Therapeutic Effect of MK2 Inhibitor on Experimental Murine Dry Eye

Yang Wu, Jinghua Bu, Yiran Yang, Xiang Lin, Xiaoxin Cai, Caihong Huang, Xiaoxiang Zheng, Weijie Ouyang, Wei Li, Xiaobo Zhang, and Zuguo Liu

Eye Institute of Xiamen University and Fujian Provincial Key Laboratory of Ophthalmology and Visual Science, Xiamen, Fujian, China

Correspondence: Zuguo Liu, Eye Institute of Xiamen University, Fujian Provincial Key Laboratory of Ophthalmology and Visual Science, Xiangang South Road, Xiamen, Fujian, China; zuguoliu@xmu.edu.cn. Xiaobo Zhang, Eye Institute of Xiamen University, Fujian Provincial Key Laboratory of Ophthalmology and Visual Science, Xiangang South Road, Xiamen, Fujian, China; xiaobaoz@xmu.edu.cn.

YW and JB contributed equally to the work present here and should therefore be regarded as equivalent authors.

ZL and XZ contributed equally to the work present here and should therefore be regarded as equivalent authors.

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Purpose: To investigate the role of mitogen-activated protein kinase-activated protein kinase-2 (MK2) in ocular surface damage of dry eye.

Methods. MK2 inhibition was performed in mice subjected to desiccating stress (DS) by topical application of MK2 inhibitor (MK2i) or vehicle eye drops. The total and phosphorylated MK2 in conjunctiva were detected by Western blot. The phenol red cotton test was used to measure tear production, and Oregon green dextran staining was performed to assess corneal epithelial barrier function. PAS staining was used to quantify conjunctival goblet cells. Immunofluorescent staining and quantitative RT-PCR were used to assess the expression of matrix metalloproteinase (MMP)-3 and -9 in corneal epithelium. Apoptosis in ocular surface was assessed by TUNEL and immunofluorescent staining for activated caspase-3 and -8. Inflammation was evaluated by CD4⁺ T-cell infiltration and production of T helper (Th) cytokines, including IFN-γ, IL-13, and IL-17A in conjunctiva.

Results. DS promoted MK2 activation in conjunctiva. Compared with vehicle control mice, MK2i-treated mice showed increased tear production, decreased goblet cell loss, and improved corneal barrier function. Topical MK2 inhibition decreased the expression of MMP-3 and -9 in corneal epithelium, and suppressed cell apoptosis in ocular surface under DS. Topical MK2 inhibition decreased CD4⁺ T-cell infiltration, with decreased production of IFN-γ and IL-17A and increased production of IL-13 in conjunctiva.

Conclusions. Topical MK2 inhibition effectively alleviated ocular surface damage via suppressing cell apoptosis and CD4⁺ T-cell-mediated inflammation in ocular surface of dry eye.

Keywords: MK2, dry eye, inflammation, ocular surface
MK2 inhibitor (MK2i) may be a suitable compound to reduce inflammation in various ocular surface diseases. Until now, the role of MK2 in the ocular surface inflammation of dry eye remains unknown.

The purpose of this study was to investigate the role of MK2 activation in the ocular surface inflammation of dry eye.

**MATERIALS AND METHODS**

**Mice**

The protocol of this research was approved by the Experimental Animal Ethics Committee of Xiamen University and it conformed to the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female C57BL/6 (B6) mice, aged 6 to 8 weeks, were purchased from Shanghai SLAC Laboratory Animal Center, Shanghai, China.

**Murine Dye Eye Model**

Desiccating stress (DS) was used to induce experimental dry eye in B6 mice by subcutaneous injection of scopolamine hydrobromide (0.5 mg/0.2 mL; catalog no. MB5860; Melonepharma, Dalian, China) four times a day (9:00, 12:00, 15:00, 18:00) and exposure to an air draft and less than 40% ambient humidity for 5 days. A group of age- and gender-matched mice that did not receive any treatment to induce dry eye served as nonstressed (NS) controls.

