

The pro-fibrogenic effect of nerve growth factor on conjunctival fibroblasts is mediated by transforming growth factor- β

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Summary

Background Nerve growth factor (NGF) and nerve growth factor receptor (NGFR) expressions have been found to be increased in sub-conjunctival scarring.

Objective The aim of this study was to investigate the *in vitro* effects of NGF on some pro-fibrogenic properties of human conjunctival fibroblasts.

Methods Expression of NGF, $\text{trkA}^{\text{NGFR}}$ and p75^{NTR} on human fibroblasts grown from conjunctival biopsies and incubated for 2 or 6 days with NGF were evaluated by immunofluorescence, RT-PCR, flow cytometry and ELISA. The fibrogenic effect of NGF on conjunctival fibroblasts was investigated by evaluating their migration (wound model), proliferation (^3H -thymidine incorporation), collagen production (^3H -proline incorporation), expression of alpha-smooth muscle actin ($\alpha\text{-SMA}$) (cell surface ELISA) and contraction of 3D collagen gels.

Results NGF induced the expression of p75^{NTR} in the fibroblasts that constitutively expressed only $\text{trkA}^{\text{NGFR}}$ and increased the migration of wounded fibroblasts, but not their proliferation and collagen production. NGF induced the conversion of fibroblasts into myofibroblasts expressing $\alpha\text{-SMA}$, and enhanced their contraction of a collagen matrix. Interestingly, chronic NGF treatment induced transforming growth factor- β 1 (TGF- β 1) production by fibroblasts, and following specific TGF- β neutralization, all the NGF-induced effects were completely abrogated.

Conclusion Our findings indicate that NGF, via TGF- β induction, is likely to be involved in the healing or fibrotic processes occurring in conjunctiva during some pathological conditions.

Keywords fibroblasts, fibrosis, nerve growth factor, tissue remodelling, wound healing

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Introduction

Nerve growth factor (NGF) is a polypeptide that plays an important role in growth, differentiation and survival of neuronal, endocrine and immune cells in physiological and pathological conditions [1, 2]. NGF effects are mediated by two cell surface receptors, $\text{trkA}^{\text{NGFR}}$ and p75^{NTR} [3, 4]. NGF widely regulates immune and inflammatory responses, being produced by and acting on T and B lymphocytes, mast cells, eosinophils, neutrophils and monocytes/macrophages [2]. In particular, NGF appears to be associated with several chronic allergic inflammatory diseases, often displaying an unbalanced tissue repair or remodelling [1, 2, 5–7].

NGF takes part in the pathophysiology of the visual system together with other growth factors (brain-derived neuro-

trophic factor, Neurotrophins 3/4, transforming growth factor- β 1 (TGF- β 1)) that have receptors expressed on the ocular surface [8–12]. Long-term topical NGF application restored stromal/epithelial integrity in patients with corneal ulcers, otherwise unresponsive to the standard therapy [11, 13]. This would imply a direct role of NGF on epithelial cells, which are known to express $\text{trkA}^{\text{NGFR}}$ and to proliferate and differentiate upon NGF stimulation [14]. In addition, NGF might influence fibroblasts and extracellular matrix regulation [15–18].

In particular, NGF and $\text{trkA}^{\text{NGFR}}$ have been observed in the normal and diseased cornea and conjunctiva [9–11, 19]. Compelling *in vitro* and *in vivo* evidences show that NGF is involved in the healing process of the ocular surface and that epithelial cells and fibroblasts are one of the source/targets of NGF [7, 14, 20]. In normal conjunctival biopsies, the basal epithelial layer expresses $\text{trkA}^{\text{NGFR}}$ whereas a weak immunoreactivity is observed in the stroma. On the other hand, a strong immunoreactivity was observed in the conjunctival stroma of chronic inflammatory disorders characterized by fibrosis and scarring such as vernal keratoconjunctivitis (VKC) and ocular cicatricial pemphigoid (OCP) [9–11]. A

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role for NGF and TGF- β 1 on fibroblast growth, collagen metabolism and mucin expression in these diseases, via their interaction with resident and infiltrating cells, has been postulated [7, 21]. Since activated fibroblasts remain the main cellular elements responsible for fibrosis [22, 23] and since NGF is usually increased in inflamed conjunctiva [9, 19], we wondered whether NGF could influence some of the pro-fibrogenic properties of the conjunctival fibroblasts.

