Therapeutic Effects of Epidermal Growth Factor on Benzalkonium Chloride–Induced Dry Eye in a Mouse Model

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PURPOSE. To investigate the therapeutic effects and possible mechanisms of epidermal growth factor (EGF) on the mouse dry eye model induced by benzalkonium chloride (BAC).

METHODS. The eye drop containing EGF was topically administered (3 ng per day) on a BAC-induced dry eye model. The following clinical indications of dry eye were evaluated on Days 2, 4, and 6: tear break-up time (BUT), corneal fluorescein staining, inflammatory index, and tear volume. Global specimens were collected on Day 6 and then the following examinations were performed: histologic investigation, TUNEL assay to measure the dead cells, periodic acid-schiff (PAS) assay to detect goblet cells, and immunostaining of antibodies of Ki-67, EGF receptor (EGFR), and MUC1 in the corneas. The levels of EGFR and p-ERK of the corneas were also measured by Western blot analysis.

RESULTS. EGF resulted in longer BUTs on Days 2 and 6, lower fluorescein staining scores on Days 4 and 6, while no significant changes in inflammatory index or tear volume. EGF induced higher EGFR expression in corneal tissues by immunofluorescent staining and Western blot analysis. EGF also upregulated p-ERK, increased Ki-67 positive cells, and decreased TUNEL positive cells. In addition, EGF significantly increased the goblet cells number and MUC1 expression in the epithelium.

CONCLUSIONS. Topical application of EGF presented clinical improvements on dry eye by stabilizing the tear film and maintaining the integrity of epithelium. The results indicate that EGF has potential as a therapeutic agent in clinical treatment of dry eye. (Invest Ophthalmol Vis Sci. 2012;53:191–197) DOI:10.1167/iovs.11-8553

Dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface. As one of the most common ocular diseases, dry eye affects 15% to 35% of the population worldwide. Patients often complain about dryness, redness, burning, and sandy-gritty eye irritation. If the condition is left untreated or aggravates, it may produce complications including corneal epithelium damage, squamous metaplasia, and neovascularization.

Among numerous managements for dry eye, the first-line choice is topical application of artificial tears which has been proven to relieve symptoms of irritation and decrease corneal drying in mild to moderate clinical conditions. However, the imperfect performance of artificial tears in severe cases may result from lack of active agents such as epidermal growth factor (EGF), hepatocyte growth factor, fibronectin, neurotrophic growth factor, and vitamin A. These essential components in the tear film play important roles in maintaining ocular surface health. Topical application of autologous serum (AS), which can supplement the active agents, leads to recovery in many severe dry eye cases. However, the relatively complicated preparation and safety concerns limit its use in individuals.

EGF is the key component of both tear film and serum. EGF is a potent polypeptide mitogen that belongs to the EGF family of growth factors. It has been demonstrated that EGF in the tear film is secreted by the lacrimal gland and plays a vital role in the corneal epithelium maintenance and wound healing. Symptoms of dry eye in patients with solid tumors treated with systemic therapy of EGF receptor (EGFR) inhibitor, led to the hypothesis that topical application of EGF may be a promising therapy for dry eye. However, the efficacy of EGF eye drops on dry eye has not been adequately discussed so far. The aim of this study was to investigate the therapeutic effects of EGF on the mouse dry eye models induced by topical administration of benzalkonium chloride (BAC) and the mechanisms underlying these effects.

METHODS AND MATERIALS

Mouse Dry Eye Model

Twenty-eight male BALB/c mice (18 to 20 g, purchased from Shanghai SLAC laboratory animal center, Shanghai, China) were used for this study. These mice were kept in the facility with standard environment throughout the study as follows: room temperature 25 ± 1°C, relative humidity 60% ± 10%, and alternating 12-hour light-dark cycles (8 AM to 8 PM). All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the experimental protocol was approved by the experimental ethics committee of Xiamen University. Both eyes of 21 mice were topically administered twice daily (9 AM, 9 PM), 5 µL of 0.2% BAC for 14 days. Based on the clinical evaluations (de-
scribed below), the mice with dry eye condition were then randomly divided into three groups: blank control group, solvent-treated group, and EGF-treated group. The other 7 mice were raised for normal control.

