Hyperosmolarity-Induced Apoptosis in Human Corneal Epithelial Cells Is Mediated by Cytochrome c and MAPK Pathways

Lihui Luo, MD,*†‡ De-Quan Li, MD, PhD,* and Stephen C. Pflugfelder, MD*

Purpose: To study whether hyperosmolarity induces apoptosis in human corneal epithelial cells through cytochrome c–mediated death pathways and by activation of mitogen-activated protein kinases (MAPKs).

Methods: Primary human corneal epithelial cells cultured in normal osmolar media (312 mOsm) were switched to hyperosmolar media (450, 500, and 550 mOsm) by adding 70, 90, and 120 mM NaCl, respectively, with or without the c-jun N-terminal kinase (JNK) inhibitor SB202190 or the extracellular-regulated kinase (ERK) inhibitor PD98059. Apoptosis was assessed by the ApopTag In Situ Oligo Ligation (ISOL) assay. Confocal microscopy was used to detect cytochrome c and active caspase-3. Total RNA was extracted and subjected to reverse transcriptase-polymerase chain reaction for apoptosis-associated genes. Western blots were performed on cell extracts for the apoptogenic molecules cytochrome c and Smac/DIABLO, and phospho-JNK and ERK.

Results: ISOL-positive apoptotic cells significantly increased from 3.3 ± 1.6% in control medium to 11.4 ± 5.8%, 18.9 ± 4.8%, and 43.9 ± 8.8% in 70, 90, and 120 mM NaCl added media, respectively. The 90 mM NaCl high saline medium notably increased release of cytochrome c and Smac/DIABLO from mitochondria; activated caspase-3, JNK and ERK; stimulated mRNA expression of interleukin-1–converting enzyme and Bax; and reduced Bcl2 expression. SB202190 and PD98059 significantly suppressed hyperosmolarity-induced JNK/ERK activation and ISOL-positive cells. In addition, PD98059 inhibited the release of cytochrome c and Smac/DIABLO from mitochondria.

Conclusions: These findings show that hyperosmolarity induces apoptosis of human corneal epithelial cells through a cytochrome c–mediated death pathway, which may be mediated by JNK and ERK MAPK signaling pathways.

Key Words: cornea, hyperosmolarity, apoptosis, cytochrome c, mitogen-activated protein kinases

(T Cornea 2007;26:452–460)
mitochondria play a crucial function in apoptosis by releasing several apoptogenic molecules (such as cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF, and endonuclease G) into the cytoplasm from the intermembrane space. Many apoptotic stimuli, such as UV radiation, promote the release of cytochrome c from the mitochondria and activate downstream destruction programs, including the caspase cascade.

The MAPKs are well-conserved signaling pathways that include extracellular signal–regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK. They are typical signaling mediators that transmit intracellular signals initiated by extracellular stimuli to the nucleus. They can regulate a variety of cellular activities, including cell growth, differentiation, survival, and death. Hyperosmolar stress and inflammatory cytokines, such as IL-1β and TNF-α, activate these MAPK signaling pathways in a variety of cell types, including fibroblasts, vascular endothelial cells, and corneal epithelial cells. JNK has also been referred to as stress-activated protein kinase (SAPK) because it is activated in response to a variety of cellular stresses. The activated MAPK kinases initiate a cascade of protein phosphorylation involving multiple other kinases and activate nuclear transcription factors, such as nuclear factor (NF)-κB, activating protein-1 (AP-1), and activating transcription factor (ATF). Apoptotic stimuli such as hyperosmotic shock or UV irradiation trigger MAPK cascades and the proteolytic cleavage of procaspases. Hyperosmosality was found to trigger activation of JNK, ERK, and caspase-3 in hyperosmolarity-induced apoptosis in mouse embryonic fibroblasts. The MAPK signaling pathways linking osmotic stress and apoptosis have not been well studied in the corneal epithelium.

The purpose of this study was to investigate whether hyperosmolar stress induces apoptosis in human corneal epithelial cells through a cytochrome c–mediated death pathway and to determine the role of MAPK signaling pathways in this process.

MATERIALS AND METHODS

Primary Human Corneal Epithelial Cell Culture

Human corneal epithelial cells were cultured from explants taken from human donor corneoscleral rims, provided by the Lions Eye Bank of Texas, using a previously described method. In brief, corneoscleral rims were trimmed, the endothelial layer and iris remnants were removed, and each limbal rim was dissected into 12 equal segments. Two segment explants were placed in each well of 6-well culture plates, and each explant was covered with a drop of fetal bovine serum overnight. The explants were cultured in a supplemented hormonal epidermal medium (SHEM, which was composed of a 1:1 mixture of high-glucose Dulbecco modified Eagle medium (DMEM) and Ham F12 medium containing 5 ng/mL of epidermal growth factor (EGF), 5 μg/mL of insulin, 5 μg/mL of transferrin, 5 ng/mL of sodium selenite, 0.5 μg/mL of hydrocortisone, 30 ng/mL of cholera toxin A, 0.5% dimethyl sulfoxide (DMSO), 50 μg/mL of gentamicin, 1.25 μg/mL of amphotericin B, and 5% FBS, at 37°C under 5% CO₂ and 95% humidity. The medium was renewed every 2 to 3 days. Epithelial phenotype of these cultures was confirmed by characteristic morphology and immunofluorescent staining with cytokeratin antibodies (AE-1/AE-3).

