Nerve Growth Factor Promotes Corneal Healing: Structural, Biochemical, and Molecular Analyses of Rat and Human Corneas

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Purpose. A recent clinical report demonstrated that topical nerve growth factor (NGF) treatment in patients affected by corneal neurotrophic ulcers induced epithelial and stromal healing restoring corneal integrity. Mechanisms(s) undergoing these clinical NGF actions are still unclear. The aim of this study was to investigate the role of NGF in human and rat cornea physiopathology.

Methods. Expression of high-affinity NGF receptors, NGF-mRNA, and NGF protein was evaluated in human and rat normal corneas, in human and rat corneal epithelial cell cultures, in human corneal organ culture, and in the rat cornea after an experimental model of epithelial injury, by means of immunohistochemistry, in situ hybridization reverse transcription–polymerase chain reaction, and enzyme-linked immunosorbent assay.

Results. The resultant data demonstrated that NGF is a constitutive molecule present and produced in normal human and rat corneas. In vitro human and rat corneal epithelial cells produce, store, and release NGF and also express high-affinity NGF receptors (TrkA). In human organ culture, epithelium, keratocytes, and endothelium have been shown to bind exogenous radiolabeled NGF, and the epithelial cells' binding was increased after epithelium injury. In vivo, after rat corneal epithelial injury, a transient increase of corneal NGF levels was observed. Inhibition of endogenous NGF activity by neutralizing anti-NGF antibodies delayed the corneal epithelial healing rate, whereas exogenous administration of NGF accelerated healing.

Conclusions. Taken together, the above findings show that NGF plays an important role in corneal physiopathology and suggest that this neurotrophin may exert therapeutic action in wide-spectrum corneal diseases. (*Invest Ophthalmol Vis Sci.* 2000;41:1063–1069)

Corneal transparency is essential for the maintenance of visual function and is contingent on the flawless integrity of all its components: the epithelium, stroma, and endothelium.[1] Disruption of the epithelial anatomic barrier activates healing and remodeling processes, which can predispose the tissue to stromal ulceration and/or cause stromal opacification, ultimately leading to irreversible visual deficit.[2] Epithelial/stromal integrity is compromised by any insult to the ocular surface: infection, trauma, chemical burns, contact lens wear, topical drug abuse, and postoperative damage.[3] Despite the numerous studies published in recent years that have indicated that cytokines, growth factors, and neuropeptides can influence the epithelial proliferations and differentiations[1] in vitro, a precise therapeutic approach to modulate the healing process has not yet been defined.[4,5]

We recently reported that the topical administration of nerve growth factor (NGF) in patients affected by neurotrophic corneal ulcer induces complete corneal recovery.[6] This type of ocular disease is characterized by an impairment of corneal sensitivity innervation associated with a deficit of epithelial metabolism and vitality, leading to inadequate healing even after minor injury.[7–9] In our study,[6] we provided consistent evidence that topical NGF treatment restored stromal and epithelial integrity, but the types of cells receptive to NGF, as well as the mechanism(s) responsible for corneal healing, were not identified. Evidence that this clinical effect was mediated by the action of NGF on the epithelium was supported by the observations that human corneal epithelium express high-affinity NGF receptors (TrkA)[10] and that in vitro NGF induce rabbit corneal epithelium to proliferate and differentiate.[11] In the present study, the role of NGF, particularly at the cellular and molecular levels, in normal and epithelial injured human and rat corneas was investigated.

Methods

Subjects and Animals

Eighteen human corneas (from 10 men and 8 women; age range, 49–72 years) were obtained from the Eye Bank of Veneto, Italy. The mean cadaver time was 6 ± 4 hours. No subject had any history of ocular surface disease. After removal

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of the corneas, they were frozen at −70°C until processed for immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), reverse transcription–polymerase chain reaction (RT–PCR), and in situ hybridization (ISH; $n = 6$). For in vitro experiments, corneal epithelium was debrided (human corneas = 4; rat corneas = 8) or cultured for autoradiographic evaluation (human corneas = 8). All procedures were conducted according to the principles expressed in the Declaration of Helsinki.

