



Aquaporin-1 expression is decreased in human and mouse corneal endothelial dysfunction

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Purpose: To determine if aquaporin-1 expression is decreased in human and mouse corneal endothelial dysfunction.

Methods: Immunohistochemistry with anti-aquaporin 1 antibody and confocal microscopy were used to study a case series of human corneal specimens, and a mouse model of corneal endothelial injury was created with injection of sterile hot water into the anterior chamber with a 33 gauge needle. Western blotting of mouse and human corneas was used to provide confirmatory evidence.

Results: Aquaporin-1 was found to be expressed in normal human cornea and decreased in human corneas with endothelial disease but not in human corneas with non-endothelial corneal disease. Aquaporin-1 was also found to be reduced in mouse corneas subjected to corneal endothelial injury. Both results were confirmed by western blot analysis.

Conclusions: Corneal endothelial injury is associated with decreased aquaporin-1 expression.

The cornea is the anterior tissue of the eye, and its transparency is essential to optical function. The corneal stroma is mildly hyperosmolar relative to the aqueous fluid of the anterior chamber, which causes fluid to enter the cornea. As corneal transparency requires, by weight, approximately 78% water content, water entering the cornea must be extruded to maintain its natural hyperosmotic state [1]. The corneal endothelium contains transporters [sodium-potassium ATPase (Na⁺/K⁺ ATPase), sodium potassium chloride (Na/KCl), and carbonic chloride (HCO₃Cl)] that pump solutes (mostly sodium and bicarbonate) out of the cornea into the aqueous fluid that in turn establishes an osmotic gradient for the flow of water through aquaporins, which serve to maintain corneal transparency in normal states. Many processes (i.e., trauma, certain diseases, iatrogenic, etc.) cause damage to the corneal endothelium and result in corneal edema. This has been hypothesized to be due to disruption of aquaporins found within the corneal endothelium [2].

Aquaporins are a class of membrane proteins which function as semi-selective pores allowing water to move in response to osmotic and hydrostatic differences and may play important roles in several ocular tissues [3]. Aquaporins are found in animals, plants, and microorganisms. Aquaporins 1 through 5 are homologs found in mammalian tissue. Rat ocular tissue contains aquaporins in the ciliary body, cornea, lens, retina, iris, and choroid with aquaporin-1 comprising the highest proportion [4]. Rat models have demonstrated that the cornea contains aquaporin-1 at 2.5 times aquaporin-5 and 3 times aquaporin-3. In the cornea, aquaporin-1 is localized to the endothelium [5].

Aquaporin-1 has been shown to be a mode of osmotic water transport from the mouse cornea [2]. In aquaporin-1 knockout mice, the rate of corneal transparency recovery after endothelial exposure to hypotonic saline was slowed relative to wild-type mice. Consequently, it has been hypothesized that up-regulation, or an increase in the function, of aquaporin-1 may reduce the swelling and opacification seen after corneal injury. However, research is lacking on the function of aquaporin-1.

Fuchs' dystrophy and bullous keratopathy (BK) are two common disease processes that involve corneal endothelial damage and subsequent progressive corneal edema and are two leading causes for corneal transplantation in the United States [6,7]. These could potentially be treated by regulation of aquaporins.

We investigated whether aquaporin-1 was reduced in patients with Fuchs' dystrophy and BK by comparing corneal button specimens from patients undergoing corneal transplantation with corneal specimens from patients with other conditions. We also performed experiments to see whether endothelial trauma in a mouse injury could be elicited using sterile hot water to mimic the human condition of BK.

METHODS

All animal experiments were approved by the Medical College of Georgia Human Assurance Committee and Institutional Animal Care and Use Committee and conformed to the Helsinki Declaration on human research and the Association for Research in Vision and Ophthalmology guidelines for animal use.

A case series of 18 consecutive patients undergoing corneal transplantation was performed. Of these, 4 had Fuchs' dystrophy, 5 had BK, and 1 had corneal graft failure due to

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endothelial decompensation. Of the remaining specimens, 2 had keratoconus and 6 had corneal scarring from trauma or infection (not affecting the endothelium). A total of 3 normal central corneal specimens from donor tissue harvested for limbal stem cell transplantation were also analyzed in conjunction with 6 limbal corneal specimens from donor tissue used for corneal transplantation.

Murine corneal endothelial injury: Wild-type C57Bl/6 mice (Harlan, Indianapolis, IN) were used. A 30 gauge needle (Becton Dickinson, Franklin Lakes, NJ) was used to nick the

cornea's anterior stroma at the limbus and then create a small pocket in the stroma. A 2.0 μ l syringe with a 33 gauge needle (Hamilton Company, Reno, NV) was used to tunnel through the corneal pocket and into the anterior chamber to inject sterile water heated to 80 °C. The triplanar wound was self-sealing. Mouse corneas were harvested at 1 and 7 days post injury or at comparable ages for normal controls.

