# Neurotrophic Factors in the Human Cornea

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**PURPOSE.** To investigate neurotrophic growth factors and corresponding receptors in human and rabbit corneal epithelium and stroma.

**METHODS.** Transcription of nerve growth factor (NGF), neurotrophin 3 (NT-3), NT-4, brain-derived neurotrophic factor (BDNF), glial cell line- derived neurotrophic factor (GDNF), and receptors Trk A-E, was investigated by reverse transcription-polymerase chain reaction. DNA dot blot analysis allowed to estimate transcription levels. Single cell proliferation assays were performed using recombinant NGF, BDNF, and GDNF. Mitogen-activated protein kinase signal transduction was investigated with Western blot analysis using antibodies against activated and total extracellular signal-regulated kinase (ERK) 1/2 and the jun N-terminal protein kinase (JNK) 1/2.

**R**ESULTS. Transcription of NGF, NT-3, BDNF, and Trk A, Trk B, Trk C, and Trk E receptors was detected in both ex vivo and cultured epithelium and stroma. Transcription of NT-4 was only detected in epithelium and transcription of GDNF only in stroma. Levels of transcription were higher for NT-3, NT-4, and the Trk receptors and lower for NGF, BDNF, and GDNF. NGF and GDNF stimulated both epithelial colony formation and proliferation, whereas BDNF only enhanced colony formation. Stromal proliferation was enhanced in serum-free medium. In epithelium, predominantly ERK 1 was activated by NGF, GDNF, and BDNF. In stromal cells NGF and GDNF stimulated phosphorylation of ERK 1 and JNK 1.

**CONCLUSIONS.** Neurotrophic factors and tyrosine kinase receptors are transcribed in the human cornea. GDNF and NGF stimulate corneal epithelial proliferation, and the effect of the latter might be mediated by activation of ERK 1. Neurotrophic factors have very specific effects on phosphorylation of ERK and JNK in epithelial and stromal cells. The differential expression of NT-4 and GDNF suggests a regulatory function within the cytokine network of the cornea. (*Invest Ophthalmol Vis Sci.* 2000;41:692–702)

he integrity of the ocular surface depends on a delicate balance between cellular proliferation and differentiation. Increasing evidence suggests that one of the prerequisites for such processes is an integrated interaction between the cells of the cornea. These cells are confined to three distinct layers: the epithelium, the stroma, and the endothelium. In general, several factors can modulate the interaction between cells, for example, direct cell contact, extracellular matrix components, and cytokines, and among them polypeptide growth factors.1 Over the recent years a multitude of cytokines have been identified in the human cornea and seem to be expressed in the form of an organized network that mediates regulatory functions between cells of the layers of the cornea.<sup>2</sup> However, the functional significance of these findings is only slowly unfolding, and it remains unclear how the cytokine network can be manipulated for therapeutic use. In this context, nerve growth factor (NGF) has recently gained attention as the first growth factor with proven efficacy for the

treatment of human corneal ulcers due to neurotrophic disease.<sup>3</sup>

NGF is a member of the neurotrophin gene family, which also includes neurotrophin 3 (NT-3), neurotrophin 4/5 (NT-4), and brain-derived neurotrophic factor (BDNF).4,5 Neurotrophins exert their biological functions by binding to high affinity transmembranous receptors belonging to the Trk family of tyrosine kinase receptors. At least four Trk receptors have been cloned and express variable capabilities to bind individual neurotrophins.<sup>6,7</sup> Although Trk A is the main receptor for NGF; Trk B binds BDNF, NT-3, and NT-4; and Trk C binds NT-3. Trk E also binds NGF. In addition a low affinity receptor, the glycoprotein p75 that belongs to the cytokine receptors, has been described.<sup>8</sup> Although a matter of controversy, experimental data suggest that the presence of a high affinity Trk receptor is sufficient for signal transduction.<sup>5</sup> Binding of a neurotrophin to its Trk receptor induces dimerization and phosphorylation that initiates a signal transduction cascade and ultimately leads to gene transcription. Neurotrophins are existing mainly as homodimers with close structural homology to each other and are related to other growth factor families such as transforming growth factor- $\beta$  (TGF- $\beta$ ).<sup>9</sup> The TGF- $\beta$  superfamily also contains a protein, glial cell line-derived neurotrophic factor (GDNF), that like neurotrophins acts as neurotrophic factor.<sup>10,11</sup>

Neurotrophic factors are regulatory molecules that play important roles in the development as well as maintenance and survival of a wide variety of cells of neuronal origin.<sup>4,5,12</sup> Several lines of evidence suggest that neurotrophic factors such as NGF also exert biological functions in cells of the

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Neurotrophic Factors in the Human Cornea 693

ocular surface: In rabbits, NGF promotes the proliferation of corneal epithelial cells in vitro and accelerates the rate of epithelial wound healing in vitro.<sup>13,14</sup> Furthermore, increased levels of NGF have been found in inflamed conjunctiva of patients with vernal keratoconjunctivitis.<sup>15</sup>

Although these findings suggest that neurotrophins participate in the regulation of physiological and pathologic processes of the ocular surface, little is known about the mechanism by which neurotrophins such as NGF modulate cells of the cornea. The two major prerequisites for a physiological role of neurotrophins in the cornea are the presence of neurotrophins and the presence of the corresponding receptors. The latter was recently demonstrated by the presence of Trk A receptors in corneal epithelium and endothelium and confirms that these cells can respond to NGF.<sup>16</sup> To further elucidate the role and origin of neurotrophins in the human cornea, we investigated the transcription of NGF, NT-3, NT-4, BDNF, and GDNF in corneal epithelial and stromal cells. Furthermore, we have shown transcription of four different Trk receptors in both ex vivo and cultured corneal epithelial and stromal cells. In addition we have compared the effects of BDNF and GDNF on epithelial and stromal proliferation to that of NGF and obtained evidence that the latter cytokine induces the mitogenactivated protein kinase (MAP kinase) signaling system by activation of extracellular signal-regulated kinase (ERK) and the jun N-terminal protein kinase (JNK).

