Serine Protease Inhibitor A3K Suppressed the Formation of Ocular Surface Squamous Metaplasia in a Mouse Model of Experimental Dry Eye

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Purpose. To investigate the effects and possible mechanisms of serine protease inhibitor A3K (SERPINA3K) on the formation of ocular surface squamous metaplasia in a mouse dry eye model induced by topical benzalkonium chloride (BAC).

Methods. The eye drops containing SERPINA3K were topically administered during the induction of BAC-induced dry eye. The clinical indications of dry eye were evaluated on day (D)16, including tear break-up time (BUT), tear volume, corneal fluorescein staining, and inflammatory index. Global specimens were collected on D16 and the following examinations were performed: histologic investigation, immunostaining of cytokeratin 10 (K10), p63 and Ki67 in the cornea, and Western blot analysis of tumor necrosis factor-α (TNF-α).

Results. Serine protease inhibitor A3K suppressed the formation of BAC-induced dry eye, presenting with longer BUTs, lower corneal fluorescein staining scores, and inflammatory index, while no significant changes in tear volume. It also reduced the severity of abnormal differentiation and proliferation on ocular surface with lower expressions of K10, p63, and Ki67, and retained the number of goblet cells in the conjunctival fornix. Serine protease inhibitor A3K significantly decreased the levels of TNF-α in the cornea.

Conclusions. Topical application of SERPINA3K ameliorated the severity of ocular surface squamous metaplasia and suppressed the formation of BAC-induced dry eye.

Keywords: squamous metaplasia, dry eye, serine proteinase inhibitor, inflammation

Squamous metaplasia is defined as a pathological transition of a nonkeratinized, stratified epithelium into a nonsecretory, keratinized epithelium. It is a commonly occurring pathological process in the mucous membrane, such as respiratory epithelium,[1] urothelium,[2] and the ocular surface epithelium.[3,4] In the eye, squamous metaplasia is commonly seen under long-term deficiency of tear film,[5] and considered as a hallmark of a variety of severe ocular surface disorders including severe dry eye, Stevens-Johnson syndrome, chemical burns, and ocular cicatricial pemphigoid.[6–8] Along with limbal stem cell deficiency, squamous metaplasia is considered as one of the two major types of ocular surface failure, which may lead to visual loss and even blindness. Unfortunately, the molecular mechanisms triggering squamous metaplasia are not well understood and it still lacks an effective approach or medication in the clinic setting. In recent years, more evidences have suggested the link between pathologic squamous metaplasia and certain types of lymphocyte/cytokine-mediated chronic inflammation of the ocular surface.[9,10] However, anti-inflammatory drugs available and surgery such as amniotic membrane patching cannot achieve satisfactory results in the management of ocular surface squamous metaplasia. At present, there is an urgent need for explorations of new potential agents with pharmacological activity on squamous metaplasia.

Serine protease inhibitor A3K (SERPINA3K), also named kallikrein-binding protein (KBP), is identified first as a member of the serine proteinase inhibitor (serpin) family.[11] It is expressed mainly in the liver, while at low levels in kidney, pancreas, and ocular tissues such as the retina.[12–14] As a specific inhibitor of tissue kallikrein, it binds with tissue kallikrein to form a covalent complex and inhibits its proteolytic activities, therefore modulating the kinin formation in vivo.[15] Later studies revealed that it has other important functions independent of inhibition of tissue kallikrein.[16] Recent evidences showed that SERINA3K has effects of antiangiogenesis, anti-inflammation, antifibrosis, and antioxidative stress in the retina.[17–19] It was further suggested that SERPINA3K could block the canonical Wnt pathway and consequently downregulate the expression of proinflammatory factors such as tumor necrosis factor α (TNF-α), which may represent one of the mechanisms underlying its anti-inflammatory effects in the retina.[17]

We have previously reported the antiangiogenic and anti-inflammatory effects of SERPINA3K in corneal alkali injury.[20] However, the understanding of the actions and mechanisms of SERPINA3K in ocular surface diseases remains limited. Specifically, no study was reported on the specific effects of SERPINA3K on dry eye and ocular surface squamous metaplasia, which are the most common ocular surface diseases in the

clinic setting. In the present study, we used a mouse dry eye model induced by benzalkonium chloride[21,22] (BAC) to investigate and focus on the specific effects of SERPINA3K on the formation of squamous metaplasia and explore its possible underlying mechanism.

Materials and Methods

Purification of SERPINA3K

The SERPINA3K/pET28 plasmid expressing SERPINA3K was introduced into *E. coli* strain BL21. The vector provides a signal peptide that enables the recombinant protein to enter the periplasmic space. The expression and purification followed the protocol recommended by GE Company with some modifications. The methodology of purification of SERPINA3K was previously reported.[20] The purity of recombinant SERPINA3K was examined by SDS-PAGE. Endotoxin concentrations were monitored using a limulus amebocyte kit. Activity of the purified protein was examined by methyl thiazolyl tetrazolium (MTT) assay using primary human umbilical vein endothelial cells.

