Objective: To investigate the protective effects of c-Jun N-terminal kinase (JNK)–1 and -2 gene knockout (KO) on the corneal epithelial response to desiccating stress.

Methods: The C57BL/6, JNK1KO, and JNK2KO mice were subjected to desiccating stress (DS) for 5 days. The effects of DS on the corneal epithelium were evaluated by measuring corneal smoothness and permeability. Expression of matrix metalloproteinases (MMP)–1, MMP-9, and cornified envelope protein precursors (small proline-rich protein [SPRR]–1a, SPRR-2a, and involucrin) in the corneal epithelia was evaluated by immunostaining and real-time polymerase chain reaction. Collagenase and gelatinase activity in corneal sections as measured with in situ fluorescent assays.

Results: The JNK2KO mice had smoother corneal surfaces and less corneal barrier disruption in response to DS than JNK1KO mice and C57BL/6 wild-type control mice. The DS increased levels of MMP-1, MMP-9, SPRR-1a, SPRR-2a, involucrin immunoreactivity, and mRNA transcripts in the corneal epithelium of JNK1KO and C57BL/6 mice, but not in JNK2KO mice. Knockout of JNK2 prevented DS-induced increase in gelatinase and collagenase activity in the cornea.

Conclusion: The JNK2 protein appears to have an essential role in desiccation-induced corneal epithelial disease by stimulating production of MMP-1, MMP-9, and cornified envelope precursors.

Clinical Relevance: The JNK2 protein could be a novel therapeutic target in dry eye disease.

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ANIMAL MODEL OF DRY EYE

This research protocol was approved by the Baylor College of Medicine Center for Comparative Medicine (Institutional Animal Care and Use Committee), and it conformed to the standards in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The JNK1 (B6.129S1-Mapk8tm1Flv/J) and JNK2 (B6.129S2-Mapk9tm1Flv/J) knockout (KO) mice in a C57BL/6 background and wild-type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) to establish breeding colonies. The genotype of JNK1KO and JNK2KO mice was confirmed according to the Jackson Laboratories protocol (data not shown). The C57BL/6 wild-type control mice were also purchased from Jackson Laboratories. Mice were used at 8 weeks of age.

Desiccating stress was induced by subcutaneous injection of scopolamine hydrobromide (0.5 mg/0.2 mL; Sigma-Aldrich, St Louis, Missouri) 4 times a day, alternating between the left and right flanks of the above 3 strains, as previously described, for 3 consecutive days (DS5). Mice were placed in modified cages with a perforated plastic screen on one side to allow airflow from a fan placed 6 in in front of them for 16 in modified cages with a perforated plastic screen on one side to allow airflow from a fan placed 6 in in front of them for 16 h/d. Room humidity was maintained at 30% to 35%. This model to allow airflow from a fan placed 6 in in front of them for 16 h/d. Room humidity was maintained at 30% to 35%. This model of dry eye disease has been used previously, with no discernible ill effects from the scopolamine treatment or low humidity. Control mice were kept in a nonstressed (NS) environment maintained at 50% to 75% relative humidity without exposure to forced air. Thirty-five animals per strain (JNK1KO, JNK2KO, and C57BL/6) per time point (NS and DS5) were used; 5 mice were used for histology sections, 10 for evaluation of corneal permeability, and 20 for gene analysis. Evaluation of corneal smoothness was performed on the same mice that were used for evaluating gene expression.

EVALUATION OF CORNEAL SMOOTHNESS

Corneal epithelial permeability to Oregon green dextran (OGD) (70,000 molecular weight [MW]; Invitrogen, Eugene, Oregon) was assessed in the NS and DS5 groups of the 3 strains (10 mice per group per experiment; 3 sets of experiments). Briefly, 0.5 µL of 50 µg/mL OGD was instilled onto the corneal surface 1 minute before euthanasia. Corneas were rinsed with phosphate-buffered saline and photographed with a stereoscopic zoom microscope (SMZ 1500; Nikon, Melville, New York) under fluorescence excitation at 470 nm. The severity of corneal OGD staining was graded in digital images in the 2-mm central corneal zone of each eye by 2 masked observers (C.S.D.P. and S.B.P.) using the MetaVue 6.24r software ( Molecular Devices, Sunnyvale, California). The mean intensity measured inside this central zone, generated by the software, was transferred to a database, and the results were averaged within each group. Results are presented as means (standard deviation) in gray levels.

