

# Hyperosmolarity-Induced Cornification of Human Corneal Epithelial Cells Is Regulated by JNK MAPK

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**PURPOSE.** To evaluate the effects of hyperosmolar stress on expression of cornified envelope (CE) precursors and transglutaminases (TGs) by primary cultured human corneal epithelial (PCHCE) cells and the regulatory effects of JNK MAPK on this process.

**METHODS.** Expression of CE precursors and TGs were evaluated in PCHCE cells exposed to media of increasing osmolarity (350–450 mOsm) for 24, 48, and 72 hours. JNK1 and -2 MAPKs were inhibited by addition of short interfering (si)RNA. Relative levels of mRNA transcripts and proteins were evaluated. TG activity, cell viability, and apoptosis were detected in PCHCE cells, with or without siRNA-JNKs.

**RESULTS.** Exposure of PCHCE cells to hyperosmolar medium increased TG activity at 3 hours, levels of the CE precursors SPRR1b and -2a and membrane-associated TG1 mRNA at 6 hours, and tissue-type TG2 mRNA at 24 hours. Osmotic stress decreased corneal epithelial cell viability, which was due in part to stimulation of apoptosis and cornification death. Inhibiting JNK2 production by siRNA in osmotically stressed PCHCE cells prevented the stimulation of SPRR and membrane-associated TG1 production and TG activity, and improved cell viability, whereas inhibition of JNK1 prevented early apoptosis.

**CONCLUSIONS.** Osmotic stress promotes production of certain CE proteins and cross-linking membrane-associated TG1 and decreases cell viability via JNK MAPK-mediated pathways. Strategies that inhibit JNK production downregulate the cornification response of PCHCE cells to osmotic stress. These findings have potential therapeutic implications for preventing cornification of the corneal epithelium in response to the hyperosmolar tear film in dry eye disease. (*Invest Ophthalmol Vis Sci.* 2008;49:539–549) DOI:10.1167/iovs.07-0569

The cornea is covered by a noncornified stratified squamous epithelium. The superficial differentiated cells of the corneal epithelium produce membrane mucins and other glycoproteins that create a hydrophilic surface that attracts the overlying tear layer. In patients with dry eye disease, the corneal surface becomes poorly hydrated and irregular. These changes may be due in part to a shift toward a cornification pattern of differentiation. The mechanism by which this shift in differentiation occurs has not been established, but may represent a stress response to increased tear osmolarity, a feature that has been implicated as an initiating factor for the corneal epithelial disease that develops in dry eye.<sup>1</sup> Exposure of the corneal epithelium to osmotic stress *in vivo* or *in vitro* stimulates activation of mitogen-activated protein kinase (MAPK)-signaling pathways—in particular, c-Jun N-terminal kinase (JNK)—that have been reported to regulate production of cornified envelope (CE) precursor proteins in the corneal epithelium.<sup>2–7</sup> Increased production of the CE precursor proteins, involucrin and small proline-rich protein (SPRR)-2 by the corneal epithelium was observed in a murine model of dry eye in which tear osmolarity was noted to double.<sup>8</sup> Hyperosmolarity has also been found to increase production of CE precursor proteins and promote cell death by cornification in an epidermal cell line.<sup>9</sup>

The epidermis of the skin has a well-developed apical cornified layer consisting of CE precursor proteins cross linked by TG enzymes. The CE precursors consist of a family of 10 members that promote epithelial cell differentiation and cornification, leading to death. SPRRs are major constituents of this family, and they consist of SPRR1a, -1b, -2a-g, -3, and -4 in humans.<sup>10</sup> We have reported that the CE precursor proteins SPRR1, SPRR2, filaggrin, and involucrin are expressed in the corneal and limbal epithelium *in situ* and that nine CE precursor genes, including involucrin, SPRR (types 1a, 1b, 2a, 2b, and 3), late envelope protein (LEP)-1 and -16, and filaggrin are expressed by cultured human corneal epithelial cells.<sup>11</sup>

Transglutaminases (TGs) are Ca<sup>2+</sup>-dependent enzymes that catalyze the formation of N<sup>ε</sup>-(γ-glutamyl) lysine bonds between proteins and incorporation of biogenic amines into proteins that function as bridges between molecules. They are involved in the cornification process through cross-linking CE precursors in epidermal keratinocytes. Seven distinct TGs have been identified in humans,<sup>12</sup> and epidermal cells produce four isoforms of TGs: 1, 2, 3, and 5.<sup>13</sup> Membrane-associated TG isoforms, which include TGs 1, 3, and 5 are expressed by the upper layers of the epidermis and play a major role in CE formation on the intracellular surface of plasma membranes in terminally differentiated keratinocytes.<sup>14–16</sup> TG2, also known as tissue-type cytoplasmic TG, is expressed by the basal layer of the epidermis and functions in guanosine triphosphate (GTP)-binding in receptor signaling<sup>17</sup> and in apoptosis.<sup>18</sup> During CE formation, membrane-associated TG is activated initially, followed by cytoplasmic TG, which promotes apoptosis.<sup>19</sup> Membrane-associated TG1 initiates the cornification process and is the major TG-controlling cornification.<sup>11</sup> In certain severe dry eye conditions, such as Stevens-Johnson syndrome, clinically evident cornification of the corneal epithelium can develop

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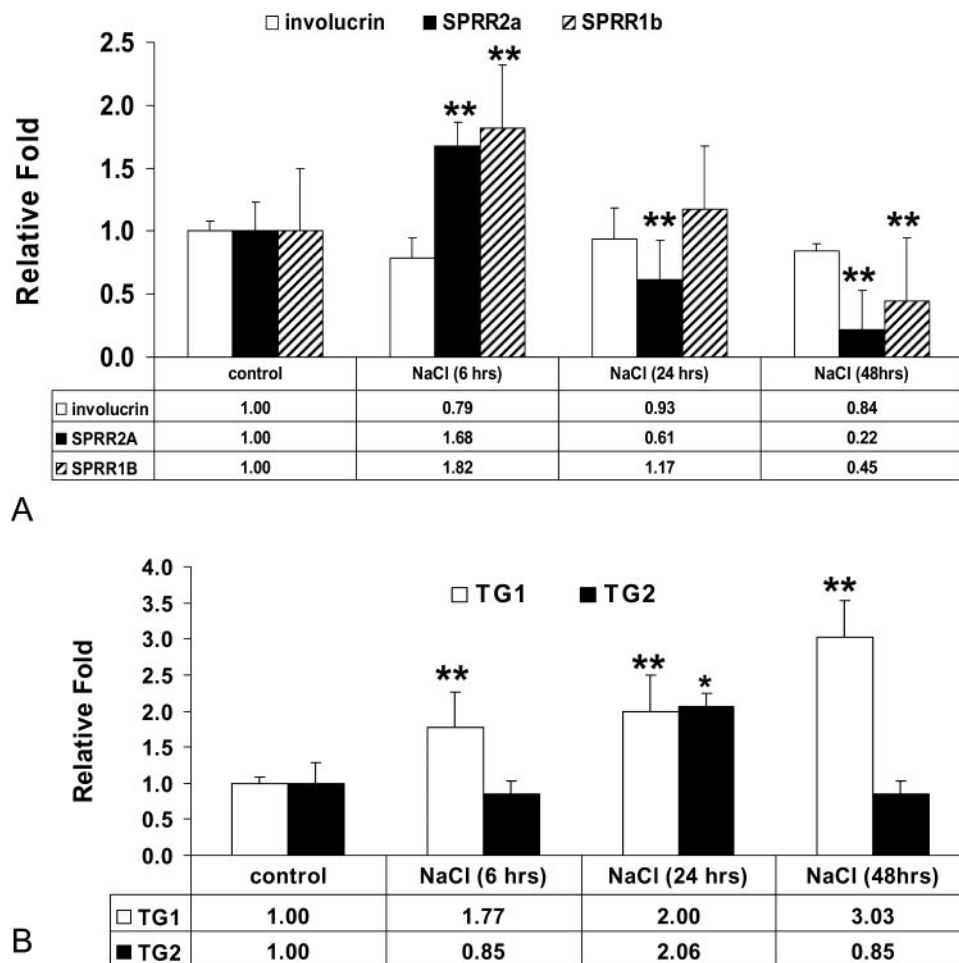
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**FIGURE 1.** Relative levels of CE precursor (**A**) and TG (**B**) mRNA transcripts evaluated by real-time PCR. PCHCE cells were treated with NaCl medium (osmolality 450 mOsm) for 6 to 48 hours. All data (mean  $\pm$  SEM) were compared to the control: \* $P < 0.05$ ; \*\* $P < 0.01$ .

that is often associated with severe eye discomfort and blindness.