Animals and Experimental Procedures

Fifty male BALB/c mice (18–20 g; purchased from Shanghai SLAC Laboratory Animal Center, Shanghai, China) were used for this study, all of which were free of clinically observable ocular surface diseases. Mice were allowed to acclimate to local conditions for at least 1 week and held in standard environment throughout the study as follows: room temperature 25°C ± 1°C, relative humidity 60% ± 10%, and alternating 12 hour light/dark cycles (8 AM to 8 PM). All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocols in this study were approved by the animal ethics committee of Xiamen University School of Medicine.

The mice were randomly assigned to five groups of 10 mice each. The first group was kept untreated as normal control (normal group). For 16 days, the remaining four groups received topical administration of 5 μL 0.2% BAC (dissolved in PBS) twice daily at the right eyes for the induction of dry eye condition[21] (8 AM, 4 PM). Meanwhile, three of the four groups received PBS (BAC + PBS group) and BSA (0.15 mg/mL; BAC + BSA group) as the control, and SERPINA3K solution (0.15 mg/mL; BAC + SA3K group) of 5 μL, respectively, at the right eye, twice daily (12 AM, 8 PM) for 16 days; and the rest one of the four groups was kept untreated (BAC + UT group) within 16 days.

On day (D)0, tear break-up time (BUT) test and Schirmer I test were performed to calculate the average BUT and tear volume of normal mice. On D16, ocular surface alterations were evaluated under slit lamp microscope, including BUTs, inflammatory index of the cornea, corneal epithelial staining scores and tear volumes, following the methods described below.

Global specimens were dissected on D16 for light microscopy, immunostaining, and Western blot following the methods described below.

Evaluation of Inflammation in the Cornea

The severity of inflammatory response was examined and scored by a single masked observer who was an ophthalmologist under slit lamp (Kanghua Science & Technology Co., Ltd., Chongqing, China) as previously described.[23] The inflammatory index was analyzed based on the following parameters: ciliary hyperemia (1 = absent; 2 = present but less than 1 mm; 2 = present between 1 and 2 mm; 3 = presented more than 2 mm); central corneal edema (0 = absent; 1 = present with visible iris details; 2 = present without visible iris details; 3 = present without visible pupil); and peripheral corneal edema (0 = absent; 1 = present with visible iris details; 2 = present without visible iris details; 3 = present with no visible iris). The final inflammatory index result was obtained by summing the scores of the different parameters divided by a factor of 9.

Tear Break-up Time and Fluorescein Staining

We dropped 1 μL of 0.1% liquid fluorescein sodium into the conjunctival sac. After three blinks, BUTs were recorded as seconds. Ninety seconds later, corneal epithelial staining was graded with a cobalt blue filter under a slit-lamp microscope. The cornea was divided into four quadrants, which was scored respectively as previously described[24] with essential modification, briefly as follows: 0 = absent; 1 = slightly punctate staining less than 30 spots, 2 = punctate staining more than 30 spots, but not diffuse, 3 = severe diffuse staining but no positive plaque; 4 = positive fluorescein plaque. The scores of each quadrant were added to arrive at a final grade (total, 16 points).

Measuring of Tear Volume

The amount of tears was measured with phenol red thread tear test using cotton threads (Zone-Quick; Yokota, Tokyo, Japan)[25] on D16, in the standard environment. Animals were kept immobile by intraperitoneal injection of 1 mg pentobarbital. The lower eyelid was pulled down slightly, and 1 mm portion of the thread was placed on the palpebral conjunctiva for 15 seconds at a specified point approximately 1/3 of the distance from the lateral canthus of the lower eyelid. The red portion of the thread is measured in millimeters. Each eye was tested for three repeats, and the average length of red portion was considered as the final length.

Immunostaining

On D16, five of the global tissues were embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Miles Inc., Elkhart, IN, USA) and frozen at −80°C. Frozen sections that were OCT-embedded (6-μm thick) were cut with a cryotome (CM 1850UV; Leica Microsystems AG, Wetzlar, Germany) and stored at −80°C. Global sections were fixed in acetone and permeated with 0.2% Triton X-100.

For immunofluorescent labeling, sections were blocked, incubated at 4°C overnight with antibody of K10 (1:300; Abcam, Cambridge, UK). After further incubation in AlexaFluor 488–conjugated secondary antibody (1:1000; Invitrogen, Carlsbad, CA, USA), sections were rinsed, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), mounted, and photographed using a confocal laser scanning microscope (FluoView 1000; Olympus Corp., Tokyo, Japan).