In the present study, we investigated the effect of short- and long-term NGF incubation of normal human conjunctival fibroblasts that express constitutively $\text{trkA}^{\text{NGFR}}$ on fibroblast migration, proliferation, differentiation and contraction of collagen matrix.

Materials and methods

Cell cultures and activations

Conjunctival biopsies were obtained from otherwise healthy patients who underwent ocular surgery (six males, 25–45 years old). Informed consent was given by the patients in accordance with the Institutional Helsinki Declaration. Fibroblasts outgrown from biopsies were expanded in T21/75 cm² flasks (<7 passages) in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin at 37 °C and 5% CO₂ (all from Biological Industries, Beit Haemek, Israel) [20]. Conjunctival fibroblast purity was assessed by anti-keratin K19 epithelial antibodies exclusion test (AE5, 1:100, Sigma Chemicals, St Louis, MO, USA). No staining for K19 was detected in the cell culture, indicating the purity of the fibroblasts.

Depending on the experiment, 24 h-serum-starved confluent fibroblasts were maintained for 2 days, short-term treatment, or for 6 days, long-term treatment. In the long-term treatment, every 2 days the medium was changed and replaced with fresh medium consisting of DMEM-2% FCS, with or without the following additives: murine salivary gland NGF (2.5–500 ng/mL) [24, 25]; purified goat anti-NGF neutralizing antibodies (α -NGF; 3 μ g/mL; R&D Systems, Minneapolis, MN, USA); human TGF- β 1 (10 ng/mL, R&D Systems); mouse anti-panTGF- β neutralizing antibodies (α -TGF- β ; 3 μ g/mL, R&D Systems); goat anti-human $\text{trkA}^{\text{NGFR}}$ neutralizing antibodies (α - $\text{trkA}^{\text{NGFR}}$; 3 μ g/mL; R&D Systems). For blocking studies, fibroblasts were pre-incubated with the specific neutralizing antibodies for 1 h at 37 °C and then stimulated with NGF (50 ng/mL) or TGF- β 1 (10 ng/mL) in the presence of the relevant neutralizing antibody. The procedure of sampling after short- or long-term treatments was 6 h later for molecular biology and 24 h later for protein detection. All the experiments were performed at least three times and in triplicates. Sterile tissue culture plastics were obtained from Falcon (Oxford, CA, USA) and NUNC (Roskilde, Denmark).

Immunofluorescence analysis

Fibroblasts, cultured until confluence on round cover slips placed in 24 wells plates and treated according to the experimental procedure, were washed in Hank's balanced

sodium solution (HBSS) and fixed in 2% buffered-paraformaldehyde (PFA). The following antisera were used: goat anti-NGF antibodies (2 μ g/mL, R&D Systems); rabbit anti-human $\text{trkA}^{\text{NGFR}}$ [26] or goat anti-human p75^{NTR} antibodies (both 2 μ g/mL, Santa Cruz Biotech., CA, USA). The specificity of antibodies was detected by incubating the slides with non-relevant isotype-matched immunoglobulin (Ig)G antibodies (Vector Laboratories Inc., Burlingame, CA, USA). Binding was detected using the indirect immunofluorescence technique (20 μ g/mL phycoerythrin (PE)-labelled anti-goat IgG antibodies for p75^{NTR}, or 2 μ g/mL avidin-FITC (fluorescein isothiocyanate) for $\text{trkA}^{\text{NGFR}}$, all from Vector Laboratories). Finally, the cell nuclei were visualized by 1 μ g/mL Hoechst dye 33342 (Sigma) diluted 1/1000 in anti-fading AF1 solution (Cityfluor, Cambridge, UK). Cells were examined using an Olympus microscope equipped with a light and fluorescence acquisition system (IAS2000 Vers. 4.00.00 software, Delta Sistemi s.a.s., Rome, Italy).