**Topical MK2i Treatment in B6 Mice Subjected to DS**

Topical MK2i treatment was performed in B6 mice subjected to DS by topical application of 5 μL MK2i (DS5+MK2i; 12.5 μg/mL, 25 μg/mL, or 50 μg/mL; catalog no. 475864; Millipore, Billerica, MA, USA) or vehicle (PBS containing 1/40,000, 1/20,000, or 1/10,000 DMSO; DS5+Vehicle) eye drops four times daily for 5 days under DS.

**Western Blot**

The conjunctivae of both eyes of one mouse were collected and pooled as one sample, and five samples were used in each group. The conjunctiva was then minced and lysed in cold radioimmunoprecipitation assay (RIPA) buffer containing a proteinase inhibitor cocktail (catalog no. 78440; ThermoFisher Scientific, Waltham, MA, USA). The total protein concentration of the supernatant was measured with a BCA protein assay kit (catalog no. 23225; ThermoFisher Scientific). Aliquots having equal protein content were subjected to electrophoresis on 10% Tricine gels and then electronically transferred to PVDF membranes (catalog no. IPVH00010; Millipore, Billerica, MA, USA). After blocking in 5% BSA for 1 hour, the membranes were incubated overnight at 4°C with primary antibodies for MK2 (1:500; catalog no. ab131531; Abcam, Cambridge, UK), Phospho-MK2 (1:500; catalog no. ab63378; Abcam) and horseradish peroxidase (HRP)-conjugated anti-β-actin antibody (1:20,000; catalog no. a5316; Sigma-Aldrich Corp., St. Louis, MO, USA). After washing three times with Tris-buffered saline containing 0.05% Tween 20 for 10 minutes, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000; catalog no. a0545; Sigma-Aldrich Corp.) for 1 hour at room temperature. The specific bands were visualized by an enhanced chemiluminescence reagent (catalog no. ECL-500; ECL, Lulong, Inc., Xiamen, China), and the image intensity was calculated with a transilluminator (ChemiDoc XRS System; Bio-Rad, Philadelphia, PA, USA).

**TABLE. Mouse Primer Sequences Used for Quantitative RT-PCR**

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<thead>
<tr>
<th>Gene</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>PCR Product, bp</th>
</tr>
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<tbody>
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<td>MMP-9</td>
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<td>AGTAAGGAAAGGGGCTCTGTA</td>
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<tr>
<td>IL-13</td>
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<tr>
<td>IL-17A</td>
<td>CGCAATAGAACCCCTGATAGAT</td>
<td>CTCTTGGTGGATGAGAAGCAGAA</td>
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<tr>
<td>IFN-γ</td>
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<td>GCTTTGCTGAAAGAAGGTAGTA</td>
<td>128</td>
</tr>
<tr>
<td>β-actin</td>
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<td>AGGCATAACAGGGACAGCAC</td>
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</table>

**FIGURE 1.** Effects of topical application of MK2i on DS-induced MK2 activation in the conjunctiva. MK2 activation was evaluated by Western blot, with β-actin as a loading control. The level of phosphorylated-MK2 (p-MK2) (A) and total-MK2 (B) in conjunctiva. (C) The ratio of the intensity of p-MK2 to total MK2 in conjunctiva. Data shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
Measurement of Tear Production

Tear production (10 eyes/five mice per group) was measured with phenol red-impregnated cotton threads (Zone-Quick; Yokota, Tokyo, Japan) at the same time point (8 PM). The thread was placed on the lower conjunctival fornix at approximately one-third of the lower eyelid distance from the lateral canthus for 15 seconds. The length of the wet red thread was measured in millimeters.