For animal experiments, 76 adult Sprague–Dawley male rats, weighing approximately 250 g, were obtained from Charles River Laboratories (Como, Italy). Rats were deeply anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (15 mg/kg). Animal care and procedures were conducted in conformity with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

NGF in Normal Human and Rat Corneas

To investigate the presence and production of NGF protein in human ($n = 6$) and rat ($n = 8$) corneas, ELISA was used to measure corneal NGF levels, RT–PCR to detect NGF mRNA, and ISH and immunohistochemistry to identify which cells expressed NGF mRNA and NGF protein, respectively.

NGF in Normal and Injured Corneas In Vitro

The production, storage, and release of NGF from human and rat epithelial cells were investigated. Epithelial cells were obtained by mechanical removal from Eye Bank corneas ($n = 4$) and rat corneas ($n = 8$). Cells were treated with trypsin 0.05% and EDTA 0.091% at 37°C for 3 hours, plated on lethally irradiated 3T3-J2 cells ($2.4 \times 10^4/mm^2$), and then cultured in 5% carbon dioxide in modified Dulbecco–Vogt Eagle's and Ham's F-12 media (3:1 mixture).[12] After 24 hours in culture, epithelial cells were fixed in 4% paraformaldehyde. Immunohistochemical analysis for NGF and TrkA and ISH for NGF mRNA were then performed.[13,14] Samples of culture medium were also collected to measure NGF concentrations by ELISA.[15]

To identify the human corneal cells that were receptive to exogenous NGF, normal and injured human corneas were cultured in the presence of 1 μg/ml of radiolabeled NGF prepared as previously described.[16] Eight human corneas were used; on four of them 3-mm-diameter epithelial lesions were created using a trephine incision and mechanical removal. Corneal discs were cultured in 60 ml of medium (minimum essential medium supplemented with 2% fetal calf serum, glutamine, HEPES buffer, antibiotics, and amphotericin B, all purchased from GIBCO SRL, Life Technologies, Milano, Italy) in conventional tissue culture bottles (Falcon; No. 3013E) and incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide.[17] After 24 hours in vitro, all corneas were fixed and evaluated by autoradiography.

In Vivo Animal Model of Corneal Epithelial Wound Healing

A total epithelial corneal debridement was performed in 30 animals using n-heptanol and mechanical removal.[18] NGF levels were then measured at various time points post-injury: 2, 12, 24, 48, and 168 hours ($n = 6$ animals/time point).

All rats were maintained on a strict 12-hour light/dark cycle (6 AM on; 6 PM off) for at least 1 week before each experiment, and throughout the duration of the experiment, to synchronize their corneal epithelial circadian rhythms.[19] Total corneal epithelial debridement was performed unilaterally. Briefly, a cotton swab saturated with n-heptanol was applied with moderate pressure to the cornea for 30 seconds. The corneal epithelium was then removed by means of a scraper, and the cornea was washed with 10 ml of physiological solution (0.9% NaCl). All wounds were made between 7 and 9 AM.

The corneal lesion was photographed immediately after the procedure and every 2 hours thereafter until the healing process was complete.[20] The circumference of the wound margin, as projected onto the photograph, was traced on a digitizer, and the lesion's area was calculated using a computer image analysis system (Videoplan, Kontron Elektronik, Germany). All measurements were counted in a masked fashion, and the size of de-epithelialized area was expressed as a percentage of the total corneal area.

Effects of Exogenous NGF or Anti-NGF Antibodies In Vivo

In two groups of animals ($n = 12$/group), the corneal epithelium was removed as previously described. In the first group, six animals were treated with topical administration of a 50 μl drop of NGF (stock solution 100 μg/ml of physiological solution) every 2 hours, and the other six rabbits (controls) were treated with physiological solution every 2 hours.[6] Highly purified active NGF form (coefficient of sedimentation = 2.5S) was prepared from submaxillary glands of adult male mice, according to the Bocchini and Angeletti method and further purified to remove all renin activity as described.[21,22]

In the second group of animals, six rabbits were treated with topical administration of a 50 μl solution containing purified anti-NGF antibody (1 mg/ml of physiological solution) every 2 hours,[23] and the other six animals (control) were treated with a 50 μl solution of nonspecific rabbit immunoglobulins at the same concentration every 2 hours.

The healing process was monitored by corneal photography every 2 hours from the time the lesion was induced. All animals were killed after the completion of corneal healing, and the corneas were histologically evaluated.