# **METHODS**

#### **Ex Vivo Corneal Tissue**

Fresh ex vivo corneal epithelium and stroma were obtained from 8 eyes undergoing enucleation for choroidal melanomas after informed consent and in conformance with the tenets of the Declaration of Helsinki. Immediately after enucleation all layers of the central and mid-peripheral corneal epithelium within an area of approximately 8 mm were removed by mechanical scraping. Within this area small samples of the stroma were excised with a diamond blade. Tissue samples were snap-frozen.

# **Cell Culture**

Human corneas stored for less than 24 hours in Likorol (Chauvin-Opsia, Labege Cedex, France) at 4°C were obtained through our eye bank. All corneas were of transplant quality but excluded from clinical use for nonocular reasons according to international eye bank criteria. Both epithelial and stromal cells were cultured on plastic dishes as outgrowth cultures with slight modifications of a previously described technique.17 For RNA extraction, explant cultures were initiated and cultured in SHEM medium (1:1 mixture of Dulbecco's modified Eagle's medium, DMEM, and Ham's nutrient mixture F-10 with 10% fetal bovine serum, FBS; GIBCO, Grand Island, NY), 5 µg/ml insulin, and 10 ng/ml epidermal growth factor (EGF) without antibiotics.<sup>18,19</sup> The epithelial phenotype of cultures was confirmed by staining with an antibody for cytokeratin K12. Because this method only vielded a very limited amount of cells we also used an SV 40-adenovirus-transformed corneal epithelial cell line.<sup>18</sup> Similar to normal corneal epithelium these cells exhibit clonal growth characteristics and display a corneal epithelial phenotype (including expression of keratin K12). The cell line was cultured as previously described

in SHEM medium.<sup>18,19</sup> Stromal fibroblasts were cultured in DMEM + 10% FBS as described previously.<sup>17</sup> All experiments were performed in triplicate and with cells obtained from different donors.

#### Isolation of Total RNA and mRNA Purification

Total RNA was isolated according to the guanidium thiocyanate-phenol-chloroform extraction method<sup>20</sup> by use of an RNAgents total RNA isolation system kit (Promega, Madison, WI) as previously described.<sup>17</sup> For mRNA isolation a Promega polyATtract system III was used as described previously.<sup>17</sup> To minimize the risk of contamination by genomic DNA, mRNA samples were digested by RNase-free DNase followed by phenol-chloroform-isoamyl alcohol extraction and isopropanol precipitation.

#### Polymerase Chain Reaction Primer Design and Reverse Transcription–Polymerase Chain Reaction

For polymerase chain reaction (PCR) primer design known coding sequences were taken from GenBank (www.ncbi.nlm.nih-.gov). Because of the high structural similarity of the sequences of all the known members of the neurotrophin gene families and the neurotrophin tyrosine kinase receptor family, all sequences in open reading frames were compared using the Clustal W multiple sequence alignment program as described previously.<sup>17</sup> Whenever possible, primers were designed to span one or more introns in the genomic sequence: NGF, sense, GAGGTGCATAGCGTAAT-GTCCA, and antisense, TCCACAGTAATGTTGCGGGTCT (product of 233 bp; GenBank accession number: V01511, X52599); NT-3, sense, TTACAGGTGAACAAGGTGATG, and antisense, GCAGCAGTTCGGTGTCCATTG (product of 298 bp; GenBank accession number: M37763); NT-4, sense, CTCTTTCTGTCTC-CAGGTGCTCCG, and antisense, CGTTATCAGCCTTGCAGC-GGGTTTC (product of 464 bp; GenBank accession number: M86528); BDNF, sense, GTGAGTTTGTGTGGACCCCGAG, and antisense, CAGCAGAAAGAGAAGAGAGGAGGC (product of 373 bp; GenBank accession number: X60201, X91251); GDNF, sense, GCCCTTCGCGTTGAGCAGTGAC, and antisense, GTCGTACGTT-GTCTCAGCTGC (product of 343 bp; GenBank accession number: NM000514); Trk A, sense, GATGCTGCGAGGCGGACGGC, and antisense, CTGGCATTGGGGCATGTGGGC (product of 570 bp; GenBank accession number: M23102); Trk B, sense, TGCAC-CAACTATCACATTTCTCG, and antisense, CACAGACGCAAT-CACCACCACA (product of 472 bp; GenBank accession number: \$76473); Trk C, sense, ACTTCGGAGCATTCAGCCCAGAG, and antisense, ACTCGTCACATTCACCAGCGTCAA (product of 484 bp; GenBank accession number: S76475, U05012); and Trk E, sense, AGGAGTACTTGCAGGTGGATC, and antisense, ACTG-GAGAAGCTGTGGTTGCT (product of 545 bp; GenBank accession number: X74979).

The first-strand cDNA was synthesized as previously described.<sup>17</sup> PCR was performed using 0.5  $\mu$ l of single-strand cDNA with 3 U Thermus aquaticus (*Taq*) DNA Polymerase, a mixture of desoxyribonucleotides (in a final concentration of 0.2 mM), 10× PCR buffer (5  $\mu$ l), and 25 pmol of upstream and downstream primers in a total volume of 50  $\mu$ l (all reagents from Takara Shuzo, Otsu, Shiga, Japan). The final concentration of MgCl<sub>2</sub> in the buffer was 1.5 mM. A PTC-100 programmable thermocycler (MJ Research, Watertown, MA) was used at 95°C for 3 minutes (predenaturation). Then 35 cycles were per-

formed including denaturation at  $94^{\circ}$ C for 1 minute, annealing at  $55^{\circ}$ C for 1 minute, and extension at  $72^{\circ}$ C for 1 minute.