Cell Treatment

Subconfluent primary human corneal epithelial cultures (grown for 12–14 days, ~4 × 10⁵ cells/well) were switched to a serum-free medium (SHEM without FBS) for 24 hours before treatment. The cells in serum-free normal osmolar medium (312 mOsM) were switched to higher-osmolarity media by adding 70, 90, or 120 mM sodium chloride (NaCl; corresponding to osmolalities of 450, 500, and 550 mOsM, respectively) for an additional 24 hours, with or without SB202190 (20 μM, an inhibitor of the JNK pathway) or PD 98059 (40 μM, an inhibitor of the ERK pathway), which were added 40 minutes before adding the NaCl. For Western blot to detect phospho-JNK and ERK, the cultures were treated with the same conditions as above for a shorter time of 30 minutes. All experiments were performed at least three times on each of three separate sets of cultures that were initiated from different donor corneas.

Apoptosis Assay

The nuclear morphology of corneal epithelial cells cultured in normal medium and in 500 mOsM medium for 24 hours was observed and photographed with a Nikon Eclipse 400 epifluorescent microscope (Garden City, NY) using a DMX 1200 digital camera after staining with 1 μg/mL of Hoechst 33342 DNA binding dye (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) for 5 minutes. The ApopTag Peroxidase In Situ Oligo Ligation (ISOL) assay (Intergen, Purchase, NY), which detects an early event in the apoptotic cascade, was performed using the manufacturer’s protocol on corneal epithelial cells treated for 24 hours in different osmolality media with or without SB202190 or PD98059. This system labels genomic DNA in apoptotic cells through use of a biotinylated hairpin oligonucleotide and T4 DNA ligase. Oligo B from the kit, which is designed to ligate only with blunt-end DNA, was used because blunt-end DNA cleavage is considered to be characteristic of apoptosis. In brief, confluent corneal epithelial cultures were fixed with 1% paraformaldehyde in PBS at room temperature for 10 minutes and postfixed with 2:1 ethanol:acetic acid solution at −20°C for 5 minutes. Endogenous peroxidases were quenched with 0.5% H₂O₂ in PBS. The samples were incubated in a mixture of equilibration buffer (12 μL/cm²) containing Oligo B and T4 DNA ligase enzyme at 22°C in a humidified chamber for 16 hours or in equilibration buffer only as a negative control. After washing in H₂O₂, streptavidin-peroxidase was applied for 30 minutes at room temperature. Finally, the samples were incubated with diaminobenzidine (DAB) peroxidase substrate to give a brown stain. After washing with H₂O₂, anti-fade Gel/Mount (Fisher, Atlanta, GA) and coverslips were applied. ISOL–labeled cells were examined with a Nikon TE200 inverted microscope. Images at ×400 magnification were captured of 6 to 8 representative fields in each group. The staining index was expressed as the number of positively stained cell/total number of cell × 100%. A t test was used to analyze data for statistically significant differences.
Corneal epithelial cells cultured in normal or high hyperosmolar saline-added media for 24 hours were incubated with 400 nM MitoTracker probes (red dye) for 30 minutes at 37°C. After washing with serum-free media, the cells were fixed with 3.7% formaldehyde in serum-free media at 37°C for 15 minutes and blocked with 5% normal goat serum in PBS containing 0.2% Triton X-100 (TPBS) for 1 hour at room temperature to reduce nonspecific labeling. The samples were incubated for 1 hour at room temperature with 1 µg/mL of primary monoclonal antibody against cytochrome c (Laboratory Vision, Fremont, CA) in PBS with 5% goat serum. After washing with TPBS, Alexa Fluor 488 (Molecular Probes, Eugene, OR)-conjugated secondary antibody [goat anti-mouse immunoglobulin G (IgG) (1:300)] was applied for 1 hour in a dark chamber. Samples were washed with TPBS and covered with antifade solution and coverslips. A sample treated without primary antibody served as a negative control. Digital images (512 × 512 pixels) were captured with an LSM-510 laser-scanning confocal microscope (Zeiss, Thornwood, NY) with 488-nm excitation and 543-nm emission filters, LP505 and LP560, respectively. They were acquired with a 40/1.3 oil-immersion objective using identical photomultiplier tube gain settings and processed using Zeiss LSM-PC software and Adobe Photoshop 7.0 (San Jose, CA).