Experimental Procedure
After grouping, the blank control group was left untreated for baseline comparison. The solvent-treated group was administered with solvent of EGF (primarily glycerol; Watsin Genetech, Shenzhen, China) and the EGF-treated group with EGF solution (200 ng/mL; Watsin Genetech) with the dosage of 5 μL three times per day (8 AM, 3 PM, 10 PM). During the treatment (on Day 2, 4, and 6), the clinical evaluations were performed by a single masked ophthalmologist. On Day 6, all mice were euthanized and the ocular or orbit tissues were carefully dissected and harvested for histologic and Western blot analysis following the methods described below.

Break-Up Time (BUT) and Fluorescein Staining
One microliter of 0.1% liquid sodium fluorescein was dropped into the conjunctival sac. After 3 blinks, BUT was recorded in seconds. Ninety seconds later, corneal epithelial damage was graded with a cobalt blue filter under a slit-lamp microscope (Kanghua Science & Technology, Chongqing, China). The cornea was divided into four quadrants, which were scored respectively. The four scores were added to a final grade (total, 16 points). The fluorescein score was analyzed as previously described13 with essential modification; it was briefly as follows: absent, 0; slightly punctuate staining <30 spots, 1; punctate staining >30 spots, but not diffuse, 2; severe diffuse staining but no positive plaque, 3; and positive fluorescein plaque, 4.

Evaluation of Inflammation
The inflammatory index was analyzed as previously described.16 Briefly, the inflammatory index was evaluated, based on three parameters: ciliary hyperemia (absent, 0; present but <1 mm, 1; present between 1 and 2 mm, 2; present >2 mm, 3); central corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present without visible pupil, 3); and peripheral corneal edema (absent, 0; present with visible iris details, 1; present without visible iris details, 2; present with no visible iris, 3). The final inflammatory index result was obtained by summing the scores of the different parameters then dividing by a factor of nine.

Measurement of Tear Volume
Tear production was measured by the phenol red thread tear test using cotton threads (Zone-Quick; Yokota, Tokyo, Japan)17 at a similar time from the lateral canthus of the lower eyelid. The red portion of the thread is measured in millimeters. Tear production was calculated by measuring the length of the red thread portion of the thread placed on the palpebral conjunctiva for 15 seconds at a specified point approximately one third of the distance from the lateral canthus of the lower eyelid. The red portion of the thread is measured in millimeters.

Immunofluorescent and Immunohistochemical Staining
Both immunofluorescent and immunohistochemical staining were performed on cryosections (6 μm thick) of the eyeballs. Sections for immunofluorescent staining were fixed in acetone at −20°C, blocked, and incubated at 4°C overnight with the EGFRI antibody (1:50,000; Abcam, San Francisco, CA) and the MUC1 antibody (1:100; Abcam). After incubation in AlexaFlour 488-conjugated IgG (1:1000; Invitrogen, Carlsbad, CA), sections were counterstained with DAPI (Vector, Burlingame, CA), mounted, and photographed using a confocal laser scanning microscope (Fluoview FV1000; Olympus, Tokyo, Japan). Corneas were scanned in the same area in each group. For immunohistochemical staining, the endogenous peroxides activity was quenched with 0.6% hydrogen peroxide in PBS for 30 minutes. After incubating with 2% BSA, the antibodies of Ki-67 (1:400; Abcam) were applied and incubated at 4°C for 14 to 18 hours. After rinsing with PBS, the sections were further incubated with biotinylated anti-rabbit IgG (1:50) according to the manufacturer’s protocol. The reaction product was then developed with diaminobenzidine (DAB), the peroxidase substrate for 60 seconds and mounted with mounting medium (H-5000; Vector) and examined under a light microscope (Nikon Eclipse 50i; Tokyo, Japan). The counterstaining with hematoxylin was not performed to avoid interference from the nuclear staining.

