

Effect of Nerve Growth Factor on the In Vitro Induction of Apoptosis of Human Conjunctival Epithelial Cells by Hyperosmolar Stress

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PURPOSE. To evaluate the effects of nerve growth factor (NGF), which is activated during inflammatory episodes of ocular diseases, on the apoptotic response in cultured human primary conjunctival epithelial cells (pHCECs).

METHODS. Levels of *NGF* transcripts and NGF protein in pHCEC grown in medium with normal osmolarity (307 mOsm/L) or hyperosmolar medium (350, 400, and 450 mOsm/L) were determined using RT-PCR or ELISA, respectively. To assess apoptosis, pHCEC were cultured in normal or 400 mOsm/L hyperosmolar medium with neutralizing anti-NGF antibody or recombinant human NGF for 6 hours before analysis by flow cytometry. Levels of Bcl-xL, Bax, phosphorylated JNK, and cleaved caspase-3 were detected using Western blotting. Levels of the inflammatory cytokine IL-6 was analyzed using ELISA.

RESULTS. Hyperosmolar conditions increased NGF levels in cultured pHCECs. Hyperosmolarity and exposure to neutralizing anti-NGF antibody significantly increased the number of apoptotic cells. Addition of recombinant human NGF to 400 mOsm/L medium decreased the number of apoptotic cells, reduced the expression of phosphorylated JNK, Bax, and cleaved caspase-3 and increased the expression of Bcl-xL. Levels of IL-6 were increased by hyperosmotic stress but decreased by exposure to recombinant human NGF.

CONCLUSIONS. Hyperosmolarity induces apoptosis of pHCECs by activating JNK signaling. Increased levels of NGF under hyperosmolar conditions may contribute, at least in part, to the reduced apoptosis of pHCECs and may be beneficial in recovering conjunctival damage due to chronic hyperosmolar stress.

Keywords: apoptosis, human primary conjunctival epithelial cells, hyperosmolarity, nerve growth factor

Dry eye is one of the most common ocular pathologies seen in ophthalmic practice; it is considered a disease of the ocular surface functional units consisting of corneal and conjunctival epithelia, lachrymal glands, meibomian glands, eyelids, and connecting neural networks.¹ Dry eye is accompanied by hyperosmolarity of the tear film and inflammation of the ocular surface.² Indeed, tear hyperosmolarity is now thought to be the key pathogenic factor behind ocular irritation, which is mediated by the inflammation of corneal epithelial cells, induced by activation of the epithelial signaling molecular cascade.³⁻⁶ Such a cascade results in the release of proinflammatory mediators into the tears,⁷⁻⁹ the death of local apoptotic cells,^{10,11} and a reduction in the number of conjunctival goblet cells,¹² as demonstrated in both experimental models and humans. Hyperosmolarity is the main pathogenic factor in keratoconjunctivitis sicca (KCS), which is accompanied by damage to the eye surface and might provide new insights into the relationship between physiological stress and inflammatory responses.

Nerve growth factor (NGF), which was originally defined as a target-derived neurotrophin, is produced during neuronal growth and differentiation and is involved in the differentiation

and survival of peripheral as well as central nerve tissue.¹³ The release of NGF by structural cells (corneal and conjunctival fibroblasts and epithelial cells) and local and infiltrating immune cells (macrophages, lymphocytes, neutrophils, mast cells, and eosinophils) during corneal healing and chronic inflammation of the ocular surface suggests that it may also function outside of the nervous system. Normal human ocular surface epithelial cells synthesize and secrete biologically active NGF and express both of the NGF receptors p75NTR and trkA.^{14,15} Corneal injury increases the levels of NGF in both the tears and corneas in animals and humans.^{16,17} Moreover, the NGF concentration in tears is increased in patients with dry eye, and serum NGF levels are higher in patients affected by vernal keratoconjunctivitis (VKC) than in non-VKC control patients.^{17,18} These chronic inflammatory diseases are characterized by important changes to the ocular surface, including accumulation of mucins and alteration of goblet cells.¹⁹⁻²¹ In addition, it has been demonstrated that rat goblet cells express NGF receptors and that in vitro NGF treatment elevates goblet cell secretion in a dose-dependent manner.²² Last, NGF treatment increases the density of conjunctival goblet cells and improves the stability of tear films in an animal model of dry eye.²³ Increased levels of NGF in