Immunohistochemistry and ISH

Human and rat corneas were fixed, cut into 10-μm-thick sections with a cryostat, and processed for immunohistochemistry using an affinity-purified NGF monoclonal antibody, which recognizes human and rodent NGFs,[24] or a specific rabbit polyclonal antibody for TrkA (trk [763]; Santa Cruz, CA),[10] which recognizes high-affinity NGF receptors. According to the manufacturer, antibody trk(763) specifically recognizes TrkA without cross-reacting with other Trk receptors.[25,26] To further assess specific binding of NGF and TrkA, corneal sections were also exposed to nonspecific purified immunoglobulins (IgGs).

ISH for NGF mRNA was performed on human and rat corneas as previously described.[14] For hybridization, a 3′-end biotin-labeled oligonucleotide complementary to bases 886 to 930 of the rat NGF mRNA sequence,[27] or to bases 703 to 742 of human NGF mRNA,[28] was used at a final concentration of 30 ng/ml.

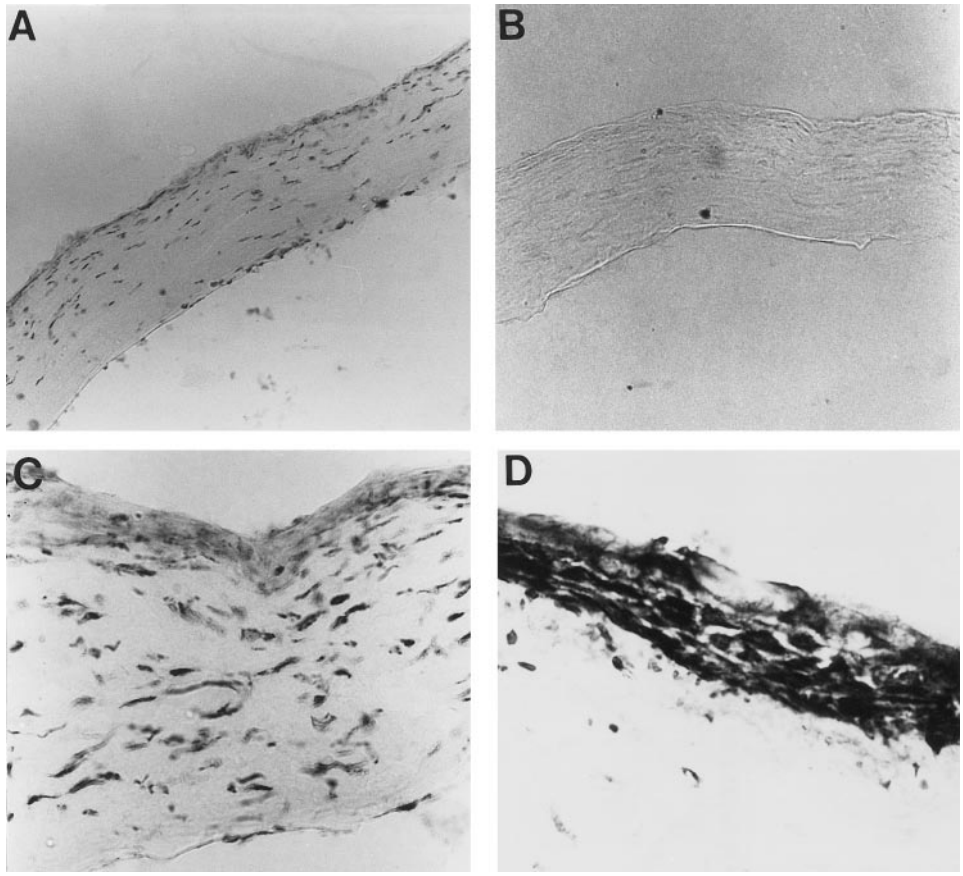

FIGURE 1. NGF mRNA and TrkA in rat cornea. ISH studies identified NGF mRNA–positive cells in the rat cornea (**A**, magnification ×20; **C**, magnification ×40). No marked cells were observed in rat cornea when slides were incubated with the oligonucleotide in the sense orientation (**B**, magnification ×20). Epithelium and keratocytes expressed high-affinity NGF receptors in the rat cornea, as demonstrated by immunohistochemistry (**D**, magnification ×40).