Semi-quantitative reverse transcription-polymerase chain reaction

RT-PCR ELISA for $\text{trkA}^{\text{NGFR}}$ and p75^{NTR} was carried out according to methods recently described [27]. Total RNA (2 μ g) was extracted from fibroblasts by Qiagen RNeasy kit (QIAGEN S.p.A., Milano, Italy) and dissolved, denatured, reverse transcribed and run for amplification with human- $\text{trkA}^{\text{NGFR}}$, 5'CTC CTT CTC GCC AGT GG^{3'}, 5'GCT CCC ACT TGA GAA TG^{3'} (367 bp product, Genebank access no. M85214) primers or human-p75^{NTR}, 5'GAG GCA CCA CCG ACA ACC TC^{3'}, 5'TGC TTG CAG CTG TTC CAC CT^{3'} (120 bp product, Genebank access no. AF187064) or human- GAPDH, 5'CAC CAC CAT GGA GAA GGC C^{3'}, 5'GAT GGA TGC CTT GGC CAG G^{3'} (190 bp product, Genebank access no. M32599) primers, RNA normalization was achieved by GAPDH gene and the specificity of PCR products was verified by Southern Blot analysis or DNA sequencing. PCR products were visualized by ethidium bromide-stained gel electrophoresis, photographed and subjected to semi-quantitative evaluation. GAPDH were used to normalize the relative differences in sample size, integrity of the individual RNA and variations in RT efficiency.

Flow cytometric analysis

Single-cell suspensions were washed (HBSS), fixed (methanol, –20 °C), permeabilized (0.1% saponin) and incubated with goat anti-human p75^{NTR} antibodies (4 μ g/mL), followed by FITC-conjugated anti-goat antibodies (1:200, Vector Laboratories). Fluorescence was quantified by an EPICS 541 flow cytometer (Coulter Electronics, Hialeah, FL, USA), equipped with a 488 nm argon-ion laser Innova 90 (50 mW, Coherent, Palo Alto, CA, USA) and a band-pass filter at 515 nm. Logarithmic and linear signals were acquired from 5000 cells/sample. Mean fluorescence intensity (MFI) of linear distribution was calculated. The specific signal for each sample was subtracted to the aspecific one, and results were expressed as increments relative to the controls calculated as follows: $\Delta\text{MFI} = (\text{specific MFI} - \text{aspecific MFI})/\text{aspecific MFI}$.

ELISA and cell-surface ELISA

NGF was measured in the supernatants by a highly sensitive ELISA, using capture and detection anti-NGF antibodies (MAB5260 and MAB5260G, Chemicon International, Temecula, CA, USA), and NGF as standard (range = 0.05 pg/mL – 1 ng/mL) [20]. NGF biological activity was confirmed by PC12 bioassay [28].

TGF- β 1 released in the supernatant was measured using a commercial ELISA kit (Quantikine DuoSet kit, R&D). The sensitivity of the assay was less than 7 pg/mL. α -SMA expression was evaluated on confluent 96 well plates (cell surface ELISA) as previously reported [20, 29]. Briefly, fibroblasts ($5 \times 10^5/0.20$ mL) were grown until confluent, and then incubated with NGF or TGF- β 1 or their specific neutralizing antibodies. After 2 or 6 days, the plates were washed in HBSS containing 0.1% CaCl₂, post-fixed in 2% buffered PFA, quenched for endogenous peroxidases and dehydrated with 95% ethanol before the assay. The plates were subsequently processed for quantification of α -SMA (mouse anti-human α -SMA antibodies, 1:250, a kind gift from Prof. Giulio Gabbiani, Dept. of Pathology, University of Geneva, Switzerland). Biotinylated anti-mouse antibodies and HRP-streptavidin specific binding (1/8000 and 1/20 000 respectively; Zymed, San Francisco, CA, USA) were developed with a ready to use TMB solution as substrate (Zymed) and quantified using an ELISA reader (Dynatech ELISA reader MR5000, PBI International, USA). The data (OD_{450/690} values) were normalized to cellular protein as assessed by a Bio-Rad protein assay, using BSA equivalent as standard.

Migration, proliferation and collagen production

Fibroblast migration was investigated in an *in vitro* wound model as previously described [30]. Fibroblast proliferation was assessed by either counting the cells after trypsinization, [³H]-thymidine uptake [31], or ICC using rabbit anti-human ki67 antibodies (1:1000, Santa Cruz). Ki67 ICC was performed on pre-fixed monolayers, as reported above. Collagen production was evaluated by [³H]-proline uptake [31].