### Laser Scanning Confocal Microscopy

Total RNA was isolated from corneal epithelial cells cultured in normal- or high-osmolality saline-added media for 24 hours by acid guanidium thiocyanate-phenol-chloroform extraction using a previously described method. The RNA concentration was measured by its absorption at 260 nm, and it was stored at –80°C until use. With a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control, the mRNA expression of apoptosis-related genes by corneal epithelia was analyzed by semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) as previously described. In brief, first-strand cDNA was synthesized from 0.5 µg of total RNA with MuLV reverse transcriptase. PCR amplification of first-strand cDNA was performed with specific primer pairs, designed from published human gene sequences (Table 1) for different markers in human gene sequences (Table 1) for different markers in

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>PCR Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE</td>
<td>X65019</td>
<td>AGGACCAAGACCTGAGAACAG</td>
<td>TCTCACCCTTCCCAGGCACT</td>
<td>547</td>
</tr>
<tr>
<td>Bax</td>
<td>L22473</td>
<td>TCCACCTGAGAGCTGAGGAGT</td>
<td>ATCCCTCTCCAGATGGTGAG</td>
<td>410</td>
</tr>
<tr>
<td>Bcl2</td>
<td>M13994</td>
<td>CACACCTGAGATCAGAGATAA</td>
<td>CCCCCCTGAGAAATGATGCT</td>
<td>379</td>
</tr>
<tr>
<td>GAPDH</td>
<td>M32599</td>
<td>GCAAAAGTCTCAGGTGACACAC</td>
<td>GTCACCCACCTGTTGCTGTA</td>
<td>498</td>
</tr>
</tbody>
</table>

### Immunofluorescent Staining for Active Caspase-3

Corneal epithelial cells cultured in normal- or high-osmolality saline-added media for 24 hours were incubated with 100% methanol at 4°C for 10 minutes, followed by incubation with TPBS for 10 minutes at room temperature. After 3 washes in TPBS, the samples were blocked with 5% normal goat serum in PBS for 30 minutes and incubated with 5 µg/mL of polyclonal rabbit anti-active caspase-3 primary antibody (Phar-Mingen, San Diego, CA) or with PBS as a negative control at 4°C overnight. After washing with PBS, the goat anti-rabbit AlexaFluor488 conjugate (1:300; Molecular Probes) was applied for 1 hour in a dark chamber, followed by 3 washes in PBS. Nuclei were counterstained using 1 µg/mL Hoechst 33342 dye in ~30 µL mounting gel, and a coverslip was applied. Sections were examined and imaged with a Nikon Eclipse 400 epifluorescent microscope with a DMX 1200 digital camera.

### Western Blot

For the detection of phospho-JNK and ERK, corneal epithelial cells cultured in normal- or high-osmolality saline-added media with or without SB202190 or PD98059 for 30 minutes were collected and lysed in RIPA buffer, containing 50 mM Tris·HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and an EDTA-free protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN). The cell extracts were centrifuged at 14,000 g for 15 minutes at 4°C, and the supernatants were used for experiments. For the detection of released cytochrome c and Smac/DIABLO, the cytosolic fraction was collected from the corneal epithelial cells treated for 24 hours after incubation with 0.3 mg/mL digitonin (Sigma) for 5 minutes at 37°C in isotonic buffer containing 20 mM potassium-HEPES (pH = 7.4), 10 mM KCl, 1.5 mM MgCl2, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). The cells were centrifuged at 12,000g for 5 minutes, and the supernatants (which contained mainly the cytosol but not the organelles) were subjected to Western blot analysis. Total protein concentrations of the cell extracts were measured by a Micro BCA protein assay kit (Pierce, Rockford, IL). The protein samples (50 µg) were mixed with 6× SDS-reducing sample buffer and boiled for 5 minutes before loading. Proteins were separated by SDS–polyacrylamide gel electrophoresis (4%–15% Tris·HCl, gradient gels; Bio-Rad, Hercules, CA) and transferred electronically to polyvinylidene

---

**TABLE 1. Human Primer Sequences Used for RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>PCR Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICE</td>
<td>X65019</td>
<td>AGGACCAAGACCTGAGAACAG</td>
<td>TCTCACCCTTCCCAGGCACT</td>
<td>547</td>
</tr>
<tr>
<td>Bax</td>
<td>L22473</td>
<td>TCCACCTGAGAGCTGAGGAGT</td>
<td>ATCCCTCTCCAGATGGTGAG</td>
<td>410</td>
</tr>
<tr>
<td>Bcl2</td>
<td>M13994</td>
<td>CACACCTGAGATCAGAGATAA</td>
<td>CCCCCCTGAGAAATGATGCT</td>
<td>379</td>
</tr>
<tr>
<td>GAPDH</td>
<td>M32599</td>
<td>GCAAAAGTCTCAGGTGACACAC</td>
<td>GTCACCCACCTGTTGCTGTA</td>
<td>498</td>
</tr>
</tbody>
</table>
difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in TTBS (50 mM Tris, pH 7.5, 0.9% NaCl, and 0.1% Tween-20) for 1 hour at room temperature and incubated for 2 hours at room temperature with a 1:100 dilution of rabbit anti–phospho-JNK (Santa Cruz Biotechnology, Santa Cruz, CA) or 1:500 dilution of mouse anti–phospho-p44/42 ERK (Santa Cruz Biotechnology), 1:133 dilution of mouse anti–cytochrome c (Laboratory Vision), or 1:400 dilution of mouse anti–Smac/DIABLO (Upstate Biotechnology, Lake Placid, NY) antibody with 5% nonfat milk in TTBS. After 3 washings with TTBS, the membranes were incubated for 1 hour at room temperature with the species-appropriate horseradish peroxidase (HRP)-conjugated secondary antibody; goat anti–rabbit IgG (1:2000 dilution; Cell Signaling, Beverly, MA) or goat anti–mouse IgG (1:5000 dilution; Pierce, Rockford, IL). Membranes were washed 4 times, and the signals were detected with an enhanced chemiluminescence (ECL) advance chemiluminescence reagent (Amersham, Piscataway, NJ), and the images were acquired and analyzed by a Kodak image station 2000R (Eastman-Kodak, New Haven, CT). Membranes were stripped in 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS and 100 mM β-mercaptoethanol at 60°C for 30 minutes and were reprobed with a 1:100 dilution of a rabbit anti-JNK (Santa Cruz Biotechnology), 1:1000 dilution of rabbit anti-ERK (Cell Signaling), or 1:5000 dilution of mouse anti–β-actin (Sigma). These anti-JNK and -ERK antibodies detect both phosphorylated and unphosphorylated forms, representing the total levels of these MAPKs. The signals were detected and captured as described above.