Corneal Permeability

Corneal epithelial permeability to Oregon green dextran (OGD) (70,000 molecular weight; catalog no. D7172; Invitrogen, Eugene, OR, USA) was assessed (10 eyes/five
mice per group) as previously described. Briefly, OGD (0.5 μL of 50 mg/mL) was instilled onto the ocular surface 1 minute before killing. Corneas were rinsed five times with saline and then photographed with a stereoscopic zoom microscope (AZ100; Nikon, Tokyo, Japan) under fluorescence excitation at 470 nm. The mean intensity of corneal OGD staining was measured in digital images using the analysis software (NIS Elements, version 4.1; Nikon, Melville, NY, USA). A 2-mm diameter circle was placed on the central cornea, and the mean fluorescent intensity in this circle was measured by the software.

Histology
The eyes and adnexa of mice were excised, embedded in optimal cutting temperature (OCT) compound (catalog no.4583; SAKURA Tissue-Tek, Torrance, CA, USA) or paraffin. OCT-embedded samples were cut into sagittal sections (6 μm thick), and then placed onto glass slides that were stored at −80°C. PAS staining and TUNEL assay were performed on paraffin sections (5 μm thick), and immunostaining was performed on frozen sections (two sections per slide, three slides per animal, five animals per group).
PAS Staining
The mucin-filled goblet cells (GCs) in the conjunctiva were stained using a PAS staining kit (catalog no. 395B-1KT; Sigma-Aldrich Corp.). Digital images of representative areas of the conjunctiva were captured with the light microscope (Eclipse 50i; Nikon, Tokyo, Japan).

Immunofluorescent Staining
Immunofluorescent staining was performed in cryosections of the eyes and adnexa. Sections were fixed in acetone at −20°C, and then incubated at 4°C overnight with polyclonal goat anti-matrix metalloproteinase (MMP)-3 antibody (1:50; catalog no. sc-6839; Santa Cruz Biotechnology, Dallas, TX, USA), goat anti-MMP-9 antibody (1:50; catalog no. sc-6840; Santa Cruz Biotechnology), rabbit anti-activated (AC) caspase-3 antibody (1:250; catalog no. ab52181; Abcam), or rabbit anti-AC-caspase-8 antibody (1:50; catalog no. sc-7890; Santa Cruz Biotechnology). Negative controls were performed at the same time by incubating a section with just PBS without any primary antibody. The next day, samples were incubated with Alexa Fluor488-conjugated donkey anti-goat (1:300; catalog no. A11055; Invitrogen, Eugene, OR, USA) or anti-rabbit IgG (1:300; catalog no. A21206; Invitrogen) for 1 hour in the dark at room temperature, followed by three washes in PBS. Sections were then counterstained with 4',6-diamidino-2-phenylindole (DAPI; catalog no. H-1200; Vector, Burlingame, CA, USA) for 5 minutes. Digital images of representative areas of the cornea or conjunctiva were captured with the Leica upright microscope (DM2500; Leica Microsystems, Wetzlar, Germany). The mean intensity of staining in each section was measured by analysis software (NIS Elements version 4.1; Nikon, Melville, NY, USA).

TUNEL Assay
To measure the end-stage apoptosis, in situ TUNEL assay (DeadEnd Fluorometric TUNEL System; catalog no. G3250; Promega, Madison, WI, USA) was performed on paraffin sections according to the manufacturer’s instructions. Sections were counterstained with DAPI (catalog no. H-1200; Vector), and digital images of representative areas of the cornea and conjunctiva were captured with the Leica upright microscope (DM2500; Leica Microsystems).

Immunohistochemistry
Immunohistochemistry was performed to detect and count the cells in the conjunctiva that stained positively for CD4. Cryosections were stained with primary rat anti-mouse CD4 antibody (1:50; catalog no. 553647; BD Pharmingen, San Diego, CA, USA), goat anti-rat antibody (1:25; catalog no. 559286; BD Pharmingen) and Vectastain Elite ABC using

![Figure 4](http://tvst.arvojournals.org/). Effects of topical application of MK2i on DS-induced cell apoptosis on ocular surface. (A) Representative images for TUNEL staining in corneal epithelium and conjunctiva; The number of TUNEL-positive cells in corneal epithelium (B) and conjunctiva (C). Data was shown as mean±SD. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars: 50 μm.
NovaRed reagents (catalog no. PK-6100; Vector). Secondary antibody alone and appropriate anti-mouse isotype (BD Pharmingen) controls were also performed. Three slides from each animal were examined and photographed with a microscope equipped with a digital camera (Eclipse 50i; Nikon, Tokyo, Japan). Positively stained cells were counted in the conjunctiva using image analysis software (NIS Elements, version 4.1, Nikon, Melville, NY, USA).