The PCR products were size-fractionated by agarose gel electrophoresis using 1.8% agarose  $1 \times$  Tris-acetate-EDTA gels stained with 0.5  $\mu$ g/ml ethidium bromide. All PCR fragments were cloned into pCR2.1 vector (Invitrogen, San Diego, CA), and sequences were confirmed by standard methods.

#### DNA Dot Blot Analysis for Detection of the Level of Gene Transcription in the Cultured Cornea

To get an estimation of the level of transcription in cultured epithelial and stromal cells we performed a DNA dot blot analysis. Because we could not culture sufficient quantities of human corneal epithelial cells, we used a corneal epithelial cell line as a source of corneal epithelium. Cloned PCR fragments corresponding to neurotrophic factor family, and Trk receptors genes were amplified using the above-mentioned primers and purified from agarose gels. A 0.1-µg aliquot of PCR product was loaded onto nylon membranes as dot. To generate the hybridization probe, 1 µg mRNA was isolated from cultured epithelial and stromal cells and transcribed with a digoxigenin probe synthesis mix (Boehringer-Mannheim, Mannheim, Germany) to synthesize first-strand cDNA labeled with digoxigenin. DNA blots were then prehybridized and hybridized with the digoxigenin-labeled cDNA probe in DIG EasyHyb buffer (Boehringer-Mannheim) at 40°C overnight. After posthybridization washing, the blots were treated with the DIG washing kit from Boehringer-Mannheim according to the manufacturer's description and exposed to ECL film (Amersham Life Science, Little Chalfont, UK). For comparison, a cDNA fragment encoding for reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) was used as positive control.

# Investigation of Components of the MAP Kinase Signal Transduction Pathways Induced by Neurotrophic Factors in the Cultured Cornea

To evaluate the effect of neurotrophic factors on the activation of signal transduction pathways in cultured corneal epithelium and stromal keratocytes, we performed Western blot analysis to investigate the accumulation of phosphorylated MAP kinases ERK and JNK in the presence of NGF, BDNF, and GDNF. Human stromal keratocytes were cultured in RPMI 1640 medium containing I-glutamine (glutaMAX) or DMEM with 10% FBS for 1 day and starved in serum-free medium for another day. Cultures were then washed with phosphate-buffered saline (PBS) and incubated in serum-free DMEM without additives or with recombinant human NGF (200 ng/ml), recombinant human BDNF (200 ng/ml), and recombinant human GDNF (200 ng/ml; all from R&D Systems, Minneapolis, MN) for 30 minutes. Some cultures were incubated with an inhibitor of MAP kinase (PD 98059; Torcris Cookson, Ballwin, MO) at 100  $\mu$ M for 1 hour before exposure to neurotrophins. After washing with PBS, cultured cells were solubilized in lysis buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride, 1% Triton X-100, and a mixture of several protease inhibitors (Complete, 1 tablet/50 ml buffer; Boehringer-Mannheim). Fifty micrograms total protein per lane was fractionated by a 10% sodium dodecyl sulfate-MOPS NuPAGE Bis-tris gel (NOVEX, San Diego, CA) and blotted onto nitrocellulose membrane. Membranes were stained with diluted polyclonal antibodies

against ERK 1, ERK 2, JNK 1, and JNK 2 (Santa Cruz Biotechnology, Santa Cruz, CA). We also used a polyclonal antibody, which recognizes the activated form of either ERK 1 and ERK 2, that was raised against the catalytic core of the phosphorylated threonine residue 183 and tyrosine residue 185 of the mammalian ERK 2. Similarly, a polyclonal antibody recognizing the phosphorylated form of JNK 1 and JNK 2 was used (both from Promega). As the last step, the membranes were visualized with the ECL Western blot analysis system (Amersham Life Science).

# Investigation of the Effect of Neurotrophic Factors on Proliferation of Corneal Epithelial and Stromal Cells

To evaluate the effect of neurotrophic factors on corneal proliferation, recombinant human NGF, BDNF, and GDNF were used (R&D Systems) and the effect compared with that of recombinant human EGF (Sigma, St Louis, MO). To evaluate the effect on corneal epithelial proliferation, a single cell clonal growth model was used that allows one to determine the effects of a given growth factor on both colony formation and clonal expansion.<sup>21</sup> We were not able to reproduce quantification of the proliferation of human epithelial cells in this model due to a shortage of good donor material. We therefore used rabbit cells which also allows one to compare the data with previous reports in the literature. New Zealand white rabbits were housed and treated according to the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research and under observation of German federal laws and the laws of the State of Baden-Württemberg. Before they were killed with an intravenous overdose of pentobarbital, the rabbits received an intramuscular injection of xylazine hydrochloride and ketamine hydrochloride. The details of the clonal growth assay have been described previously.<sup>21</sup> Five thousand viable cells were seeded in each 60-mm dish in serum-free medium MCDB 151 with a supplement of insulin (5  $\mu$ g/ml), transferrin (5 µg/ml), selenium (5 ng/ml), and hydrocortisone (5  $\mu$ g/ml; all from Sigma). This seeding density resulted in a single cell clonal growth that could be quantified under the phase contrast microscope (on day 6) by determination of the number of colonies per dish and the number of cells per colony. This quantification was facilitated by use of dishes that contained a grid on the bottom that was roughly 2-mm wide (Sarstedt, Newton, NC). For data collection the entire surface areas of four randomly selected dishes for each condition were screened. Furthermore, the number of cells per colony was determined in 75 randomly selected colonies for each condition. To stimulate cellular proliferation, NGF (50 or 200 ng/ml), BDNF (50 or 200 ng/ml), GDNF (50 or 200 ng/ml), or EGF (10 ng/ml) was added to the medium. To get an estimation about the rate of proliferation after 12 days (a time when neighboring colonies started to grow into each other and therefore prevented numerical quantification) dishes were fixed in -20°C methanol and stained with methylene blue.