For immunohistochemical staining, the activity of endogenous peroxidases was quenched with 0.6% hydrogen peroxide for 30 minutes. After incubating with 2% BSA, the antibodies of p63 (1:250) and Ki67 (1:400) were applied and incubated at 4°C for 14 to 18 hours. Sections were further incubated with biotinylated anti-rabbit or anti-rat IgG (1:50) using tissue staining kits (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. The reaction product was then developed with diaminobenzidine. Sections were mounted and examined with a light microscope (Eclipse 50i; Nikon Instruments, Tokyo,

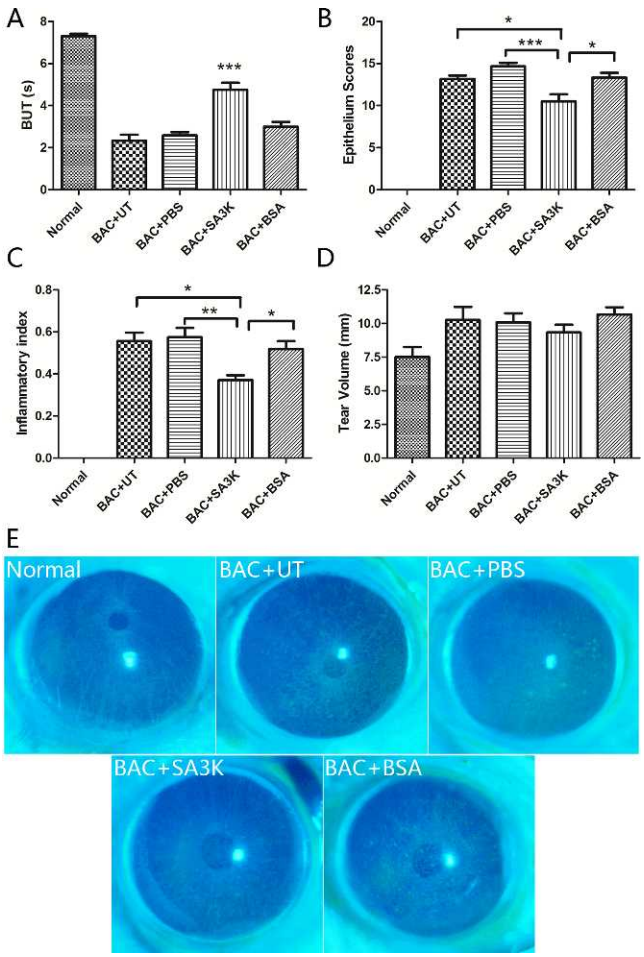

Figure 1. Dry eye severity eye ameliorated with SERPINA3K. Serine protease inhibitor A3K significantly suppressed the formation of dry eye on D16 after medication, with the manifestations of (**A**) prolonged BUT, (**B**) less fluorescein sodium staining scores, and reduced (**C**) inflammatory index in the cornea. (**D**) No significant changes of tear volume were observed among groups. (**E**) Representative images were also provided showing the corneal fluorescein staining in all of the five groups on D16. Data were presented as mean ± SEM; $n = 10$. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$.

Japan). The counterstaining with hematoxylin was not performed to avoid the possible interference of nuclear staining during observation.

Western Blot Assay

Proteins of the cornea and conjunctiva from each group were extracted with cold radioimmunoprecipitation assay buffer. Equal amounts of proteins of the cell lysates were subjected to electrophoresis on 8% SDS-PAGE and then electrophoretically transferred to PVDF membranes. After 1 hour of blocking in 5% BSA, the membranes were incubated with primary antibodies for TNF-α (1:400, Abcam) and β-actin (1:10,000; Sigma-Aldrich, St. Louis, MO, USA) as a loading control. After three washes with Tris-buffered saline with 0.05% Tween 20 for 10 minutes each, the membranes were incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (1:10,000, Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 1 hour. The specific binds were visualized by enhanced chemiluminescence reagents and recorded on film.

Light Microscopy and Periodic Acid Schiff (PAS) Assay

Hematoxylin and eosin (H&E) staining was performed in cryosections. Five specimens of the whole orbit tissue in each group were embedded in paraffin, cross-sectioned, and stained with PAS reagents (PAS Staining System 395B-1 KT; Sigma-Aldrich) and hematoxylin. The number of goblet cells in the conjunctival fornix was counted in six representative slices of homologous positions from each orbit tissue. These sections were examined using the light microscope mentioned above.

Statistical Analysis

Analysis of the significance of differences between groups was performed by using the Two-way ANOVA (Bonferroni posttest). Values of $P < 0.05$ were considered statistically significant.

Results

SERPINA3K Ameliorated the Severity of Dry Eye

We first evaluated the effects of SERPINA3K on the formation of dry eye condition, by using the BAC-induced experimental dry eye setting that we established and previously reported.[21] We made necessary modification of the setting in this study. Briefly, 0.2% BAC was topically administrated twice daily for 16 days, simultaneously with the following groups: untreated (BAC + UT group); PBS (BAC + PBS group); BSA solutions (BAC + BSA group); and SERPINA3K-treated group (BAC + SA3K group; Fig. 1).