IN SITU ZYMOGRAPHY AND COLLAGENASE ASSAYS

In situ zymography and collagenase assays were performed to localize the gelatinase/collagenase activity in corneal cryosections using a previously described method. Corneal sections were assayed individually either for gelatinases (MMP-2, MMP-9) or collagenase I (MMP-1) (n=5 per strain per assay). Sections were thawed and incubated overnight with reaction buffer, 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl2, and 0.2 mM NaN3, pH 7.6, containing 40 µg/mL of either fluorescein isothiocyanate conjugate (FITC)–labeled dye-quenched (DQ) gelatin or FITC–labeled collagen 1, which were available in a gelatinase/collagenase assay kit (EnzChek; Molecular Probes, Eugene, Oregon). As a negative control, 50 nM of 1,10-phenanthroline, a metalloproteinase inhibitor, was added to the reaction buffer before applying the FITC-labeled DQ/collagen 1 to frozen sections. Proteolysis of the FITC-labeled DQ gelatin or FITC-DQ collagen substrate yields cleaved FITC peptides that are fluorescent. The localization of fluorescence indicates the sites of net gelatinolytic/collagenase activity, and the intensity is proportional to the amount of activity in the tissue. After incuba-
tion, the sections were washed with phosphate-buffered saline and counterstained with propidium iodide (2 µg/mL in phosphate-buffered saline; Sigma, St Louis, Missouri) in an antifade GelMount (Fisher, Atlanta, Georgia), and a coverslip was applied. Areas of gelatinolytic/collagenase activity of MMPs were viewed with a Nikon Eclipse E400 fluorescent microscope (Melville, New York), and images were captured by a Nikon DS-QiMc digital camera.

MEASUREMENT OF FLUORESCENCE INTENSITY IN CORNEA

Fluorescence intensity in the corneal sections used for either immunostaining (MMP-1, MMP-9, SPRR-1a, SPRR-2a, and involucrin) or in situ assays (zymography and collagenase I) were measured in digital images using NIS Elements Software (version 3.0, BR [basic research]; Nikon). At least 6 images per time point per strain were analyzed. The epithelial layer of the stained corneas was circumscribed by 2 masked observers. The fluorescence intensity was calculated by the software and entered into an Excel spreadsheet (Microsoft Corp, Redmond, Washington). Data are presented as mean (standard deviation) in gray levels.

RNA ISOLATION AND REAL-TIME POLYMERASE CHAIN REACTION

Total RNA from the corneal epithelium from the 3 strains per time point (NS and DS5) was extracted using an acid guanidium thiocyanate-phenol-chloroform method, as previously described.21 Four samples per strain per time point were used, and 1 sample consisted of pooled samples from 5 mice. Samples were treated with DNase to prevent genomic DNA contamination, according to the manufacturer’s instructions (Qiagen, Valencia, California).

First-strand complementary DNA was synthesized from 1 µg of total RNA using random hexamers and M-MuLV reverse transcriptase (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Piscataway, New Jersey), as previously described.21 Real-time polymerase chain reaction was performed using gene expression assay primers and MGB probes specific for glyceraldehyde 3-phosphate dehydrogenase (GADPH), MMP-1, MMP-3, MMP-9, involucrin, SPRR-1a and SPRR-2a (assay identification numbers, Mm9999915, Mm00473493, Mm00440295, Mm00473493, Mn00515219, Mm01962902, and Mm00845122, respectively), and a TaqMan Universal PCR Master Mix AmpErase (Applied Biosystems, Foster City, California), in a commercial thermocycling system (Mx3005P QPCR System; Stratagene, La Jolla, California), according to the manufacturer’s recommendations. Assays were performed in duplicate, and there were 4 independent samples. A non-template control was included in all of the experiments to evaluate DNA contamination of the reagents used. The GAPDH gene was used as an endogenous reference for each reaction. The results of quantitative polymerase chain reaction were analyzed by the comparative cycle threshold (Ct) method in which target change = 2−ΔΔCt (User Bulletin, No. 2, P/N 4303859; Applied Biosystems). The Ct was determined using the primary (fluorescent) signal as the cycle at which the signal crossed a user-defined threshold (the middle point of the amplification curve, usually 40% of the maximum fluorescence). The results were normalized by the Ct value of GAPDH, and the mean Ct of relative mRNA level in the NS group of each strain was used as the calibrator for the strain.

STATISTICAL ANALYSIS

The unpaired t test was used to compare the controls (NS vs DS5) with each mouse strain. P ≤ .05 was considered statistically significant. Analyses were performed using GraphPad Prism 4.0 software (GraphPad, San Diego, California).