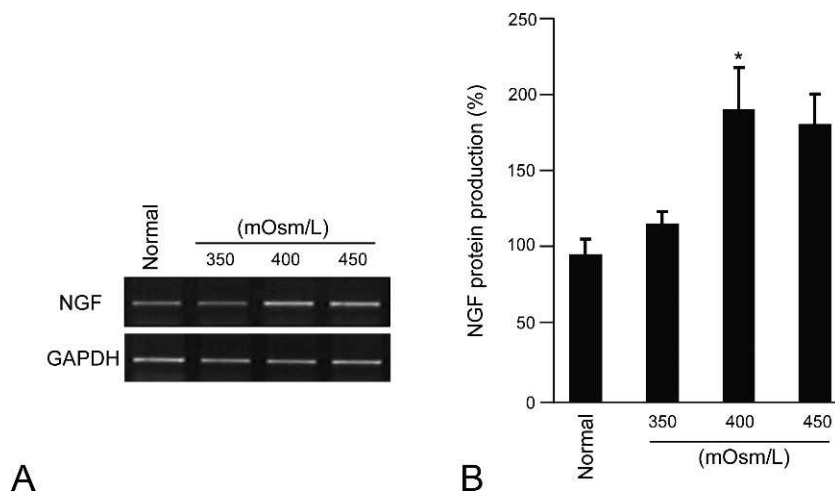


FIGURE 1. (A) NGF mRNA expression level is shown by semiquantitative RT-PCR using GAPDH as an internal control. (B) NGF protein production is shown by ELISA in human primary conjunctival epithelial cells cultured in normal and hyperosmolar medium (350, 400, or 450 mOsm, generated by adding 25, 50, or 75 mM NaCl, respectively, for 24 hours). * $P < 0.05$ compared with normal osmolarity medium.

tears from KCS patients were found to be decreased by treatments with anti-inflammatory corticosteroid.^{17,24} These findings suggest an additional role for NGF in the corneal epithelium, which links hyperosmotic stress to the production of proinflammatory cytokines.

Given that conjunctival epithelial cells cover >90% of the ocular surface, they could be important sources of cytokines and chemokines. Thus, these cells play an important role in inflammation at the ocular surface.^{25,26} However, the role of conjunctival epithelial cells in the pathogenesis of dry eye has not been clearly demonstrated. The aim of this study, therefore, was to evaluate whether a direct link exists between hyperosmolarity and the NGF response in cultured human primary conjunctival epithelial cells (pHCECs) thereby determine the potential for using conjunctival epithelial cells to assess dry eye-induced damage of the ocular surface. On the basis of our objective, we determined the antiapoptotic and anti-inflammatory effects of NGF in pHCECs.

MATERIALS AND METHODS

pHCEC Isolation and Culture

Human conjunctivas were isolated from 6 eyes donated by 3 expired or brain-dead individuals. The study protocol was approved by the institutional review board of Asan Medical Center. The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained from the appropriate family members. Conjunctival specimens were incubated for 1 hour at 37°C with 0.1% protease (Sigma-Aldrich, Inc., St. Louis, MO) in a 1:1 mixture of Dulbecco modified Eagle's medium and Ham's F12 nutrient mixture (DMEM/F12; Gibco, Life Technologies, Inc., Carlsbad, CA) supplemented with 1% penicillin-streptomycin (Gibco). The loosened cells were scraped with a pipette, washed 3 times, and suspended in DMEM/F12 supplemented with 1% penicillin-streptomycin (Gibco) and 10% fetal bovine serum (FBS; Gibco). Cells were preplated on 60-mm plastic culture dishes for 1 hour at 37°C in a humidified, 5% CO₂ atmosphere in order to eliminate fibroblasts by differential attachment. Suspended epithelial cells were seeded at 3 × 10⁴ cells per dish in 60-mm plastic culture dishes. Two cultures were seeded from each biopsy specimen. The culture medium used was bronchial epithelial growth

medium (Clonetics Corp., Walkerville, MD) supplemented with insulin (5 mg/mL [Clonetics Corp.]), hydrocortisone (0.5 mg/mL [Clonetics Corp.]), epinephrine (0.5 mg/mL [Clonetics Corp.]), triiodothyronine (6.5 ng/mL [Clonetics Corp.]), transferrin (10 ng/mL [Clonetics Corp.]), retinoic acid (10 ng/mL [Clonetics Corp.]), bovine pituitary extract (0.13 mg/mL [Clonetics Corp.]), a gentamicin-amphotericin solution (50 mg/mL:50 ng/mL mixture [Clonetics Corp.]), human epidermal growth factor (10 ng/mL; Sigma-Aldrich, Inc.), and BSA (0.15 mg/mL; Sigma-Aldrich, Inc.). Culture medium was changed 1 day after seeding and every other day thereafter until the cultures reached ~60% to 70% confluence for 5 to 6 days, at which time they were dissociated with 0.25% trypsin-EDTA (Clonetics Corp.) as previously described.^{27,28}