To investigate the effect of neurotrophic factors on the proliferation of cultured stroma, keratocytes were passaged from DMEM + 10% FBS into DMEM + 1% FBS or in DMEM without FBS at a density of  $5 \times 10^4$  cells/60-mm dish. Some cultures received recombinant human NGF, BDNF, or GDNF in concentrations as shown above. Proliferation was measured after 6 days by counting cells under the phase contrast microscope (50 fields at  $100 \times$  per condition) as well as trypsinized

cells. Also a CellTiter 96AQueous One Solution proliferation assay was performed according to the manufacturer's description (Promega). For this colorimetric assay 500 or 1000 cells were grown for 6 days in 96-well plates (Falcon). On addition to the culture well a tetrazolium dye is bioreduced by cells into a colored formazan product and the absorbance is quantified at 490 nm. The quantity of the formazan product should be proportional to the number of living cells in the well and can therefore serve to estimate proliferation.

#### **Statistical Analysis**

All experiments examining the effect of neurotrophic factors on corneal proliferation were performed in triplicate with cells from different donors. The influence of growth factors on colony formation, colony size, and cell number was studied using one-way ANOVA. The log transformation was used as necessary to affect homogeneity of variance and normality in these data. Student's *t*-test was used to determine which differences were significant after ANOVA.

# RESULTS

# Transcription of Neurotrophic Factors in the Human Cornea

The transcription of neurotrophic factors was detected in freshly harvested cells from human corneal epithelium (Fig. 1A) and stroma (Fig. 1B) by reverse transcription-polymerase chain reaction (RT-PCR). In Figure 1A the result of a representative RT-PCR shows that the specific cDNA fragments of NGF (lane 1, 233 bp), NT-3 (lane 2, 298 bp), NT-4 (lane 3, 464 bp), and BDNF (lane 4, 373 bp) could be amplified from ex vivo human corneal epithelium. However, we could not detect transcription of GDNF (lane 5) using the primers shown in the Methods section. This result has been confirmed by three independent experiments using cDNA from primary cultured epithelial cells and a human corneal epithelial cell line immortalized with SV 4018 (data not shown). In contrast, ex vivo corneal stroma contained mRNA encoding for the above-mentioned proteins including GDNF (lane 5, 343 bp) as shown in Figure 2. However, we could not detect transcription of NT-4 (lane 3) using the primers shown in the Methods section. The results of the RT-PCR were identical when cDNA from cultured corneal stromal keratocytes was used (data not shown).

All the above-mentioned PCR products were cloned into *Escherichia coli* and sequenced. Comparison of the resulting DNA sequences with known genes via the blast search program of GenBank revealed a 100% sequence identity with the expected neurotrophic factors in all experiments. Likewise, there were only very low scores of sequence homology to other known genes (data not shown).

# Transcription of Tyrosine Kinase Receptors Specific for Neurotrophic Factors in the Human Cornea

The ex vivo corneal epithelium (Fig. 2A) and stroma (Fig. 2B) also contained mRNA encoding for tyrosine kinase receptors that are necessary for binding and signal transduction of neurotrophic factors. Figure 2A shows the RT-PCR result after amplification of cDNA fragments specific for Trk A (lane 1, 570 bp), Trk B (lane 2, 472 bp), Trk C (lane 3, 484 bp), and Trk E (lane 4, 545 bp) from ex vivo corneal epithelium. Figure 2B



**FIGURE 1.** Shown are results of agarose gel electrophoresis (1.8%) of PCR products amplified from the cDNA generated from mRNA extracted from ex vivo corneal epithelium (**A**) and stroma (**B**) stained with ethidium bromide. Both epithelium and stroma expressed NGF (233 bp; *lane 1*), NT-3 (298 bp; *lane 2*) and BDNF (373 bp; *lane 4*). NT-4 (464 bp; *lane 3*) was expressed only in corneal epithelium. GDNF (343 bp; *lane 5*) was expressed only in corneal stroma. M, DNA molecular weight marker (Phi  $\times$  174 DNA/*Hin*fl fragments).

indicates the same result using mRNA from ex vivo corneal stroma. When cultured corneal epithelial cells (primary cultures or corneal epithelial cell line) or cultured stromal keratocytes were used, the spectrum of RT-PCR was not changed (data not shown). All these Trk gene fragments have also been cloned, sequenced, and analyzed by the blast search program for further confirmation.