Immunohistochemistry: For each specimen, 4 μ sections were cut from paraffin blocks and mounted on treated slides (Superfrost plus, VWR Scientific Products, Suwanee, GA).

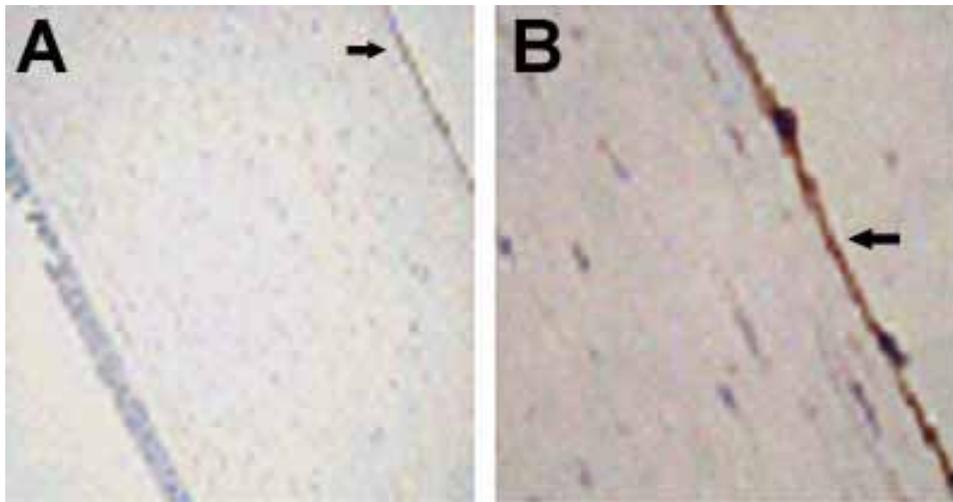


Figure 1. Aquaporin-1 expression in normal human cornea. Representative photographs of aquaporin-1 expression in normal human corneal specimens. The magnification of **A** was 20x and the magnification of **B** was 100x. Arrows identify the endothelium.