Three dimensional collagen gel contraction

Fibroblasts were added to bacteriological 35 mm dishes (10⁵ cells/dish) containing DMEM-2% FCS and 100 mM NaOH, immediately after the addition of type I collagen (obtained from rat tail tendon, and reconstituted in 18 mM acetic acid at a final concentration of 2 μ g/mL), in medium alone or with additives (final pH 7.5), as described [32, 20]. Gel diameters were estimated in a blind fashion on days 2, 4 and 6 by placing the dishes on a graduate ruler over a black surface.

Statistical analysis

The statistical package used was StatView II for PC (Abacus Concepts Inc., Barkley, CA, USA). ANOVA followed by Tukey/Kramer post hoc was employed to analyse the data represented in the graphics as mean $\pm$ SEM [33].

Results

Conjunctival fibroblasts incubated with nerve growth factor express *trkA*^{NGFR} and *p75*^{NTR}

We first assessed the presence of *trkA*^{NGFR} and *p75*^{NTR} on conjunctival fibroblasts monolayers by IF. As shown in Fig. 1, conjunctival fibroblasts expressed constitutively *trkA*^{NGFR} protein (B) and messenger (C). However, *p75*^{NTR} was not detected both at the protein and mRNA levels (Fig. 2).

Upon quantification by FACS analysis, *trkA*^{NGFR} was found to be significantly increased after long-term treatment with NGF in comparison with control fibroblasts ($P < 0.05$; $n = 6$, for each experimental group) (A). Also, *p75*^{NTR} was found to be expressed both at protein (A) and messenger (B) levels from day 2, although to a lesser degree than *trkA*^{NGFR}.

Nerve growth factor increases the migration of wounded conjunctival fibroblasts beyond a wound line

The effect of NGF on fibroblast migration was examined on monolayer 1 day after their wounding. As shown in Fig. 3, the addition of NGF (50–200 ng/mL) induced a significant increase in the migration of fibroblasts beyond the wound line in comparison with the fibroblasts cultured in the absence of NGF (control) (50 ng/mL: 3.8-fold increase vs. control; $P < 0.005$; $n = 6$). At the lowest concentrations tested (5, 10, 25 ng/mL), we observed a slight but not significant effect of NGF on fibroblast migration. The effect of NGF was clearly concentration dependent. The addition of neutralizing α -*trkA*^{NGFR} antibodies completely abrogated the NGF-enhancing effect.

Nerve growth factor does not influence fibroblast proliferation and collagen production

To assess the NGF effect on fibroblast proliferation, three different methods, i.e. trypan blue exclusion test, [³H]-

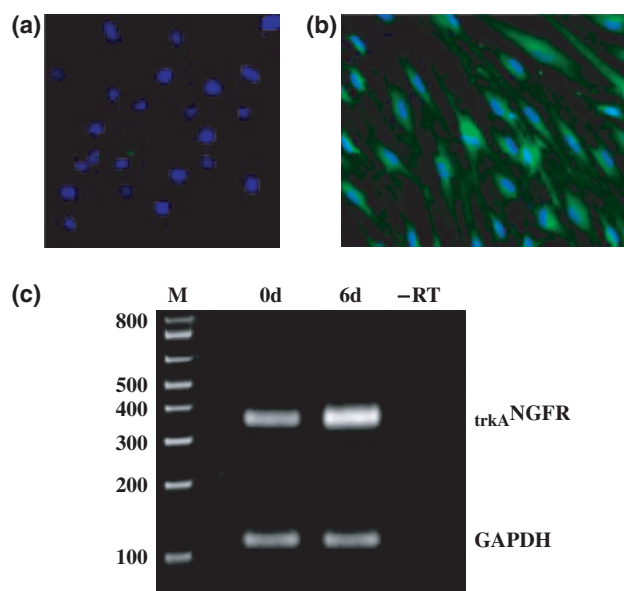


Fig. 1. Conjunctival fibroblasts express *trkA*^{NGFR}. Immunofluorescence analysis of *trkA*^{NGFR} protein (fluorescein isothiocyanate): Internal control (a) and specific staining of *trkA*^{NGFR} (b). Nuclei were visualized with Hoechst ($\times 40$). (c) RT-PCR analysis of *trkA*^{NGFR} mRNA on fibroblasts at day 0 and following 6 days of treatment with nerve growth factor. – RT indicates the negative control of amplification.

