A Mouse Model of Keratoconjunctivitis Sicca

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PURPOSE. To evaluate the effects of pharmacologic inhibition of aqueous tear production and desiccating environmental stress on aqueous tear production, tear clearance, corneal epithelial permeability, and conjunctival epithelial morphology, proliferation, and conjunctival goblet cell differentiation.

METHODS. Aqueous tear production was inhibited by applying transdermal scopolamine (scop) patches to the depilated mid-tail of female MC, CBA mice. Desiccating environmental stress was created by placing mice in a hood with a continuous airflow blower. Aqueous tear production and volume, tear clearance, and corneal barrier function were compared in four experimental groups: untreated control mice, mice placed in the blower hood, mice treated with a scop patch, and mice treated with a scop patch and blower hood (scop patch + blower). Cotton threads were used to evaluate aqueous tear production and volume. Tear clearance was assessed by fluorometric measurement of collected tear fluid 15 minutes after instillation of 1% sodium fluorescein. Corneal epithelial barrier function was assessed by fluorometric measurement of carboxyfluorescein uptake. Conjunctival morphology and goblet cell density were evaluated in stained histologic sections. Conjunctival epithelial proliferation was assessed by bromodeoxyuridine (BrdU) labeling.

RESULTS. Significant decreases in cotton thread wetting and tear clearance were observed in mice treated with a scop patch (P < 0.001) or with a scop patch and blower desiccation (P < 0.001), with a greater reduction in tear clearance in the latter group. Significantly increased corneal carboxyfluorescein uptake was noted in the scop patch group compared with untreated mice (P = 0.05) and in the scop patch + blower group compared with all the other groups (P < 0.0001). Changes in conjunctival epithelial morphology and a significant decrease in conjunctival goblet cell density (P < 0.001) were observed in the scop patch + blower group compared with the untreated control group. The number of proliferating conjunctival epithelial cells was significantly greater in the scop patch + blower group.

CONCLUSIONS. Pharmacologic inhibition of tear production in mice is accompanied by ocular surface epithelial changes resembling human keratoconjunctivitis sicca (KCS) that are exacerbated by desiccating environmental stress. Cholinergic stimulated tear production appears to be essential for maintaining a healthy ocular surface. (Invest Ophthalmol Vis Sci. 2002; 43:652–658)

Dry eye is a common condition that affects 10% of the population between the ages of 30 and 60 years, increasing in prevalence to 15% of the population aged more than 65 years.2 Dry eye results from decreased production, increased evaporation, or decreased clearance of tears.2,3 Dry eye produces ocular irritation and ocular surface disease, termed keratoconjunctivitis sicca (KCS), that causes blurred and fluctuating vision and increases the risk of sight-threatening corneal infection and ulceration.4 The histologic features of KCS are abnormal proliferation and differentiation of the ocular surface epithelium with decreased density of conjunctival goblet cells and decreased and abnormal production of mucus by the ocular surface epithelium.5 These cellular changes are accompanied by altered epithelial barrier function with increased corneal fluorescein uptake.6,7 The most severe KCS has been reported to develop in conditions in which there is loss of the ability to tear in response to neural stimulation.8–10

The mechanism by which dry eye causes KCS has not been established. A number of factors have been implicated in its pathogenesis, including increased tear film osmolarity and decreased tear concentrations of lacrimal-gland–secreted factors that support the ocular surface epithelium, such as epidermal growth factor.11–14 There is also mounting evidence that inflammation plays a role in this process. Increased expression of a number of inflammatory mediators has been identified on the ocular surface of eyes with KCS. These include increased concentrations of proinflammatory cytokines in the conjunctival epithelium and tear fluid13–15; increased concentration and activity of proteases, such as plasmin and MMP-9, in the tear fluid14,16; increased concentration of lipid peroxidases in the tear fluid17; and increased expression of immune activation markers, such as HLA-DR, intercellular adhesion molecule (ICAM)-1, and CD-40, by the conjunctival epithelium.18–20 The importance of inflammation in the pathogenesis of dry eye is underscored by reports that the signs and symptoms of dry eye markedly improve with anti-inflammatory therapy.21,22