Serine protease inhibitor A3K statistically significantly suppressed the formation of dry eye on D16 after medication, with the manifestations of prolonged BUT (Fig. 1A), less epithelial damage (Fig. 1B) and reduced inflammatory response in the cornea (Fig. 1C). However, the medications of 16 days did not induce significant changes of tear volume among four groups (Fig. 1D). The average values of BUT and tear volume of normal mice were 7.3 seconds and 7.5 mm, respectively.

Morphological Changes of Ocular Surface

We then examined the morphological changes of ocular surface of the experimental animals. Staining with H&E (Fig. 2) demonstrated that the corneal epithelium was intact and smooth with four to five layers of epithelial cells in the normal eyes. In BAC-treated corneal tissues without SERPINA3K medication, cell layers and thickness of the corneal epithelium both increased apparently with some areas showing nine cell layers on D16. Larger cells with irregular shapes could be observed at the superficial epithelium in the corneas of BAC + UT, BAC + PBS and BAC + BSA groups. In SERPINA3K-treated eyes, the corneal epithelium remained in five to six layers and was slightly altered morphologically. In addition, the BAC-treated eyes without SERPINA3K had much more inflammatory infiltrations in the corneal stroma than those of SERPINA3K-treated group, which was consistent with the results of corneal inflammatory index (Fig. 1C).

Similar trends of stratification were observed in the bulbar and fornical conjunctiva. More cell layers of conjunctival epithelium were present in BAC-treated eyes without SERPINA3K, while fewer layers were observed in the SERPINA3K-treated eyes and normal eyes. In conjunctival fornix, larger cells with irregular shapes were also observed at the superficial layers. Meanwhile, HE staining revealed the putative outlines of goblet cells with the present of sporadic or clustered vacuoles in the fornix.

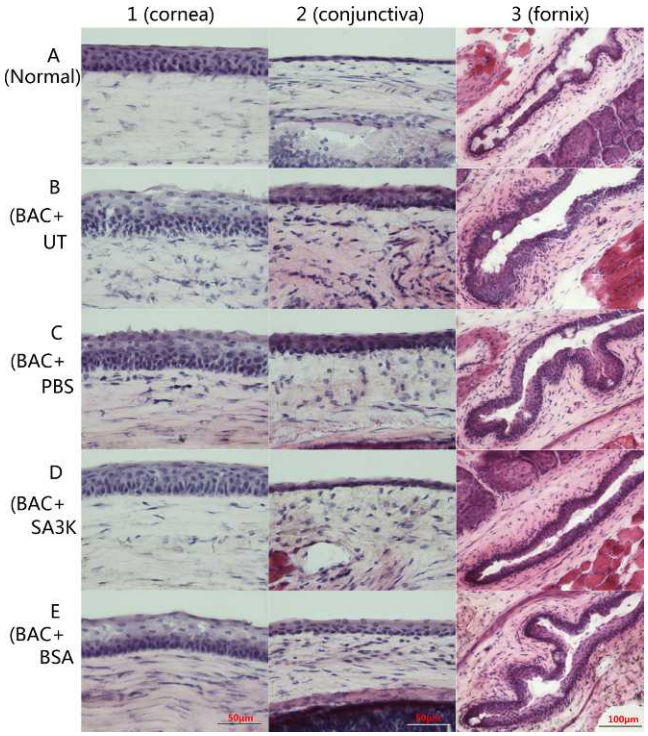

Figure 2. Morphological changes of ocular surface. Representative images of H&E staining showed the morphological alterations of the cornea (*column 1*), bulbar conjunctiva (*column 2*) and fornical conjunctiva (*column 3*) on D16. The ocular surface epithelium was intact and smooth in the (**A**) normal eyes with four to five cell layers in the cornea, one to two layers in the bulbar conjunctiva, and three to four layers in the fornical conjunctiva. In BAC-treated eyes without SERPINA3K medication, cell layers and thickness of the ocular surface epithelium both increased apparently and larger cells with irregular shapes could be observed at the superficial epithelium in the (**B**) BAC + UT, (**C**) BAC+ PBS, and (**E**) BAC + BSA groups. More inflammatory infiltrations could be observed in the corneal stroma of BAC-treated eyes without SERPINA3K. In the (**D**) BAC + SA3K group, the cell layers and thickness of ocular surface epithelium remained similar to that of the normal eyes and was slightly altered morphologically. *Scale bars*: 50 μm in column 1 and column 2, 100 μm in column 3.

SERPINA3K Inhibited Squamous Metaplasia

The inhibitory effects of SERPINA3K on squamous metaplasia were investigated by immunostaining assays. The expression of K10 keratin, an epidermal keratinocyte-specific intermediate filament, was negative in normal ocular surface epithelium. In BAC-treated eyes without SERPINA3K, K10-positive cells were apparently present in superficial and suprabasal cells of both the corneal and conjunctival epithelium on D16, while K10-positive cells were extremely sparse in SERPINA3K-treated eyes (Fig. 3).