RESULTS

Hyperosmolarity Induces Apoptosis of Human Corneal Epithelial Cells

Apoptosis was detected in corneal epithelial cells exposed to normal osmolar (312 mOsM) or hyperosmolar media (450–550 mOsM) generated by adding 70, 90, or 120 mM NaCl for 24 hours with an ApopTag ISOL assay, which specifically detects DNA fragmentation. The percentage of ISOL-positive cells significantly increased from 3.3 ± 1.6% in normal media to 11.4 ± 5.7% (P < 0.05) in 70 mM NaCl–added media, 18.9 ± 4.8% (P < 0.01) in 90 mM NaCl–added media, and 43.9 ± 8.8% (P < 0.001) in 120 mM NaCl–added media, using the means of 5 repeated experiments (Fig. 1). Because 90 mM NaCl–added media (500 mOsM) significantly promoted apoptosis, this high-saline concentration was used to compare with the normal osmolar group (312 mOsM) in all of the subsequently described experiments. Nuclei in cornea epithelial cells exposed to 90 mM NaCl–added media and stained with Hoechst 33342 DNA binding dye had a small fragmented appearance (Fig. 2) compared with the nuclei of cells cultured in control media of normal osmolarity.

Immunofluorescent staining for active caspase-3 revealed that corneal epithelial cells exposed to 500 mOsM high saline for 24 hours had more active caspase-3 than cells cultured in normal media (Fig. 3), which had minimal to no staining. Staining was not observed in the secondary antibody negative control (data not shown).

FIGURE 1. ApopTag ISOL assay in representative fields showing the increased ISOL-positive apoptotic cells in corneal epithelial cultures exposed to high-osmolarity saline-added media (+70, 90, or 120 mM NaCl) for 24 hours, compared with cells cultured in normal medium. The percentage of positive cells in each group (n = 5) is shown in the graph. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with control medium.

Hyperosmolarity Induces Apoptosis Through a Cytochrome c–mediated Death Pathway

Release of cytochrome c from mitochondria in corneal epithelial cells cultured in normal- or high-osmolarity media

FIGURE 2. Representative Hoechst 33342 nuclear staining of the corneal epithelial cells in control and high-osmolarity saline–added (+90 mM of NaCl) media.
for 24 hours was detected by immunofluorescent laser scanning confocal microscopy using dual-color probes for mitochondrial cytochrome c (green) and MitoTracker (red) (Fig. 4). A greater number of dual labeled (yellow stained) points, indicating cytochrome c localized to mitochondria, was observed in corneal epithelial cells cultured in control media of normal osmolarity. In contrast, there were more green-stained points, indicating cytochrome c released from mitochondria, into the cytoplasm of cells treated with high-osmolarity media (500 mOsM, 90 mM NaCl added). The level of cytochrome c and Smac/DIABLO released from mitochondria was also evaluated in cytoplasmic preps by Western blot using antibodies specific to these proteins and β-actin as an internal control. As shown in Figure 5, the release of cytochrome c (15 kd) and Smac/DIABLO (25 kd) from mitochondria were notably increased in corneal epithelial cells exposed to high-osmolarity (90 mM NaCl added) media for 24 hours. This hyperosmolarity-stimulated increase of cytochrome c and Smac/DIABLO proteins was blocked by PD98059 but not by SB202190 (see below for details).

Semiquantitative RT-PCR showed that the levels of mRNA transcripts of some cytochrome c–related genes, such as caspase1/IL-1β–converting enzyme (ICE) and Bax were increased, whereas the anti–apoptotic factor Bcl2 was decreased in corneal epithelial cells exposed to high-osmolarity media (Fig. 6). These experiments suggest that hyperosmolarity induces apoptosis through a cytochrome c–mediated death pathway.

Hyperosmolarity Activates JNK and ERK MAPK Pathways in Human Corneal Epithelial Cells

To determine whether hyperosmolarity activates MAPK signaling pathways in human corneal epithelial cells, a JNK pathway inhibitor, SB202190, and an ERK pathway inhibitor, PD98059, were added individually into the culture media 40 minutes before NaCl was added. As shown in Figure 7, Western blot analysis revealed that the activated (phosphorylated) forms of JNK [p-JNK1 (46 kd), p-JNK2 (54 kd)] and ERK [p-ERK1 (44 kd) and p-ERK2 (42 kd)] were markedly increased in cells exposed to 500 mOsM hyperosmolar media. The addition of 20 μM SB202190 almost abolished the hyperosmolarity-induced increase in p-JNK1 and p-JNK2 (Fig. 7A). The stimulated increases in p-ERK1 and p-ERK2 were also markedly inhibited by 40 μM PD98059 (Fig. 7B). In contrast, a similar intensity of total JNK1, JNK2 (Fig. 7A, bottom) and total ERK1, ERK2 (Fig. 7B, bottom) was detected in these samples by using an antibody that recognized both unphosphorylated and phosphorylated JNK and ERKs.