A dry-eye animal model that mimics the human dry-eye disease would be a useful tool for investigating the multiple factors that have been implicated in the pathogenesis of KCS. Several different animal models of dry eye have been proposed.23–29 Androgen deficiency after orchietomy in rats has been reported to increase the volume of secreted tear fluid, while it reduces the concentration of secretory component and of Ig A in the tear fluid.23–24 Testosterone reverses these changes, whereas hypophysectomy prevents this reversal. Another method for inducing dry eye is surgical excision of tear-producing glands. In rabbits, surgical extirpation of the lacrimal gland, hardier gland, and nictitating membrane resulted in increased tear osmolarity, increased corneal epithelial desquamation, decreased corneal epithelial glycogen, decreased conjunctival goblet cell density and increased ocular surface staining with rose bengal dye.25 Dry eye has also been induced by surgical removal of the exorbital lacrimal glands in monkeys and mechanical inhibition of blinking with a bleph-
arthropat for 1 to 3 hours in rabbits.28,29 Progressive lacrimal gland inflammation resembling Sjögren syndrome develops in certain strains of mice; however, it has not been established that KCS develops in these mice.30,31 Pharmacologic blockade of cholinergic muscarinic receptors in the lacrimal glands with topically administered atropine sulfate has been reported to decrease tear production and cause KCS in rabbits.26 The presence of depressed lacrimal gland secretion in response to neural stimulation in many forms of severe dry eye makes cholinergic blockade relevant to human disease. The greater knowledge of the murine inflammatory response, coupled with the ability to experimentally manipulate gene expression in mice, makes them a better choice for an animal model than rabbits.

The purpose of this study was to determine whether an experimentally induced decrease in tear production and clearance would cause phenotypic ocular surface epithelial changes resembling human KCS.

METHODS

Transdermal scopolamine patches (scop patch) were obtained from Novartis (Summit, NJ); wicks (Transorb) from American Filtrona (Richmond, VA); phenol red impregnated cotton threads (Zone-quick) from Oasis (Glendora, CA); sodium fluorescein from Alcon Laboratories, Inc. (Fort Worth, TX); 0.3% carboxyfluorescein (CF) from Holles Laboratories (Cohasset, MA); and bromodeoxyuridine (BrdU) and anti-BrdU monoclonal antibody from Roche Molecular Biochemicals (Indianapolis, IN). Female MC, CBA mice were obtained from Charles River Laboratories (Wilmington, MA).

Aqueous tear production and volume, tear clearance, and corneal barrier function were compared in four experimental groups: untreated control mice, mice placed in a blower hood, mice treated with the anticholinergic agent scopolamine (scop), and mice treated with scop and placed in a blower hood.

Tear Fluorescein Clearance

Tear fluorescein clearance was determined as previously reported in humans.3,14,32 One microliter 1% fluorescein was applied to the ocular surface. After 15 minutes, fluorescein-stained tear fluid was collected atraumatically with a porous polyester rod (Transorb; American Filtrona) from the tear meniscus in the lateral canthus under a dissecting microscope (SMZ-1; Nikon, Tokyo, Japan), minimizing irritation of the ocular surface or lid margin (Fig. 1, top left). Immediately after tear collection, the rods were placed in sealed polypropylene tubes and were protected from light until fluorophotometric analysis. The volume of the collected tear fluid was determined by the weight difference between the rod containing the sample and the precollection empty rod (Model GA110 scale; Ohaus, Bern, Switzerland). A volume of phosphate-buffered saline (PBS; weight of rod in micrograms) was added to the end of the rod, the tubes were spun at 12,000 cycles per minute for 5 minutes, and the fluid was transferred to wells of a 96-well polycarbonate microtiter plate (Corning 96; Corning Glassworks; Corning, NY). Fluorescence was measured with a fluorescence multiplate reader (Cytofluor II; PerSeptive Biosystems, Framingham, MA) as previously described.3

Measurement of Aqueous Tear Production

Tear production was measured with cotton threads (Zone-quick; Oasis). The threads were held with jeweler forceps and applied to the ocular surface in the lateral canthus for 60 seconds. Wetting of the thread was measured in millimeters, using the scale on the cotton thread (Fig. 1, top right).


**Induction of Dry Eye with Cholinergic Receptor Blockade and Blower Hood Desiccation**

Two methods were used to deliver anti-cholinergic agents to block lacrimal gland muscarinic cholinergic receptors: topically applied atropine sulfate and transdermally delivered scopalamine. Topically treated mice received 1 μL of 1% atropine sulfate in each eye. A separate group of mice served as a negative control and received 1 μL sterile saline. Transdermal scopalamine ( scop ) patches were applied by cutting the patches into four pieces, wrapping them around the depilated midtail, and securing them with cellophane tape ( Fig. 1, bottom left ). Patches were reapplied on days 0 and 2. To induce environmental stress, some mice were placed in a blower hood ( Lab-Safety Hood; Labconco, Kansas City, MO ), with a flow rate of 300 fl/min at 7 seconds, for 1 hour, 3 times per day for 4 days ( scop patch + blower; Fig. 1, bottom right ). Tear production and clearance were evaluated before treatment, then 1, 4, 12, 24, and 48 hours after treatment.