Immunostaining of p63 was performed to elucidate epithelial proliferative status. Expression of p63 was recorded in basal and some suprabasal epithelial cells in normal cornea, bulbar and fornical conjunctiva. In BAC-treated corneas without SERPINA3K, the number of p63-positive nuclei was dramatically increased on D16. Meanwhile, more large cells with irregular shape were recorded in the superficial layers of the corneas of blank control. However, in the corneas treated with SERPINA3K, p63-positive nuclei were confined to the basal and some suprabasal epithelium on D16 with slightly increased number of p63-positive nuclei when compared with the normal eye. In the bulbar and fornical conjunctiva, similar trends were observed among groups (Fig. 4).

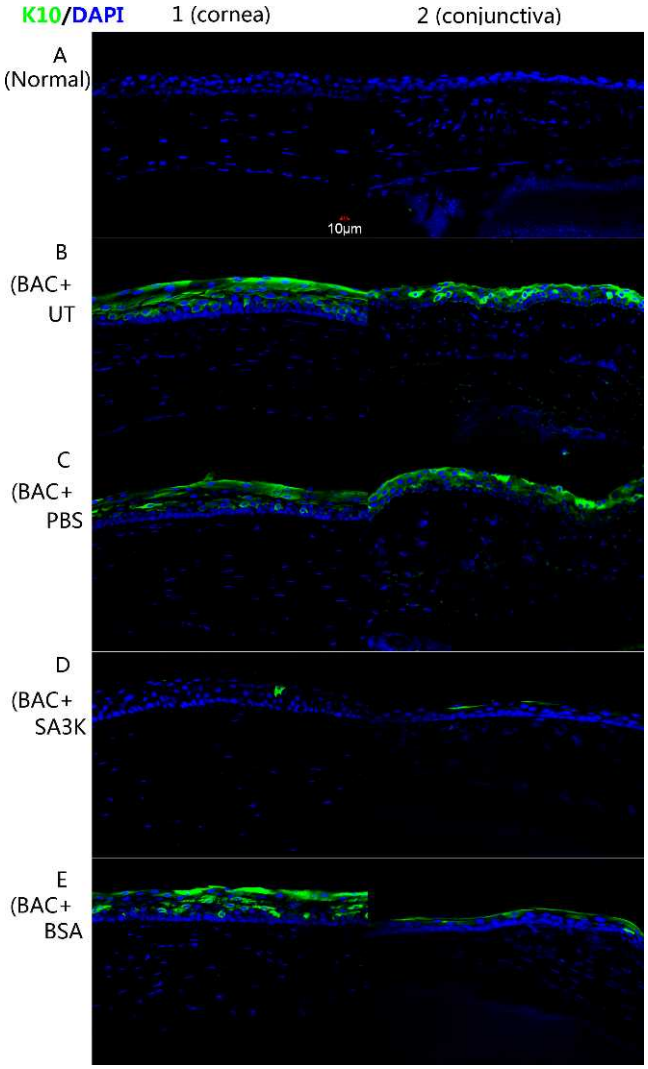

Figure 3. Expression of K10 inhibited by SERPINA3K. Representative immunostaining images with antibody of K10 in the cornea (*column 1*) and bulbar conjunctiva (*column 2*) on D16. No K10 expression could be recorded in the normal eyes (**A**). In the eyes of the (**B**) BAC + UT, (**C**) BAC + PBS, and (**E**) BAC + BSA groups, K10-positive cells were apparently present in superficial and suprabasal cell layers of both the corneal and conjunctival epithelium on D16, while extremely sparse in (**D**) SERPINA3K-treated eyes. *Scale bar*: 10 μm.

We also performed immunohistological staining of Ki67, which is known to be present during active phases of the cell cycle (G1, S, G2, and mitosis) but absent from resting cells (G0), thus is extensively used as a cell proliferation marker.[26] It was demonstrated that the number of Ki67 positive cells was low in the basal layer of normal cornea and conjunctiva. Similar to p63, an obvious increase of Ki67-positive cells was observed in the basal and suprabasal epithelium of BAC-treated eyes without SERPINA3K on D16, while only a slight increase in the SERPINA3K-treated group (Fig. 5).

SERPINA3K Retained Goblet Cell Density

The activity of SERPINA3K on the goblet cells in conjunctiva was also examined by PAS staining. It was revealed that the normal conjunctival fornix had only two to three layers of epithelium in BALB/c mice. Cells that were PAS-positive in normal mouse resided mainly in the superficial epithelium of