Western Blot Analysis
Proteins of corneas from each group were extracted with cold RIPA buffer. Equal amounts of proteins of the cell lysates were subjected to electrophoresis on 8% or 11% SDS-PAGE and then electrohoresically transferred to PVDF membranes. After 1-hour blocking in 2% BSA, the membranes were incubated with primary antibodies for EGF (1:50,000; Abcam), Phospho-p44/42 ERK and p44/42 ERK (1:1000; Cell Signaling Technology, Inc., Danvers, MA) and β-actin (1:10,000; Sigma, St. Louis, MO) 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 HRP-conjugated goat anti-rabbit IgG (1:10,000; Bio-Rad, Hercules, CA) for 1 hour. The specific bands were visualized by enhanced chemiluminescence reagents (Lulong Inc., Xiamen, China) and recorded by the transilluminator (Chemidoc XRS; Bio-Rad).

In Situ Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)
To measure end-stage apoptosis, in situ TUNEL was performed in frozen sections using an assay (DeadEnd Fluorometric TUNEL System G3250; Promega, Madison, WI) according to manufacturer’s instructions. Sections were counterstained with DAPI (Vector), mounted, and the photo images were taken with a confocal laser scanning microscope (Fluoview FV1000; Olympus). For the positive control, sections were incubated in DNase I before the addition of equilibration buffer, while DDW was used instead of TdT reaction mix in the negative control.

Periodic Acid-Schiff (PAS) Staining
The goblet cells in conjunctival fornices were stained by a PAS staining system (PAS Staining System 395B-1KT; Sigma) in the paraffin sections of the whole orbit tissue. Goblet cells were counted in six representative slices of homologous positions of tissues from each group. The average numbers of goblet cells were included for comparison. The counterstaining with hematoxylin was performed.

Statistical Analysis
Two-way ANOVA (Bonferroni posttest) was applied to make comparisons between groups at different time points. Differences with P values < 0.05 were considered statistically significant.

Results
EGF Ameliorated the Stability of Tear Film and Epithelium Damage
To determine whether EGF has beneficial effects on the dry eye, we used the BAC-induced experimental setting that we established and reported recently.13 Briefly, BAC was topically administered at a dosage of 0.2 μg daily for 14 days. The clinical evaluations of BUT, corneal fluorescein staining, inflammatory index, and tear volume were identical in three groups: the blank control group, the solvent-treated group, and the EGF-treated group (D0; Fig. 1).

It was demonstrated that there was a general trend of extending BUT (Fig. 1A) and decreasing fluorescein staining
scores (Fig. 1B) of each group after the BAC administration stopped. However, it was shown that EGF at the dose of 3 ng daily statistically significantly increased BUT on Day 2 and Day 6 while decreased fluorescein staining scores on Days 4 and 6, compared with both the blank control group and the solvent-treated. Meanwhile, we also measured inflammatory index and tear volume changes after EGF treatment. It was shown that inflammatory indexes decreased since the termination of BAC administration while phenol red thread tear volume tests kept at the same level to the baseline of this study (Figs. 1C, 1D). EGF did not induce significant changes of inflammatory index and tear volume among 3 groups at all time points during the treatment (Figs. 1C, 1D). In addition, it was revealed by the H&E staining that EGF-treated corneas had smoother epithelium compared with the blank control group and solvent-treated group (Fig. 2).

Alteration of Epidermal Growth Factor Receptor

To elucidate the mechanism underlying the above effects of EGF on the dry eye, we focused on the key factor of the EGF pathway: EGFR with immunofluorescent staining and Western blot assay. It was demonstrated that BAC induction reduced the volume of corneal EGFR in the blank control group and the solvent-treated group, compared with normal control (Figs. 3A–F), meanwhile, interestingly the treatment of EGF resulted in a high density of EGFR expression in the corneal epithelium (Figs. 3G, 3H). In addition, the Western blot analysis results also showed EGFR level of the cornea was lower in the blank control group and the solvent-treated group than that of normal ones. EGF administration induced a high level of EGFR (Fig. 3I), which was consistent with the results of immunofluorescent staining images.

Effect of EGF on Phosphorylation of ERK, Cell Proliferation, and Apoptosis

To further investigate the mechanism of EGF’s effects on dry eye, we conducted studies by examining the changes of downstream pathway of EGF (i.e., the phosphorylation of ERK, the alterations of cell proliferation and apoptosis).
ment induced more Ki-67 positive cells (Fig. 4B4). Our data suggested that EGF increased the cell proliferation in the dry eye condition.