#### Level of Transcription of Neurotrophic Factors and Corresponding Tyrosine Kinase Receptors in Cultured Human Corneal Epithelium and Stromal Keratocytes

To confirm the results of the initial PCR and to get an estimation about the level of gene transcription, we performed a DNA dot blot analysis (Fig. 3). Because the hybridization probe for the DNA dot blot was first-strand cDNA generated from 1  $\mu$ g mRNA of cultured epithelial cells or stromal keratocytes, the result of the DNA dot blot allows one to estimate and compare the transcription level of neurotrophic factors and corresponding tyrosine kinase receptors in different cells. Figure 3A shows the spectrum of the transcription levels of neurotrophic factors and corresponding tyrosine kinase receptors in the human corneal epithelial cell line. The transcriptions of NGF (lane 1), BDNF (lane 4), and Trk E (lane 9) were significantly weaker than those of the other neurotrophic factors and tyrosine kinase receptors. The levels of transcription of NT-3 (lane 2),



**FIGURE 2.** Shown are results of agarose gel electrophoresis (1.8%) of PCR products amplified from the cDNA generated from mRNA extracted from ex vivo corneal epithelium (**A**) and stroma (**B**) stained with ethidium bromide. Both epithelium and stroma expressed Trk A (570 bp; *lane 1*), Trk B (472 bp; *lane 2*), Trk C (484 bp; *lane 3*), and Trk E (545 bp; *lane 4*). M, DNA molecular weight marker (Phi  $\times$  174 DNA/*Hint*I fragments).

NT-4 (lane 3), Trk A (lane 6), Trk B (lane 7), and Trk C (lane 8) were lower than that of GAPDH, which was used as a positive control (lane 10). No transcription of GDNF (lane 5) was detected in the DNA dot blots from cultured corneal epithelial cells. Furthermore, Figure 3B shows that transcription of NT-4 (lane 3), which was clearly present in corneal epithelial cells, could not be detected in cultured stromal keratocytes. In contrast GDNF (lane 4) that was not transcribed in epithelial cells showed a positive signal in cultured stromal keratocytes. The level of transcription of the remaining neurotrophic factors and tyrosine kinase receptors in stromal cells was approximately the same as in corneal epithelial cells. This result confirmed the data obtained from RT-PCRs as shown in Figures 1 and 2.

### Effect of Neurotrophic Factors on the Phosphorylation of MAP Kinase in Cultured Corneal Epithelium and Stromal Keratocytes

The activation of the MAP kinase signaling cascade is essential for mediating the effect of various growth factors on cellular proliferation and differentiation. Therefore, the intracellular accumulation of phosphorylated MAP kinases ERK and JNK is an indication for the activation of the MAP kinase pathway in response to neurotrophic factors. To correlate the investigation of the signal transduction with the results of the functional analysis, we studied the induction of members of the MAP kinase cascade in human corneal epithelium and stroma. To ensure that the signals corresponded to phosphorylation we

also used the inhibitor PD 98059, which inhibits MAP kinase. Figure 4A shows that the phosphorylated forms of ERK 1 and 2 can be induced in cultured human epithelial cells. As compared with the serum-free control medium (lane 7) 200 ng NGF induced phosphorylation of ERK 1, and to a lesser extent of ERK 2 (lane 1). This induction was prevented by addition of the inhibitor PD 98059. The levels of phosphorylated ERK 1and ERK 2 were also increased by BDNF (200 ng/ml; lane 3), but this increase was not inhibited by PD 98059. Similar to NGF, GDNF (200 ng/ml) also induced phosphorylation of ERK 1 and ERK 2 (lane 5), and this effect could also be prevented by PD 98059. Data presented in Figures 4B, 4C, and 4D show the same expression level of total (phosphorylated and nonphosphorylated) ERK 1 and ERK 2 (Fig. 4B), activated JNK 1 and JNK 2 (Fig. 4C), and total (phosphorylated and nonphosphorylated) JNK 1 and JNK 2 (Fig. 4D) in human corneal epithelial cells when incubated with or without the neurotrophic factors under investigation. This result indicates that phosphorylation of ERK1 and ERK 2 (but not JNK 1 or JNK 2) can be induced by NGF, GDNF, and BDNF in cultured rabbit corneal epithelial cells.

Figure 5 shows that the effect of neurotrophic factors on phosphorylation of MAP kinases is different in cultured human corneal stromal keratocytes. Data presented in Figure 5A indicate that in comparison to the control in stromal keratocytes (lane 7) phosphorylation of ERK 1, and to a lesser extent of ERK 2, was induced by 200 ng/ml NGF (lane 1), and this increase was inhibited by PD 98059 (lane 2). In contrast 200 ng/ml BDNF did not induce phosphorylation of ERK 1 or ERK 2 compared with the control (lane 3) and remained unchanged with PD 98059 (lane 4). GDNF (200 ng/ml) induced ERK 1



**FIGURE 3.** Level of transcription of neurotrophic factors and corresponding tyrosine kinase receptors in cultured human corneal epithelial cells (**A**) and stromal keratocytes (**B**). Each DNA dot shown in *lanes 1* through *10* represents 0.1  $\mu$ g PCR product specific for NGF (*lane 1*), NT-3 (*lane 2*), NT-4 (*lane 3*), BDNF (*lane 4*), GDNF (*lane 5*), Trk A (*lane 6*), Trk B (*lane 7*), Trk C (*lane 8*), Trk E (*lane 9*), and GAPDH (*lane 10*). In concordance with the results shown in Figure 1, the transcription of NT-4 was only detectable in cultured human corneal epithelial cell line, and the transcription of GDNF was only detectable in cultured human corneal stromal keratocytes. For comparison, *lane 10* represents GADPH as positive control, which shows the strongest signal. NT-3, NT-4, Trk A, Trk B, and Trk C showed a higher level of transcription than NGF, BDNF, GDNF, and Trk E in corneal cells.