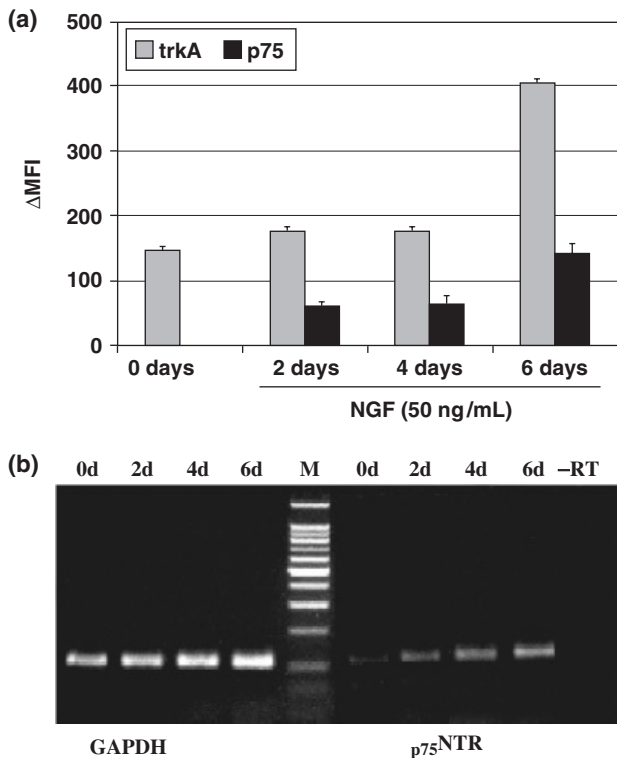


Fig. 2. NGF induces the expression of p75^{NTR}: (a) The histogram represents mean fluorescence intensity (ΔMFI, mean ± SEM of six experiments) of anti-trkA^{NGFR} and anti-p75^{NTR} staining in fibroblasts treated with nerve growth factor (NGF) (50 ng/mL) for 2, 4 and 6 days. (b) RT-PCR analysis of p75^{NTR} mRNA on fibroblasts at day 0 and following 2, 4 and 6 days of treatment with NGF. – RT indicates the negative control of amplification. **P* < 0.05 represents significance vs. 2 and 4 days. Five thousand cells were analysed.

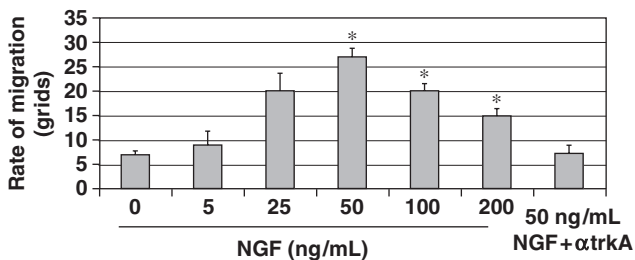


Fig. 3. Nerve growth factor (NGF) induces conjunctival fibroblasts migration across a wound line. Rate of migration (grids) of fibroblasts cultured in the presence of different concentrations of NGF (0–200 ng/mL) for 1 day. Neutralizing α-trkA^{NGFR} antibodies (3 μg/mL) were used to neutralize NGF (50 ng/mL). Data represent mean ± SEM of six experiments performed in triplicates. **P* < 0.05 indicates significance vs. control.

thymidine incorporation and Ki67 staining were employed. Addition of exogenous NGF did not influence the index of proliferation by conjunctival fibroblasts, as detected by all the techniques employed (data not shown).

Likewise, NGF (short-term treatment) did not increase collagen production by fibroblasts, as evaluated by [³H]-proline incorporation (data not shown).

Conjunctival fibroblasts produce and release nerve growth factor and transforming growth factor β1

NGF and TGF-β1 were released spontaneously in the medium by conjunctival fibroblasts (control, Figs 4a and b).