We also examined changes of the cell death by the TUNEL assay. It was revealed that dry eye condition resulted in more cell death in corneal epithelium in both the blank control group and the solvent-treated group compared with the normal control group, meanwhile EGF treatment alleviated the cell death in corneal epithelium (Fig. 4C).

Effects of EGF on Density of Goblet Cells and Mucins

To determine whether EGF has effects on the mucins of tear film, we studied the MUC5AC which is secreted by goblet cells, and the MUC1 which is tethered to the epithelial membrane. We investigated MUC5AC by counting the number of goblet cells with PAS staining (Fig. 5E). It was demonstrated that PAS positive cells in the conjunctiva was decreased in the dry eye model (Figs. 5A–C), while EGF treatment significantly increased the number of goblet cells (Fig. 5D).

As one of membrane tethered mucins on the ocular surface, MUC1 was studied with immunofluorescent staining. It was revealed that MUC1 was expressed in the whole corneal epithelium (Figs. 6A, 6B) while the expression was weak in the dry eye condition (Figs. 6C, 6D). Meanwhile EGF treatment increased the level of MUC1, compared with that of solvent-treated group (Figs. 6E–H).

DISCUSSION

Dry eye is a common ocular surface disease initiated by various stresses. Numerous animal models19,20 have been developed to imitate certain pathophysiologic mechanisms to evaluate various promising therapies. The BAC-induced model was introduced in this study because its pathogenesis is identical with some initial stresses of dry eye, such as administration of eye drops with preservative in the chronic disease, toxic gas from worsened environment dissolved in the tear film, and the mild chemical burn. Furthermore, BAC-induced dry eye model shared the same pathologic changes with human dry eye, for example, epithelial apoptosis, goblet cell loss, tear film defect and the inflammation, which were revealed in the previous reports.14,21 Epidermal growth factor has been widely used in managing many ocular surface diseases, such as trauma and infection.8–11 However the efficacy of EGF eye drop on the dry eye has not been adequately discussed. Our study demonstrated the therapeutic effects of EGF to treat dry eye in vivo. The mechanisms underlying these effects include epithelium maintenance by promoting epithelium proliferation and inhibiting apoptosis, as well as tear film supplement by increasing goblet cell number and maintaining MUC1 expression. As the key component in the tear film, EGF has great potential as an agent for the treatment of dry eye.

The EGF belongs to the receptor tyrosine kinase family which is widely expressed in the ocular surface. The activation of EGFR was involved in physiological procedures of cell proliferation, migration, differentiation, and apoptosis through multiple signal transduction pathways, particularly mitogen-activated protein kinases (MAPKs).22–23 Administration of BAC resulted in inflammation of the ocular surface, leading to the decrease of EGFR on the epithelial cell membranes, which may also occur in other types of dry eye. It was noticed that topical application of EGF induced the expression of EGFR (Fig. 3), which might be due to the signaling transduction and its proliferative effect on renewing the epithelium. However, specific mechanisms were not clear and it remains to be further investigated and elucidated. Abundant EGFR on the cell performed immunohistochemical staining that the number of Ki-67 positive cells was low in normal conditions (Fig. 4B1). Few cells were labeled by Ki-67 in both the blank control group and solvent-treated group (Figs. 4B2, 4B3). Meanwhile EGF treatment induced more Ki-67 positive cells (Fig. 4B4). Our data suggested that EGF increased the cell proliferation in the dry eye condition.

It was shown by Western blot analysis that p-ERK level was low in the BAC-induced dry eye models, while treatment of EGF upregulated p-ERK level (Fig. 4A), indicating that the effects of EGF is mediated with the p-ERK.