Imposition of Hyperosmolar Stress on pHCECs

To induce hyperosmotic stress, subconfluent pHCECs in normal osmolarity medium (307 mOsm/L) were transferred to a higher osmolarity medium (350, 400, and 450 mOsm/L) generated by adding 25, 50, or 75 mM NaCl, respectively, for 24 hours. Medium samples were collected and centrifuged (1,000g for 10 minutes), and the supernatants were stored at -70°C. For RT-PCR analysis of NGF and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), these cultures were subjected to the same conditions described above. All experiments were performed at least 3 times with each of 3 separate sets of cultures that were initiated from different donors.

Semiquantitative RT-PCR

Total RNAs were isolated from conjunctival epithelial cells cultured in normal or high-osmolarity saline-added medium for 24 hours by using the RNeasy mini kit according to the manufacturer's protocol (Qiagen, Inc., Hilden, Germany). First-strand cDNAs were synthesized from 0.5 μg of total RNA with murine leukemia virus (MuLV) reverse transcriptase (Fermentas, Inc., Foster, CA). The primer sequences specific for NGF transcripts (GenBank accession no. NM_002506) were 5'-CAC ACT GAG GTG CA-3' (forward) and 5'-TGA TGA CCG CTT GCT CCT GT-3' (reverse) and 5'-GCC AAG GTC ATC CAT GAC AAC-3' (forward) and 5'-GTC CAC CAC CCT GTT GCT GTA-3' (reverse) for GAPDH transcripts (GenBank accession no. NM_002046.3).

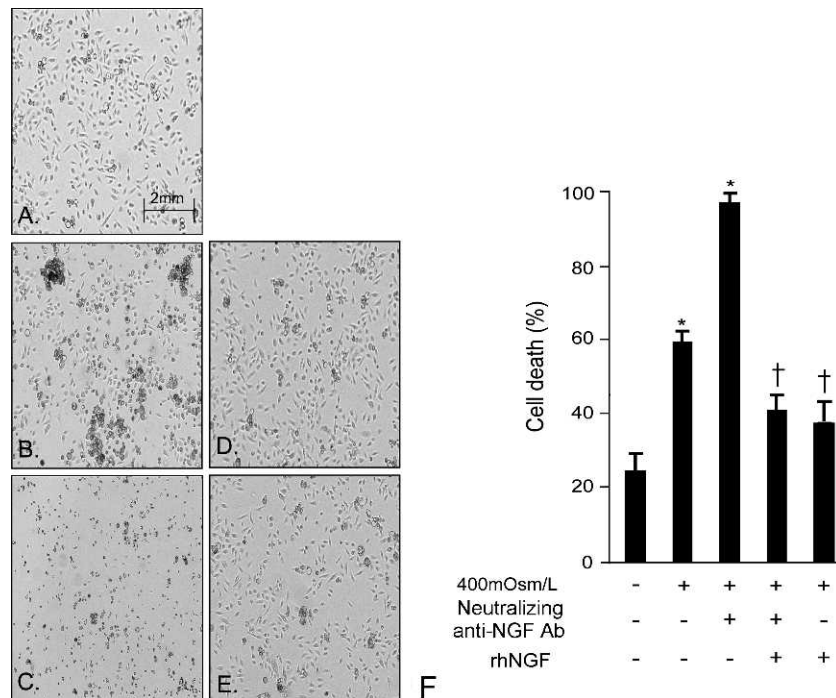


FIGURE 2. Representative levels of cell death are shown in human primary conjunctival epithelial cells cultured for 24 hours in normal medium (A), high-osmolarity medium (400 mOsm) containing additional NaCl (B), 400 mOsm medium supplemented with neutralizing anti-NGF antibody (C), 400 mOsm medium supplemented with recombinant human NGF added (D), or 400 mOsm medium supplemented with both neutralizing anti-NGF antibody and recombinant human NGF (E). Three different fields from each sample were quantitatively estimated and analyzed by counting the cell number (F). Magnification: $\times 100$. * $P < 0.05$ compared with the normal osmolarity medium. † $P < 0.05$ compared with NaCl or NaCl plus neutralizing anti-NGF antibody treatment.

