Nerve Growth Factor Attenuates Apoptosis and Inflammation in the Diabetic Cornea

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Apoptosis and inflammation are enhanced in the diabetic cornea; NGF attenuates these responses—both in vivo and in vitro. Therefore, NGF therapy may represent a novel approach for the management of diabetic keratopathy.

Keywords: apoptosis, inflammation, diabetes, cornea, nerve growth factor

Diabetes is a major endocrine disease worldwide, and its prevalence is increasing.1 The most important ocular complication associated with diabetes is diabetic retinopathy; nonetheless, diabetic keratopathy is also a common complication.2 Among the well-recognized corneal changes associated with diabetic keratopathy,3 epithelial wound healing and nerve function have been most widely studied, mainly because they are associated with severe sight-threatening complications of diabetic keratopathy, such as neurotrophic ulcer, stromal opacification, surface irregularities, and microbial keratitis.4 However, the exact pathophysiology of diabetic keratopathy remains unknown, and it is difficult to explain the various aspects of the disease by focusing solely on wound healing and nerve function.

The symptoms of this condition are similar to those of dry eye syndrome, for example, foreign body sensation and changes in vision.5,6 Diabetic keratopathy is also similar to dry eye syndrome in terms of its clinical manifestations5,6: superficial punctate keratitis,7 recurrent erosions, and persistent epithelial defects.8 In mild cases of diabetic keratopathy, the cornea may even appear disease-free on slit-lamp biomicroscopy; nevertheless, patients complain of various symptoms that may be related to apoptosis and mild inflammation. Currently, such patients receive no targeted therapy for these symptoms.

Several factors have been proposed to cause diabetic complications across many tissues: upregulation of the polyol pathway, activation of protein kinase C, hexosamine pathway flux, increased levels of advanced glycation end products (AGEs) arising from nonenzymatic reactions, and so on.9–11 However, the recent literature suggests that all diabetic complications are the result of the following mechanism: Hyperglycemia leads to reactive oxygen species (ROS) production, and the resulting oxidative stress in turn induces apoptosis.12 Indeed, several studies have reported that increased ROS production is associated with pathologic diabetic consequences in many tissues.13,14 Our own investigations have confirmed that high levels of glucose promote apoptosis and inflammation in conjunctival epithelial cells.15 Moreover, the ROS-induced apoptosis observed in high-glucose environments may delay epithelial wound healing in the diabetic cornea.16,17

With specific regard to the cornea, nerve growth factor (NGF) plays an important role in pathophysiology, and exogenous NGF accelerates epithelial healing—both in rats and in humans.18 Moreover, NGF levels in tears are elevated in patients with keratoconjunctivitis sicca.19 and plasma NGF levels are raised in patients with vernal keratoconjunctivitis.20 Similarly, serum NGF may be associated with altered corneal nerve fiber...
length and nerve branch density in patients with diabetic peripheral neuropathy.\textsuperscript{21} We recently performed an in vitro study in which NGF may have attenuated apoptosis in cultured primary human conjunctival epithelial cells (HCECs) exposed to hyperosmolar stress.\textsuperscript{22} These findings suggested that NGF plays an important additional role in the regulation of innervation in the diabetic cornea, using human corneal epithelial cells (HCECs) and the streptozotocin (STZ)-induced diabetic rat cornea.

\section*{MATERIALS AND METHODS}

\subsection*{Corneal Epithelial Cell Line}
Telomerase-immortalized HCECs were kindly donated by James V. Jester (Gavin Herbert Eye Institute, University of California, Irvine, CA, USA).\textsuperscript{23} The cells were cultured in bronchial epithelial growth medium (Lonza, Walkersville, MD, USA) and the important additional role in the regulation of innervation in diabetes enhances apoptosis and inflammation by promoting ROS production in the cornea, and that this effect can be reversed by NGF Therefore, the aim of the current study was to examine the antiapoptotic and anti-inflammatory effects of NGF on the diabetic cornea, using human corneal epithelial cells (HCECs) and the streptozotocin (STZ)-induced diabetic rat cornea.