**Corneal Fluorescein Staining**

Corneal fluorescein staining was evaluated and photographed with a slit lamp biomicroscope ( Humphrey-Zeiss, Dublin, CA ) using a cobalt blue light 10 minutes after application of 1 μL 1% sodium fluorescein.

**Permeability to CF**

Uptake of CF by corneas in normal and dry-eye mice was determined by modifying a previously reported technique. 35,36 CF ( 1 μL of a 0.3% solution ) was applied to the ocular surface. After a 10-minute delay, the mice were killed with high-dose carbon dioxide. Corneas without scleral rims were then excised, rinsed twice with 200 μL balanced salt solution ( BSS; Alcon Laboratories, Inc. ), cut into four pieces, and placed in 200 μL BSS. The solution containing the corneal tissue was protected from light and placed on an orbital shaker for 90 minutes. The CF concentration ( in units per microliter ) was measured with a fluorometer ( excitation wavelength-bandwidth, 485 nm; emission wavelength-half bandwidth, 530 nm; gain, 40; Cytoflour II; PerSeptive Biosystems ).

**Conjunctival Histopathology and Epithelial Proliferation**

Conjunctival biopsy specimens, measuring approximately 1 × 2 mm, were obtained from the same regions in the superior and the inferior bulbar conjunctiva, in control eyes and eyes of mice treated with the scop patch + blower. Tissues were fixed immediately in 2% formaldehyde and embedded in paraffin. Six- to eight-micrometer-thick sections were cut and stained with hematoxylin and eosin, or with the periodic acid–Schiff ( PAS ) reagent.

Conjunctival epithelial morphology was evaluated, and the number of conjunctival epithelial goblet cells was counted along the length of three separate tissue sections that spanned the entire length of a microscope field ( Axiophot 2; Nikon ), by two independent masked observers, using a ×20 objective. Three mice were evaluated in each treatment group.

As a control to study the direct effects of scopalamine on the ocular surface, 1 μL 0.25% scopalamine solution was applied to the ocular surface, 4 hours before the biopsy was performed. Conjunctival biopsies were then performed to evaluate conjunctival goblet cell density and epithelial morphology.

Conjunctival epithelial proliferation was assessed by injecting BrdU ( 0.5 mg in 0.2 mL ) subcutaneous in the flank 24 and 2 hours before death. BrdU was immunodetected in 5-μm frozen tissue sections using an anti-BrdU monoclonal antibody after treating sections with 2 N HCl for 60 minutes at 37°C and then 0.1 M borate buffer. The number of labeled cells was counted in three separate sections in specimens from three mice in each treatment group.

All procedures in the study protocol adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**RESULTS**

**Aqueous Tear Production and Clearance**

The initial phase of this study compared the effects of topically applied atropine to the eye and transdermal scop patches applied to the tail of mice, to determine which method produces the greatest pharmacologic inhibition of tear production. Topical atropine produced a significant reduction in aqueous tear production 1 hour after instillation, compared with mice that were treated with a saline drop ( P = 0.001 ). However, the effect was short-lived with tear production returning to baseline levels within 4 hours. In contrast, the transdermal scop patches produced a statistically significant decrease in aqueous production ( mean, 0.5 ± 0.7 mm ) and tear clearance ( mean, 985.625 ± 312 fluorescence units [ FU ] ) at 24 hours, compared with control animal ( mean, 2.98 ± 0.6 mm and 283 ± 96 FU, P = 0.001 for each ). Placement of mice treated with the scop patch into a blower hood further worsened tear clearance ( P < 0.001, Table 1 ), but it did not cause a greater reduction in the cotton thread test results than that in mice treated with the scop patch alone.