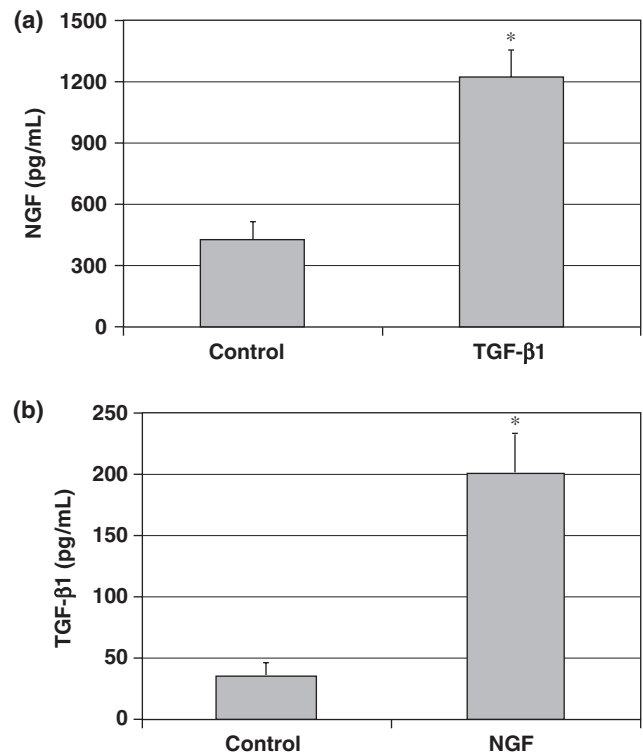


Fig. 4. Nerve growth factor (NGF) and transforming growth factor-β1 (TGF-β1) are increased in long-term treated fibroblasts: (a) NGF production by conjunctival fibroblasts exposed to TGF-β1 (10 ng/mL) for 6 days. (b) TGF-β1 production by conjunctival fibroblasts exposed to NGF (50 ng/mL) for 6 days. Data represent mean ± SEM of three experiments performed in triplicates. **P* < 0.05 indicates significance vs. control.

Interestingly, NGF release increased significantly when the fibroblasts were treated with TGF-β1 (10 ng/mL) for 6 days (2.8-fold increase vs. control; *P* < 0.01) (A). Likewise, a significant increase in TGF-β1 was detected after long-term treatment with NGF (50 ng/mL) (5.6-fold increase vs. control; *P* < 0.01) (B). The addition of neutralizing α-trkA antibodies completely abrogated the NGF enhancing effect (data not shown).

Nerve growth factor induces α-SMA expression

The addition of NGF for 6 days to conjunctival fibroblasts increased their α-SMA expression in a dose-dependent manner (*P* < 0.05; *n* = 12) (Fig. 5a). At the concentration of 250 ng/mL, NGF induced the expression of α-SMA that was similar to that of 10 ng/mL TGF-β1. At concentrations higher than 250 ng/mL, NGF did not further significantly increase this effect (*P* > 0.05), and at concentrations lower than 50 ng/mL there was a slight but not significant α-SMA increase. Specific neutralization of trkA^{NGFR} completely inhibited the effects observed by NGF (50 ng/mL, *P* < 0.05).

Next, blocking experiments were performed to investigate the mechanism of α-SMA induction by NGF. As shown in Fig. 5b, the addition of neutralizing α-TGF-β antibodies to NGF completely inhibited NGF effects on α-SMA expression, while addition of α-NGF to the cultures treated with TGF-β1 had no influence on TGF-β1 induction of α-SMA.