NGF and NGF mRNA Determination

NGF levels were measured in the human and rat corneas and in the culture medium of human epithelial cells by a highly sensitive, two-site ELISA with a sensitivity of 5 pg/ml, using anti-NGF antibodies (Clone 27/21, Boehringer Mannheim, Mannheim, Germany), according to the protocol previously described.[15]

To further verify whether the NGF was locally produced, the presence of its mRNA was also evaluated using a RT–PCR procedure on human corneas. Total RNA was extracted by using the method of Chomczynski and Sacchi[29] as modified in the TRIZOL kit (GIBCO SRL, Life Technologies, Milano, Italy), and RT–PCR was performed as described previously.[30] The PCR product, obtained using the sense 5′-CAGGACTCACAGGAGCAAGC-3′ and anti-sense 5′-GCCTTCCTGCTGAGCACACA-3′ primers, was a 349 base–long fragment corresponding to 511 to 889 of the human NGF gene.[31]

^{125}I-NGF Incorporation in Human Corneal Organ Culture

Human corneas were cultured in a medium containing 0.1 μg/ml of ^{125}I-NGF. NGF was radioiodinated with ^{125}I-Na (IMS30, 1 mCi; Amersham, Milan, Italy) by the chloramine-T procedure and purified by Sephadex G-25 column chromatography.[16] The specific activity was 1.0 to 1.5 Ci/mmol. To assess specific NGF binding to noninjured ($n = 2$) and injured ($n = 2$) corneas they were preincubated with a 100-fold excess of non-radiolabeled (cold) NGF. After 24 hours in vitro, all corneas were fixed overnight with 4% paraformaldehyde in phosphate buffer 100 mM, pH 7.4. The tissues were subsequently placed in 30% buffered sucrose solution for 24 hours. Sections of cornea were cut at a thickness of 15 μm for autoradiography. Slides were coated with the nuclear tracking emulsion, Ilford K2 (Ilford Scientific Product, Knutsford, UK), and, after 1 month of exposure, developed using a Kodak D19 developer.[16] Sections were counterstained with toluidine blue and observed under light microscope (Axiophot; Zeiss, Oberkochen, Germany) at magnifications ×40 and ×100.

Statistical Analysis

ANOVA was calculated using the StatView package for Macintosh (SAS Institute, Cary, NC). The effects of NGF or anti-NGF antibody (Ab-NGF) on epithelial healing were analyzed by ANOVA considering the wound area over time in the various treatment groups (saline versus NGF or nonspecific IgGs versus Ab-NGF). Post-hoc comparisons within logical sets of means were performed by the Tukey HSD test. Differences in NGF concentration during the healing process were evaluated with the nonparametric Mann–Whitney U test. $P < 0.05$ was considered statistically significant.

RESULTS

NGF in Normal Human and Rat Corneas

Under normal physiological conditions, human and rat corneas produced and stored NGF. ELISA showed that NGF was present in normal human corneas at a concentration of 1154 ±