Quantification of β -NGF

Concentrations of β -NGF in the supernatants were determined using a human ELISA kit (Quantikine; R&D Systems, Inc., Minneapolis, MN) in accordance with the manufacturer's protocol.

Cell Morphology

The morphologies of conjunctival epithelial cells cultured for 24 h in either normal medium, 400 mOsm/L medium, 400 mOsm/L medium plus human β -NGF polyclonal neutralizing anti-NGF antibody (25 ng/mL; catalog no. AB-256-NA; R&D systems, Inc.), 400 mOsm/L medium plus recombinant human NGF (10 ng/mL; catalog no. 256-GF/CF; R&D Systems, Inc.), or 400 mOsm/L medium plus neutralizing anti-NGF antibody with recombinant human NGF were observed and photographed using upright microscopy (Zeiss, Inc., Thornwood, NY). The neutralizing anti-NGF antibody was added 1 hour before adding NaCl to increase the osmolarity. Three different fields from each sample were quantitatively estimated and analyzed by counting the cell number using an Image-Pro Plus 6 (Olympus, Inc., Tokyo, Japan). The efficacy of the neutralizing activity of the anti-NGF antibody (R&D Systems, Inc.) was demonstrated with the TF-1 human erythroleukemic cell line.²⁹

Assessment of Apoptosis by Flow Cytometry

pHCECs were cultured in normal or high-osmolarity saline medium with neutralizing anti-NGF antibody and recombinant human NGF for 6 hours. To measure apoptosis, cells were harvested and washed with ice-cold PBS. Cell pellets were collected by centrifugation, resuspended in binding buffer, and incubated with FITC-labeled Annexin V and propidium iodide

(BD Biosciences, Inc., San Diego, CA) for 15 min at room temperature in the dark. The cells were then analyzed using flow cytometry (FACSCalibur; BD Biosciences, Inc.).

Western Blot Analysis

To detect levels of Bcl-xL, Bax, phosphorylated JNK (p-JNK), and cleaved caspase-3, conjunctival epithelial cells cultured in normal or hyper-osmolarity saline medium with or without neutralizing anti-NGF antibody and with or without recombinant human NGF for 24 hours were collected and lysed by the addition of a lysis solution (10 mM Tris, 10 mM NaCl, 2 mM EDTA, 25 mM NaF, 2 mM Na_3VO_4 , 1 mM PMSF, protease inhibitors, 0.5% Triton X-100, pH 7). The cell extracts were centrifuged at 14,000g for 15 minutes at 4°C, and the supernatants were used for subsequent analysis. Total protein concentrations in the supernatants were determined using the Bradford method. Aliquots of protein (30 μg) were boiled in an equal volume of Laemmli sample buffer and resolved by 12% SDS-PAGE. Proteins were then transferred to nitrocellulose (NC) membrane (Immobilon; Millipore, Inc., Billerica, MA). After the membranes were blocked for 1 hour in 5% nonfat dry milk and 0.1% Tween-20, the blots were treated with primary antibodies against Bcl-xL, Bax, p-JNK, and cleaved caspase-3 (Cell Signaling Technology, Inc., Danvers, MA), with an antibody for β -actin (Sigma-Aldrich, Inc.) used as a loading control. After 3 washes with Tris-buffered saline with 0.1% Tween-20 for 10 minutes each, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG (1:10,000 dilution). The target proteins that were specifically recognized by the antibodies were visualized using enhanced chemiluminescence reagents (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