**Ocular Surface Fluorescein Staining and CF Uptake**

Visual evaluation of corneal fluorescein staining and corneal CF uptake were used as measures of the effect of dry eye on corneal epithelial barrier function. Minimal scattered punctate staining or no staining was observed on the corneas of control mice, after instillation of fluorescein dye ( Fig. 2, top left ). Mild central punctate fluorescein staining was observed in most mice treated with a scop patch ( Fig. 2, top right ). Patches of punctate and diffuse corneal fluorescein staining were observed in all scop patch + blower-treated mice ( Fig. 2, bottom ). Consistent with the visually observed staining was an increase in CF uptake by the
corneas of mice treated with a scop patch compared with untreated mice \( (P = 0.05) \) and in the scop patch + blower-treated mice compared with the other groups \( (P < 0.0001; \) Table 1). These results indicate that the experimental manipulations decreased aqueous tear production and tear clearance and altered corneal epithelial barrier function. Based on findings that the worst KCS developed in mice treated with a scop patch and blower, this combination was used to evaluate the effects of dry eye on the conjunctival epithelium.

Figure 2. Mouse corneas viewed and photographed under cobalt blue light 10 minutes after application of 1 \( \mu \)l 1% sodium fluorescein to the ocular surface. Top left: normal control mouse. Top right: mouse 4 days after application of scop patch. Bottom left: mouse 4 days after application of scop patch and placement in a blower hood 1 hour per day. Bottom right: mouse treated with scop patch + blower. This mouse blinked infrequently and developed a central corneal epithelial defect.

Figure 3. Histologic sections of paraffin-embedded mouse conjunctiva stained with periodic acid Schiff (PAS) reagent. (A) Superior bulbar conjunctiva of normal mouse. Goblet cells are stained pink and are located in the superficial epithelium. (B) Inferior bulbar conjunctiva of normal mouse. (C) Superior bulbar conjunctiva of mouse after 4 days of treatment with scop patch + blower. Epithelium was thinned with loss of goblet cells. (D) Inferior bulbar conjunctiva of mouse after 4 days of treatment with scop patch + blower. Epithelium had an increased number of cell layers and showed loss of goblet cells.
Conjunctival Epithelial Morphology, Goblet Cell Density, and Proliferation

The conjunctiva had a cuboidal basal epithelial layer with three to four overlying squamous layers with interspersed goblet cells in control untreated mice (Figs. 3A, 3B). The conjunctival epithelial morphology in dry-eye mice showed two patterns: a thinner-than-normal layer with loss of the cuboidal basal layer or a hyperplastic appearance with six or more epithelial cell layers (Figs. 3C, 3D). Goblet cell density was significantly reduced in the scop patch + blower-treated mice compared with normal mice (Table 2, compare Fig. 3A, 3B with Fig. 3C, 3D). Mice treated with topical scopolamine showed normal epithelial cell morphology and conjunctival goblet cell density. The number of proliferating conjunctival epithelial cells labeled with BrdU (Fig. 4) was significantly greater in the scop patch + blower–treated eyes (41.00 ± 17.69 cells per 20 microscope field) than in untreated control mice (11.00 ± 2.71 cells per 20 microscope field, *P* = 0.02).

Table 2. Average Conjunctival Goblet Cell Density

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<th>Superior</th>
<th>Inferior</th>
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<tr>
<td>Control (n = 4)</td>
<td>10.5 ± 4.7</td>
<td>20.5 ± 9.2</td>
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<tr>
<td>Scop patch + blower (n = 4)</td>
<td>0.75 ± 0.93</td>
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The mean ± SD number of goblet cells in three separate ×20 microscopic fields in three mice in each group in the superior and inferior bulbar conjunctiva. The *t*-test was used for statistical comparisons.

**DISCUSSION**

In this study, experimentally induced dry eye resulted in ocular surface epithelial changes, with features that mimic human KCS, such as corneal fluorescein staining, altered corneal epithelial barrier function, reduced conjunctival goblet cell density, and increased conjunctival epithelial proliferation. Pharmacologic inhibition of tear secretion with the anti-cholinergic agent scopolamine significantly decreased tear production and clearance compared with untreated mice and caused a significant increase in corneal epithelial permeability to CF within 4 days of treatment. Exposing the scopolamine-treated mice to desiccating environmental stress significantly worsened the severity of the ocular surface disease. This finding suggests that normal production and clearance of tears is required for maintaining a healthy, well-lubricated ocular surface and for protecting it from environmental insults.

Regulated tear secretion by the lacrimal glands is controlled by the autonomic nervous system. The rat lacrimal gland is densely innervated with parasympathetic nerves that contain acetylcholine and vasoactive intestinal peptide (VIP). These nerves were observed to surround the basolateral membranes of most lacrimal gland secretory acini.