SB202190 and PD98059 Inhibit Apoptosis of Corneal Epithelial Cells in Response to Hyperosmolar Media

To study whether JNK and ERK MAPKs affected hyperosmolarity-induced apoptosis, cultured human corneal epithelial cells were treated with medium containing 90 mM NaCl for 24 hours, followed by addition of SB202190 or PD98059, and apoptosis was detected by activated caspase-3 staining. As shown in Figure 8, the addition of SB202190 significantly decreased the number of activated caspase-3 stained cells (yellow) compared to control media (Fig. 8A). Similarly, the number of activated caspase-3 stained cells in medium containing PD98059 was also decreased (Fig. 8B). These results suggest that both JNK and ERK MAPKs are involved in the hyperosmolarity-induced apoptosis of corneal epithelial cells.

Semiquantitative Western blot analysis revealed that the expression levels of caspase-3, caspase-9, and PARP were increased in cells treated with hyperosmolar media (Fig. 9). The addition of SB202190 or PD98059 inhibited the expression of these apoptosis-related proteins (Fig. 9A). These results suggest that hyperosmolarity activates the JNK and ERK MAPK pathways, leading to the activation of caspase-3, caspase-9, and PARP.

Hyperosmolarity also activates the NF-κB pathway, which is a critical regulator of inflammation. As shown in Figure 10, the addition of SB202190 or PD98059 partially inhibited the expression of the NF-κB target gene, IL-6 (Fig. 10A). These results suggest that hyperosmolarity activates the NF-κB pathway, which may contribute to the hyperosmolarity-induced inflammatory response in corneal epithelial cells.

In conclusion, hyperosmolarity activates JNK and ERK MAPK pathways and induces apoptosis in human corneal epithelial cells. This study provides new insights into the molecular mechanisms underlying hyperosmolarity-induced corneal epithelial cell apoptosis and inflammation, and may have implications for the development of therapeutic strategies to prevent or reverse the effects of hyperosmolarity in the ocular surface.

FIGURE 3. Representative immunofluorescent staining (left column) for activated caspase-3 in corneal epithelial cells cultured in normal (top) and high-osmolarity 90 mM NaCl-added (bottom) media for 24 hours. Hoeschst 33342 nuclear counterstaining is shown in the right column.

FIGURE 4. Representative immunofluorescent staining for cytochrome c (green) and MitoTracker red (red) in mitochondria of human corneal epithelial cells cultured in normal and hyperosmolar 90 mM NaCl-added media for 24 hours detected by laser scanning confocal microscopy. In merged color images (right), arrows point to the cytochrome c released from mitochondria. Original magnification, ×400.
Epithelial cells were exposed to a high-osmolarity environment (+90 mM NaCl) in the presence or absence of specific inhibitors of these pathways, and apoptosis was evaluated by the ISOL assay. As shown in Figure 8, osmotic stress for 24 hours caused a 5.7-fold increase in the percentage of ISOL-positive epithelial cells (P < 0.01 compared with normal medium), which was reduced ~68%, from 18.9 ± 4.8% in high-saline media to 6.0 ± 1.0% when JNK was inhibited by SB202190. The blockade of ERK with inhibitor PD98059 had a similar effect. The percentage of ISOL-positive cells was significantly reduced to 5.4 ± 2.7% in the PD98059-treated group compared with the high-osmolarity saline-added group (P < 0.01). These findings suggest that the ERK and JNK MAPK pathways are involved in hyperosmolarity-induced apoptosis.

Western blot performed to detect the release of cytochrome c and Smac/DIABLO from mitochondria into cytoplasm of corneal epithelia treated with high-osmolarity 90 mM NaCl-added media for 24 hours and the effects of SB20210 (SB) and PD98059 (PD) on this process.

**FIGURE 5.** Representative Western blots showing the increased release of cytochrome c and Smac/DIABLO from mitochondria into cytoplasm of corneal epithelia treated with high-osmolarity 90 mM NaCl-added media for 24 hours and the effects of SB20210 (SB) and PD98059 (PD) on this process.

The corneal epithelium is an essential barrier to noxious environmental stresses, such as desiccation and microbial agents. Corneal barrier function may be compromised by disruption of the tight junctions, epithelial cell volume shrinkage, or cell death. Dry eye is perhaps the most common disease affecting corneal barrier function. Dry eye may develop from decreased tear secretion or excessive tear evaporation, and it results in a hyperosmolar tear film. Apoptosis of the ocular surface epithelial cells has been implicated in the pathogenesis of the ocular surface disease that develops in dry eye. Apoptosis of the corneal and conjunctival epithelia has been detected in animal models of dry eye. It is difficult to

**FIGURE 6.** Expression of ICE (547 bp), Bax (410 bp), and Bcl2 (379 bp) mRNA in corneal epithelia cultured in normal (M) and hyperosmolar (H) media for 24 hours by semiquantitative RT-PCR using GAPDH (498 bp) as an internal control.