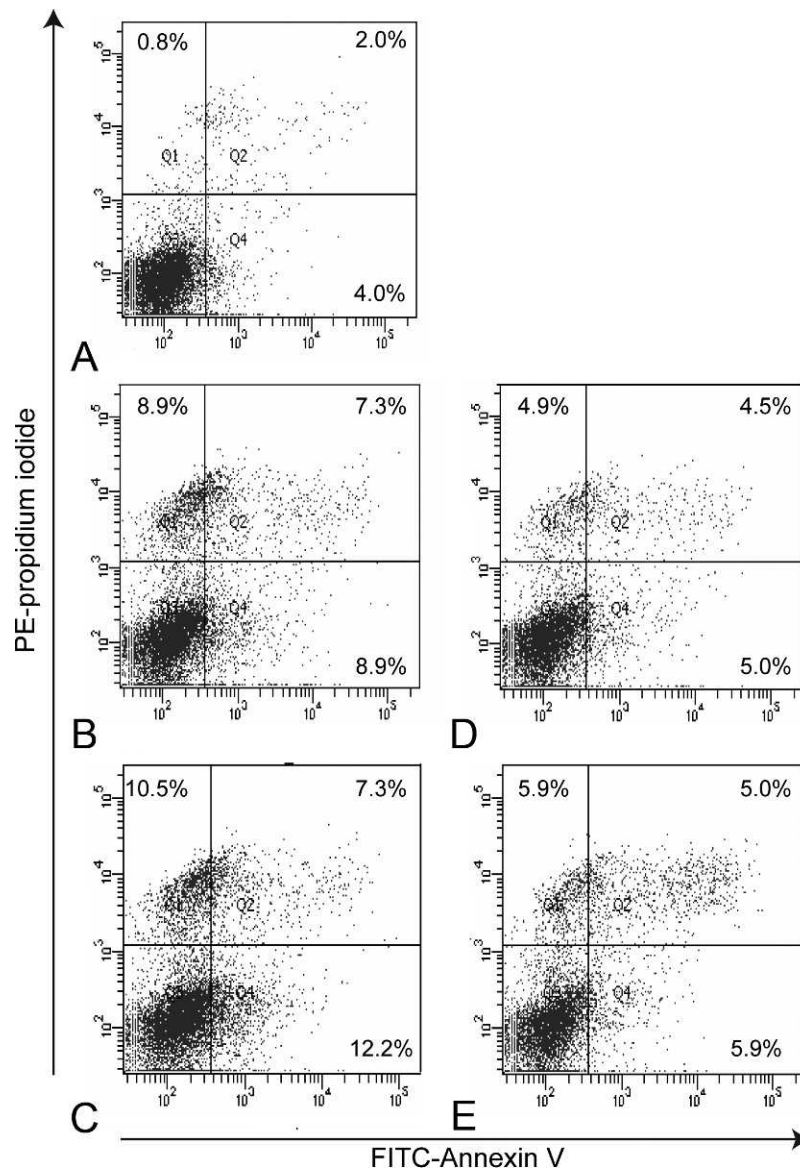


FIGURE 3. Flow cytometric analysis conducted at 6 hours after treatment shows an apoptotic response in human primary conjunctival epithelial cells exposed to normal medium (**A**), high-osmolarity medium (400 mOsm) generated by the addition of NaCl (**B**), 400 mOsm medium supplemented with neutralizing anti-NGF antibody (**C**), 400 mOsm medium supplemented with recombinant human NGF (**D**), or 400 mOsm medium supplemented with both neutralizing anti-NGF antibody and recombinant human NGF (**E**). (**A-E**) The percentage of total cells in quadrants corresponded to early apoptotic cells (annexin V-positive and PI-negative cells, Q4, *lower right*), late apoptotic cells (annexin V-positive and PI-positive cells, Q2, *upper right*), and necrotic cells (annexin V-negative and PI-positive cells, Q1, *upper left*).

Multiplex Cytokine Analysis

Concentrations of the proinflammatory cytokines IL-6 and IL-8 in the supernatants of conjunctival epithelial cells cultured in normal or high-osmolarity saline-added medium with or without neutralizing anti-NGF antibody and/or recombinant human NGF for 24 hours were determined using a custom-designed cytokine/chemokine kit that uses multiplex bead technology (Luminex, Inc., Austin, TX). Assays were conducted in accordance with the manufacturer's instructions.

Statistical Analysis

All quantitative experiments were performed at least in triplicate, and the data shown are means \pm SDs of 1 representative experiment. Statistical significance was deter-

mined using the Student *t*-test between highlighted groups, and a 95% confidence level was taken at a *P* value of <0.05 .