\subsection*{NGF and NGF mRNA Levels in HCECs}
Human CECs were cultured in glucose-added medium (0, 5, 25 mM) for 24 hours; total RNA was then isolated using the RNeasy mini kit in accordance with the manufacturer’s protocol (Qiagen, Inc., Hilden, Germany). First-strand cDNA was synthesized from 0.5 µg total RNA using murine leukemia virus reverse transcriptase (Fermentas, Inc., Foster, CA, USA). The primer sequences specific for the NGF transcripts (GenBank accession no. NM_002506) were 5'-CAG GGT CA-3' (forward) and 5'-TGA TGA CCG GCT CTT GCT GTT AT-3' (reverse); for the tropomyosin receptor kinase A (TrkA) transcripts, the primers were 5'-GGC AAG GTC ATC CAT AAC-3' (forward) and 5'-TGG CAT GTC CAT GGG AF-3' (reverse); and for the tropomyosin receptor kinase A (TrkA) transcripts, the primers were 5'-GGC AAG GTC ATC CAT AAC-3' (forward) and 5'-TGG CAT GTC CAT GGG AF-3' (reverse); GenBank accession no. NM_001012331); for the tropomyosin receptor kinase A (TrkA) transcripts, the primers were 5'-GGC AAG GTC ATC CAT AAC-3' (forward) and 5'-TGG CAT GTC CAT GGG AF-3' (reverse); GenBank accession no. NM_001012331); for the tropomyosin receptor kinase A (TrkA) transcripts, the primers were 5'-GGC AAG GTC ATC CAT AAC-3' (forward) and 5'-TGG CAT GTC CAT GGG AF-3' (reverse); GenBank accession no. NM_001012331). The concentrations of β-NF in the supernatant were determined using a human ELISA kit (Quantikine; R&D Systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer’s protocol.

\subsection*{Measuring Intracellular ROS in HCECs}
The HCECs were seeded onto six-well plates. After cell attachment and growth for 1 day, the cells were treated with the respective concentrations of glucose (0, 5, or 25 mM) with or without 20 ng/mL human recombinant NGF; catalog no. 256-GE; R&D Systems, Inc.) for 24 hours at 37°C. The cells were then incubated to allow treatment-induced ROS production. We measured intracellular ROS levels in HCECs by incubating the cells with the redox-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Life Technologies, Grand Island, NY; 10 µM) at 37°C for 30 minutes in the dark; the cells were then detached from the culture wells using 0.25% trypsin-EDTA and washed twice using ice-cold phosphate-buffered saline (PBS). Flow cytometric measurements (BD Biosciences, Inc., San Diego, CA, USA) were performed six times for each treatment. The mean fluorescence intensity was quantified using CELLQuest software (BD Biosciences, Inc.). Cells that had been incubated without DCFH-DA were used as the negative control. The distribution of fluorescent DCF on the cell monolayer was visualized and photographed under a fluorescence microscope (Olympus, Tokyo, Japan).

\subsection*{Assessing Apoptosis in HCECs}
The HCECs were cultured in media with the respective glucose concentrations, with or without human NGF, for 24 hours. Cells were then harvested and washed using ice-cold PBS. Cell pellets were collected by centrifugation, resuspended in binding buffer including 0.01 M HEPES/NaOH (pH 7.4), 0.14 M NaCl, 2.5 mM CaCl₂, and incubated with fluorescein isothiocyanate (FITC)-labeled annexin-V (BD Biosciences, Inc.) and propidium iodide (PI; BD Biosciences, Inc.) at room temperature in the dark for 15 minutes. Cells were then analyzed using fluorescence-activated cell sorting Calibur flow cytometry (BD Biosciences, Inc.).