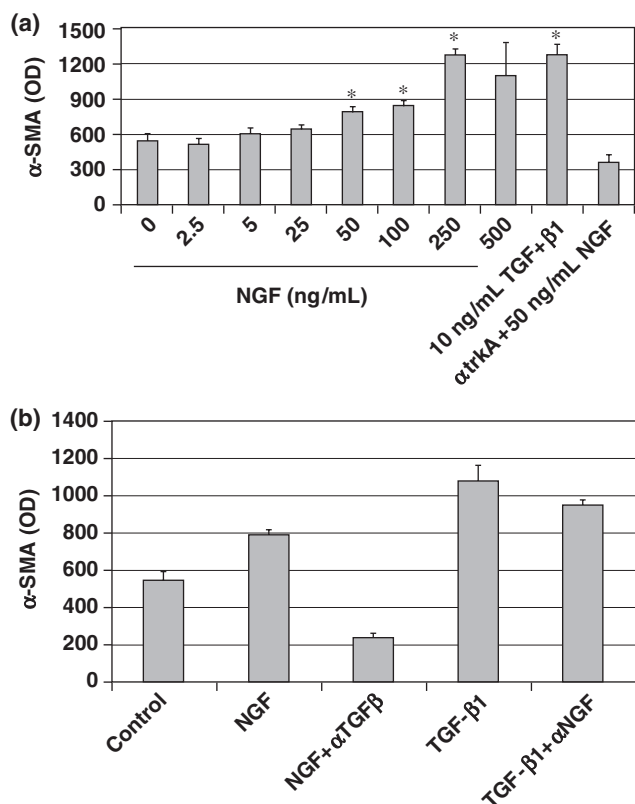


Fig. 5. Nerve growth factor (NGF) induces α -SMA expression in conjunctival fibroblasts through transforming growth factor- β (TGF- β): (a) α -SMA expression on fibroblasts exposed to different concentrations of NGF (0–500 ng/mL) or TGF- β 1 (10 ng/mL) for 6 days. Neutralizing α -trkA^{NGFR} antibodies (3 μ M) were used to neutralize NGF (50 ng/mL). (b) α -SMA expression on conjunctival fibroblasts cultured with medium alone (control), or with NGF (50 ng/mL) in the presence or absence of α -TGF- β (3 μ g/mL), or with TGF- β 1 (10 ng/mL) in the presence or absence of α -NGF (3 μ g/mL) for 6 days. α -SMA expression was evaluated by cell surface ELISA. Data represent mean $\pm$ SEM of twelve experiments performed in triplicates. * P < 0.05 indicates significance vs. control.

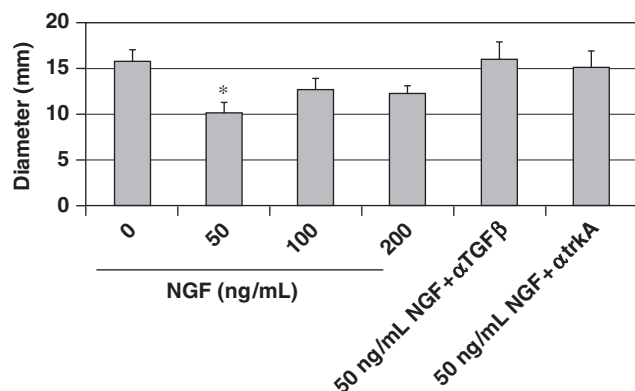


Fig. 6. Nerve growth factor (NGF) induces conjunctival fibroblast contraction of a collagen gel. Rate of contraction of conjunctival fibroblasts embedded in collagen gel in the presence of different concentrations of NGF (0–200 ng/mL) for 6 days. α -transforming growth factor- β (3 μ g/mL) or α -trkA^{NGFR} (3 μ g/mL) were added in the presence of NGF (50 ng/mL). Data represent mean $\pm$ SEM of six experiments performed in triplicates. * P < 0.05 indicates significance vs. control.

Nerve growth factor increases the ability of conjunctival fibroblasts to contract a collagen gel

Collagen is the major protein of the extracellular matrix and its contraction is a physiological step that occurs during the

last stages of repair [21]. Fibroblasts were seeded in a 3D gel and incubated with increasing NGF concentrations (0–500 ng/mL) for 6 days. As shown in Fig. 6, NGF significantly contracted the gel at 50 ng/mL, when compared to fibroblasts incubated in medium alone (1.5-fold increase contraction vs. control; P < 0.01; n = 6). The addition of neutralizing α -TGF β antibodies on fibroblasts treated with NGF (50 ng/mL) completely abolished the effect of NGF. The blocking experiments performed with neutralizing α -trkA^{NGFR} showed that contraction induced by NGF was mediated by trkA^{NGFR}.

Discussion

In this study, we demonstrated that NGF modulates cultured human conjunctival fibroblast activities via TGF- β .

NGF and trkA^{NGFR} have been observed in the normal and pathological cornea and conjunctiva [9–11, 21]. Compelling *in vitro* and *in vivo* evidences show that NGF is involved in the healing process of the ocular surface and that epithelial cells and fibroblasts are both the source and target of NGF [7, 14, 19]. The increased expression of NGF and its receptors has been previously observed in chronic inflammatory and fibrotic disorders of ocular surface (VKC and OCP) [9–11].

Since activated fibroblasts are key cells in fibrotic processes [22, 23], we investigated whether NGF can influence some of the pro-fibrogenic properties of conjunctival fibroblasts.