RESULTS

Hyperosmolarity Induces NGF Expression in pHCECs

Levels of NGF transcripts were determined in pHCEC exposed to normal osmolar (307 mOsm) or hyperosmolar medium (350, 400, or 450 mOsm) for 24 hours. Secretion levels of both NGF mRNA and NGF were significantly increased after exposure to hyperosmolar medium with an osmolarity of 400 mOsm ($P < 0.05$) (Figs. 1A, 1B), as assessed by determining the mean values obtained from triplicate experiments. Given that 400 mOsm medium significantly promoted the accumulation of

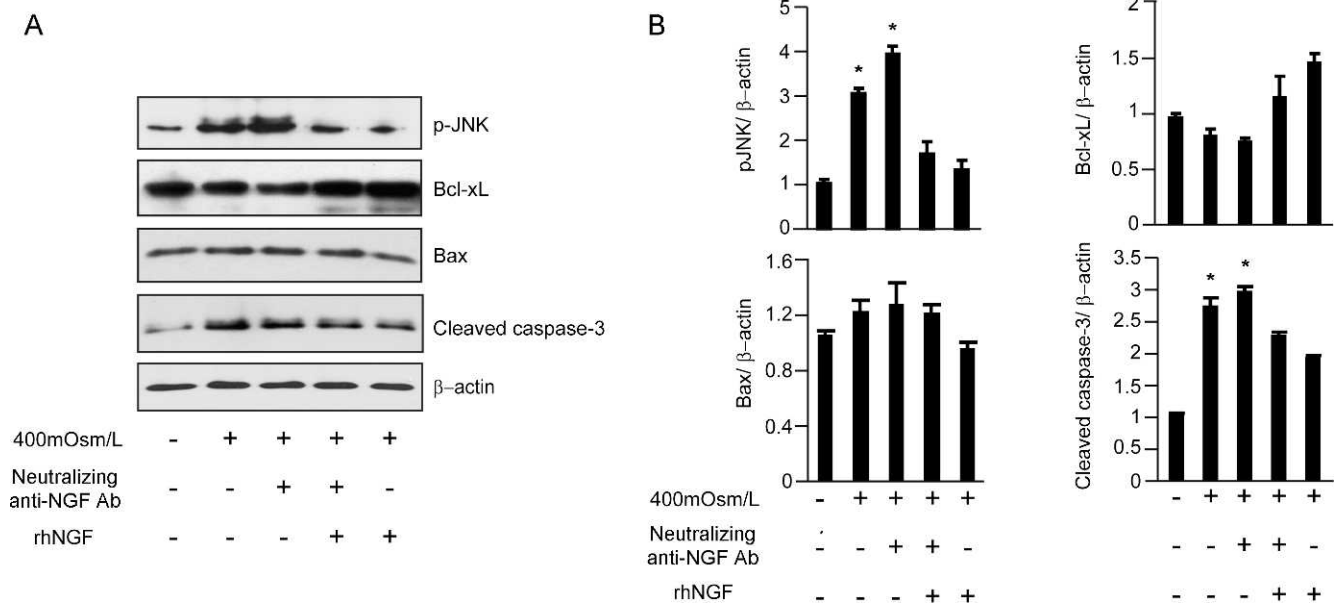


FIGURE 4. Representative Western blot analysis shows the levels of phosphorylated JNK (p-JNK), Bcl-xL, Bax, and cleaved caspase-3 in normal medium, 400 mOsm medium supplemented with neutralizing anti-NGF antibody, 400 mOsm medium supplemented with recombinant human NGF, and 400 mOsm medium supplemented with both neutralizing anti-NGF antibody and recombinant human NGF (A–B). * $P < 0.05$ compared with the normal osmolarity medium.

NGF transcripts, this hyperosmolar condition was used in all subsequent experiments for comparisons with cells grown in medium with a normal osmolarity (307 mOsm).