With this knowledge in mind, we hypothesized that the hyperosmolar stress of dry eye promotes expression of CE precursors and TGs, leading to cell death by cornification in human corneal epithelial cells via the JNK MAPK signaling pathway. Furthermore, we propose that the cell signaling pathways controlling cornification and apoptosis in the corneal epithelium in response to hyperosmolar stress are different. In this work, we have evaluated the effects of hyperosmolar stress on expression of CE precursors and TGs by primary cultured human corneal epithelium (PCHCE) and the regulatory effects of JNK MAPK on this process.

## MATERIAL AND METHODS

### Material and Reagents

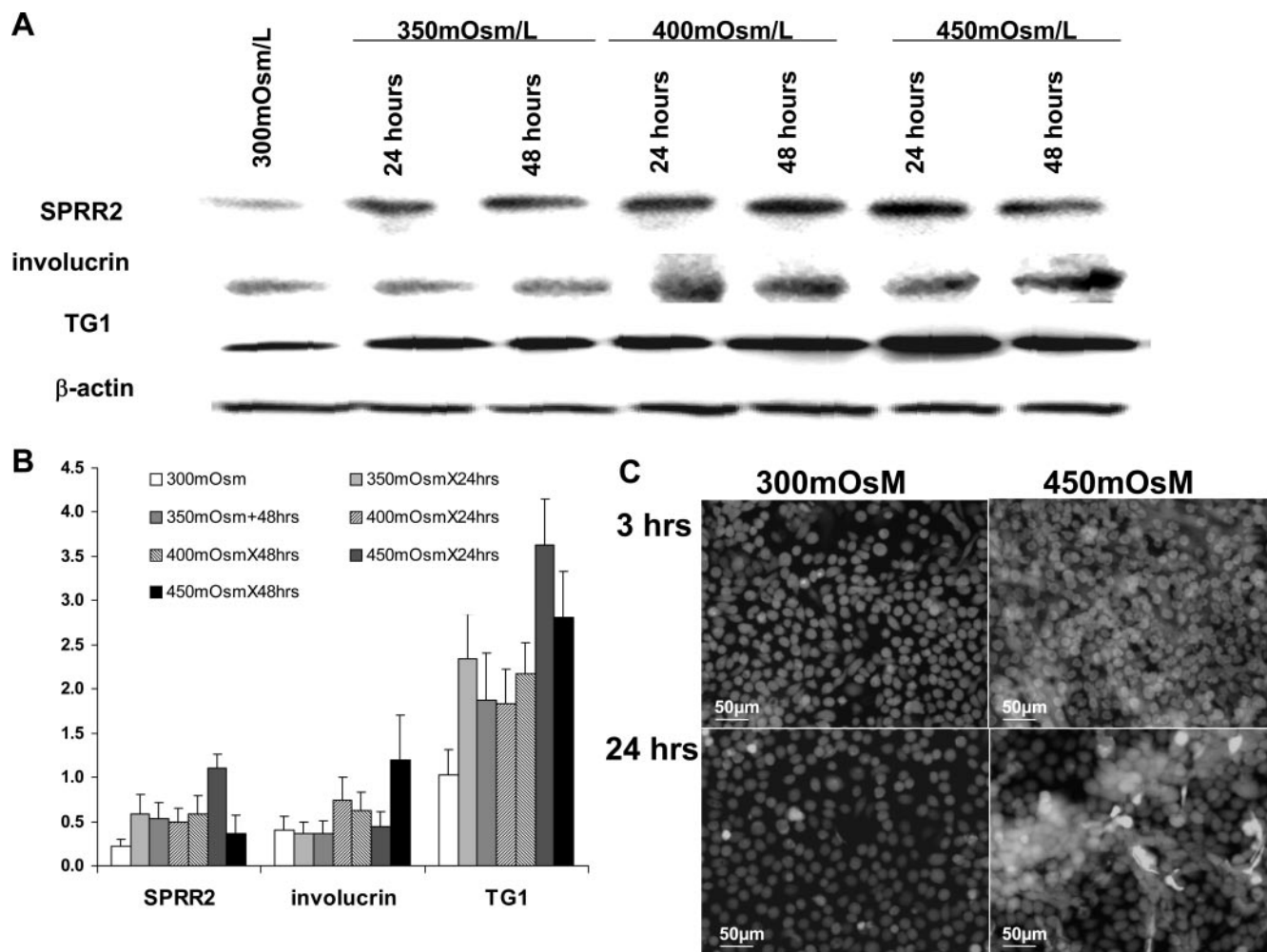
Reagents were obtained from the following sources: keratinocyte serum-free medium (KFSM) from Invitrogen-Gibco (Grand Island, NY); rabbit anti-SPRR2, clone ab18580, from Abcam, Inc. (Cambridge, MA); clone APO-25N-001 from Alexis Biochemical (San Diego, CA); mouse anti-involucrin (clone SY5) from Laboratory Vision (Fremont, CA); mouse anti-human keratinocyte TG monoclonal antibody (clone B.C1) from Biomedical Technologies Inc. (Stoughton, MA); and mouse monoclonal anti-actin antibody (clone AC-15) from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 488-conjugated goat IgG secondary antibodies and

fluorescein cadaverine (F-CDV) from Invitrogen-Molecular Probes (Eugene, OR); Hoechst 33342, propidium iodide (PI), and DNA size markers from Sigma-Aldrich; RNA extraction kits (RNeasy Mini Kit, RNAi kit, and siRNA-fluorescein, siRNA-F) from Qiagen (Valencia, CA); a PCR kit (GeneAmp RNA-PCR kit and Taqman Universal PCR master mix; Applied Biosystems, Foster City, CA); a cDNA synthesis kit (Ready-To-Go-Primer First-Strand Beads) from GE Health Care, Inc. (Piscataway, NJ); nuclear and cytoplasmic extraction reagents (NE-PER) and a BCA protein assay kit from Pierce (Rockford, IL); gels for protein electrophoresis (4%–15% Tris-HCl; Ready Gel) from Bio-Rad (Hercules, CA); polyvinylidene difluoride (PVDF; Immobilon-P) membrane from Millipore (Billerica, MA); a chemiluminescent reagent (Luminol) from Santa Cruz Biotechnology (Santa Cruz, CA); an in situ TUNEL kit from Roche (Basel, Switzerland); MTT cell proliferation assay kit from R&D Systems, Inc. (Minneapolis, MN); a JNK cell signaling assay (Beadlyte) and an anti-phosphatidylserine-conjugated Alexa 488 antibody from Upstate Biotechnology (Charlottesville, VA); and an Annexin-V FITC cell viability kit from ApoTarget (Camarillo, CA).