**FIGURE 7.** Representative Western blots showing the levels of (A) phospho-JNKs (p-JNK1, p-JNK2), total JNKs (JNK1, JNK2) and (B) phospho-ERKs (p-ERK1, p-ERK2) and total ERKs (ERK1, ERK2) in corneal epithelia cultured in normal and hyperosmolarity 90 mM NaCl-added media treated for 30 minutes without or with SB202190 (SB) or PD98059 (PD).

**DISCUSSION**

The corneal epithelium is an essential barrier to noxious environmental stresses, such as desiccation and microbial agents. Corneal barrier function may be compromised by disruption of the tight junctions, epithelial cell volume shrinkage, or cell death. Dry eye is perhaps the most common disease affecting corneal barrier function. Dry eye may develop from decreased tear secretion or excessive tear evaporation, and it results in a hyperosmolar tear film. Apoptosis of the ocular surface epithelial cells has been implicated in the pathogenesis of the ocular surface disease that develops in dry eye. Apoptosis of the corneal and conjunctival epithelia has been detected in animal models of dry eye. It is difficult to
Our study found that Representative ApopTag ISOL assay showing/C15 although a It has been found that cytochrome c release results 0.01 compared The threshold of 450 mOsm necessary to 47 Caspase- 45,46 Cornea 11, Our study in corneal epithelial cells, 6 2007 Lippincott Williams & Wilkins It is well recognized that apoptosis occurs with 90 mM NaCl media. We previously detected osmolarities of up to 41 In our study with dry eye; however, expression of proapoptotic markers with dry eye model.41 The threshold of 450 mOsm necessary to detect apoptosis in cultured corneal epithelial cells in our study could be caused by the sensitivity of the ISOL assay we used to detect apoptotic events or to the greater resistance of cultured corneal epithelial cells to hyperosmolar stress than the ocular surface epithelia in vivo. Other studies have found that many types of cultured mammalian cells can survive moderately hypertonic environments (up to 500 mOsm) because of a specific adaptation process that eventually results in accumulation of compatible osmolytes such as taurine, betaine, and myoinositol.42,43 It is well recognized that apoptosis occurs through mitochondrion-dependent pathways, including an extrinsic pathway involving the interaction of death ligands (eg, TNF-α, Fas ligand) with their respective cell surface receptors and an intrinsic pathway that is initiated by DNA damaging, such as UV light and chemotherapeutic agents.44 Both pathways eventually result in mitochondrial damage with release of cytochrome c and downstream activation of caspas, such as caspase-3. The apoptotic process is also mediated through mitochondrion-independent pathways, which converge on the proteolytic activation of caspase-3. In our study, release of cytochrome c and Smac/DIABLO from the mitochondria to the cytoplasm (Figs. 4, 5) was detected by immunofluorescent staining and Western blot, indicating hyperosmolarity-induced apoptosis of corneal epithelial cells through a cytochrome c–mediated death pathway. A previously reported study found that hypertonic shock caused reversible fragmentation of the mitochondrial network in Vero cells.45 It has been found that cytochrome c release results from perturbation of mitochondrial membrane permeability. The Bcl-2 family of proteins is made up of antiapoptotic members, such as Bcl-2 and Bcl-XL, and proapoptotic members, such as multidomain Bax or Bak and single-domain BH3-only proteins, which directly modulate outer mitochondrial membrane permeability during apoptosis.46–48 Caspase-1/ICE also affects mitochondrial membrane permeability, and its production precedes the disruption of the mitochondrial inner transmembrane potential to induce the release of cytochrome c.47 In our study in corneal epithelial cells, osmotic stress increased the expression of ICE and Bax and suppressed Bcl2 (Fig. 5), events that may disrupt adequately sample corneal epithelial cells in human patients with dry eye; however, expression of proapoptotic markers (Fas, Fas ligand, APO2.7, CD40, and CD40 ligand) by the conjunctival epithelium has been found to be significantly higher in dry eye than in normal eyes.19 Our study found that hyperosmolarity induced apoptosis of corneal epithelial cells through a cytochrome c–mediated death pathway, which was mediated by MAPK signaling pathways. This study establishes a link between the hyperosmolar tear film of dry eye and the induction of apoptosis by the ocular surface epithelial cells.