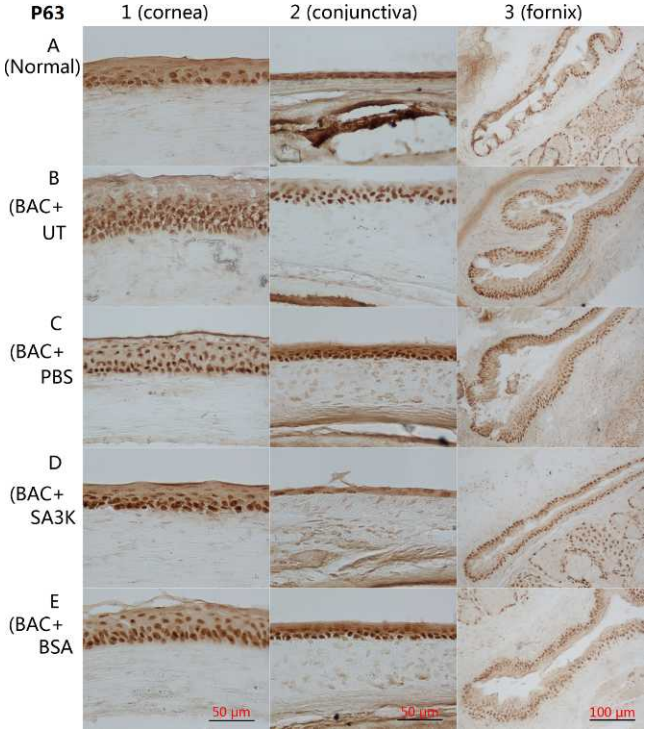

FIGURE 4. Expression of p63 suppressed by SERPINA3K. Representative immunostaining images with antibody of p63 in the cornea (*column 1*), bulbar conjunctiva (*column 2*), and fornical conjunctiva (*column 3*) on D16. Expression of p63 was recorded at low density in basal and some suprabasal epithelial cells in (**A**) normal eyes. In BAC-treated eyes without SERPINA3K, the number of p63-positive nuclei of the ocular surface was apparently increased in the (**B**) BAC + UT, (**C**) BAC + PBS, and (**E**) BAC + BSA groups. In (**D**) SERPINA3K-treated eyes, p63-positive cells were confined to the basal epithelium with slightly increased number of p63-positive nuclei when compared with the normal eye. *Scale bars*: 50 μm in columns 1 and 2, 100 μm in column 3.

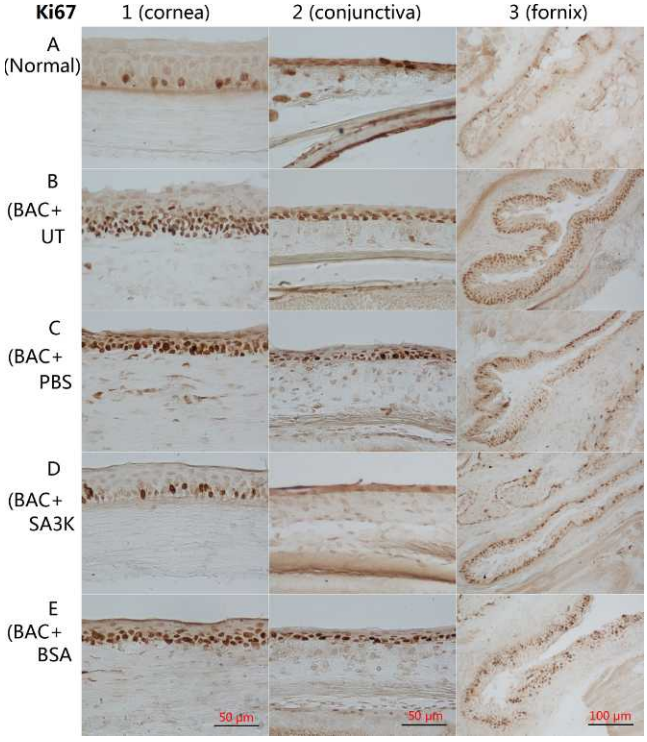

FIGURE 5. Expression of Ki67 was inhibited by SERPINA3K. Representative immunostaining images with antibody of Ki67 in the cornea (*column 1*), bulbar conjunctiva (*column 2*) and fornical conjunctiva (*column 3*) on D16. Expression of Ki67 was recorded at low density in basal epithelial cells in normal eyes (**A**). In BAC-treated eyes without SERPINA3K, the number of Ki67-positive nuclei of the ocular surface was apparently increased in the (**B**) BAC + UT, (**C**) BAC + PBS, and (**E**) BAC + BSA groups on D16, and these cells resided in both of the basal and suprabasal layers. In SERPINA3K-treated eyes (**D**), Ki67-positive cells were confined to the basal epithelium with slightly increased number of Ki67-positive nuclei when compared with the normal eye. *Scale bars*: 50 μm in columns 1 and 2, 100 μm in column 3.

conjunctival fornix, which were abundant in number and distributed in a continuous homogeneous pattern (Fig. 6A). The data showed that the number of PAS-positive cells significantly decreased in all BAC-treated eyes without SERPINA3K treatment, while the SERPINA3K treatment did rescue the number of goblet cells significantly (Figs. 6B–F). In addition, the fornical conjunctival epithelium reached four to six layers in BAC-treated eyes without SERPINA3K treatment, while SERPINA3K treatment induced remaining of two to three layers in the majority of the fornical epithelium. These results were consistent with that of H&E staining. Interestingly, the PAS-positive cells resided mainly in the superficial layer of SERPINA3K-treated fornical conjunctiva. In contrast, the majority of PAS-positive cells could be observed in the basal and suprabasal epithelium of fornical conjunctiva in BAC-treated eyes without SERPINA3K.