### Human Corneal Epithelial Cell Culture

Fresh human corneoscleral tissues (<72 hours after death) that were not suitable for clinical use, from donors aged 19 to 67 years, were obtained from the Lions Eye Bank of Texas (Houston, TX). They were prepared for explant culture by a previously described method.<sup>20,21</sup>

The confluent PCHCE cells were passaged at  $4 \times 10^4$  cells/cm<sup>2</sup> into 12-well plates containing KFSM for 24 hours, and then NaCl was added



**FIGURE 2.** Effects of osmotic stress on levels of CE precursor and TG proteins in PCHCE cells by Western blot, using  $\beta$ -actin as a control (A). The ratio of band intensity (y-axis) of CE precursors and membrane-associated TG1 compared with  $\beta$ -actin (mean  $\pm$  SD in three experiments) in osmotically stressed PCHCE cells at 24 and 48 hours (B). TG activity measured by F-CDV incorporation at 3 and 24 hours in osmotically stressed PCHCE cells (C).

to wells in concentrations of 50, 70, and 100 mM for 3, 6, 24, 48, and 72 hours. The osmolarity of KSFM medium is 290 mOsm. NaCl added in concentrations of 50, 70, and 100 mM produced media osmolarities of 350, 400, and 450 mOsm, respectively, measured by a vapor pressure osmometer (model 3300; Advanced Instruments, Inc., Norwood, MA) at the clinical core laboratory of the Department of Pathology, Methodist Hospital (Houston, TX). Total RNA was extracted for reverse transcription (RT) and real-time PCR from cultures treated for 6, 24, 48, or 72 hours. Proteins were extracted separately from 24-, 48-, and 72-hour 450 mOsm-treated groups for Western blot analysis and immunofluorescence staining.

### Transfection of siRNA-JNK

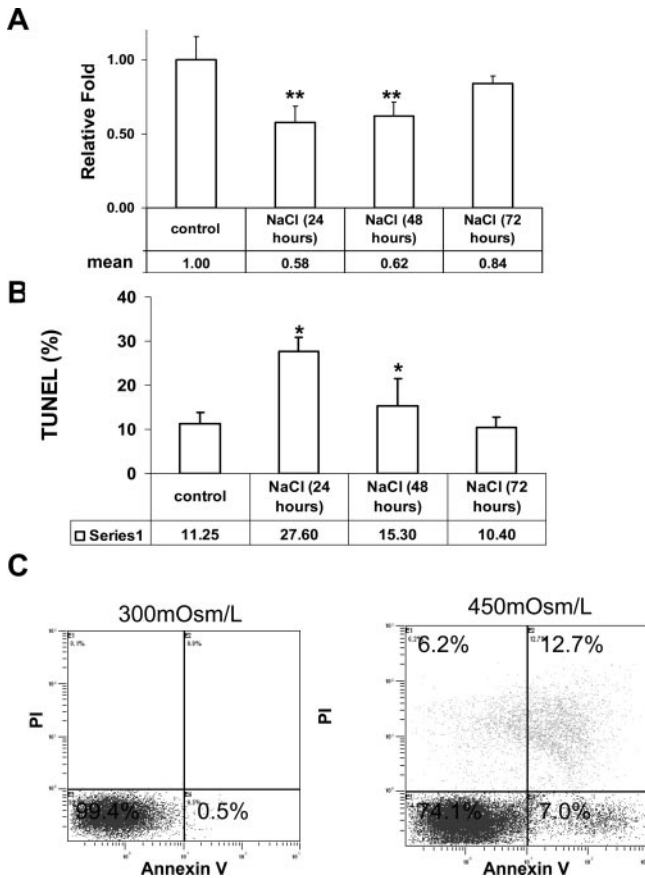
To design target-specific siRNA duplexes, a sequence of the type AA (N<sub>9</sub>) dTdT was selected from the open reading frame of the MAPK-specific mRNA to obtain 21-nt antisense strands with symmetrical-2 nt 3' identical overhanging sequences. Each selected siRNA sequence was submitted to a BLAST search against the human genome sequence to ensure that only one gene was targeted. The dsRNA sequences targeting two different regions in exon 8 of MAPK8 (JNK1; GenBank accession number NM\_002750; <http://www.ncbi.nlm.nih.gov/Genbank>; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) and exon 7 in MAPK9 (JNK2) mRNA (accession number in GenBank: NM\_002752) were designed for each gene and synthesized by Ambion (Austin, TX).

PCHCE cells, passaged into wells containing KSFM in 12-well plates at a density of  $4 \times 10^4$  cells/cm<sup>2</sup> were transfected with annealed dsRNA at a final concentration of 67 nM (Fast-Forward protocol; Qiagen). Certain cultures were treated with transfection reagent alone or with the nonsilencing siRNA-F (sequences, sense: r[UUC UCC GAA CGU GUC ACG U]dTdT-Fluorescein; antisense: r[ACG UGA CAC GUU CGG AGA A]dTdT) as negative controls for 24 hours, followed by treatment with 100 mM NaCl (450 mOsm) for 24, 48, or 72 hours. RNA and protein were extracted for real-time PCR and Western blot, respectively. Other cultures were prepared for immunofluorescent staining.

### RNA Extraction and Real-Time PCR

Total RNA was isolated from different groups of PCHCE cells by a selective silica-gel-based membrane extraction method (Qiagen).

Reverse transcription (RT) and relative quantitative real-time PCR were performed as previously described.<sup>22</sup> Primers and reporter probe for JNK1 (NM\_139046.1, sequence 125-149: TCAAGGAATAGTATGCCG-CAGCTTAT), JNK2 (NM\_139068.1, sequence 10-133: CAAGGGATTGTT-TGTGCTGCATTG), SPRR2a (NM\_005988.2, sequence 323-347: TGGCT-CACCTCGTCCACAGCTCCA), SPRR1b (NM\_003125, sequence 30-54: CTCTCTTCACACCAGGACCAGCCA), involucrin (NM\_005547.2, sequence 388-412: AAGGGATCAGCAGCTAAACAACAG), membrane-associated TG1 (NM\_000359, sequence 2339-2363: CTCACGTTGGGGA-CATTGGAGGCA), and tissue-type TG2 (NM\_198951, sequence 278-302:



**FIGURE 3.** Effects of osmotic stress (exposure to 450-mOsm medium) on corneal epithelial viability: MTT (A) and TUNEL-positive cells (B). All data were compared to the control: \**P* < 0.05; \*\**P* < 0.01. (C) Representative results of flow cytometry analysis of Annexin V and PI staining on osmotically stressed PCHCE cells for 24 hours (right) compared with controls (left). Cells undergoing early apoptosis are in the bottom right quadrant and those with late apoptosis are in the top right quadrant.

AGTGTGCGTGACCGGCCAGCCCTA) (TaqMan Gene Expression Assays; Applied Biosystems). Assays were performed in duplicate. A non-template control was included in all the experiments, to evaluate for DNA

**TABLE 1.** Results of Flow Cytometry of PCHCEs for Markers of Apoptosis

	PI (%)	PI + Annexin V (%)	Annexin V (%)	Anti-phosphatidylserine (%)
Control (300 mOsm/L)	0	0	0.4 ± 0.03	0.4 ± 0.07
Control + NaCl (450 mOsm/L)	5.9 ± 1.4*	12.1 ± 2.1*	8.2 ± 1.7*	10.5 ± 1.7*
siRNA-F	0	0	1.1 ± 2.4	4.5 ± 0.8
siRNA-F + NaCl	2.5 ± 0.4†	8.3 ± 2.5*	11.2 ± 2.2*	21.3 ± 3.9*
siRNA-JNK1	0	0	8.9 ± 1.6	6.5 ± 1.2
siRNA-JNK1 + NaCl	2.7 ± 0.6†	5.7 ± 1.9*‡	8.5 ± 1.8§	9.5 ± 1.3†§
siRNA-JNK2	0	0	5.9 ± 1.8	5.5 ± 1.1
siRNA-JNK2 + NaCl	3.7 ± 1.8†‡	6.1 ± 1.4*‡	7.9 ± 1.9‡	15.2 ± 1.31*‡¶
siRNA-JNK1 + 2	0	0	6.2 ± 0.40	10.1 ± 1.3
siRNA-JNK1 + 2 + NaCl	3.4 ± 1.10†‡	6.4 ± 1.13*‡	6.9 ± 2.11§	10.5 ± 1.15§