**FIGURE 4.** Effect of neurotrophic factors on phosphorylation of MAP kinase in cultured rabbit human corneal epithelium. Western blot analysis with antibodies against phosphorylated ERK 1 (44 kDa) and ERK 2 (42 kDa; **A**), total ERK 1 and ERK 2 (phosphorylated and nonphosphorylated; 44 and 42 kDa; **B**), phosphorylated JNK 1 (46 kDa), and JNK 2 (54 kDa; **C**) and against total JNK 1 and JNK 2 (phosphorylated and nonphosphorylated; 46 and 54 kDa; **D**) demonstrated that phosphorylated ERK 1, and to a lesser extent ERK 2, was induced in human epithelial cells cultured in NGF (*lane 1*), BDNF (*lane 3*), or GDNF (*lane 5*) in comparison to cells cultured in serumfree control medium (*lane 7*). Phosphorylation of ERK 1 by NGF and GDNF but not by BDNF was inhibited by addition of the MEK inhibitor PD 98059 (*lanes 2, 6,* and *4,* respectively). In contrast JNK 1/2 were not induced by NGF (*lane 1*), BDNF (*lane 2*), or GDNF (*lane 3*) compared with the control (*lane 4*).

(lane 5), and this increase was inhibited by PD 98059 (lane 6). Figure 5B shows the same level of total ERK in stromal keratocytes cultured either in serum-free medium or with the neurotrophic factors under investigation. Compared with ERK 1, the expression of both activated JNK 1 and JNK 2 in stromal keratocytes was relatively weak. There were slight differences concerning the expression of activated JNK 1, which was lower in cells cultured in serum-free DMEM (lane 4) than in cells stimulated with NGF (lane 1), BDNF (lane 2), or GDNF (lane 3). Among the neurotrophic factors, BDNF seemed to have the strongest effect on the activation of JNK 1 (lane 2). Activated JNK 2 in stromal keratocytes was not induced by neurotrophic factors (Fig. 5C). Figure 5D shows that the expression of total JNK 1 and JNK 2 was the same in stromal keratocytes regardless of the culture conditions. This result indicates that NGF, GDNF, and BDNF have different effects on the accumulation of phosphorylated forms of ERK 1 and JNK 1 in cultured human corneal stromal keratocytes.

#### Functional Role of Neurotrophic Factors in Cultured Corneal Epithelia and Stroma

All the neurotrophic factors under investigation had a significant effect on the proliferation of corneal epithelial cells: As shown in Figure 6A the number of colonies per dish increased significantly on the addition of recombinant human NGF (200 ng, P > 0.05), BDNF (200 ng, P < 0.05), or GDNF (50 ng, P <0.05, and 200 ng, P < 0.0001). This indicates that the ability of corneal epithelial cells to form colonies was enhanced by NGFs. However, this effect was much smaller than that of EGF, a growth factor that uses a signal transduction pathway similar to that of NGF as shown in Figure 6B. Perhaps more important is the effect on the clonal proliferation, which is reflected by the number cells within each colony (Fig. 7). As previously demonstrated, corneal epithelial cells were continuously entering cellular proliferation, which on day 6 resulted in a spectrum of colonies, ranging from very small colonies to very large colonies. This observation explains the relatively large SD and the requirement to count a large number of colonies (75) in each dish to obtain statistically meaningful data. The clonal proliferation of corneal epithelial cells was significantly stimulated by both recombinant NGF (50 and 200 ng/ml, P < 0.001) and GDNF (50 and 200 ng/ml, P < 0.001) as shown in Figure 7A. However, the addition of BDNF to the culture medium did not result in a significant increase in the number of cells per colony compared with the control (Fig. 7A). Again, the magnitude of the stimulatory effect on corneal epithelial proliferation was much smaller than that of EGF as shown in Figure 7B. These findings were observed not only on day 6 but also on day 12, a time point when neighboring colonies became confluent, thus precluding a numerical analysis. Figure 8 shows representative dishes stained with crystal violet. In serum-free control



**FIGURE 5.** Effect of neurotrophic factors on phosphorylation of MAP kinase in cultured human corneal stromal keratocytes. Western blot analysis with antibodies against phosphorylated ERK 1 (44 kDa) and ERK 2 (42 kDa; **A**), total ERK 1 and ERK 2 (phosphorylated and nonphosphorylated; 44 and 42 kDa; **B**), phosphorylated JNK 1 (46 kDa) and JNK 2 (54 kDa; **C**), and total JNK 1 and JNK 2 (phosphorylated and nonphosphorylated; 46 and 54 kDa; **D**) demonstrate that phosphorylation of ERK 1, and to a lesser extent of ERK 2, was induced by NGF (*lane 1*) and GDNF (*lane 5*) in comparison to serum-free control medium (*lane 7*) or BDNF (*lane 3*). Phosphorylation of ERK 1 by NGF and GDNF was inhibited by addition of the MEK inhibitor PD 98059 (*lanes 2* and 6). Phosphorylation of JNK 1 (**C**) was weakly induced by NGF (*lane 1*), BDNF (*lane 2*), and GDNF (*lane 3*) compared with serum-free medium (*lane 4*).



**FIGURE 6.** Effect of recombinant human NGF, BDNF, and GDNF (**A**) and EGF (**B**) on the colony formation of primary rabbit corneal epithelial cells on day 6 (mean values and standard deviations). Cells were cultured in a clonal density in supplemented serum-free MCDB. Addition of 200 ng/ml NGF, 200 ng/ml BDNF, or 200 ng/ml GDNF resulted in a statistically significant (P < 0.005, *asterisks*) increase of the total number of colonies. The effect of 10 ng/ml EGF on colony formation was significantly greater than the effect of the neurotrophic factors.

medium (Fig. 8A) only very few and very small colonies could be detected. In the presence of EGF, the number and size of the colonies had significantly increased (Fig. 8B). Similarly, but to a much weaker extent, the presence of NGF in a low concentration of 50 ng/ml led to an increase in number and size of colonies that became more obvious at a concentration of 200 ng/ml (Fig. 8D). In contrast neither 50 ng/ml nor 200 ng/ml BDNF led to a detectable increase in size or number of colonies in comparison to the serum-free control (Figs. 8E and 8F). Similar to NGF, GDNF in concentrations of 50 or 200 ng/ml also slightly enhanced the number and size of the colonies on day 12 (Figs. 8G and 8H). In summary, both NGF and GDNF stimulated the clonal proliferation of corneal epithelial cells, whereas BDNF had no effect.