SERPINA3K Had Anti-Inflammatory Effect

We previously have showed the anti-inflammatory effects of SERPINA3K on corneal injury after alkali burn through downregulation of inflammatory factors such as TNF-α.[20] We therefore investigated if SERPINA3K has similar anti-inflammatory activity in the mouse dry eye in this study, by measurement of inflammatory responses using Western blot (Fig. 7). As shown, BAC solution significantly induced expression of TNF-α after treatment for 16 days, compared with normal eyes. The data confirmed the downregulation of inflammatory factor TNF-α level in SERPINA3K-treated corneas, compared with those BAC-treated eyes without SERPINA3K on D16. In addition, the TNF-α level in the BAC + SA3K group was close to that of the normal group.

DISCUSSION

It has been reported that SERPINA3K has various effects in the retina, such as anti-inflammatory, antiangiogenic, antioxidant, and antifibrotic.[17–19,27] In ocular surface, we previously demonstrated the beneficial effects of SERPINA3K in corneal alkali burn,[20] the protection of corneal endothelium from barrier function disruption[28] and protection of corneal epithelium from oxidative stress.[29] In this study, we provided novel evidences that SERPINA3K ameliorated the severity of squamous metaplasia and suppressed the formation of dry eye in a murine model. This study also indicated that the beneficial effects of SERPINA3K might be mediated through the downregulation of proinflammatory factor.

Increased production and activation of proinflammatory cytokines such as TNF-α by stressed ocular surface as well as by the inflammatory cells that infiltrate the tissues have been extensively reported in dry eye.[30–33] In fact, inflammation is the key mechanism of ocular surface injury and dryness, as both the cause and consequence.[34] The inflammatory molecules may reduce the trigeminal sensitivity, increased the tear

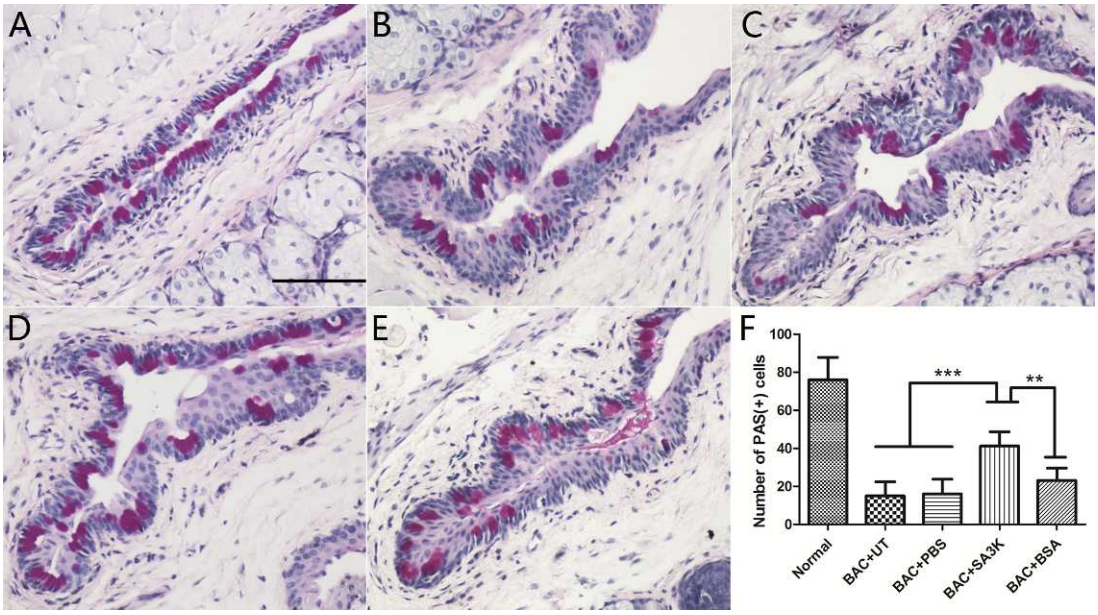

FIGURE 6. Goblet cell density retained by SERPINA3K. Representative images of PAS staining showed the goblet cells in the conjunctival fornix on D16. The goblet cells in normal mouse resided mainly in the superficial epithelium of conjunctival fornix and distributed in a continuous homogeneous pattern in line (**A**). The number of PAS-positive cells was significantly decreased in the (**B**) blank control, (**C**) PBS, and (**E**) BSA groups, but partially maintained in the (**D**) SERPINA3K-treated group. (**F**) Shows the average number of goblet cells in each group. Data were presented as mean ± SEM; $n = 6$. $^{**}P < 0.01$. $^{***}P < 0.001$. *Scale bar*: 50 μm.