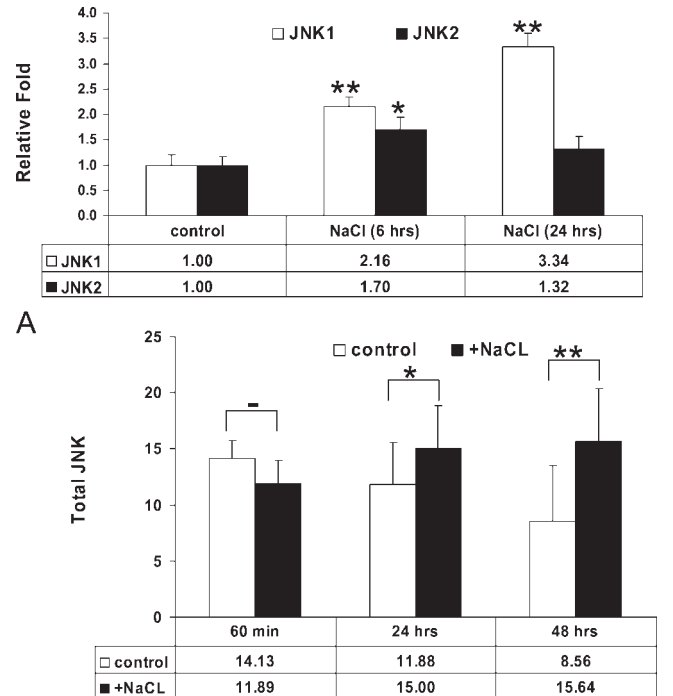
Data are the percentage of cells positively stained for PI (propidium iodide), PI + Annexin V, Annexin V or anti-phosphatidylserine and are expressed as the mean ± SEM.

\* *P* < 0.01.

† The osmotically stressed treatment group compared to non-treatment group, *P* < 0.05.

Difference between osmotically stressed and unstressed cells treated with JNK-specific siRNA compared with the difference observed in the siRNA-F groups: ‡*P* < 0.05; §*P* < 0.01.

Difference between osmotically stressed and unstressed cells treated with JNK 2+ or 1 + 2-specific siRNA compared with the difference observed in the JNK1-specific siRNA groups: ||*P* < 0.05; ¶*P* < 0.01.

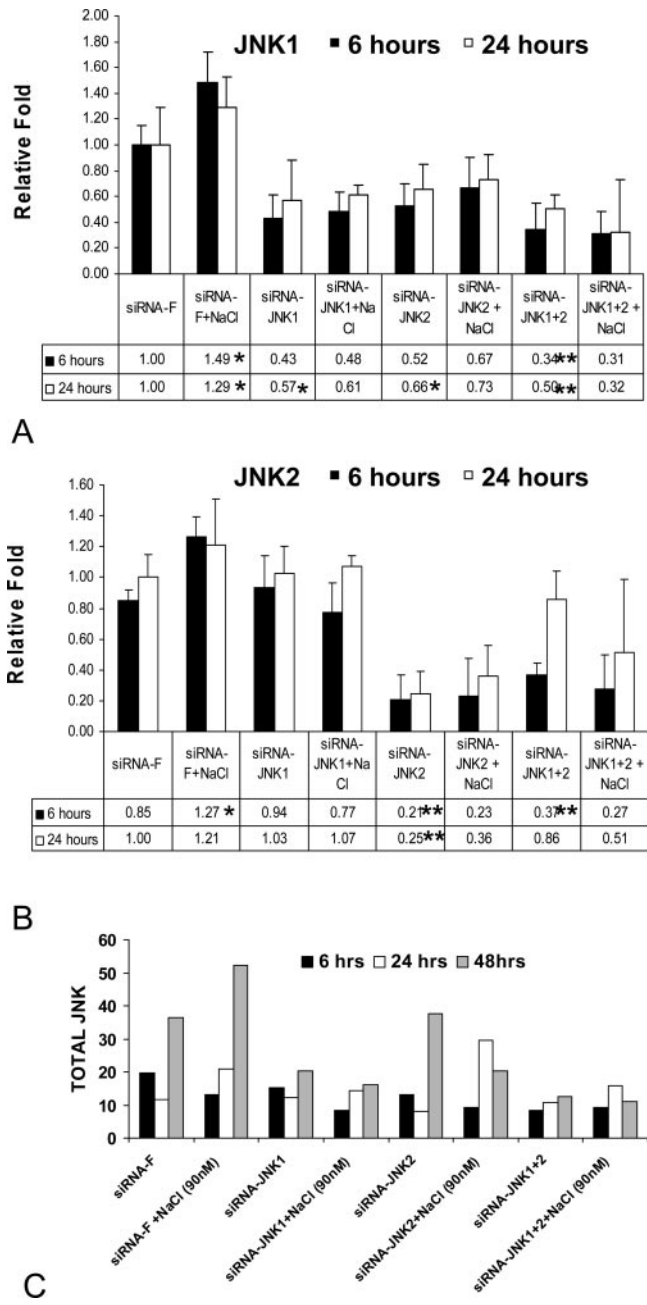


**FIGURE 4.** Effects of osmotic stress in PCHCE cells in 450-mOsm medium on JNK mRNA level as shown by real-time PCR (A) and total JNK protein measured by immunobead assay (B). Data are the mean ± SEM. All data were compared with the control: \**P* < 0.05; \*\**P* < 0.01.

contamination of the sample. The results of the relative quantitative real-time PCR were analyzed by the comparative threshold cycle (CT) method<sup>23</sup> and normalized with *GAPDH* as an internal control.

**Western Blot**

Western blot was performed by using a previously described method,<sup>24</sup> with modification. Primary antibodies were anti-SPRR2 (clone APO-25N-001; 1:500), anti-involucrin (1:100), or TGase 1 (clone B.C1; 1:20), and the secondary antibody was horseradish peroxidase-conjugated IgG (1:1000 dilution).



**FIGURE 5.** Effects of RNA silencing on the level of JNK. JNK mRNA measured by real-time PCR (JNK1: **A**; JNK2: **B**) and total JNK protein measured by immunobead assay (**C**). (**A**, **B**) Mean  $\pm$  SEM; (**C**) mean. Results from siRNA-JNKs (siRNA-JNK1, -2, and -1+2) were compared to that of siRNA-F. Data from the siRNA-F and siRNA-JNK groups with NaCl added were compared to the corresponding siRNA-F and siRNA-JNK groups; \* $P < 0.05$ ; \*\* $P < 0.01$ .

### Immunofluorescent Laser Scanning Confocal Microscopy

Immunofluorescent staining was performed with anti-SPRR2 (clone ab18580, 1:50), anti-involucrin (1:50), and mouse anti-human keratinocyte TG monoclonal (clone B.C.1, 1:20) antibodies, as previously reported.<sup>25</sup>

### Cell-Signaling Assay

Levels of total JNK were measured with an immunobead assay (Beadlyte Cell-Signaling Assay; Upstate Biotechnology). The cells were lysed

in cell-signaling buffer B, included in the kit, and the total protein concentrations of cell lysates were measured by a BCA protein assay. Each sample (20  $\mu$ g/25  $\mu$ L) was pipetted into a well of a 96-well plate and incubated with 25  $\mu$ L of beads coupled to JNK-specific capture antibodies overnight. The beads were washed and mixed with biotinylated JNK-specific reporter antibodies followed by streptavidin-phycoerythrin. The concentration of total JNK was measured (model 100, Luminex system; Luminex Corp., Austin, TX). Fifty events per bead were obtained, and data were exported to a spreadsheet program (Excel; Microsoft, Redmond, WA) for further analysis.