In contrast to the stimulatory effect on corneal epithelial proliferation, the neurotrophic factors under investigation slightly inhibited the proliferation of human keratocytes when DMEM + 1% FBS was used as control (data not shown). However, when serum-free DMEM was used as control, the addition of either 20 or 100 ng/ml NGF, GDNF, or BDNF significantly enhanced the proliferation of stromal keratocytes (P < 0.005) as shown in Figure 9. These data indicate that

members of the neurotrophin family can enhance the proliferation of human stromal keratocytes in serum-free medium.

# DISCUSSION

Several lines of evidence suggest that corneal nerves are important for corneal function: Dysfunction of the sensory innervation for various reasons causes breakdown of the corneal epithelium and ulceration.<sup>22-25</sup> Such neurotrophic ulcers can be experimentally induced, and the assumption has been made that corneal nerves might release factors that are important for corneal function.<sup>22,26</sup> More specifically, NGF can regulate corneal sensitivity in mice which lack high-affinity NGF receptors display impaired corneal sensitivity.<sup>27,28</sup> Because NGF can heal human neurotrophic ulcers it might be released from corneal nerves to modulate proliferation of corneal epithelial cells.<sup>16</sup> However, transcription of neurotrophins in sensory nerve endings within the cornea has not been proven. Neurotrophins might also derive from other sources such as tears because NGF can be transcribed in lacrimal glands.<sup>29</sup> Data from the present study indicate that four cytokines encoded by the



FIGURE 7. Effect of recombinant human NGF, BDNF, and GDNF (A) and EGF (B) on the clonal proliferation of primary rabbit corneal epithelial cells on day 6 (mean values and standard deviations). Cells were cultured in a clonal density in supplemented serum-free MCDB. Addition of NGF (50 or 200 ng/ml) or of GDNF (50 or 200 ng/ml) but not of BDNF (50 or 200 ng/ml) resulted in a statistically significant (P < 0.005, asterisks) increase in the number of cells per colony. The effect of 10 ng/ml EGF on clonal proliferation was significantly greater than the effect of the neurotrophic factors.



**FIGURE 8.** Effect of recombinant human NGF, BDNF, and GDNF and EGF on proliferation of primary rabbit corneal epithelial cells on day 12. Cells were cultured in a clonal density in serum-free MCDB medium, and dishes were stained with crystal violet. In control medium (**A**) colonies were small and barely visible. In the presence of 10 ng/ml EGF (**B**) large colonies developed. NGF at a concentration of 50 ng/ml (**C**) or 200 ng/ml (**D**) dose-dependently increased the size of colonies over the control. In contrast BDNF did not stimulate clonal proliferation at 50 ng/ml (**E**) or 200 ng/ml (**F**). GDNF only slightly enhanced colony size at 50 ng/ml (**G**) but led to a significant increase in colony size at 200 ng/ml (**H**). Both NGF and GDNF failed to enhance clonal proliferation as much as EGF.

neurotrophin gene family (NGF, NT-3, NT-4, and BDNF) and corresponding receptors (Trk A-E) are present in the cornea. Furthermore, we found that GDNF, a member of the TGF- $\beta$  gene family that is related to neurotrophins,<sup>9,10</sup> is also expressed. These findings suggest that cytokines with pronounced effects on neuronal cells are also members of the corneal cytokine network.

Corneal cytokines have been classified on the basis of their expression.<sup>2,30</sup> Although most growth factors are ex-

pressed in both stroma and epithelium some are confined to only one cell type. These cytokines might be of importance for the interaction between epithelium and stroma.<sup>31</sup> Hepatocyte and keratocyte growth factors (HGF and KGF, respectfully), two paracrine mediators of epithelial function are expressed exclusively in stromal keratocytes.<sup>2,30,32,33</sup> We have recently shown that growth and differentiation factor-5 (GDF-5), a member of the TGF- $\beta$  family, is also exclusively expressed in stromal keratocytes.<sup>17</sup> In contrast to HGF and KGF, which stimulate corneal epithelial proliferation, GDF-5 was inhibitory. Our present data suggest that another member of the TGF- $\beta$  family, GDNF is also exclusively expressed in stromal keratocytes but stimulates proliferation of corneal epithelial cells. As with GDF-5 the proliferation of stromal keratocytes was not significantly affected by GDNF, which suggests a role as epithelial modulator. In contrast, NT-4 was exclusively expressed in epithelial cells. Therefore, this growth factor belongs to the same group of cytokines as transforming growth factor- $\alpha$  (TGF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and platelet-derived growth factor-B (PDGF-B), which are also exclusively expressed in the corneal stroma.<sup>2</sup> Interestingly, both TGF- $\alpha$  and IL-1 $\beta$  can upregulate the transcription of neurotrophins, such as NGF in 3T3 mouse fibroblasts. $^{\overline{34}}$  Although we did not carry out a functional characterization of either NT-3 or NT-4, the latter cytokine might be important for the regulation of stromal keratocytes.