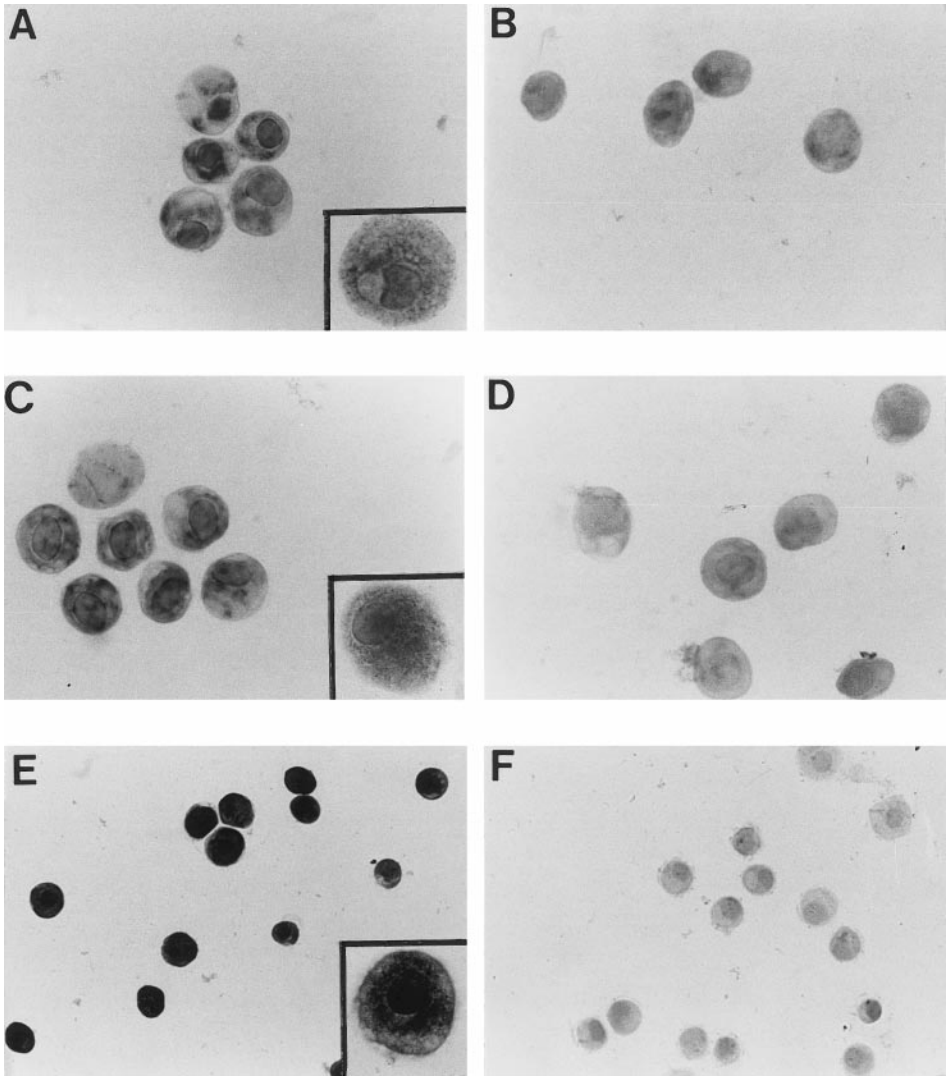

FIGURE 2. NGF mRNA, NGF and TrkA in human corneal epithelial cells. ISH demonstrated the expression of NGF mRNA in human corneal epithelial cells (**A**, magnification ×40; *inset*, magnification ×100). No marked cells were observed when incubated with the sense oligonucleotide (**B**, magnification ×40). Epithelial cells in culture expressed immunoreactivity for NGF (**C**, magnification ×40; *inset*, magnification ×100) and high-affinity NGF receptors (**E**, magnification ×20; *inset* magnification ×100). (**D**) (magnification ×40) and (**F**) (magnification ×20) show the unspecific stain (control) for NGF and TrkA, respectively.

376 pg/mg and in the rat at a concentration of 388 ± 121 pg/mg. RT–PCR and in situ hybridization for NGF mRNA and NGF immunohistochemistry indicated that NGF was produced by epithelium, keratocytes, and endothelium in humans and rats (Figs. 1A, 1C). Indeed, as shown in Figure 1D, rat corneal epithelium, keratocytes, and endothelium all expressed immunopositivity for TrkA.

NGF in Normal and Injured Corneas In Vitro

Results of ISH, immunohistochemistry, and ELISA showed that NGF concentration in the growth media of in vitro human and rat corneal epithelial cells were more than two times greater than control media (27 ± 6 and 12 ± 4 pg/ml, respectively, versus <5 pg/ml), evidence that these cells synthesized, stored, and released NGF (Figs. 2A, 2B, 2C, 2D). Human and rat epithelial cells were also shown to express TrkA (Figs. 2E, 2F).

In corneal organ culture, cells of the epithelium, keratocytes, and endothelium of human normal corneas were shown to bind radiolabeled NGF. Monitoring the rate of healing after creation of a 3-mm-diameter epithelial lesion revealed the healing process of the wounded area (7.1 mm^2 and a marked NGF positivity within 24 hours. As illustrated in Figures 3A and 3B, lesioned NGF-positive epithelial cells showed a stronger binding of radiolabeled NGF than that seen in control non-lesioned tissue, suggesting that these cells were highly responsive to NGF activity. NGF-positive epithelial cells were not observed when corneal tissue was preexposed to an excess of "cold" NGF, further suggesting that these cells specifically bound NGF.

In Vivo Animal Model of Corneal Epithelial Wound Healing

To learn more about NGF's role in the corneal healing process, an animal model of epithelial debridement was used in which healing was initiated after 4 hours and completed within 24 hours. Time-course studies showed that the corneal lesion induced a progressive increase of NGF levels, reaching the highest value at 48 hours (3010 ± 284 pg/mg; $P < 0.01$). Thereafter, NGF concentrations progressively decreased, arriving at baseline values (706 ± 123 pg/mg; $P > 0.05$; Fig. 4) 7 days after induction of the lesion.