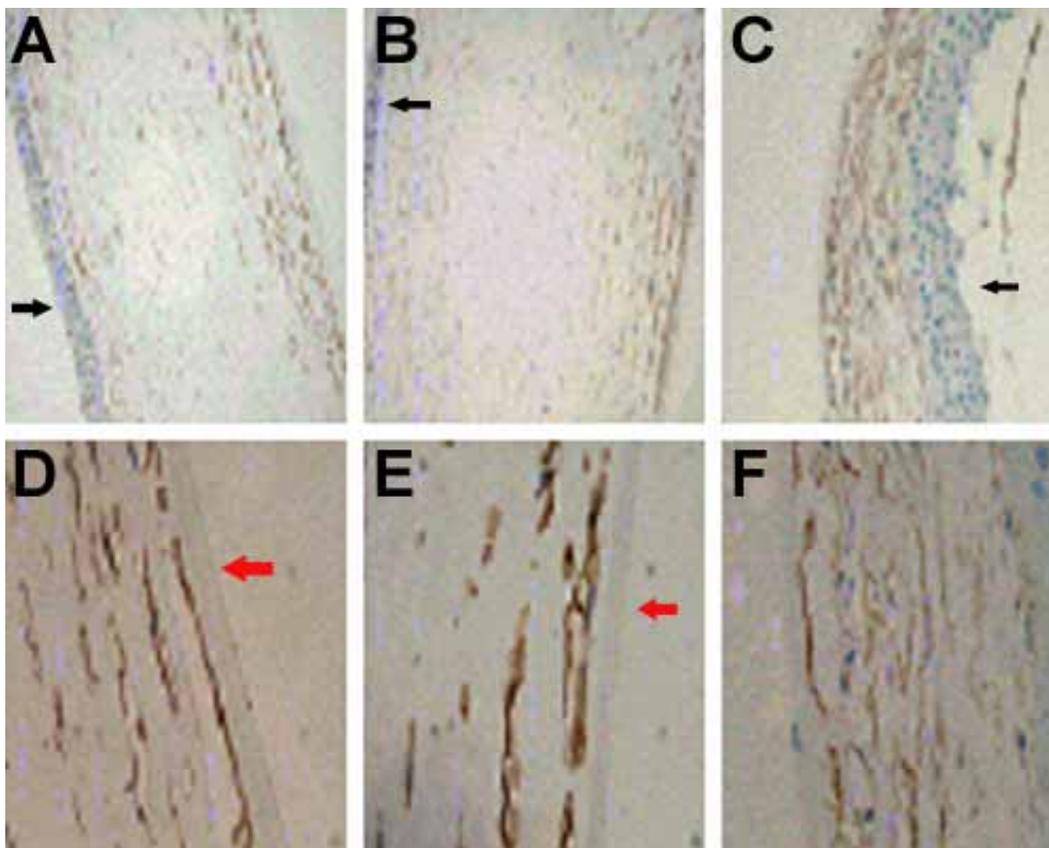


Figure 2. Aquaporin-1 expression in diseased or damaged human corneas. Photographs of aquaporin-1 expression in human corneas with Fuchs' dystrophy (**A,D**), BK (**B,E**), and graft failure (**C,F**). The magnification of **A**, **B**, and **C** was 20x and the magnification of **D**, **E**, and **F** was 100x. In **A**, **B**, and **C** the arrows identify the epithelium.

Slides were air dried overnight and placed in a 60 °C oven for 30 min. Slides were then deparaffinized in two changes of xylene for 7 min and ran through 2 changes of absolute ethanol for 2 min each, 2 changes of 95% ethanol for 2 min, 80% ethanol for 2 min, 70% ethanol for two min, and finally distilled water. Slides were pretreated for primary antibody with Target Retrieval Solution (pH 6.0, Dako Corp, Carpinteria, CA.) using a steamer, then rinsed in distilled water. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide (H₂O₂) in distilled water for 5 min followed by distilled water for 2 min. Slides were incubated in Power Block (Biogenex Laboratories Inc., San Ramon, CA), rinsed in distilled water, and placed in 1X phosphate-buffered saline (PBS) for 5 min. Slides were then incubated with primary antibody [Rabbit Antiwater Channel (AQP-1; Aquaporin 1, BD Pharmingen, San Jose, CA)] at a dilution of 1:1000 for 2 h at room temperature followed by two changes of 1X PBS. Slides were then incubated with secondary biotin-labeled, affinity-isolated goat anti-rabbit and goat anti-mouse immunoglobulins (LSAB2-HRP kit, Dako) for 10 min and rinsed in two changes of 1X PBS. Slides were then incubated in Streptavidin-

HRP (LSAB2, Dako) for 10 min and rinsed in two changes of 1X PBS. Bound antibody was detected with DAB substrate kit (DAB substrate kit for peroxidase-HRP, Dako) followed by counterstaining with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI).

Corneal microscopy: Images of human and mouse corneal slices stained with Aquaporin 1 antibody were captured using a Spot 1.3 Cooled Color Digital camera (Spot Diagnostic Instruments, Inc., Sterling Heights, MI) attached to Olympus AX 70 True Research System microscope (Olympus America, Inc., Melville, NY) controlled by Spot 3.4 software. General morphology images were captured with 20x magnification; endothelial and stromal images were captured at 40x and 100x oil immersion magnification. All images required 9.0-voltage bright field. The images were fed into a Sony Trinitron MultiScan 500PS computer (Dell Computer Corporation, Round Rock, TX) and saved as joint photographic experts group (.jpeg) files.

Western blot analysis: Human corneal tissue, cut in half, was placed in 150 µl RIPA buffer (Tris-HCl, NaCl, NP-40, Na-deoxycholate, and protease inhibitors) and ground using a