\subsection*{Western Blot Analysis in HCECs}
The HCECs were cultured in glucose media with or without NGF for 24 hours; they were then collected and lysed using a lysis solution (10 mM Tris, 10 mM NaCl, 2 mM EDTA, 25 mM NaF, 2 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF), proteinase and phosphatase inhibitor cocktail, 0.5% Triton X-100, pH 7) to detect the levels of cleaved caspase-3, Bcl-2-associated X protein (BAX), NF-kB inhibitor α (IkBα) and nuclear factor-kB (NF-kB)-p65. In each case, the total protein concentration in the supernatant was determined using the Bradford method. Aliquots of protein (30 µg) were boiled in equal volumes of Laemmli sample and were loaded into a 12% or 15% acrylamide gel and SDS-PAGE and were electrophoretically transferred to nitrocellulose filters (Amersham, Little Chalfont, UK). The blots were treated with antibodies against cleaved caspase-3 (1:1,000; catalog no. 9661; Cell Signaling Technology, Danvers, MA, USA), BAX (1:1,000; catalog no. 2772; Cell Signaling Technology), IkBα (1:1,000; catalog no. 9247; Cell Signaling Technology), NF-kB-p65 (1:1,000; catalog no. sc-109; Santa Cruz Biotechnology; Santa Cruz, CA, USA), and with an antibody for β-actin (1:1,000; Sigma-Aldrich Corp.) used as a loading control for overnight at 4°C. The membranes were incubated with horseradish peroxidase–conjugated anti-IgGs (1:10,000) (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The respective target proteins of the antibodies were visualized using enhanced chemiluminescence reagents (Santa Cruz Biotechnology).

\subsection*{Multiplex Cytokine Analysis in HCECs}
Concentrations of the proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in the supernatants of HCECs cultured for 24 hours in normal or high-glucose medium, with or without NGF, were determined using inflammatory cytokine human magnetic 5-plex panel (catalog
The control and diabetic control groups received one drop (40 μL) of PBS three times a day. The diabetic NGF group received one drop of rat NGF-β (200 μg/mL; catalog no. N2513; Sigma-Aldrich Corp.) three times a day.

**Animal Model**

Six-week-old male Sprague-Dawley rats with an initial weight of 150 to 160 g were acclimated for 1 week. The rats were randomly divided into three groups: diabetic (n = 6), diabetic NGF (n = 6), and age-matched normal control (n = 6) groups. Diabetes was induced by intraperitoneal injection of 65 mg/kg STZ in ice-cold 0.5 M citrate buffer (pH 4.5). The control group received the citrate buffer only. After 24 hours, the glucose levels were determined in the rats that had received the injection. A glucose concentration > 300 mg/dL in heparinized tail vein blood (measured using a glucometer) was considered as indicating successful induction. All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were reviewed and approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences at the Asan Medical Center. The committee abides by the Institute of Laboratory Animal Resources guide.

All animals received their respective eye drops three times a day during the experimental period, beginning 1 day after the injection. The control and diabetic control groups received one drop (40 μL) of PBS three times a day. The diabetic NGF group received one drop of rat NGF-β (200 μg/mL; catalog no. N2513; Sigma-Aldrich Corp.) three times a day.

**Histopathology and Immunohistochemistry in the Rat Cornea**

In all three groups, 3 weeks after intraperitoneal injection, the eyes were enucleated; all the corneas were buttoned and fixed in 10% phosphate-buffered formalin for 24 hours before being embedded in paraffin wax. The eyes were then cut into 4-μm-thick sections and stained using hematoxylin and eosin. Cleaved caspase-3 and IL-1β expression was analyzed by immunohistochemistry (IHC; Envision-HRP detection system; Dako, Carpinteria, CA, USA). After deparaffinization and antigen retrieval using 0.01 M sodium citrate buffer (pH 6.0) in a pressure cooker at full power for 10 minutes, tissue sections were treated with 3% H2O2 for 5 minutes. The control group received the citrate buffer only. After 24 hours, the blood glucose levels were determined in the rats that had received the injection. A glucose concentration > 300 mg/dL in heparinized tail vein blood (measured using a glucometer) was considered as indicating successful induction. All experiments conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were reviewed and approved by the Institutional Animal Care and Use Committee of the Asian Institute for Life Sciences at the Asan Medical Center. The committee abides by the Institute of Laboratory Animal Resources guide.