Effects of Exogenous NGF and Anti-NGF Antibodies In Vivo

Figure 5A illustrates that exogenous NGF treatment accelerated healing of the epithelium when compared with saline-treated

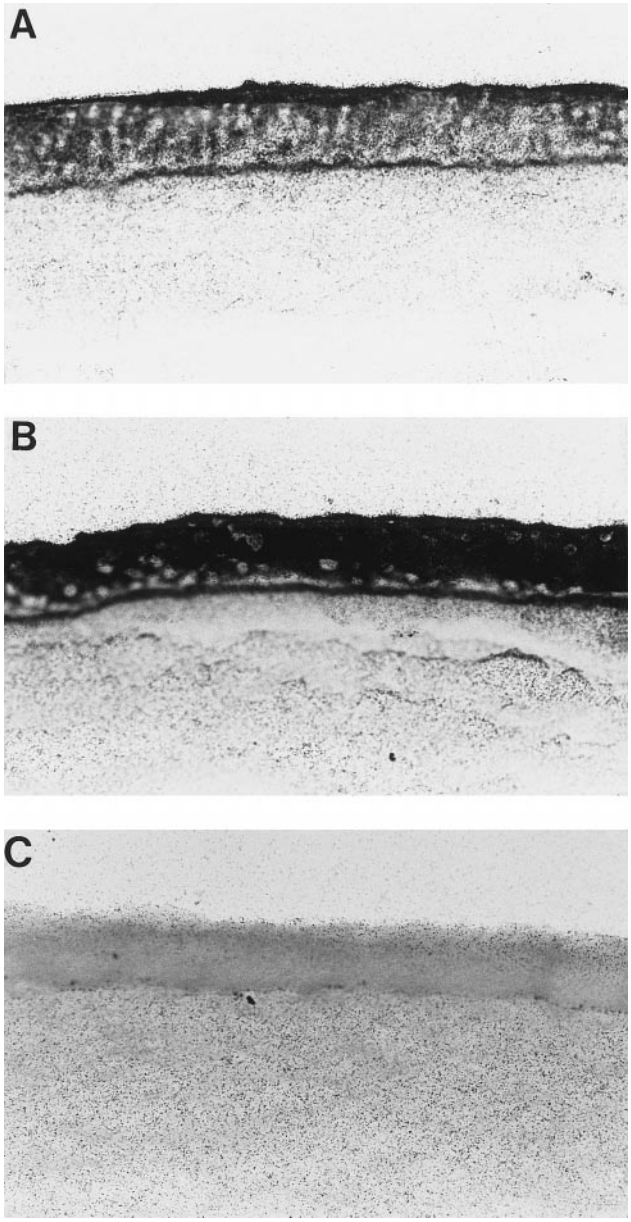

FIGURE 3. Exogenous NGF uptake by human corneal epithelium. An autoradiographic evaluation of NGF uptake by normal unlesioned human cornea in organ culture revealed that epithelial cells bound ^{125}I-NGF (**A**, magnification ×40). Epithelial cells in the area of corneal injury (**B**, magnification ×40) showed a much stronger binding of ^{125}I-NGF than that seen in control nonlesioned tissue, suggesting that these cells were highly responsive to NGF activity. No radiolabeling was observed in corneal epithelium incubated with ^{125}I-NGF and an excess of "cold" NGF (**C**, magnification ×40).

eyes. This statistical difference was confirmed by ANOVA (repeated measures × treatment: $F_{5,50} = 20.615$; $P = 0.0001$) and post-hoc comparisons ($P < 0.05$). Four hours after creation of the lesion, the NGF-treated cornea showed a reduction of wound area (59% ± 11% of the cornea was de-epithelialized), whereas the control eyes showed no sign of healing. The time occurring to complete healing was 10 ± 1 hours in NGF-treated eyes versus 16 ± 2 hours in the control eyes. None of the NGF-treated corneas showed changes in anterior stromal transparency, whereas 2 of the 6 saline-treated eyes presented a mild opacification.