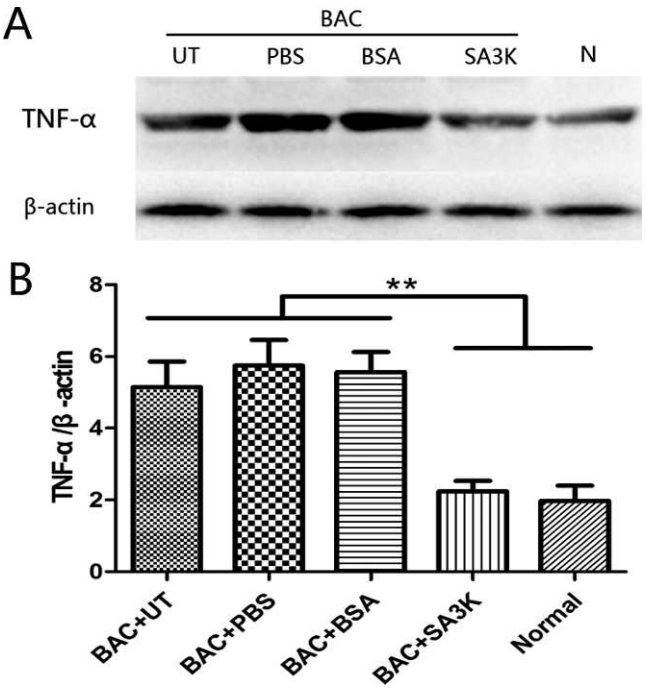

FIGURE 7. Anti-inflammatory effects of SERPINA3K. Representative bands of Western blot data. (**A**) Shows that BAC solution significantly induced expression of TNF-α after treatment for 16 days, compared with normal eyes. The protein level of TNF-α in SERPINA3K-treated corneas was downregulated and significantly lower than those in the BAC + UT, BAC + PBS, and BAC + BSA groups. In addition, the TNF-α level in the BAC + SA3K group was close to that of the normal group. (**B**) Shows the statistical analysis of bands of groups by density values. Data were presented as mean ± SEM; $n = 6$ in each group. $^{**}P < 0.01$.

film osmolarity, thus leading to the "reactivation" of inflammatory cascades.[34–36] Several proinflammatory cytokines such as TNF-α and interleukin-1 beta (IL-1β) are able to recruit more inflammatory cells and trigger downstream inflammatory responses.[33,37] Regardless of the initiating cause, a vicious cycle of inflammation may develop on the ocular surface and cause aggravation of dry eye condition.[34] Therefore, anti-inflammatory therapy has been considered as one of the key points in management of dry eye.[38] Our data indicated that SERPINA3K has the ability to ameliorate the severity of dry eye, probably through the inhibition of critical inflammatory cytokines TNF-α.

Squamous metaplasia is a hallmark of a variety of severe ocular surface disorders including chronic dry eye,[8] and always happens under chronic inflammatory infiltration of ocular surface.[10] T cells that are CD4 positive might be one of the key cells that trigger squamous metaplasia. Adoptive transfer of CD4–positive T cells could cause advanced ocular surface keratinization.[39] Inflammatory factors such as TNF-α and IL-1β are critical in the formation of squamous metaplasia.[39] Our data revealed the emergence of epidermis-specific K10 expression in the cornea and conjunctiva, indicating that the nonkeratinized epithelium was replaced by squamous epithelium. However, no CD4-positive cells were recorded in the cornea (data not shown). Our previous study indicated that CD4-positive cells may be recorded after BAC treatment for 4 weeks.[40] Therefore, CD4-positive cells might not be necessary for squamous metaplasia, and inflammatory cytokines such as TNF-α might be the key participants in epithelial squamous metaplasia. Long-term deficiency of lacrimal secretion is considered as another major cause of squamous metaplasia.[5]

Goblet cells are highly specialized epithelium located in the apical surface of the conjunctival fornix in BALB/c mouse. The main functions of goblet cells are synthesizing, storing, and secreting some of the mucous component of the tear film.[41] The cell number could be significantly reduced under chronic or severe ocular surface insults such as BAC application.[21,22] Our data showed that the number of goblet cells could be

apparently maintained by SERPINA3K while significantly reduced in the other three BAC-treated groups. Increased goblet cells, as well as decreased inflammatory cytokines, might be two of the factors improving the tear film stability. Unfortunately, there is no study exploring the direct effects of SERPINA3K to cultured goblet cells so far and this area would be of great interest.

In summary, topical application of SERPINA3K showed clinically observable suppression to the formation of BAC-induced dry eye by stabilizing the tear film, decreasing the inflammatory response, and alleviating the ocular surface squamous metaplasia, which were supported by histologic assessment. Furthermore, it was revealed for the first time that SERPINA3K could alleviate the severity of dry eye by reducing the expression of TNF-α. Our study indicated that SERPINA3K may be of great potential as a preventive agent to patients with high risk of dry eye.

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Investigative Ophthalmology & Visual Science

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