RNA Extraction and Quantitative RT-PCR

Murine corneal epithelium was scraped with a scalpel, conjunctiva was surgically excised, and total RNA was isolated from the corneal epithelium or conjunctiva by a PicoPure RNA isolation kit (catalog no. KIT0204; Arcturus, Mountain View, CA, USA). Five samples per group were used, and one sample consisted of pooled corneal epithelium or conjunctiva of both eyes of the same animal. cDNA was synthesized using a reverse transcription kit (catalog no. RR047A; TaKaRa, Shiga, Japan). Real-time PCR was performed on StepOne Real-Time PCR System (Applied Biosystems, Alameda, CA, USA) using a SYBR Premix Ex TaqKit (catalog no. RR420A; TaKaRa), and the primer sequences are summarized in the Table. The amplification program included an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, and 60°C for 30 seconds, after which a melt curve analysis was conducted to check amplification specificity. The results of real-time PCR were analyzed by the comparative cycle threshold (Ct) method where target change $\Delta \Delta Ct$ was normalized with $\beta$-actin as an endogenous reference, and calibrated against the NS group.

Mouse IL-17A, IL-13, and IFN-γ ELISA Assay

The conjunctiva proteins of each group (five samples per group, and one sample consisted of pooled conjunctiva of both eyes of the same animal) were extracted with cold RIPA buffer (catalog no. R0278; Sigma-Aldrich Corp.), and total protein concentration of the cell extract was measured with a BCA protein assay kit (catalog no. 23225; ThermoFisher Scientific). The same dilution ratio was used in all groups. ELISA kits were used to detect the protein concentrations of IL-17A (catalog no. BMS6001; eBioscience, San Diego, CA, USA), IL-13 (catalog no. BMS6015; eBioscience), and IFN-γ (catalog no. BMS606; eBioscience) in conjunctiva according to manufacturer’s instructions. The optical absorbance was measured at 450 nm with a microplate reader (Bio TekElx800; Bio-Tek Instruments, Winooski, VT, USA), and the protein concentrations were calculated according to the standard curve.

Statistical Analysis

One-way ANOVA with Tukey’s post hoc test was conducted for statistical comparison between groups using GraphPad Prism 5.0 software (GraphPad Software, Inc, San Diego, CA, USA). $P \leq 0.05$ was considered statistically significant.

RESULTS

The Activation of MK2 in Ocular Surface During DS

As shown in Figure 1, DS promoted MK2 activation in the conjunctiva, and topical application of MK2i eye drops sup-
pressed the activation of MK2 in the conjunctiva. In this study, 25 μg/mL was chosen as the adopted concentration to assess the effect of MK2i on ocular surface damage in dry eye induced by DS.

Topical Application of MK2i Alleviated DS-Induced Ocular Surface Damage

Topical application of MK2i eye drops improved tear production (Fig. 2A), increased the number of conjunctival GCs (Figs. 2B, 2C), and alleviated the corneal barrier dysfunction shown by OGD staining (Figs. 2D, 2E) in experimental dry eye.

Topical Application of MK2i Decreased the Expression of MMP-9 and MMP-3 in Corneal Epithelium During DS

Previous studies have shown that disruption of the corneal barrier in dry eye is associated with increased production of MMPs, particularly MMP-9 and MMP-3.17–20 This study investigated the expression of MMP-9 and MMP-3 in corneal epithelium using immunofluorescent staining and quantitative RT-PCR, and found that topical application of MK2i eye drops suppressed the expression of MMP-9 and MMP-3 in corneal epithelium during DS (Fig. 3).