RESISTANCE TO DESICCATION-INDUCED CORNEAL SURFACE ABNORMALITY

The results revealed that JNK2KO, but not JNK1KO, is resistant to desiccation-induced corneal surface abnormality. Dry eye is often accompanied by an altered corneal epithelial barrier and corneal surface irregularity. Fluorescent dyes such as sodium fluorescein and OGD have been used with success to evaluate corneal barrier disruption.20,21,24 In humans, corneal regularity can be assessed by videokeratoscopy.22 Mice blink very infrequently, and it is not possible to assess the tear breakup time. Therefore, in mice, the regularity of the light ring reflected off the corneal surface has been used as a measure of corneal smoothness and compromise of tear film integrity.20

We evaluated the corneal permeability to OGD and corneal smoothness of the 3 mouse strains before and after experimental DS. The results are presented in Figure 1A, and B. The 3 strains had similar uptake of OGD by the corneal epithelium at baseline; however, DS induced significant corneal barrier disruption in C57BL/6 and JNK1KO mice, but not in the JNK2KO mice (Figure 1A). Of the 3 strains, wild-type C57BL/6 mice...
showed the greatest barrier disruption. A similar response pattern between strains was observed when evaluating corneal smoothness (Figure 1, C and D). The C57BL/6 and JNK1KO mice showed increased corneal irregularity after 5 days of desiccating stress, while JNK2KO DS5 mice maintained their corneal smoothness at baseline levels.

INCREASE IN GELATINASE AND COLLAGENASE LEVELS AND ACTIVITY BY DS

The results show that DS increases gelatinase and collagenase levels and activity in wild-type and JNK1KO mouse strains.

We have previously reported that MMPs are implicated in desiccation-induced disruption of barrier function, including cleavage of tight junction proteins such as occludin and zonula occludens 1.9-11 To better evaluate the role of JNK SAPK in corneal epithelial function, we performed immunostaining for the gelatinase MMP-9 and the collagenase MMP-1 and in situ gelatinase and collagenase assays in sequential corneal sections of the 3 strains. Our results are presented in Figure 2.

Desiccating stress increased immunoreactivity of MMP-9 (Figure 2A) and MMP-1 (Figure 2E) in the corneal epithelium, notably in the most apical cell layers, in both C57BL/6 and JNK1KO mice. The MMP-9 and -1 fluorescent intensity levels were significantly higher in the DS5 than the NS sections from C57BL/6 and JNK1KO mice (Figure 2, C and G; P < .001, P < .001, and P = .02, respectively). In situ gelatinase and collagenase activity increased significantly in DS5 corneal epithelium of C57BL/6 mice and showed a moderate, non-statistically significant increase in JNK1KO and no change in JNK2KO corneas. Analysis of MMP-9, -3, and -1 gene expression in the corneal epithelium is presented in the Table. Levels of MMP-9 mRNA transcripts significantly increased in C57BL/6, but decreased in both JNKKO strains at DS5. Levels of MMP-1 and -3 mRNA transcripts significantly increased in C57BL/6 and JNK1KO strains at DS5, while they showed a non-statistically significant decrease in JNK2KO mice. These findings suggest that the increased levels of MMP-9 protein observed by immunofluorescent staining at DS5 in the JNK1KO may occur at the posttranscriptional level.

LEVELS OF CORNIFIED ENVELOPE PROTEIN PRECURSOR TRANSCRIPTS

High levels of cornified envelope protein precursor transcripts were observed in JNK1KO, but not JNK2KO mice.

We have previously demonstrated that DS increased expression of cornified envelope precursor proteins.21 To further investigate the role of JNK SAPK pathway in this stress response, we examined the expression of the cornified envelope precursors SPRR-1a, SPRR-2a, and involucrin in the corneal epithelium by immunostaining and real-time polymerase chain reaction.
Our immunofluorescent staining results are presented in Figure 3. The intensity of SPRR-1a and SPRR-2a was similar for the 3 strains in the NS control group. Increased staining intensity for involucrin was noted in the basal epithelium of the JNK1KO and JNK2KO corneas at baseline compared with C57BL/6; however, this was not statistically significant.

Immunoreactivity of SPRR-1a, SPRR-2a, and involucrin significantly increased in the corneas of DS5 C57BL/6 and JNK1KO, while no change in immunoreactivity was observed in JNK2KO mice (Figures 3, A-C).

The levels of mRNA transcripts for these cornified envelope precursors are presented in the Table. Levels of SPRR-1a transcripts increased in all 3 strains following 5 days of DS, with the greatest relative increase from baseline in the C57BL/6 strain. The C57BL/6 mice also had a significant increase in SPRR-2a and involucrin transcripts. Involucrin transcripts also increased in JNK1KO, while there was no change in SPRR-2a and a statistically significant decrease in involucrin transcripts in the JNK2KO mice.

### Comment

Our model of DS has previously been noted to induce activation of JNK SAPK in the corneal and conjunctival epithelium, with the greatest increase noted for JNK2. We hypothesized that SAPK signaling pathways are activated by the ocular surface desiccation and hypertonic extracellular environment that results from decreased tear production in our model. Consistent with this theory, we found increased levels of active phosphorylated JNK1 and JNK2 (JNK2 > JNK1) in ocular surface epithelium treated with hypertonic saline in vivo and in cultured human corneal epithelial cells exposed to hyperosmolar media. Because there are no specific inhibitors for each JNK isoform, we opted to evaluate mice deficient in either JNK1 or JNK2 to better dissect the specific contribution of each JNK isoform to the epithelial response of dry eye.