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**Apoptosis Assay in the Rat Cornea**

Apoptosis was detected under different conditions using the TdT-mediated dUTP nick-end labeling (TUNEL) technique (Cell Death Detection Kit; Roche Diagnostics, Seoul, South Korea), which labels the cut ends of DNA fragments in the nuclei of apoptotic cells. The nuclei of all cells were stained by 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

**Statistical Analysis**

All quantitative experiments were performed at least six times, and data are shown as the mean ± standard deviation. Statistical significance was determined using Student’s t-test, and P values < 0.05 were considered statistically significant.
RESULTS

Human NGF mRNA in HCECs Exposed to High Glucose Levels

Levels of NGF transcripts and secreted NGF were determined in HCECs exposed to normal-glucose (5 mM) or high-glucose medium (25 mM) for 24 hours. Levels both of NGF mRNA and of NGF secretion were significantly lower after exposure to high glucose levels ($P < 0.05$; Figs. 1A, 1B). Tropomyosin receptor kinase A expression was checked by RT-PCR, which mediates NGF activity as receptor, and the mRNA was significantly lower in high glucose ($P < 0.05$; Fig. 1C).

FIGURE 2. Fluorescence of 2',7'-dichlorofluorescin diacetate (DCFH-DA) oxidized by intracellular ROS was detected by flow cytometry and quantitatively measured. The mean fluorescence intensity of the flow cytometry was quantified as mean ± SD. $^*$ $P < 0.05$ compared with normal glucose. $^{**} P < 0.05$ compared with high glucose. High glucose generates dichlorofluorescin (DCF)-sensitive reactive oxygen species (ROS) in human corneal epithelial cells (HCECs), and nerve growth factor (NGF) reduces high glucose–induced ROS. HCECs were cultured for 24 hours in medium containing normal glucose (NG; 5 mM), high glucose (HG; 25 mM), or high glucose with NGF (20 ng/mL); they were then incubated with cell-permeable DCFH-DA (10 μM) for 45 minutes. Cells were detached using trypsin digestion and washed with PBS. (A) Distribution of fluorescent DCF on the cell monolayer, photographed under a fluorescence microscope. Representative data of one of six experiments are shown. (B) Quantitative flow cytometric measurements.

FIGURE 3. Hematoxylin and eosin staining of the corneas of the three experimental groups, control (Con), diabetic (DM), and diabetic NGF (DM+NGF), and quantitative analysis of total corneal thickness in the three groups. (A) Histopathologic analysis in the cornea of diabetic rat showing perinuclear clear areas and thickened basement membranes in the corneal epithelium, as well as stromal edema. These changes in the diabetic cornea were partially reversed by NGF treatment. Representative data of one of six experiments are shown. Magnification: ×100. (B) Mean differences in total corneal thickness in the control, diabetic, and diabetic with NGF treatment groups.
ROS in HCECs Exposed to High Glucose Levels

When HCECs were exposed to high glucose levels (25 mM), more cells had high-intensity fluorescence than when the cells were cultured in normal glucose (5 mM; Figs. 2A, 2B). Furthermore, fluorescence intensity was greater in cells exposed to high glucose levels than in those incubated in normal glucose, a conclusion that could be confirmed using fluorescent microscopy (Figs. 2A, 2B). The increased fluorescence intensity by high glucose levels was attenuated by NGF treatment (Figs. 2A, 2B).

Histopathologic Analysis in the Rat Cornea

In the corneal epithelium of the STZ-induced diabetic rat, perinuclear clear areas and thickened basement membranes were observed (Fig. 3A). Furthermore, the corneal stroma was thicker due to edema (Fig. 3A). The total thickness of the diabetic cornea was significantly higher than that of the normal cornea (Fig. 3B). These changes in the diabetic cornea were consistently reversed by NGF treatment (Figs. 3A, 3B).