Induction of NGF by Hyperosmolarity Inhibits Apoptosis of pHCECs

pHCECs were cultured in normal or hyper-osmolar medium with neutralizing anti-NGF antibody or recombinant human NGF for 24 hours. Cells decreased in size and were detached from culture plates when exposed to high-osmolarity medium and showed significantly higher rates of cell death than pHCECs grown in medium with a normal osmolarity (Figs. 2A, 2B, 2F). Moreover, the death of pHCECs induced by hyperosmolarity was aggravated by exposure to NGF-neutralizing antibody (Figs. 2C, 2F). Treatment with recombinant human NGF rescued pHCECs from the cell death response induced by hyperosmolarity or exposure to NGF-neutralizing antibodies (Figs. 2D–2E, 2F). Analysis of apoptosis involved culturing pHCECs in medium with a normal or high osmolarity in the presence of NGF-neutralizing antibody or recombinant human NGF for 6 hours, incubation with FITC-labeled Annexin V and propidium iodide, and analysis using flow cytometry (Figs. 3A–3E). The percentage of FITC-positive cells, which was 4.0% in normal medium, increased to 8.9% in high-osmolarity medium and significantly increased to 12.2% in high-osmolarity medium containing neutralizing anti-NGF antibody (Figs. 3A–3C). The observation that the apoptosis induced by hyperosmolarity or the addition of NGF neutralizing antibodies was rescued partially by treatment with recombinant human NGF (Figs. 3D–3E) suggested that NGF inhibited hyperosmolarity-induced cell death in pHCECs.

NGF Inhibits Activation of p-JNK and Cleavage of Caspase-3 in pHCECs Exposed to Hyperosmolarity

To investigate the possible mechanisms underlying the anti-apoptotic effects of NGF, Western blotting was performed using p-JNK and cleaved caspase-3 antibodies. As shown in Figures 4A

to 4B, Western blot analysis revealed that the levels of p-JNK and cleaved caspase-3 are significantly increased upon exposure to hyperosmolar stress and incubation in medium containing neutralizing anti-NGF antibody ($P < 0.05$). However, the addition of human recombinant NGF markedly abolished the hyperosmolarity-induced increase in levels of p-JNK and cleaved caspase-3. The levels of the proapoptotic protein Bax increased and those of the anti-apoptotic factor Bcl-xL decreased in pHCECs exposed to high-osmolarity medium and to medium containing the neutralizing anti-NGF antibody. However, treatment with recombinant human NGF treatment reduced Bax expression and increased the levels of the Bcl-xL protein.

NGF Inhibits Induction of IL-6 by Hyperosmolarity

There was a significant increase in IL-6 secretion from pHCECs exposed to high-osmolarity medium (381.23 ± 5.75 pg/mL) compared with normal osmolarity medium (215.48 ± 2.79 pg/mL; all, $P < 0.05$) (Fig. 5A). There was a significant reduction in the IL-6 levels measured in pHCECs exposed to hyperosmotic medium plus recombinant human NGF (215.76 ± 1.93 pg/mL) and hyperosmotic medium plus neutralizing anti-NGF antibody plus recombinant human NGF (246.32 ± 3.29 pg/mL) compared with cells exposed to high-osmolarity medium alone (381.23 ± 5.75 pg/mL) and hyperosmotic medium plus neutralizing anti-NGF antibody (391.58 ± 12.6 pg/mL; all $P < 0.05$). The increase in IL-8 levels induced by hyperosmolarity was not significantly reduced by simultaneous exposure to recombinant human NGF (Fig. 5B).

DISCUSSION

Apoptosis of ocular surface epithelial cells has been implicated in the pathogenesis of the ocular surface disease associated with dry eye.^{30,31} Conjunctival cells from patients with moderate-to-severe KCS overexpress markers related to inflammation and apoptosis.²⁷ In another study, NGF was found to induce epithelial differentiation, increase the number of goblet