Activation of the JNK pathway has been implicated in the increased production of MMPs and cornified envelope precursor proteins that has been observed in the corneal epithelium following desiccating or hyperosmotic stress. The activation of MAPK p38 alone has been shown to induce expression of MMP-1 and -3 in an activator protein 1-independent manner by stabilizing the corresponding mRNAs in human skin fibroblasts. To confirm the relative roles of JNK1 and JNK2 in the corneal epithelium stress response in vivo, we compared corneal epithelial barrier function and smoothness, as well as production of MMPs and cornified envelope precursors, following 5 days of experimental desiccation in JNK1 and JNK2 gene-deficient and wild-type C57BL/6 strains.

We observed that JNK2KO mice were resistant to corneal epithelial barrier disruption and maintained a regular corneal surface in response to desiccation, whereas JNK1KO mice behaved like wild-type control mice, with significant disruption of barrier and a non-statistically significant increase in surface regularity. Consistent with the lack of worsening of clinical parameters of dry eye, JNK2KO mice showed no change in immunoreactivity to MMP-9 and -1 and no increase in gelatinase or collagenase activity in their corneal epithelium. Furthermore, levels of MMP-3, a physiological activator of MMP-9, remained constant in JNK2KO mice. Previously, MMP-9 has been found to have a role in corneal barrier disruption in dry eye by degrading tight junction proteins in the apical corneal epithelium and accelerating apical cell desquamation. We observed that tear MMP-9 activity increased with severity of corneal epithelial disease in dry eye and positively correlated with corneal fluorescein staining scores inversely correlated with low-contrast visual acuity.

To distinguish proteolytic activation of MMP by JNK2 from synthesis of new MMP in cultured corneal epithelial cells, a drug such as cycloheximide, which inhibits protein synthesis, could be used in the future. The JNK pathway may also upregulate expression of cytokines capable of regulating MMP production (ie, interleukin 1 and tumor necrosis factor α), so it is reasonable to say that the consequent effector of MMP upregulation may be a direct or indirect effect of JNK.

Cornified envelope precursor proteins are usually expressed in low levels in the wet ocular surface mucosal epithelia, as opposed to differentiated skin epidermal cells, which have a thick cornified envelope that functions to maintain barrier function. This and previous studies suggest that the corneal epithelium responds to desiccating...
or osmotic stress by producing cornified envelope precursor proteins, perhaps as a mechanism to form an alternative skinlike protective barrier.21,28,33 Certain cornified envelope precursor genes such as involucrin and SPRR-2a have been noted to be regulated by JNK14,28; however, there are no studies showing any specificity for JNK2 over JNK1 in the promoter-binding sites of SPRR and MMP genes. Similar to MMPs, the production of these factors, particularly SPRR-2a and involucrin, was also attenuated in JNK2KO mice.

Our findings suggest that certain aspects of the regulated response of the corneal epithelium to desiccating stress are modulated by the JNK2 signaling pathway. This implies that JNK2 inhibitors may have therapeutic potential for treatment of the corneal epithelial disease that develops in dry eye. Indeed, clinical studies have found that corticosteroids, which inhibit activator protein 1 gene transcription, have improved corneal epithelial disease.24 Furthermore, doxycycline, a tetracycline antibiotic with JNK inhibitory properties, has been reported to significantly improve corneal barrier function and smoothness in our murine model of dry eye.10,20,21

**Figure 3.** Evaluation of cornified envelope protein precursors in the corneal sections of the 3 strains of mice. Laser scanning immunofluorescent confocal microscopy of corneal sections stained for small proline-rich protein (SPRR)–1a (A, green), SPRR-2a (B, green), or involucrin (C, green) with propidium iodide nuclear counterstaining (red) in nonstressed control conditions (NS) and after 5 days of desiccating stress (DS5) in C57BL/6, c-Jun N-terminal kinase knockout 1 (JNK1KO), and JNK2KO mice. Graphical data are the mean (standard deviation) of measured fluorescence levels for each protein. *P < .05 compared with NS in the same strain; **P < .01 compared with NS in the same strain; and ***P < .001 compared with NS in the same strain. Scale bars = 50 µm.

**Figure 4.** Schematic showing the proposed sequence of events after exposure of ocular surface tissue to desiccating stress. JNK indicates c-Jun N-terminal kinase. Smaller font of JNK1 indicates its possible smaller role.
Taken together, our results demonstrate clearly different roles for JNK2 and JNK1 in the pathogenesis of corneal epithelial disease (Figure 4) and that inhibiting JNK2 could be a novel therapeutic target in dry eye disease.

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