Topical Application of MK2i Suppressed DS-induced Apoptosis in Ocular Surface

A growing body of clinical and experimental studies has shown that pathologic apoptosis has a key role in the pathogenesis of dry eye disease.21–25 In this study, the number of TUNEL-positive cells was decreased in both the corneal epithelium and conjunctiva in the MK2i-treated group compared with the vehicle control group (Fig. 4). In addition, this study found that topical application of MK2i eye drops suppressed the immunoreactivity of AC-Caspase-3 and AC-Caspase-8 in ocular surface under DS (Figs. 5, 6).

Topical Application of MK2i Suppressed CD4+ T-Cell–Mediated Inflammation in Conjunctiva During DS

Dry eye is an inflammatory ocular surface disease characterized by infiltration of CD4+ T cells producing IFN-γ and IL-17A in conjunctiva.17,24,25 The results of this study indicated that topical application of MK2i effectively suppressed the DS-induced infiltration of CD4+ T cells in conjunctiva (Figs. 7A, 7B). Topical application of MK2i decreased the production of IL-17A and IFN-γ and increased the production of IL-13 in conjunctiva under DS (Figs. 7C, 7D).

DISCUSSION

MK2 is a kinase that is exclusively activated on stress by p38. The activation of MK2 can be stimulated in various inflammatory conditions, and MK2-induced production of proinflammatory cytokine has been observed in several inflammatory conditions.8,10,12 It has been confirmed that MK2 activation plays a crucial role in a vast amount of functions linked with inflammation and may thus be a potential drug target for...
inflammatory diseases, such as arthritis, atherosclerosis, and cancer.\textsuperscript{26} In this study, we present evidence that topical application of MK2i eye drops could effectively alleviate epithelial damage, reduce cell apoptosis, and suppress CD4\textsuperscript{+} T-cell–mediated inflammation in the ocular surface in an experimental murine model of dry eye.

Dry eye is a chronic inflammatory disease characterized by infiltration of CD4\textsuperscript{+} T cells producing IFN-\gamma and IL-17A in conjunctiva.\textsuperscript{17,24,25} Adoptive transfer of CD4\textsuperscript{+} T cells isolated from mice subjected to DS to the nude mice induces dry eye-like ocular surface damage in the nude mice, suggesting that CD4\textsuperscript{+} T-cell-mediated inflammation plays a vital role in the pathogenesis of dry eye.\textsuperscript{24} The pathogenetic mechanisms responsible for CD4\textsuperscript{+} T-cell–induced ocular surface damage in dry eye are not completely understood; however, there is increasing evidence indicating that an altered balance of Th cytokines are capable of altering ocular surface epithelial homeostasis. It has been reported that inflammatory cytokines (IFN-\gamma and IL-17A) released by the resident intraepithelial lymphocytes and infiltrating CD4\textsuperscript{+} T cells are obviously increased, whereas the production of Th2 cytokine IL-13, which has a homeostatic function in maintaining conjunctival GCs, is notably decreased in the ocular surface of murine dry eye.\textsuperscript{17,25,27–29} This study investigated the role of MK2 activation in ocular surface inflammation during DS, and found that topical application of MK2i significantly alleviated DS-