Topical treatment with anti-NGF antibody (Ab-NGF) immediately after epithelial debridement caused a significant delay in healing. ANOVA (repeated measures × treatment: $F_{5,50} = 6.397$; $P = 0.0001$; Fig. 5B) and post-hoc comparisons further indicated that this treatment delayed corneal healing ($P < 0.05$), reaching a maximum difference compared with the control eyes, treated with nonspecific IgGs, 6 hours after creation of the lesion (79% ± 9% versus 56% ± 9%; $P < 0.05$; Fig. 5B). Moreover, 4 of 6 Ab-NGF–treated eyes and 2 of 6 nonspecific IgG-treated eyes displayed a weak opacification of the anterior stroma.

DISCUSSION

It was recently demonstrated that topical NGF treatment in patients with corneal neurotrophic ulcers induces healing and restoration of corneal sensitivity without any local or systemic side effects.[6] To characterize this effect, the role of NGF in normal corneas and in the healing process was investigated using in vitro and in vivo models of corneal physiology and injury. These experiments demonstrated that NGF was stored and produced in human and rat normal corneas and that after corneal epithelial injury, acceleration in healing was associated with an increase of NGF levels. ISH and immunohistochemical analysis identified that cells of the epithelium, keratocytes, and endothelium synthesized and stored NGF. Furthermore, human and rat epithelial cells in vitro not only produced, stored, and released NGF but also expressed TrkA, supporting the hypothesis that its effect on corneal healing was mediated by NGF–NGF receptor interaction.

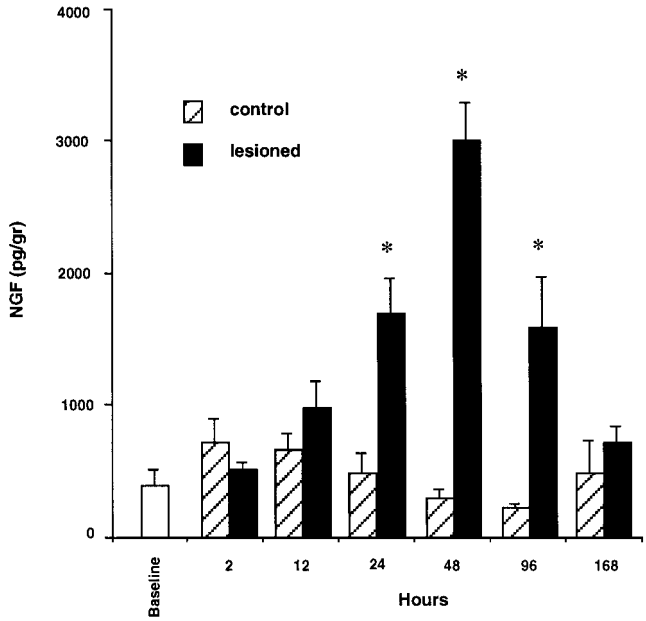

FIGURE 4. Time course of corneal NGF levels after epithelial injury. NGF protein levels were quantified in the cornea over time after complete epithelium debridement. Six animals were evaluated per time point: results in one lesioned eye were compared with the contralateral, untreated, control eye. NGF levels were significantly increased in the wounded eye after 24 hours, reaching a peak at 48 hours, and returning close to control levels after 168 hours.