In the present study, we observed that conjunctival fibroblasts constitutively expressed trkA^{NGFR}, while p75^{NTR} was induced by the addition of NGF both after short- and long-term incubations. Similarly, trkA^{NGFR} expression was enhanced during the chronic stimulation. This effect indicates that NGF is able to modulate its receptors, inducing an autocrine pathway in conjunctival fibroblasts.

Short-term NGF treatment did not induce fibroblast proliferation or collagen production but it significantly increased their migration across wound line, an early event in tissue repair [34]. The effect of NGF on fibroblast migration was completely abolished by the addition of α -trkA^{NGFR} antibodies. Similar data were previously obtained with human skin and lung fibroblasts [20], suggesting that in spite of the known heterogeneity of fibroblasts in different anatomical localizations, fibroblast response to NGF is qualitatively similar [20, 35]. Following short-term treatment, NGF did not significantly increase TGF- β 1 levels. Therefore, we can hypothesize that the absence of increase in collagen synthesis is due to the lack of a direct effect of NGF together with the absence of TGF- β 1 in the cultures.

During conjunctival fibrotic diseases, several cytokines and growth factors such as TGF- β 1,2,3, CTGF, NGF, IFN- γ are produced by inflammatory, epithelial and stromal cells and exert their effects on fibroblasts [7, 8, 36–38]. For example, Cordero et al. [36] have shown the direct effect of TGF- β on tenon's fibroblasts contraction, proliferation and migration *in vitro* and a rapid onset of an exaggerated scarring response *in vivo* [37]. Treatment with anti-TGF- β 2 inhibited conjunctival scarring in an *in vivo* model of glaucoma surgery, indicating an active and pivotal role of TGF- β in pathological wound healing. In the present study we investigated the possible

interaction between NGF and TGF- β . We found that conjunctival fibroblasts released in their supernatants small amounts of NGF and TGF- β 1, and this spontaneous release was increased following their reciprocal stimulation.

The next experiments were planned to evaluate the long-term effects of NGF on fibroblast differentiation into myofibroblasts, an important step during tissue repair [34]. Therefore, the expression of α -SMA as well as fibroblast contraction of a collagen matrix was investigated.

Long-term NGF treatment of the fibroblasts resulted in an increase in α -SMA expression, triggered by $\text{trkA}^{\text{NGFR}}$. According to recent studies [39, 40], p75 seems to be related to apoptosis, especially in the brain during the development of the visual system. In contrast, trkA seems to influence cell proliferation, differentiation and survival *in vitro*. Conjunctival fibroblasts basically express trkA , while p75 receptors appear only after NGF stimulation, together with their differentiation into myofibroblasts. Therefore, we can hypothesize that NGF-induced p75 expression on myofibroblasts causes these cells to be susceptible to apoptosis (unpublished results with lung fibroblasts). This could be a possible explanation of how NGF limits its profibrogenic effect during the late phase of tissue remodelling. The expression of α -SMA in conjunctival fibroblasts paralleled their functional activity. In fact, NGF promoted fibroblast contraction of collagen gel in a free floating 3D model [32], indicating its active role during the late phase of reparative processes also.

Next, we investigated the specific contribution of TGF- β , the most important pro-fibrogenic factor [41], in some of the NGF induced pro-fibrogenic effects on conjunctival fibroblasts. Neutralization of endogenous TGF- β in α -SMA expression and gel contraction experiments almost completely abolished the pro-fibrogenic effects induced by NGF, clearly indicating that these long-term NGF effects are mediated by TGF- β . On the other hand, the neutralization of NGF was not able to counteract the effects induced by TGF- β 1. These results strengthen the possibility that interactions between NGF and TGF- β 1 take place as demonstrated in the Pheochromocytoma cell line [42]. Moreover, they show once more the key role of TGF- β 1 in different ocular pathological conditions.

In conclusion, conjunctival fibroblasts in addition to lymphocytes, mast cells and eosinophils might be considered another target and source of NGF [1, 2, 7]. A better understanding of the molecular relationships between NGF and TGF- β in controlling fibroblast properties during the development of fibrosis, especially in the ocular surface, might be useful in the effort to develop new therapeutic strategies to prevent or treat the development of fibrotic lesions.

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