### Detection of the TG Activity with Fluorescein Cadaverine Incorporation

TG activity was evaluated by fluorescein cadaverine (F-CDV) incorporation by using a previously reported protocol.<sup>26-28</sup> F-CDV was added to PCHCE cells at a concentration of 0.5 mM for 3 to 24 hours in the dark. After incubation, the cells were washed four times in PBS for 2 minutes each and were examined by fluorescence microscopy (Nikon, Tokyo, Japan). The relative fluorescence intensity, as subjectively graded in digital images served as a measure of the relative TG activity.

### Evaluation of Apoptosis

Apoptosis was assessed by the terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labeling (TUNEL) assay, which detects DNA fragmentation, as well as by immunodetection of cell membrane Annexin-V FITC and phosphatidylserine by flow cytometry. The TUNEL assay was performed with a kit (Roche, Penzberg, Germany), using a modification of the manufacturer's protocol.

Annexin-V is a 35 to 36 kDa, calcium-dependent, phospholipid-binding protein with high affinity for phosphatidylserine. In concert with PI, Annexin-V can detect cells undergoing apoptosis. The staining followed the manufacturer's protocol (ApoTarget). After the PCHCE cells were washed with PBS twice and resuspended at  $2$  to  $3 \times 10^6$  cell/mL in binding buffer, Annexin-V FITC and PI were added to the cells and incubated 15 minutes at room temperature in the dark. The reaction was then diluted with binding buffer, and flow cytometry analysis was performed.

Phosphatidylserine molecules are shifted from the inner to outer leaflet of the cell membrane during early apoptosis.<sup>29</sup> For detection of phosphatidylserine, cells were fixed in 37% formaldehyde at 37°C for 10 minutes. After they were washed in PBS, the cells were incubated with anti-phosphatidylserine-conjugated Alexa Fluor 488 antibody for 1 hour at 4°C, washed with PBS, and resuspended in flow cytometry buffer. The cells were analyzed by flow cytometry.

### MTT Assay

The number of viable cells was measured by an MTT assay. The assay measures the activity of the mitochondrial enzyme succinyl dehydrogenase, which is expressed in living cells. The signal generated is proportional to the number of metabolically active cells.<sup>30</sup> This assay was performed according to the manufacturer's instructions (R&D Systems, Inc.).<sup>31</sup>

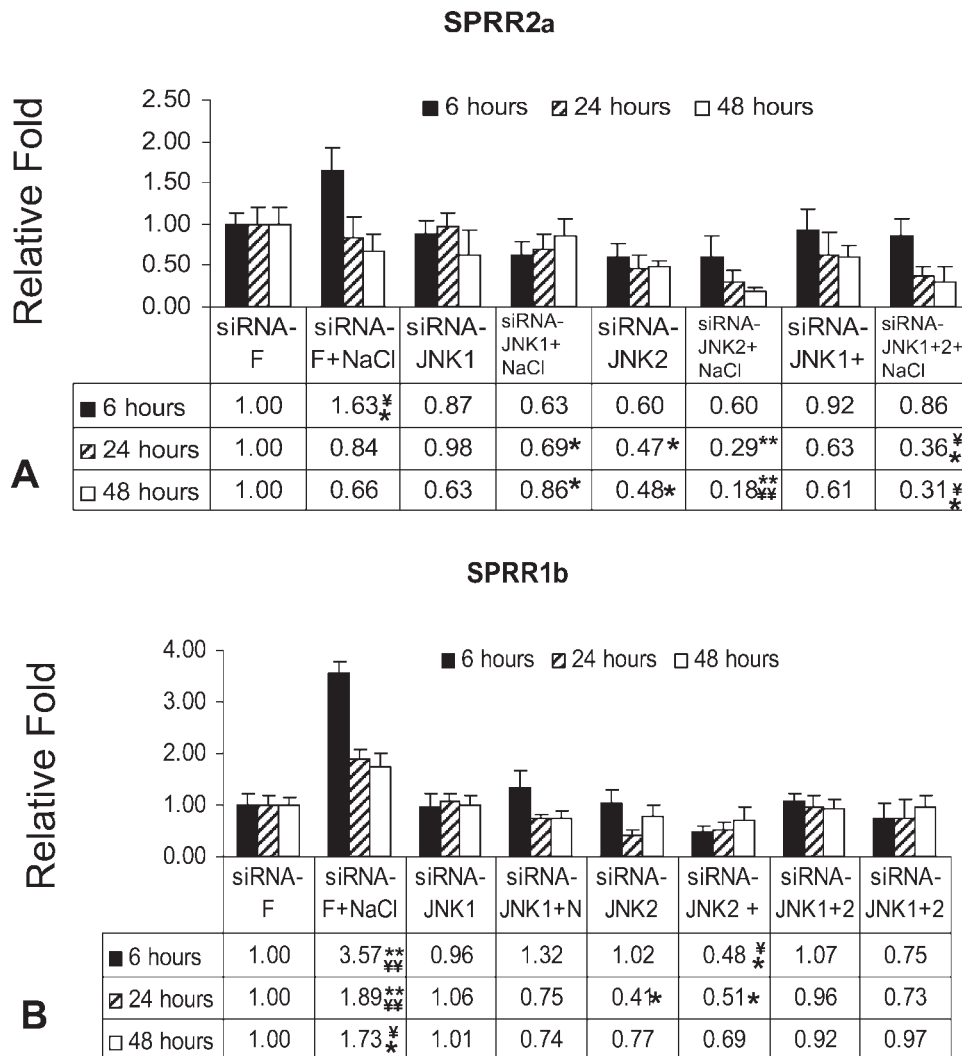
### Statistical Analysis

Statistical comparisons of control and treatment groups for real-time PCR, Western blot, flow cytometry, and MTT data were performed with ANOVA (Prism 4.0 software; GraphPad Software, Inc., San Diego, CA), with  $P \leq 0.05$  considered statistically significant.

## RESULTS

### Effects of Osmotic Stress on Expression of CE Precursors and TGs in PCHCE Cells

Relative quantitative RT-PCR was performed to evaluate expression of CE precursor and TG mRNA transcripts in PCHCE cells exposed to media of increasing osmolarity (350, 400, and



**FIGURE 6.** Effects of JNK-specific siRNAs on levels of SPRR2a (**A**) and SPRR2b (**B**) mRNA transcripts in control and osmotically stressed PCHCE cells (450 mOsM) measured by real-time PCR. Data are the mean  $\pm$  SEM. All data were compared to siRNA-F; <sup>\*</sup> $P < 0.05$ ; <sup>\*\*</sup> $P < 0.01$ . siRNA-JNKs with osmotic stress groups were compared to corresponding groups without osmotic stress; <sup>‡</sup> $P < 0.01$ ; <sup>‡‡</sup> $P < 0.05$ .

450 mOsM) for increasing lengths of time (6, 24, and 48 hours). The CE precursors involucrin, SPRR1b and -2a were evaluated because we have noted these to be abundantly produced by the corneal epithelium, and production of involucrin and SPRR2 by the corneal epithelium are noted to increase in response to desiccating stress.<sup>9,11</sup> Levels of SPRR1b and -2a transcripts significantly increased after 6 hours of hyperosmolar stress, but SPRR2a decreased to below baseline levels at 24 hours and both SPRR1b and -2a were lower than baseline at 48 hours. There was no significant change in the level of involucrin transcripts at any time point (Fig. 1A). Membrane-associated TG1 mRNA progressively increased up to 48 hours, while tissue-type TG2 levels were significantly greater at 24 hours (Fig. 1B).

Levels of SPRR2 and membrane-associated TG1 protein evaluated by Western blot increased after 24 and 48 hours of all levels of osmotic stress (350 to 450 mOsM), whereas involucrin increased only after exposure to 400 and 450 mOsM media (Fig. 2A).

Increased TG activity measured by F-CDV incorporation after exposure to 450-mOsM medium was noted by 3 hours, and strong fluorescence was noted at 24 hours (Fig. 2B).