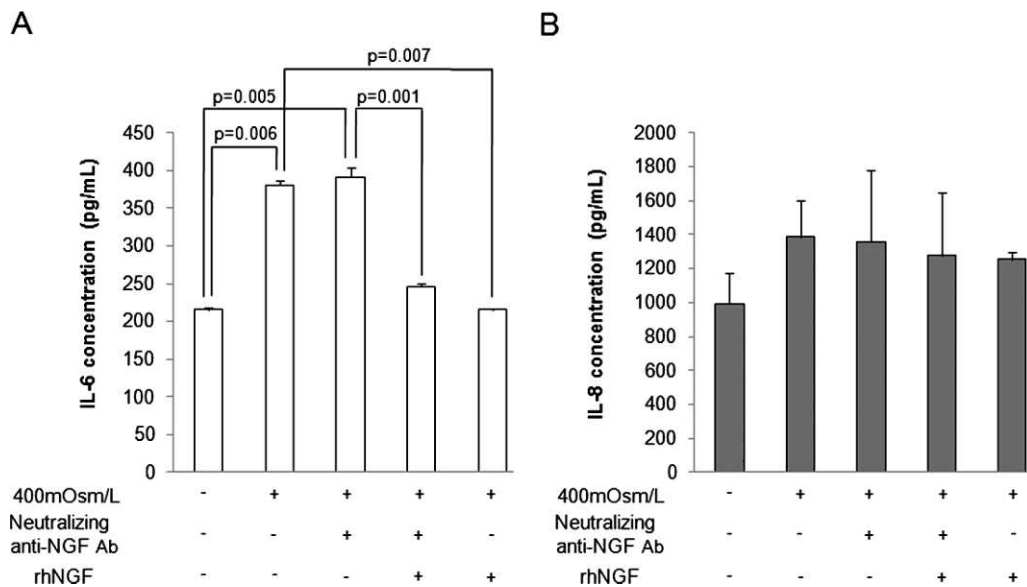


FIGURE 5. Cytokine secretion by human primary conjunctival epithelial cells is shown after 24 hours of culture in normal medium, high-osmolarity medium (400 mOsm) containing supplemental NaCl, 400 mOsm medium containing neutralizing anti-NGF antibody, 400 mOsm medium containing recombinant human NGF, or 400 mOsm medium containing both neutralizing anti-NGF antibody and recombinant human NGF.

cells, and increase MUC5AC expression in both cell lines and primary human conjunctival epithelial cells in culture.³² However, the mechanism of NGF production and its role under hyperosmolar stress conditions have not been established in pHCECs. Our current study findings show that the exposure to hyperosmolar stress elevates the expression of the NGF gene at both the mRNA and protein levels in ex vivo pHCECs and that the increased levels of NGF expression prevents apoptosis of these cells. To our knowledge, this is the first report showing that NGF expression significantly increases in pHCECs exposed for 24 hours to medium in which hyperosmolarity was increased by the addition of NaCl (Fig. 1). As shown in Figures 2 and 3, hyperosmolarity-induced apoptosis was aggravated by blocking NGFs with NGF-neutralizing antibodies but could be rescued by treatment with recombinant human NGF. These findings provide strong evidence that NGF prevents the hyperosmolarity-induced apoptosis of conjunctival epithelial cells, which indicates a protective effect of this factor against hyperosmolarity-stimulated epithelial damage.

MAPKs are important cell-signaling mediators that play vital roles in the cellular response to stress. The JNK and p38 MAPK cascades are strongly activated by cellular stress and by proinflammatory agents such as endotoxin, IL-1, and TNF- α .³³⁻³⁵ Hyperosmolar stress has been reported to activate the JNK cascade pathway in mammalian cells.³⁶ In our current study, p-JNK was found to be activated in pHCECs exposed to hyperosmolar medium (400 mOsm). Activated JNK may down-regulate Bcl-xL and up-regulate Bax. These changes in protein regulation increased mitochondria-dependent caspase activity, leading to apoptosis. Considering our results, the inhibition of JNK phosphorylation by NGF under hyperosmolar stress conditions at least partly contributes to the down-regulation of Bax and the subsequent decrease of caspase activation. Although our present findings suggest that hyperosmolarity induces apoptosis and activates the intracellular MAPK pathway in cultured pHCECs, further studies are necessary to establish a direct link between the elevated osmolarity of tears, apoptosis of ocular surfaces, and MAPK activation in patients with dry eye.

Dry eye disease is known to be caused in part by an increase in the osmolarity of the tear film, which results in inflammation and subsequent cell damage.¹⁴ Both animal- and cell-based studies have shown that hyperosmotic stress induces the expression and production of proinflammatory cytokines in the ocular surface cells.^{17,20,21} The key finding of our present study was that treatment with recombinant human NGF significantly inhibits the hyperosmolarity-induced secretion of the proinflammatory cytokine IL-6 in pHCECs but does not inhibit IL-8 secretion. It is assumed that IL-6 is the primary proinflammatory cytokine induced by hyperosmotic stress in pHCECs. We show that there is a difference between the inhibitory effects of recombinant human NGF on IL-6 and IL-8 under conditions of hyperosmotic stress.

In summary, hyperosmotic stress induces apoptosis of pHCECs as well as corneal epithelial cells, and NGF acts as an antiapoptotic and anti-inflammatory agent in pHCECs challenged by exposure to hyperosmolarity stress. Hence, NGF may have therapeutic applications for the treatment of ocular disorders, such as dry eye.

Acknowledgments

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