We detected the level of Ki-67 to determine the growth fraction of epithelium, because Ki-67 is known to be present during active phases of the cell cycle (G1, S, G2, and mitosis) but absent from resting cells (G0).18 It was demonstrated with immunohistochemical staining that the number of Ki-67 positive cells was low in normal conditions (Fig. 4B1). Few cells were labeled by Ki-67 in both the blank control group and solvent-treated group (Figs. 4B2, 4B3). Meanwhile EGF treatment induced more Ki-67 positive cells (Fig. 4B4). Our data performed immunohistochemical staining that the number of Ki-67 positive cells was low in normal conditions (Fig. 4B1). Few cells were labeled by Ki-67 in both the blank control group and solvent-treated group (Figs. 4B2, 4B3). Meanwhile EGF treatment induced more Ki-67 positive cells (Fig. 4B4). Our data
membrane of the EGF-treated group were inclined to bind with extra EGF, resulted in autophosphorylation of tyrosine residues, and prompted downstream activations. It was confirmed by our in vivo study that the ERK1/2, also known as classical MAPKs, was activated by EGF through autophosphorylation (Fig. 4A), leading to DNA synthesis and cell proliferation25 (Fig. 4B) and antiapoptosis26 (Fig. 4C). Other functions of EGF, such as modulation of cell migration, adhesion, and immune response, might also be present.27,28

Goblet cells are highly specialized epithelial cells located in the apical surface of the conjunctiva. The main functions of goblet cells are synthesizing, storing, and secreting MUC5AC for the mucous component of the tear film. Chronic or severe inflammation in the dry eye condition can reduce the goblet cells,29,30 which was confirmed in our study (Fig. 5). We demonstrated that goblet cells increased significantly after 6 days treatment of EGF, which was consistent with the previous in vitro study31 in that EGF activated p44/p42 MAPK and promoted proliferation in human and rat goblet cells. It is believed that goblet cell number is associated with the secretion of MUC5AC,32,33 which contribute to the lubrication and infection prevention of the ocular surface. The improvement of BUT (Fig. 1) may also result from more MUC5AC being secreted from goblet cells in the EGF-treated group. The direct observation of MUC5AC with immunolabeling was not performed because MUC5AC is a secretory protein, and the pattern may not reflect its real functional status. In addition to MUC5AC, several other membrane-spanning mucins of corneal epithelium are important components of mucous layer of tear film, such as

**FIGURE 4.** ERK was phosphorylated with the effect of promoting cell proliferation and inhibiting apoptosis. (A) The phosphorylation of ERK was valued by Western blot and the expression of p-ERK was upregulated in the EGF-treated cornea. (B) Immunohistochemical staining of Ki-67 was performed to observe the growth fraction of epithelium (magnification, ×400). More proliferative epithelial cells were marked in EGF-treated group (B4), compared with normal control (B1), blank control group (B2) and solvent-treated group (B3). (C) TUNEL assay was applied to measure the cell death of corneal epithelium. Few cells were marked in normal control (C1, C2) and EGF-treated cornea (C7, C8), while massive dead cells were detected in the blank control group (C3, C4) and the solvent-treated group (C5, C6).

**FIGURE 5.** The alterations of goblet cells in conjunctiva observed by PAS staining (magnification, ×400). (A) Normal conjunctival fornix. (B) Conjunctival fornix of the blank control group. (C) Conjunctival fornix of the solvent-treated group. (D) Conjunctival fornix of the EGF-treated group. (E) Mean PAS positive cell numbers of each group. **P < 0.01, ***P < 0.001.
MUC1, MUC4, MUC16, and MUC19. We evaluated the expression of MUC1 by immunolabeling (Fig. 6). It was demonstrated that MUC1 was impaired in the BAC-induced dry eye compared with the normal specimens, which was consistent with a previous study of Sjögren’s syndrome. The effect of EGF on promoting epithelial (including goblet cells) proliferation and supplementing the mucous layer could maintain the MUC1 expression in dry eye condition, which contributed to the tear film stability in return. And then, the improvement of tear film offered better protection of corneal epithelium. In summary, topical application of EGF could supplement tear film by increasing the number of goblet cells and maintaining MUC1 expression. Our study indicated that EGF had a great potential to be a therapeutic agent in the clinical treatment of dry eye.

**FIGURE 6.** Representative images of MUC1 immunofluorescent staining for each group. MUC1 was expressed in the whole epithelium of normal cornea (A, B) while the expression was weak in dry eye condition of the blank control group (C, D) and the solvent-treated group (E, F). Meanwhile high epithelial MUC1 expression can be observed in the EGF-treated group (G, H).

**References**