Our results suggest a functional role of neurotrophins in the cornea. This is supported by the effects of neurotrophins on other tissues outside the central nervous system like, for example, the skin. NGF is produced in murine and human keratocytes, and mRNA and protein for NGF were detected in the wound margin.<sup>35-37</sup> In skin organ cultures NGF increased proliferation.<sup>38</sup> Furthermore, NGF accelerates the rate of wound contraction and healing in normal and diabetic mice.<sup>37,39</sup> These findings indicate that NGF plays an important role in cutaneous wound healing. NGF might also modulate corneal wound healing, and its expression might be upregulated in the context of wounding. This hypothesis is supported by recent observations in rats that demonstrated a transient increase in corneal NGF levels after wounding and by a rabbit model in which exogenous NGF stimulated the rate of corneal wound healing.14,40 During corneal wound healing several cytokines are released and modulate epithelial and stromal cells. One of the most versatile modulatory cytokines is IL-1,<sup>41</sup> which has been shown to upregulate the synthesis of NGF in cultured rat fibroblasts and keratinocytes and therefore provides a possible link between wounding and NGF expression.37,42 Also cutaneous wounding of mice leads to an increase in the NGF production of the salivary gland, which results in increased serum levels.<sup>37</sup> Although NGF has not been demonstrated in tears, corneal wounding might lead to an increase of NGF and possibly other neurotrophins in the lacrimal gland.

In human keratinocytes the proliferative effect of NGF has shown to be mediated by high affinity Trk receptor.<sup>35</sup> Protein for the Trk A receptor has been detected previously only on corneal epithelial cells and not in the stroma.<sup>16</sup> In contrast, our results indicate that ex vivo and cultured stromal cells express mRNA encoding for Trk A and that the expression level in both cell types is similar. Furthermore, corneal epithelial and stromal cells possess high affinity receptors for all members of the neurotrophin family. These receptors mediate physiological functions such as the observed mitogenic effect on corneal



epithelial cells. A comparison of the mitogenic effect of NGF with that of other growth factors (e.g., EGF) shows that NGF has only a weak effect. In contrast, NGF seems to have a significant (therapeutic) in vivo effect. This raises the question of its mode of action, including the mechanism of signal transduction. NGF and other neurotrophins may not only modulate transcription of cytokines but also modulate apoptosis of corneal epithelial cells.<sup>37,43</sup> The latter hypothesis is based on the finding that the ERK and JNK signal transduction pathways can have opposing effects on apoptosis.<sup>44</sup>

Binding of members of the neurotrophin gene family to tyrosine kinase receptors activates several distinct signaling pathways mediated by MAP kinases<sup>45,46</sup>: The Ras/ERK pathway involves activation of MAP kinase and ERK 1, ERK 2, or both, which then leads to the phosphorylation of a given transcription factor (such as Elk-1 or SAP-1).47 The second pathway dependent on MAP kinase involves phosphorylation of JNK 1, JNK 2, or both and is distinct from the ERK pathway because it phosphorylates transcription factors (such as Jun) at a different S/T-P motif.46 Each of the possible ligands of the membranous tyrosine kinase receptor can induce a different signaling cascade, and the exact composition of the cascade also depends on the cell type. In PC 12 cells and oligodendrocytes NGF has shown to bind to the Trk A receptor and to phosphorylate ERK 1 but not ERK 2.48,49 Similarly, our results demonstrate that NGF, BDNF, and GDNF predominantly phosphorylate ERK 1. The latter might induce additional signaling pathways such as, for example, the phosphatidylinositol-3 kinase pathway.<sup>50</sup> Interestingly, NGF, BDNF, and GDNF, which can phosphorylate JNK also in other cells,<sup>51</sup> have more effect on stromal than on epithelial JNK.

Further analysis of the signal transduction pathways might define the role of neurotrophic factors within the cytokine network of the cornea. Both NGF and EGF induce the MAP kinase cascade but differ in their effect on proliferation of the corneal epithelium. One possible explanation might be that different components of the MAP kinase system can lead to transcription of factors with opposing physiological effects. One of the initial steps in the MAP kinase cascade is the phosphorylation of the oncogenes Ras and Raf before activa**FIGURE 9.** Effect of recombinant human NGF, BDNF, and GDNF on proliferation of primary human corneal stromal cells on day 6. After culture in DMEM + 10% FBS, cells were plated at a low density in DMEM without FBS as processed (mean and SD). All neurotrophic factors led to a statistically significant induction of absorbance, which reflects the cell density compared with the control (P < 0.005, *asterisks*).

tion of ERK or JNK.<sup>52,53</sup> Both Ras and Raf can induce transcription of, for example, TGF- $\beta$ ,<sup>54</sup> and NGF can increase transcription and secretion of TGF-\beta1 in nonocular cells.55 TGF-\beta inhibits corneal epithelial cell proliferation.<sup>13,56</sup> Furthermore, not only TGF-\u00df1 through TGF-\u00bf3 but also bone morphogenetic proteins, growth, and differentiation factors, activins/inhibins, and receptors are transcribed in the cornea.<sup>17,57,58</sup> Although it has not been demonstrated that NGF induces transcription of other cytokines in corneal epithelial cells, neurotrophins might induce TGF- $\beta$  family members. This might explain the difference in the effects of neurotrophins and EGF on corneal epithelial proliferation. In addition, downstream components of the MAP kinase cascade can interfere with other signaling systems. Activation of ERK induced by EGF also results in phosphorylation of signaling components induced by TGF-B such as the protein "similar to mothers against decapentaplegic-1" (Smad-1).<sup>59,60</sup> In response to members of the TGF- $\beta$  super family, the carboxyl-terminal domain of Smads is essential for the phosphorylation of Smad 1,5 or Smad 2 and 3, association with Smad 4, translocation into the nucleus, and transcriptional response.<sup>61-63</sup> It has been suggested that the Smad and JNK signaling pathways converge at AP1-binding promoter sites of several genes.<sup>64</sup> Further investigations are needed to determine possible links between the NGF signaling and TGF- $\beta$  signaling pathways in cells of the cornea.

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