\textbf{FIGURE 7.} Effects of topical application of MK2i on DS-induced inflammation in conjunctiva. (A) Representative images of immunohistochemical staining for CD4\textsuperscript{+} T cells in conjunctiva; (B) The number of CD4\textsuperscript{+} T cells in conjunctiva; (C) The mRNA levels of IFN-\gamma, IL-13 and IL-17A in conjunctiva; (D) The protein levels of IFN-\gamma, IL-13 and IL-17A in conjunctiva detected by ELISA assay. Data was shown as mean ± SD. *\textit{P} < 0.05, **\textit{P} < 0.01, ***\textit{P} < 0.001. Scale bars: 50 \textmu m.
induced inflammation in conjunctiva by suppressing infiltration of CD4+ T cells and production of IFN-γ and IL-17A, suggesting that MK2 activation has a crucial role in CD4+ T-cell-induced inflammation in the ocular surface of dry eye. It is well recognized that cell apoptosis on the ocular surface has a key role in the pathogenesis of dry eye disease and apoptosis has been defined as a therapeutic target for dry eye.21–25,30–32 Apoptosis occurs through two pathways: the extrinsic pathway mediated by the binding of death ligands (e.g., TNF-α, Fas) with their corresponding cell surface receptors and the activation of caspase-8; and the intrinsic pathway mediated by DNA damage and subsequent activation of caspase-9. Both pathways result in downstream activation of effector caspases, such as caspase-3.33–35 Previously, it has been reported that DS induces conjunctival apoptosis mainly through the caspase-8-mediated extrinsic pathway.53,54 This study found that topical application of MK2i notably suppressed the immune-reactivity of AC-Caspase-3 and -8, and reduced the number of TUNEL-positive cells in ocular surface under DS, suggesting that topical application of MK2i could suppress the DS-induced cell apoptosis via extrinsic apoptosis pathway in ocular surface.

Disruption of corneal epithelial barrier function is a distinct clinical feature of dry eye disease. Corneal barrier dysfunction can lead to ocular irritation, ocular surface irregularity, blurred vision, and visual morbidity.50–55 MMP-9 has been found to have a central role in DS-induced corneal barrier disruption, as MMP-9-deficient mice were resistant to barrier disruption in DS.18–20 It has been reported that IL-17 neutralization during DS decreased the expression of MMP-9 and MMP-3 and improved corneal barrier function.21–23,30–32 Apoptosis occurs through two pathways: the extrinsic pathway mediated by DNA damage and subsequent activation of caspase-9. Both pathways result in downstream activation of effector caspases, such as caspase-3.33–35 Previously, it has been reported that DS induces conjunctival apoptosis mainly through the caspase-8-mediated extrinsic pathway.53,54 This study found that topical application of MK2i notably suppressed the immune-reactivity of AC-Caspase-3 and -8, and reduced the number of TUNEL-positive cells in ocular surface under DS, suggesting that topical application of MK2i could suppress the DS-induced cell apoptosis via extrinsic apoptosis pathway in ocular surface.

Conjunctival GCs are highly secretory epithelial cells that secrete the gel-forming mucin, MUC5AC, that lubricates the ocular surface and stabilizes the tear film. It has been confirmed that the imbalance between Th1 and Th2 cytokines plays an essential role in conjunctival GC loss during DS. Th1 and Th2 cytokines have been found to have opposing effects on conjunctival GC development. IL-13, the predominant Th2 cytokine, appears to have a homeostatic function in promoting GC development.56–58 It is well recognized that cell apoptosis on the ocular surface has a key role in the pathogenesis of dry eye disease and apoptosis has been defined as a therapeutic target for dry eye.21–25,30–32 Apoptosis occurs through two pathways: the extrinsic pathway mediated by the binding of death ligands (e.g., TNF-α, Fas) with their corresponding cell surface receptors and the activation of caspase-8; and the intrinsic pathway mediated by DNA damage and subsequent activation of caspase-9. Both pathways result in downstream activation of effector caspases, such as caspase-3.33–35 Previously, it has been reported that DS induces conjunctival apoptosis mainly through the caspase-8-mediated extrinsic pathway.53,54 This study found that topical application of MK2i notably suppressed the immune-reactivity of AC-Caspase-3 and -8, and reduced the number of TUNEL-positive cells in ocular surface under DS, suggesting that topical application of MK2i could suppress the DS-induced cell apoptosis via extrinsic apoptosis pathway in ocular surface.

In conclusion, this study provides evidence that topical application of MK2i could effectively alleviate ocular surface damage via suppressing cell apoptosis and CD4+ T-cell-mediated inflammation on the ocular surface in an experimental murine dry eye model. This study provides new insight for the pathogenesis of dry eye, and MK2 is a potential therapeutic target of dry eye.

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**References**