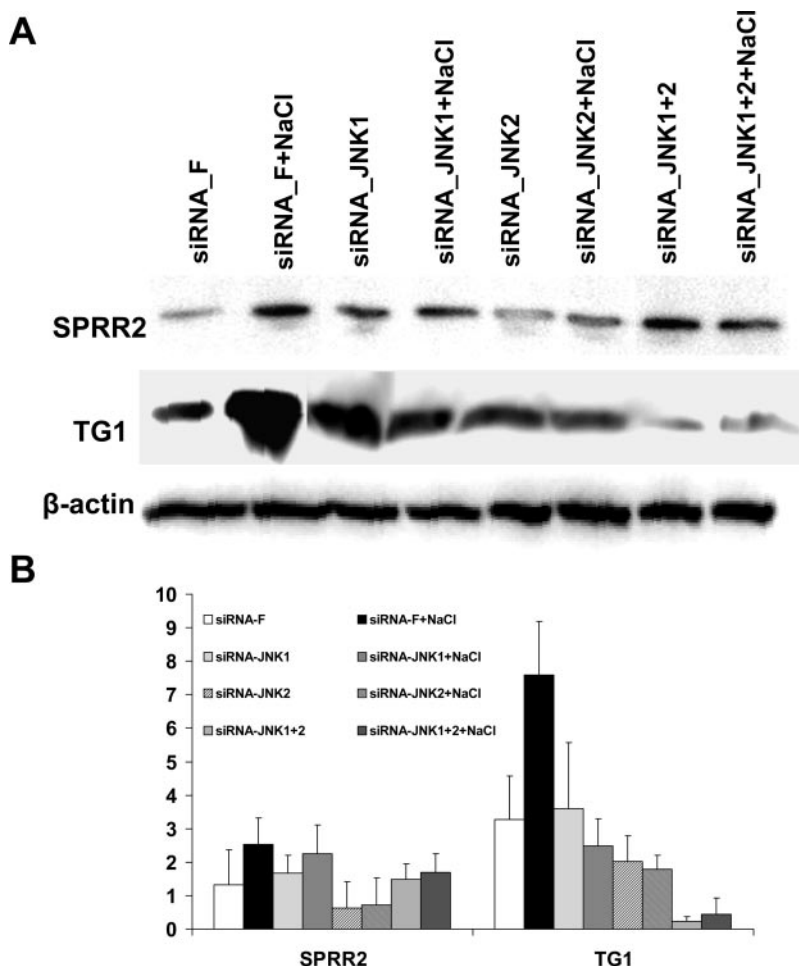
### Effects of Osmotic Stress on Corneal Epithelial Viability

Cell viability measured by an MTT assay (Fig. 3A) significantly decreased up to 48 hours after exposure to 450-mOsM medium. This decrease was accompanied by a significantly increased number of TUNEL-positive cells measured by flow cytometry (Fig. 3B).

Flow cytometry analysis of Annexin V with PI showed evidence of early apoptosis in  $8.2\% \pm 1.7\%$  of cells and late apoptosis in  $12.1\% \pm 2.1\%$  of cells 24 hours after exposure to 450-mOsM medium (Table 1; a representative flow cytometry is shown in Fig. 3C). In support of these findings,  $10.5\% \pm 1.7\%$  of cells were reactive to anti-phosphatidylserine, a marker of early apoptosis, by flow cytometry.

### Increased CE Precursor and TG Production by Osmotic Stress

Relative quantitative RT-PCR showed an increase in JNK1 and -2 mRNA transcripts in PCHCE cells exposed to hyperosmotic medium (450 mOsM) for 6 hours (Fig. 4A). At 24 hours, JNK1 transcripts remained significantly elevated, whereas JNK2 lev-



**FIGURE 7.** Effect of JNK-specific siRNAs on levels of expression of SPRR2 and membrane-associated TG1 protein in PCHCE cells grown in hyperosmolar medium (450 mOsM) for 24 hours.  $\beta$ -Actin was used as the control (A). Ratio of SPRR or membrane-associated TG1 with  $\beta$ -actin (y-axis); mean  $\pm$  SD detected by Western blot (B).

els fell to baseline levels. Total JNK protein measured by immunobead assay was significantly increased 24 and 48 hours after osmotic stress (Fig. 4B).

Treatment with JNK1- or -2-specific short interfering (si)RNA effectively knocked down levels of their respective mRNA transcripts after 6 hours and prevented an increase in JNK1- and/or -2-specific mRNA levels in response to osmotic stress in the PCHCE cells (Fig. 5A, 5B). The transfection efficiency was  $73.5\% \pm 2.1\%$ , similar to that observed in our previously reported study.<sup>31</sup> JNK siRNA also prevented an increase in total JNK protein in osmotically stressed cells (Fig. 5C).

We also evaluated the effect of JNK-specific siRNAs on mRNA levels of CE precursors SPRR1b and -2a in osmotically stressed PCHCE cells. The siRNA-F treated cells exposed to 450 mOsM had a significant increase in SPRR2a transcripts at 6 hours and in SPRR1b transcripts at 6, 24, and 48 hours. In contrast, cells treated with JNK1- or -2-specific siRNA showed no change or significant decreases in levels of SPRR transcripts compared with the control. Treatment with JNK-1-specific siRNA showed less inhibition of SPRR transcripts than JNK2-specific siRNA (Fig. 6).

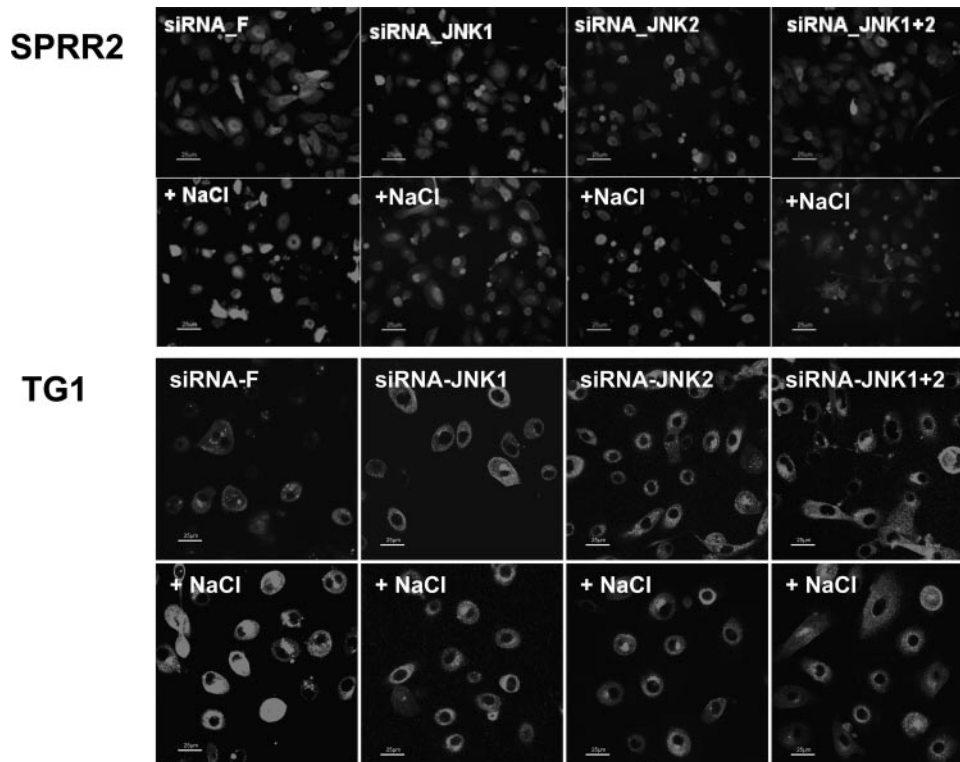
Western blot showed that treatment of osmotically stressed cells (450 mOsM for 24 hours) with JNK-specific siRNAs (for 24 hours) prevented the increase in SPRR2 and membrane-associated TG1 protein (Fig. 7).