Immunocytochemical examination of lacrimal gland neural innervation in mice shows that most of their nerve fibers are also parasympathetic, containing both acetylcholine and VIP. It has been demonstrated that sympathetic nerves containing norepinephrine are also present in the lacrimal gland, innervating some acini and blood vessels, although the density of sympathetic nerves is less than in the parasympathetics.

Systemic administration of anti-cholinergic drugs, such as atropine, is well recognized to decrease tear secretion. Decreased tear production and the development of dry spots on the corneal surface, after 2 days of topical atropine sulfate administration in rabbits, were reported by Burgafassi et al. Indeed, in our preliminary studies, we observed that topical administration of atropine sulfate also decreased aqueous tear production in mice. However, this effect was short-lived, lasting for only a few hours before returning to normal.

The short effective duration of topical atropine sulfate prompted us to evaluate the effectiveness of a transdermal delivery system that would control absorption and the rate of drug delivery into the bloodstream over an extended period. A transdermal patch delivery system is available for the anticholinergic medication scopolamine. Scopolamine transdermal patches have become a frequently used and effective means of delivering this medication for treatment of motion sickness and for nausea and vomiting related to chemotherapy.

Similar to atropine, scopolamine is a competitive inhibitor of acetylcholine for muscarinic cholinergic receptors. Quantitatively, scopolamine is more potent than atropine in its action on the iris, ciliary body, and certain secretory (salivary, bronchial, and sweat) glands but is less potent in its actions on the heart, and intestinal and bronchial smooth muscle. Transdermal scop patches were noted to significantly decrease tear production in the CBA mice that were used in our study. Maximum decrease in aqueous tear production was noted by 12 hours, and it persisted for more than 24 hours. By 48 hours,

**FIGURE 4.** BrdU labeling of proliferating conjunctival epithelial cells in a normal untreated mouse (top) and a mouse treated with scop patch + blower for 4 days (bottom). Dotted line: junction of the conjunctival epithelium and substantia propria; arrows: BrdU-labeled cells.
the cotton thread test results tended to return to normal. Based on these results, scop patches were reapplied after 2 days.

The air draft in the blower hood created an environmental stress that increased evaporation and further desiccated the ocular surface. The cotton thread test, tear fluorescein clearance, and corneal CF uptake data in mice placed in the blower hood alone were similar to those in untreated mice. It is possible that the air draft stimulated reflex tearing in these mice that compensated for the increased evaporative tear loss. This hypothesis is supported by the finding that the worst ocular surface disease was observed in the mice that were placed in the blower hood and treated with a scop patch that blocked their ability to produce reflex tears. The aqueous tear production in the scop patch + blower group was not different from that in the group that received the patch alone. However, their tear clearance was significantly decreased, probably because fluorescein tear clearance is a better measure of dynamic movement of tear fluid across the ocular surface, indirectly assessing tear production, tear drainage, tear volume, and evaporative loss. It is commonly noted that among human patients with dry eye who have the same level of aqueous tear production, some have more severe ocular surface disease, whereas others have minimal or none. We have recently reported that tear clearance shows a stronger correlation with the severity of corneal fluorescein staining in human patients with dry eye than does the level of aqueous tear production measured with the Schirmer test. Certainly, our findings in this mouse model of dry eye support the concept that poor tear clearance leads to ocular surface disease.

A striking finding in the dry-eye mice was a marked decrease in the number of conjunctival goblet cells compared with those in the untreated control mice. Cytologic studies have indicated that decreased goblet cell density is a consistent finding accompanying aqueous tear deficiency in humans and the rabbit. Confluent goblet cell clusters are a main source of the ocular surface mucoproteins that lubricate and protect the ocular surface. It is likely that the poorly lubricated ocular surface resulting from reduced mucin production by the goblet cells and nongoblet ocular surface epithelia in KCS, makes the ocular surface more susceptible to environmental insults. The reason for the disappearance of PAS-stained goblet cells in our mice was not investigated. It is possible that these cells are still present but are not visualized, because their mucin production is inhibited by conditions on the ocular surface. Alternatively, the dry-eye environment may promote conjunctival epithelial proliferation rather than goblet cell differentiation. This is supported by our finding of a significantly greater number of conjunctival epithelial cells in the S phase of the cell cycle in the mice in which KCS developed. This mouse model provides an excellent tool to study these alternative hypotheses.

Taken together, our findings indicate that there are many similarities between this mouse model of dry eye and human aqueous tear deficiency. This model may be a useful tool for investigating the pathophysiological mechanisms for KCS.

References