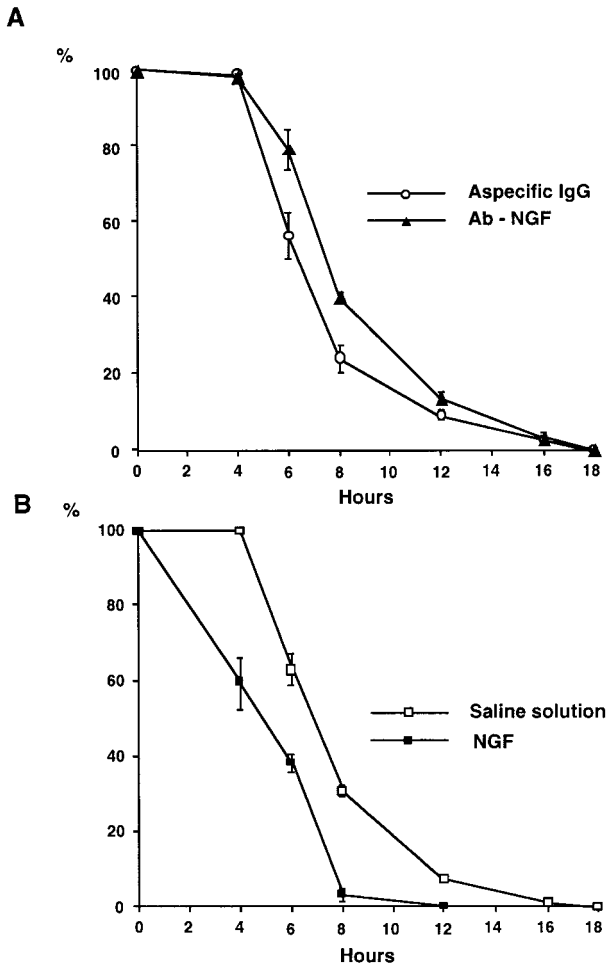

FIGURE 5. Effects of exogenous and endogenous NGFs on epithelial healing. Topical administration of exogenous NGF accelerated healing ($P < 0.01$) and decreased the overall time to complete healing when compared with the control group treated with saline solution (**A**). Conversely, topical treatment with Ab-NGF attenuated healing of the created epithelial lesion compared with nonspecific IgG ($P < 0.05$; **B**).

These findings suggest that in normal corneas endogenous NGF is an essential factor for the trophism and integrity of the corneal epithelium. Moreover, the coexpression of NGF and its receptor on the same corneal cell suggests the presence of an autocrine and/or paracrine circuit that supports the survival and/or function of epithelial cells. A similar mechanism has been reported in neuronal and immune cells.[32–36] A clinical study has demonstrated that NGF treatment accelerates the healing of corneal ulcers that had developed subsequent to dysfunction of corneal sensitivity innervation.[6] It is well known that impairment of corneal sensitivity leads to decreased vitality and metabolism of the corneal epithelium.,[7–9,37] with frequently associated epithelium rupture and delay or absence of spontaneously healing.

Corneal NGF levels were also shown to increase after corneal epithelial injury in the rat. It is possible that endogenous NGF plays an important role in epithelial healing by modulating the proliferation and differentiation of epithelial cells, a hypothesis also supported by results of in vitro studies in rabbit corneal epithelium.[11] Evidence from various studies further strengthen this argument: in the present study, in vivo inhibition of endogenous NGF by Ab-NGF significantly delayed the epithelial healing process; animals with a targeted mutation for the NGF receptor develop ulcers and mutilation of the feet and corneal opacification[38,39]; and concentrations of NGF are decreased in ulcer tissue from patients affected by diabetes mellitus, leprosy, and nerve trauma.[40,41]

Both the previously reported clinical study and the findings of the present study have indicated that NGF was essential for the epithelial healing process and that its binding to corneal NGF receptor cells was a crucial event triggering its pharmacological activity. This hypothesis is consistent with the observation that in vitro human corneal cultures exposed to radiolabeled NGF demonstrated epithelial binding to exogenous NGF and that this binding was greatly enhanced in injured tissue. In addition, exogenous NGF treatment in vivo in rats with lesioned corneas significantly accelerated epithelial healing. Although these data clearly indicated the existence of a direct action of NGF on epithelial cells, the possibility of an indirect effect of NGF through the production and release of specific neuropeptides, which then acted as cell mediators capable of stimulating the healing process, cannot be excluded.[4,5,42,43]

A relevant observation in our in vivo studies was the presence of a mild anterior stromal opacification in 4 of 6 Ab-NGF–treated corneas, compared with 2 of 6 control eyes (both nonspecific IgG– and saline-treated eyes) and 0 of 6 NGF-treated corneas. This finding suggests a role for NGF in epithelium–stroma communication leading to the induction of stromal healing and remodeling mechanisms, and/or the promotion of a rapid epithelial healing to avoid the onset of a stromal opacity. This attractive possibility needs to be explored in both in vitro and in vivo studies.

Cumulatively, our structural, biochemical, and molecular evidence support the previous clinical findings that NGF plays a crucial role in the corneal healing process.[6] They also provide new insight into the pathophysiological mechanisms of NGF effects and into a potential therapeutic use of this neurotrophin in a wide spectrum of corneal injuries. Our working hypothesis is that NGF treatment promotes the healing process, avoiding the onset of the stromal remodeling mechanisms that lead to superficial stroma opacification in corneal disease.

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