Immunofluorescence of osmotically stressed PCHCE cells for SPRR2 and membrane-associated TG1 showed a pattern similar to that of the PCR and Western blot with JNK-specific siRNAs blunting the increase in these proteins (Fig. 8).

The effects of JNK-specific siRNAs on TG activity and levels of membrane-associated TG1 transcripts were evaluated in PCHCE cells subjected to osmotic stress. TG activity, measured by F-CDV incorporation, increased in osmotically stressed cells treated with siRNA-F; however, JNK1- and -2-specific siRNAs prevented an increase in TG activity (Fig. 9A). siRNA-F-treated control cultures showed a significant increase in levels of membrane-associated TG1 transcripts at 6, 24, and 48 hours after osmotic stress. This increase was not observed in siRNA-JNK1- and JNK2-treated cultures after 24 and 48 hours (Fig. 9B).

### Increased Viability of Osmotically Stressed PCHCE Cells by JNK Inhibition

Osmotic stress with high-saline medium decreased the number of viable corneal epithelial cells in siRNA-F-treated cultures when measured by MTT; however, there was no significant change in the number of viable cells in osmotically stressed cultures treated with siRNA-JNK1 and -2 (Fig. 10). Cells treated with JNK2- or JNK1- and 2-specific siRNA showed a greater



**FIGURE 8.** Immunofluorescence staining for SPRR2 (*top*) and membrane-associated TG1 (*bottom*) in siRNA (siRNA-F- or JNK-specific) treated PCHCE cells for 24 hours grown in control or hyperosmolar sodium chloride (+NaCl) medium (450 mOsm for 24 hours).

percentage of viable cells at 48 and 72 hours than siRNA-JNK1-specific siRNA treated cells (Fig. 10;  $P < 0.05$ ).

To determine whether the decrease in the number of viable cells was due to apoptosis, flow cytometry was performed on cells stained with PI, anti-Annexin V or anti-phosphatidylserine. Osmotic stress increased the number of cells showing evidence of apoptosis and cell death. Similar findings were noted in the siRNA-F-treated groups. Treatment of cells with JNK1-specific siRNA significantly decreased the percentage of cells exhibiting early apoptosis (Annexin V or anti-phosphatidylserine positive) or late apoptosis (PI+Annexin V positive). Treatment of cells with JNK2- or/and JNK1-specific siRNA also decreased early and late apoptosis compared with the siRNA-F control group, but the siRNA-JNK 2 groups was not as effective as JNK1 in decreasing signs of early apoptosis (Table 1).

## DISCUSSION

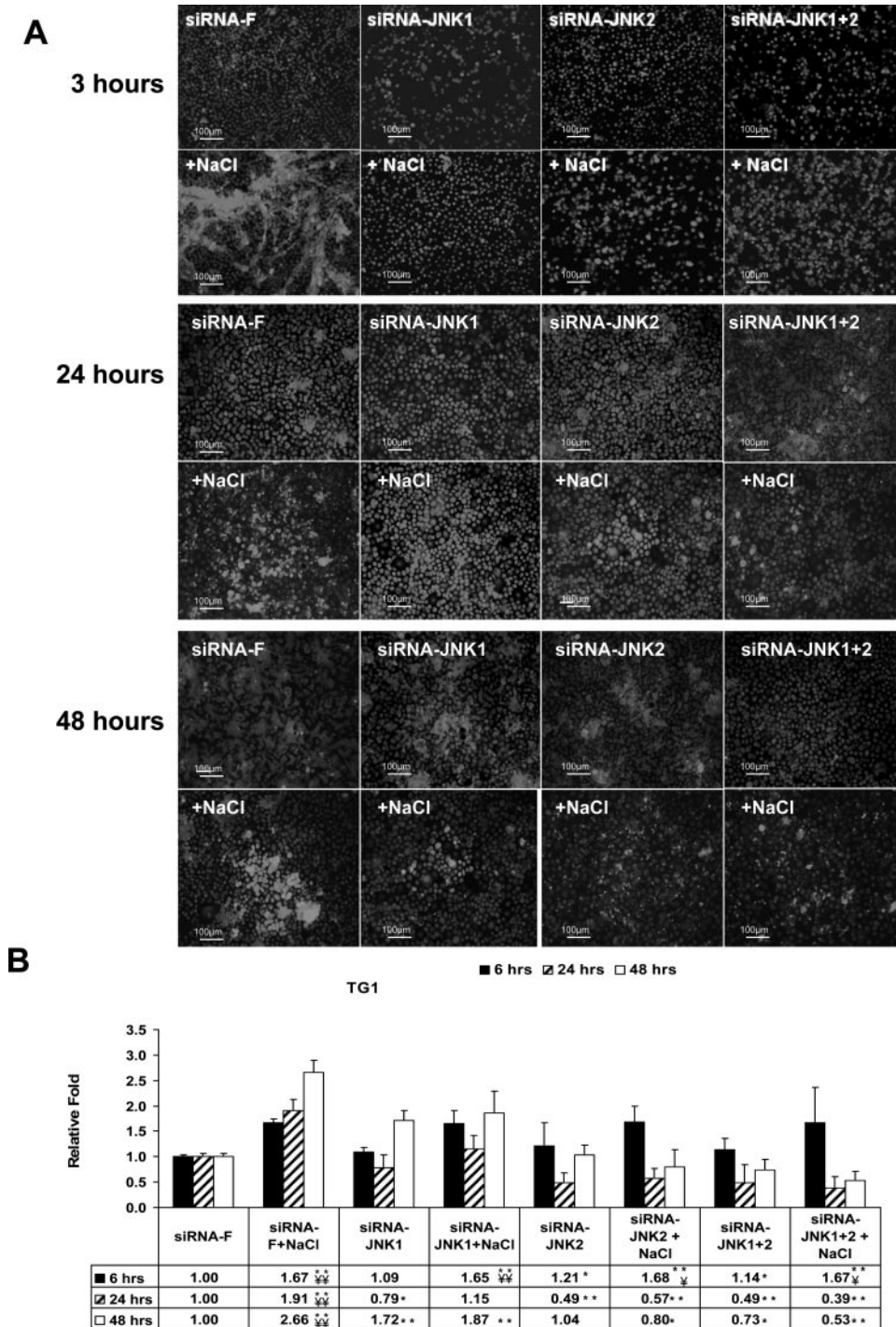
We evaluated the effects of osmotic stress on production of CE precursors and membrane-associated TG1 by primary cultured human corneal epithelial cells. The corneal epithelium is the most exposed mucosal surface in the human body. It is normally protected and hydrated by tear fluid that has an osmolarity of approximately 305 mOsm.<sup>1,8</sup> Hyperosmolarity was chosen as a corneal epithelial stressor in this study, because these cells are exposed to increased tear osmolarity in dry eye conditions when tear production decreases or tear evaporation increases. We found that osmotic stress transiently increased expression of the CE precursors, SPRR1b and -2a, with RNA transcripts peaking at 6 hours, and elevated protein levels persisting until 48 hours. Levels of involucrin transcripts did not significantly change under osmotic stress; however, protein levels were elevated at 24 and 48 hours in cells exposed to the higher media osmolarities. Osmotic stress caused a more

sustained elevation of membrane-associated TG1 transcripts and protein, both of which were elevated at 48 hours. The accompanying increase in TGs activity would enhance cross-linking of the newly synthesized CE precursors. Before 48 hours, the increased expression of CE precursors was related to the stronger TG activity evaluated by F-CDV. Therefore, osmotic stress appears to induce a coordinated increase of the structural and cross-linking components that are necessary for cornification. Under nonstress conditions *in vivo*, the corneal epithelial barrier is maintained by the precorneal mucin layer and epithelial tight junctions; production of cornifying factors by the corneal epithelium occurs at low levels. The increased production of CE precursors and membrane-associated TG1 by the corneal epithelium in the face of osmotic stress found in our study may represent a protective response to establish a more robust barrier under desiccating conditions. It is likely that production of this skinlike, poorly hydrated barrier *in vivo* may have adverse consequences for comfort and vision.

