis of the conjunctival biopsy specimens revealed the presence of an increased number of eosinophils and mast cells, some of them degranulated, indicating the presence of mast cell-activating factors. Degranulated mast cells associated with an increased number of eosinophils are hallmarks of allergic inflammatory conditions.<sup>29</sup> It is worth mentioning that the number of lymphocytes—that is, the



**FIGURE 2.** Double immunohistochemistry for NGF on eosinophils and mast cells in IJCN conjunctival biopsy specimens. (A) Positive dark brown-red-black double staining for NGF (brown, \*) and EG<sub>2</sub> (red) demonstrated that most of the eosinophils in the specimen expressed NGF protein. Arrows: colocalization. (B) Positive dark brown-red-black double staining (arrows) for NGF (brown, \*) and for AA1 (red) also demonstrated that the majority of mast cells were a source of conjunctival NGF. Magnification: (A, B)  $\times 40$ .



**FIGURE 3.** NGF's effects on peripheral blood eosinophils of patients with IJCN seeded on lesional and extralesional IJCN fibroblast monolayers. Adherence (A) and survival (B) of eosinophils and release of EPO (C) were evaluated on day 4 of coculture. Data represent the mean  $\pm$  SEM of six experiments performed in triplicate.

presence of lymphoid follicles and germinal center in 30% of our patients—is always significantly larger than that of eosinophils. In contrast, eosinophils are a typical finding, occurring in 77% of these patients, suggesting an allergic

etiology. Other chronic allergic conditions are also characterized by prominent lymphocyte response and a smaller number of eosinophils.

In particular, mast cells are known to play a central role in the early phase of the allergic reaction and to exert influence also in the late phase, mostly through interactions with the eosinophils,<sup>30,31</sup> and NGF has recently been proposed as a marker for allergy.<sup>32</sup> Nevertheless, the presence of eosinophils in the lesions is not a sine qua non in allergy. Eosinophils are also found in nonallergic lesions, such as Hodgkin lymphoma, eosinophilic granuloma, and sympathetic ophthalmia.

In the healthy conjunctiva, no eosinophils are observed, whereas a few mast cells are usually present. Normal conjunctiva often contains lymphocytes in the substantia propria, which represents a local immune response to foreign materials and antigens. It is conceivable that eosinophils would occasionally be seen as part of a mucosa-associated cellular immune response. The presence of a very small number of eosinophils, in our control group (noninflamed conjunctival compound nevi), can be explained by the fact that we included in our control group patients who may have had earlier mild or resolved lesions of IJCN.

The immunohistochemical analysis of IJCN sections showed an increased immunoreactivity for NGF mRNA and NGF protein, surrounding and inside the nevus. In particular, double immunohistochemical analysis, demonstrating that NGF positivity is found in most eosinophils and mast cells, indicates that these cells actively contribute to the conjunctival NGF in this ocular entity. Compound nevus biopsy specimens that did not show any sign of inflammation were only faintly positive for NGF, which further strengthens the association of NGF with IJCN. These data represent the first evidence of an *in situ* expression of NGF in human conjunctiva, because the local expression of NGF protein has not been investigated in VKC or other ocular diseases. In inflamed tissues, a major source of biologically active NGF is represented by lymphocytes—both B and T—macrophages, eosinophils, and mast cells, besides structural cells, such as fibroblasts.<sup>4,14,16,26</sup> In addition, because in the previous retrospective study on clinical and histopathologic features of IJCN we observed lymphocytic infiltrations, germinal centers, and plasma cells,<sup>2</sup> we cannot exclude lymphocytes, macrophages, and even fibroblasts as additional sources of conjunctival NGF. It is possible that eosinophils and NGF both represent an immune process that is triggered by the nevus itself, rather than by an allergic disease. The observation of the presence of trkA immunoreactivity in eosinophils and mast cells in the conjunctiva of IJCN (Micera A, Levi-Schaffer F, unpublished data, 2001) indicates that these cells may also respond to NGF. In the healthy conjunctiva, trkA positivity is confined to basal epithelial cells, endothelium, and, in rare cases, stromal cells.<sup>13</sup> Our findings on the increased expression of trkA in the IJCN biopsy tissue are comparable to those found in VKC and OCP.<sup>14</sup> It is possible that the nevus cells themselves are responsible for attracting mast cells and therefore eosinophils, independent of any systemic or localized allergic response. This is suggested also by the fact that mast cells and eosinophils express trkA receptor and that nevus cells contain elevated levels of NGF. It is noteworthy that several lines of evidence indicate mast cells and eosinophils to be active producers of several growth factors, including NGF and stem cell factor (SCF).<sup>33</sup> Because melanocytes of the skin express specific receptors for both factors,<sup>34</sup> we cannot exclude that in IJCN the rapid progression of the nevus lesions may also be due in part to the release of mast cell and eosinophil-derived growth factors. This hypothesis is supported by the close localization among mast cells, eosinophils, and nevus cells.<sup>2</sup>