Hyperosmolarity Induces Apoptosis Through a Cytochrome c–Mediated Death Pathway

Apoptosis plays an important role in various biologic events in metazoa, including development, maintenance of tissue homeostasis, and elimination of malignant cells. Our study evaluated apoptosis in corneal epithelial cells exposed to high-osmolarity saline–added (70, 90, and 120 mM NaCl) media for 24 hours compared with cells cultured in normal osmolar media. Hoechst staining showed nuclear morphologic changes, the characteristics of apoptosis, such as nuclear shrinkage and fragmentation, in the high-osmolarity group (Fig. 2). These experiments indicated that human corneal epithelial cells will undergo apoptosis during prolonged osmotic stress. In this study, all high saline–treated groups were conducted at media osmolarities of 450 mOsm or greater because lower media osmolarity did not significantly induce apoptosis in cultured corneal epithelial cells. This level of osmolarity is at the upper end of what has been detected in tear fluid collected from the inferior tear meniscus of patients with dry eye,8 although studies have suggested that the osmolarity of the precorneal tear layer of patients with dry eye in areas of thinning could be much higher.40 We previously detected osmolarities of up to 600 mOsm in tear fluid collected in an experimental murine dry eye model.41 The threshold of 450 mOsm necessary to detect apoptosis in cultured corneal epithelial cells in our study could be caused by the sensitivity of the ISOL assay we used to detect apoptotic events or to the greater resistance of cultured corneal epithelial cells to hyperosmolar stress than the ocular surface epithelia in vivo. Other studies have found that many types of cultured mammalian cells can survive moderately hypertonic environments (up to 500 mOsm) because of a specific adaptation process that eventually results in accumulation of compatible osmolytes such as taurine, betaine, and myoinositol.42,43 It is well recognized that apoptosis occurs through mitochondrion-dependent pathways, including an extrinsic pathway involving the interaction of death ligands (eg, TNF-α, Fas ligand) with their respective cell surface receptors and an intrinsic pathway that is initiated by DNA damaging, such as UV light and chemotherapeutic agents.44 Both pathways eventually result in mitochondrial damage with release of cytochrome c and downstream activation of caspas, such as caspase-3. The apoptotic process is also mediated through mitochondrion-independent pathways, which converge on the proteolytic activation of caspase-3.

In our study, release of cytochrome c and Smac/DIABLO from the mitochondria to the cytoplasm (Figs. 4, 5) was detected by immunofluorescent staining and Western blot, indicating hyperosmolarity-induced apoptosis of corneal epithelial cells through a cytochrome c–mediated death pathway. A previously reported study found that hypertonic shock caused reversible fragmentation of the mitochondrial network in Vero cells.45 It has been found that cytochrome c release results from perturbation of mitochondrial membrane permeability. The Bcl-2 family of proteins is made up of antiapoptotic members, such as Bcl-2 and Bcl-XL, and proapoptotic members, such as multidomain Bax or Bak and single-domain BH3-only proteins, which directly modulate outer mitochondrial membrane permeability during apoptosis.46–48 Caspase-1/ICE also affects mitochondrial membrane permeability, and its production precedes the disruption of the mitochondrial inner transmembrane potential to induce the release of cytochrome c.47 In our study in corneal epithelial cells, osmotic stress increased the expression of ICE and Bax and suppressed Bcl2 (Fig. 5), events that may disrupt manner in corneal epithelial cells exposed to high-osmolarity saline–added (70, 90, and 120 mM NaCl) media for 24 hours compared with cells cultured in normal osmolar media. Hoechst staining showed nuclear morphologic changes, the characteristics of apoptosis, such as nuclear shrinkage and fragmentation, in the high-osmolarity group (Fig. 2). These experiments indicated that human corneal epithelial cells will undergo apoptosis during prolonged osmotic stress. In this study, all high saline–treated groups were conducted at media osmolarities of 450 mOsm or greater because lower media osmolarity did not significantly induce apoptosis in cultured corneal epithelial cells. This level of osmolarity is at the upper end of what has been detected in tear fluid collected from the inferior tear meniscus of patients with dry eye,8 although studies have suggested that the osmolarity of the precorneal tear layer of patients with dry eye in areas of thinning could be much higher.40 We previously detected osmolarities of up to 600 mOsm in tear fluid collected in an experimental murine dry eye model.41 The threshold of 450 mOsm necessary to detect apoptosis in cultured corneal epithelial cells in our study could be caused by the sensitivity of the ISOL assay we used to detect apoptotic events or to the greater resistance of cultured corneal epithelial cells to hyperosmolar stress than the ocular surface epithelia in vivo. Other studies have found that many types of cultured mammalian cells can survive moderately hypertonic environments (up to 500 mOsm) because of a specific adaptation process that eventually results in accumulation of compatible osmolytes such as taurine, betaine, and myoinositol.42,43 It is well recognized that apoptosis occurs through mitochondrion-dependent pathways, including an extrinsic pathway involving the interaction of death ligands (eg, TNF-α, Fas ligand) with their respective cell surface receptors and an intrinsic pathway that is initiated by DNA damaging, such as UV light and chemotherapeutic agents.44 Both pathways eventually result in mitochondrial damage with release of cytochrome c and downstream activation of caspas, such as caspase-3. The apoptotic process is also mediated through mitochondrion-independent pathways, which converge on the proteolytic activation of caspase-3. In our study, release of cytochrome c and Smac/DIABLO from the mitochondria to the cytoplasm (Figs. 4, 5) was detected by immunofluorescent staining and Western blot, indicating hyperosmolarity-induced apoptosis of corneal epithelial cells through a cytochrome c–mediated death pathway. A previously reported study found that hypertonic shock caused reversible fragmentation of the mitochondrial network in Vero cells.45 It has been found that cytochrome c release results from perturbation of mitochondrial membrane permeability. The Bcl-2 family of proteins is made up of antiapoptotic members, such as Bcl-2 and Bcl-XL, and proapoptotic members, such as multidomain Bax or Bak and single-domain BH3-only proteins, which directly modulate outer mitochondrial membrane permeability during apoptosis.46–48 Caspase-1/ICE also affects mitochondrial membrane permeability, and its production precedes the disruption of the mitochondrial inner transmembrane potential to induce the release of cytochrome c.47 In our study in corneal epithelial cells, osmotic stress increased the expression of ICE and Bax and suppressed Bcl2 (Fig. 5), events that may disrupt
Mitochondrial membrane permeability and modulate the release of cytochrome c. There is evidence that mitochondrial cytochrome c has dual functions in controlling both cellular energy metabolism and apoptosis. Once released from the mitochondria, cytochrome c interacting with apoptotic protease-activating factors (Apaf) activates the execution caspases that subsequently lead to apoptosis. Increased immunoreactivity to active caspase-3 was observed in corneal epithelial cells exposed to high saline (Fig. 3). Active caspase-3 is capable of cleaving various cellular proteins to cause apoptotic death. The mechanisms by which osmotic stress triggers release of apoptogenic factors such as cytochrome c and Smac/DIABLO from the mitochondria into the cytoplasm are not fully understood, but our studies indicate that MAPK pathways are involved in this process.