We previously reported that JNK MAPK was activated in the corneal epithelium in response to experimental dryness and osmotic stress.<sup>4,32</sup> Therefore, we evaluated the effects of JNK inhibition via RNA interference on the production of these cornification factors in response to osmotic stress. We found that JNK signaling pathways are involved in the stimulated production of CE precursors and membrane-associated TG1 by cultured human corneal epithelial cells. Both JNK- and -2-specific siRNAs prevented an increase in SPRR2a and -1b production, whereas JNK2 and JNK1+2 siRNAs inhibited membrane-associated TG1 transcripts and TG activity in osmotically stressed cells.

Osmotic stress caused a decrease in the number of viable corneal epithelial cells for up to 48 hours after exposure, corresponding to the increased production of CE precursors. Inhibition of both JNK1 and -2 improved cell viability com-





**FIGURE 9.** TG activity measured by F-CDV incorporation in siRNA-treated PCHCE cells grown in control or hyperosmolar medium (+NaCl) for 3, 24, and 48 hours (A). Levels of membrane-associated TG1 mRNA transcripts measured by real-time PCR (B) in PCHCE cells grown in control or hyperosmolar sodium chloride (+NaCl) medium with JNK1-, -2-, or -1+2-specific siRNA for 6 to 48 hours. Data are the mean  $\pm$  SEM. All siRNA-JNK groups (siRNA-JNK1, -2, and -1+2) were compared to siRNA-F (\* $P < 0.05$ ; \*\* $P < 0.01$ ) and all siRNA-JNKs+NaCl groups (siRNA-JNK1, -2, and -1+2 +NaCl) were compared to the corresponding siRNA-JNK groups (siRNA-JNK1, -2, and -1+2;  $\forall P < 0.01$ ;  $\forall P < 0.05$ ), respectively.

pared with cells treated with control siRNA, with a slightly greater inhibitory effect seen in JNK2 siRNA-treated cells. This decrease in viability of osmotically stressed cells appears to be due in part to apoptosis. JNK1 siRNA treatment caused a greater decrease in markers of early apoptosis (Annexin V and anti-phosphatidylserine) than JNK2 siRNA; in contrast, inhibi-

tion of JNK2 caused a greater decrease in CE precursor production. Therefore, it is possible that some of the decrease in cell viability induced by osmotic stress is due to the cornification process (see Fig. 11). After the initial decrease in cell viability, the number of viable cells returned to near-normal at 72 hours. This rebound may represent proliferation of cells

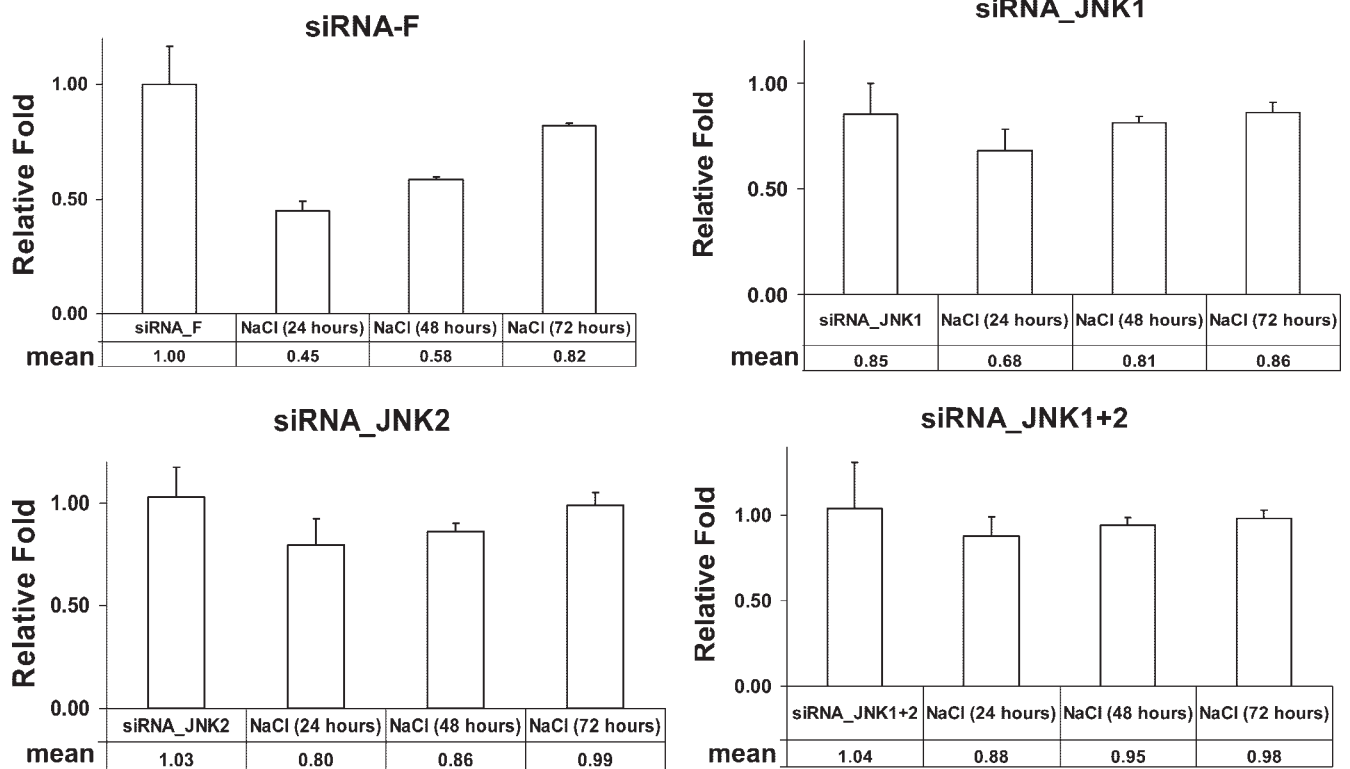


FIGURE 10. Number of viable cells measured by MTT in control (siRNA-F) and JNK-specific siRNA-treated PCHCE cells exposed to 450-mOsm sodium chloride (+NaCl) medium for up to 72 hours.

that survived the osmotic stress, perhaps through osmo-adaptation. It is well recognized that to avoid high intracellular ion concentration and to balance intra- and extracellular osmolarity, cells typically adapt to sustained hyperosmotic stress by preferential accumulation of compatible osmotic solutes. The osmo-transporters involved in this process are regulated by multiple signaling pathways, including JNK2.<sup>33</sup>

Inhibiting the JNK pathway appears to prevent production of CE precursors and maintain corneal epithelial viability in response to osmotic stress. These findings have potential implications for treating dry eye and ocular surface conditions that are associated with elevated tear osmolarity.

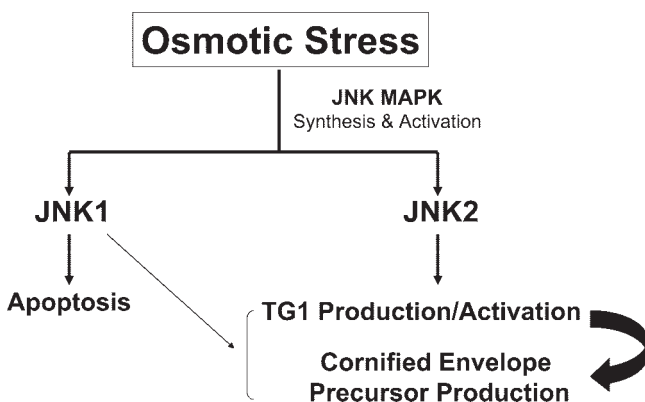


FIGURE 11. A summary diagram of the signaling pathways controlling apoptosis and cornification that are mediated by JNK MAPK pathways in osmotically stressed cells.

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