Apoptosis in HCECs and Rat Cornea

The percentage of FITC-positive cells, which was 12.1% in normal-glucose medium, increased to 25.2% in high-glucose medium (Fig. 4A). In high-glucose medium with NGF treatment, the percentage of FITC-positive cells was 12.4%, implying that the apoptosis induced by high glucose had been partially suppressed by treatment with NGF (Fig. 4A). The percentage of PI-positive cells, which was 4.7% in normal-glucose medium, increased to 14.2% in high-glucose medium. The necrosis induced by high glucose had been partially suppressed (7.9%) by NGF treatment (Fig. 4A). Western blot analysis revealed that the levels of cleaved caspase-3 and BAX were significantly increased in a high-glucose environment.
(Fig. 4B). However, NGF markedly suppressed the high glucose-induced increase in levels of cleaved caspase-3 and BAX (Fig. 4B).

In the corneas of the control group, few TUNEL-positive nuclei were detected (Fig. 5). In the diabetic corneas, many TUNEL-positive cells, as well as some fragmented nuclei, were observed across the entire cornea; TUNEL-positive cells were seen in many corneal epithelial cells in particular (Fig. 5). Conversely, barely any TUNEL-positive nuclei were observed in the diabetic NGF group (Fig. 5). That is, the NGF treatment markedly reversed the apoptotic response in the diabetic cornea.

Immunohistochemically, the epithelium, stroma, and endothelium of the diabetic cornea showed intense staining for cleaved caspase-3 (Figs. 6A, 6C). However, no immunohistochemical staining for cleaved caspase-3 was observed in the diabetic cornea with NGF treatment (Figs. 6B, 6D).

**DISCUSSION**

Previous studies have confirmed that dry eye symptoms in diabetes are correlated with the severity of the diabetic retinopathy. In relation to this, several investigators have suggested that dry eye symptoms in diabetes are associated with peripheral polyneuropathy caused by reduced corneal sensitivity, altered homeostasis of the cornea, or ocular surface dysfunction caused by tear film instability and reduced tear secretion. Nevertheless, the exact pathophysiological changes that occur in the diabetic cornea remain controversial.

Recently, several studies have suggested that the initial step in the immunopathogenic mechanism responsible for dry eye syndrome is desiccating stress on ocular surface epithelial cells. The glucose concentration in tears is tightly linked to blood glucose, as in dry eye syndrome. Therefore, we hypothesized that high glucose-induced biochemical and ultrastructural changes in the cornea, especially in the corneal epithelium, lead to desiccating stress, and that such changes are the most important factor leading to dry eye in diabetic patients whose corneas look disease-free—more important than structural changes in the nerves or basement membrane.

It has been suggested that NGF plays a role in ocular surface healing and sensitivity in corneal and conjunctival cells. Moreover, Kang et al. showed that NGF has an antiapoptotic effect in hyperosmotic stress-induced apoptosis of conjunctival epithelial cells. The results show that NGF mRNA and NGF production are significantly reduced in HCECs cultured in high glucose. Next, to assess whether high glucose levels enhance ROS, apoptosis, and inflammation in corneal epithelial cells, as well as whether NGF can reverse these pathologic changes, we evaluated ROS, apoptosis, and inflammation in HCECs cultured in high glucose, with or without NGF. With a view to obtaining in vivo evidence, we then investigated histopathologic, apoptotic, and inflammatory changes in the corneas of diabetic rats, with or without NGF treatment.