MAPKs Mediate the Hyperosmolarity-Induced Apoptosis in Human Corneal Epithelial Cells

MAPKs are important cell signaling mediators that play vital roles in the cellular response to stress. The different MAPKs can be activated in response to specific stimuli, and they in turn initiate specific downstream events. The JNK and p38 MAPK cascades are strongly activated by cellular stresses and by proinflammatory agents such as endotoxin, IL-1, and TNF-α. In contrast, ERK MAPK is strongly activated by growth factors such as platelet derived growth factor (PDGF), EGF, transforming growth factor (TGF)-α, and other stimuli that mediate cell proliferation, differentiation, and survival. Hyperosmolar stress has been reported to activate the JNK cascade pathway in mammalian cells. In our study, Western blot was performed using antibodies specific for the phosphorylated (active) forms of JNK and ERK. We found that JNK-1/2 and ERK-1/2 were activated in human corneal epithelial cells exposed to hyperosmolar media (500 mOsm) for 30 minutes (Fig. 7). No effect on total levels of JNKs and ERKs was observed. This phenomenon was confirmed by treatment with 20 μM SB202190, a JNK pathway inhibitor, which was found to dramatically inhibit the hyperosmolarity-stimulated phosphorylation of JNK-1/2, and with 40 μM PD98059, an ERK signaling inhibitor, which blocked the hyperosmolarity-induced activation of ERKs (Fig. 7). Activation of the JNK cascade in other mammalian cells by osmotic stress may occur through cross-linking growth factor, TNF, and IL-1 receptors. The mechanism of MAPK activation in human corneal epithelial cells by osmotic stress remains to be determined. JNK was found to be needed for UV-induced apoptosis in primary murine embryonic fibroblasts. p38 MAPK was noted to mediate TNF-α–induced apoptosis in rat fetal brown adipocytes, whereas active ERK and JNK both exhibited regulatory functions in hyperosmolarity-induced apoptosis in mouse embryonic fibroblasts (MEFs). In this study, the role of JNK and ERK activation in hyperosmolarity-induced apoptosis in the corneal epithelium was evaluated by treating with the JNK pathway inhibitor SB202190 or the ERK pathway inhibitor PD98059. We found that both inhibitors significantly reduced osmotic stress–induced apoptosis as shown in Figure 8. These data suggest that JNK and ERK MAPK mediate this stress-induced apoptosis in corneal epithelial cells, perhaps by promoting release of cytochrome c from the mitochondria of stressed cells. Activation of JNK and p38 MAPK were previously noted to play an important role in triggering apoptosis in response to extracellular stress (such as UV) through a cytochrome c–mediated death pathway. Our study found that release of cytochrome c and Smac/DIABLO were markedly increased in corneal epithelial cells exposed to high-osmolarity media and that PD98059 dramatically suppressed the stimulated release of these factors into the cytoplasm (Fig. 5). In contrast, SB202190 did not show a measurable effect on the stimulated release of these factors. These results suggest that the ERK signaling pathway may mediate hyperosmolarity-induced apoptosis in corneal epithelium through a cytochrome c death pathway, whereas the JNK signal pathway may work through different apoptogenic molecules or pathways. Although these studies suggest that hyperosmolarity induces apoptosis and activates intracellular MAPK pathways in cultured corneal epithelia, further studies are necessary to establish a direct linkage between elevated tear osmolarity, ocular surface apoptosis, and MAPK activation in human patients with dry eye.

In conclusion, our findings provide direct evidence that hyperosmotic stress induces corneal epithelial cell apoptosis through a cytochrome c–mediated death pathway, which seems to be mediated in part by activation of JNK and ERK MAPK signaling pathways.

ACKNOWLEDGMENT

The authors thank the Lions Eye Bank of Texas for providing the human corneoscleral tissues.

REFERENCES


© 2007 Lippincott Williams & Wilkins