Fibroblasts, previously viewed as simple structural cells providing the cellular and extracellular scaffold to tissues, are now recognized as regulatory cells exerting active roles in tissue homeostasis.<sup>35</sup> In addition, fibroblasts contribute to the progression of pathologic conditions through the release of several factors capable of influencing virtually all the cellular types locally present.<sup>35</sup> In this context, we have previously described the reciprocal influence of fibroblasts and eosinophils that play a role in the pathophysiology of allergic inflammation.<sup>22,11</sup> Moreover, we have recently reported that NGF is produced by and can modulate human lung and skin fibroblasts.<sup>16</sup>

Our observation that IJCN lesional fibroblasts produced biologically active NGF in much higher amounts than extralesional fibroblasts raised the question of whether eosinophils could be influenced by the IJCN fibroblasts themselves, also through the increased production of NGF.

To address this question, eosinophils purified from the peripheral blood of patients with IJCN were seeded on fibroblasts derived from lesional and extralesional IJCN biopsy specimens, and their behavior in coculture was observed. Lesional fibroblasts increased the adherence of the eosinophils significantly. Because the addition of exogenous NGF also increased eosinophil adherence to both lesional and extralesional fibroblasts, it is possible to speculate that NGF enhances the expression of adhesion molecules on the eosinophils. There is some evidence to indicate the increasing effect of NGF on the expression of neuronal adhesion molecules, whereas few data are available on the effect of this factor on the expression of adhesion molecules on immune cells.<sup>36,37</sup>

Thirty percent of the originally seeded eosinophils were still alive when cocultured on lesional and extralesional fibroblasts. In another study we have reported that conjunctival fibroblasts decrease peripheral blood eosinophil apoptosis, possibly through the release of the three eosinophil survival cytokines, granulocyte-macrophage colony-stimulation factor (GM-CSF), IL-3, and IL-5.<sup>33</sup> In the present study, neutralization of endogenous NGF or the addition of exogenous NGF did not alter eosinophil survival in the coculture, indicating that factors other than NGF are involved in this fibroblast property.<sup>11</sup>

We have found that NGF can directly activate eosinophils to release EPO.<sup>11</sup> In these cocultures the addition of exogenous NGF significantly increased the eosinophil degranulation due to LPS in both lesional and extralesional fibroblasts, and conversely the addition of neutralizing anti-NGF antibodies decreased eosinophil activation on lesional monolayers. This indicates a "priming" activity of NGF in addition to a direct activating capacity on human peripheral blood eosinophils. In summary, these findings indicate some differential activities of lesional and extralesional fibroblasts toward eosinophils partially mediated by NGF.

In conclusion, our data showing that NGF and trkA are highly expressed together with increased numbers of eosinophils and mast cells in the IJCN conjunctiva and that NGF and eosinophils are increased in blood of patients with IJCN are further evidence of a link between NGF and ocular surface diseases, including allergy.

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