The results show that NGF mRNA and NGF production are significantly reduced in HCECs cultured in high glucose, and that high glucose levels affect mitochondria-dependent caspase activity, enhancing ROS production and increasing the number of apoptotic cells. In addition, high glucose levels significantly enhance expression of NF-kB-p65, as well as of the proinflammatory cytokines IL-1β and TNF-α in HCECs. This suggests that ROS is associated with NF-kB activation, and that it may enhance the inflammatory response in HCECs, as well as in the tissues or cells of diabetic patients. That is, we have demonstrated that high glucose levels enhance ROS, apoptosis, and inflammation in HCECs. Furthermore, we have shown that imaging mitochondria-dependent caspase activity—enhanced apoptosis in the corneas of STZ-induced diabetic rats. Notably,
although apoptotic cells were detected across the entire cornea, the strongest apoptotic responses were detected in basal corneal epithelial cells, perhaps because apoptosis is an intracellular reaction, and the basal corneal epithelial cell layers are the greatest in number and have the strongest cellular activity. Our results also show that enhanced inflammation in the cornea of STZ-induced diabetic rats is associated with IL-1β. Unlike apoptosis, immunoreactivity for IL-1β was detected evenly across the entire cornea, suggesting that secreted inflammatory cytokines from damaged cells are distributed across the cornea. Histopathology demonstrated corneal stromal edema and active apoptotic changes in corneal epithelium. That is, enhanced apoptosis and inflammation in the diabetic cornea can be demonstrated both histopathologically and immunohistochemically.

Nevertheless, the most important finding in our current study is that NGF can reverse all these phenomena—both in vitro and in vivo. Taken together, our results indicate that diabetic hyperglycemia enhances apoptosis and inflammation in the cornea—especially in the corneal epithelium—and that this occurs without any severe neurotrophic ulcerative changes. This may explain the unusually severe symptoms of dry eye that occur in patients with diabetes whose corneas appear disease-free. In addition, our results confirmed that NGF can successfully suppress this enhanced apoptosis and inflammation in the diabetic cornea.

There is evidence that high glucose can enhance apoptosis, ROS production, and inflammation. And a number of studies have been done in both humans and animal models to investigate the ability of topical NGF to accelerate the healing
process in cornea ulcers. However, to our knowledge, the current study is the first to identify that NGF can act as an antiapoptotic and anti-inflammatory agent in the diabetic cornea, which has been demonstrated by both in vitro and in vivo experiments.

In summary, high glucose in diabetes induces apoptosis and inflammation in the cornea by promoting ROS formation; these changes are most apparent in the corneal epithelial cells. Furthermore, the biochemical changes associated with apoptosis and inflammation occur without severe structural change, and this may explain the dry eye symptoms seen in diabetic patients. We have provided evidence that these pathologic changes can be reversed by NGF treatment. Hence, NGF could be used to treat mild to severe diabetic keratopathy, and targeted prevention of apoptosis may represent a novel therapeutic approach to managing the diabetic cornea. The main limitation of our current study was its in vitro and animal model design; in future, nonrandomized and randomized clinical trials will be needed to confirm our findings.

**Figure 7.** Western blot analysis for nuclear factor (NF)-κB-p65 and IκBα, and multiplex cytokine analysis for interleukin (IL)-1β and tumor necrosis factor (TNF)-α, in human corneal endothelial cells (HCECs) treated with the indicated concentrations of glucose. Nerve growth factor (NGF) inhibits high glucose–activated NF-κB and inflammatory cytokines in HCECs. (A) The expression of NF-κB-p65 (nuclear fraction) and IκBα was analyzed and quantified by Western blotting, using β-actin as a control, after treatment for 24 hours with normal glucose (NG; 5 mM), high glucose (HG; 25 mM), or high glucose with NGF (20 ng/mL). Representative data of one of six experiments and quantitative mean densitometry results are shown. (B) After identical treatment, IL-1β and TNF-α expression were quantified using multiplex cytokine analysis. The data are presented as the mean ± SD of at least six independent experiments. *P < 0.05 compared with normal glucose. **P < 0.05 compared with high glucose.

**Figure 8.** Immunofluorescence staining for interleukin (IL)-1β in the cornea of the three experimental groups: control (Con), diabetic (DM), and diabetic NGF (DM+NGF). (A) Representative photomicrographs of the corneas of control rats (magnification: ×100). (B) Representative photomicrographs of the corneas of diabetic rats (magnification: ×100). (C) Representative photomicrographs of the corneas of diabetic rats after NGF treatment (magnification: ×100). Diabetic corneal epithelial, stromal, and endothelial cells showed strong immunoreactivity for IL-1β. Only weak or no immunoreactivity for IL-1β was seen in the diabetic cornea after NGF treatment.
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