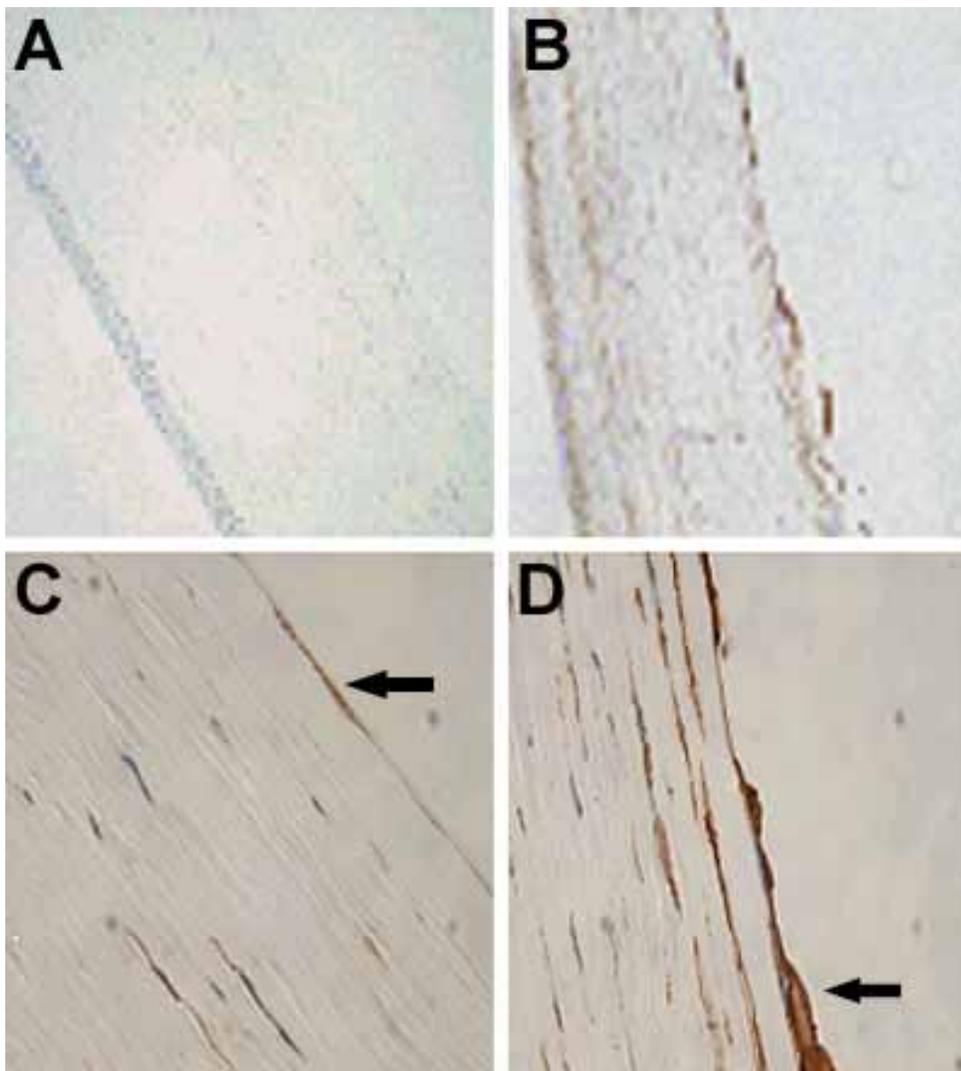


Figure 3. Aquaporin-1 expression in human corneas with keratoconus and corneal scarring. Photographs of aquaporin-1 expression in human corneas with keratoconus (A,C) and corneal scarring (B,D). The magnification of A and B was 20x and the magnification of D and E was 100x. The arrows in C and D identify the endothelium.

Kontes tissue grinder (Fisher Scientific, Pittsburgh, PA). Mouse corneal tissues were pooled together, pulverized using Bessman tissue pulverizer (Fisher Scientific), and transferred into 60 µl RIPA buffer. Immediately afterwards, tissue samples were sonicated on ice 4 times for 15 s intervals at level 7 intensity.

Following centrifugation, samples were loaded onto a 10% SDS-PAGE, transferred, and probed for AQP-1 protein. Membranes were blocked for 1 h at room temperature with 5% milk in PBST, followed by overnight incubation at 4 °C in a concentration of 1:1000 AQP-1 primary antibody (BD Bio-

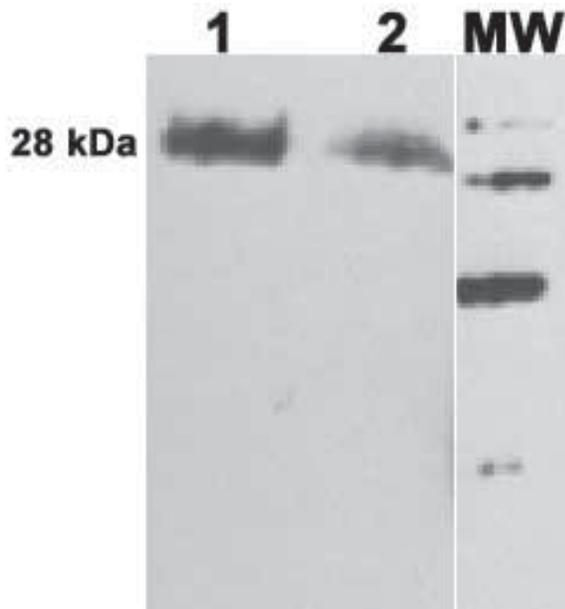


Figure 4. Western analysis of human cornea. Western blot analysis (anti-AQP-1; 1/1000) of human tissue from normal corneal limbal ring (lane 1) and bullous keratopathy (lane 2). The lane labeled MW contains a standard molecular weight standard.

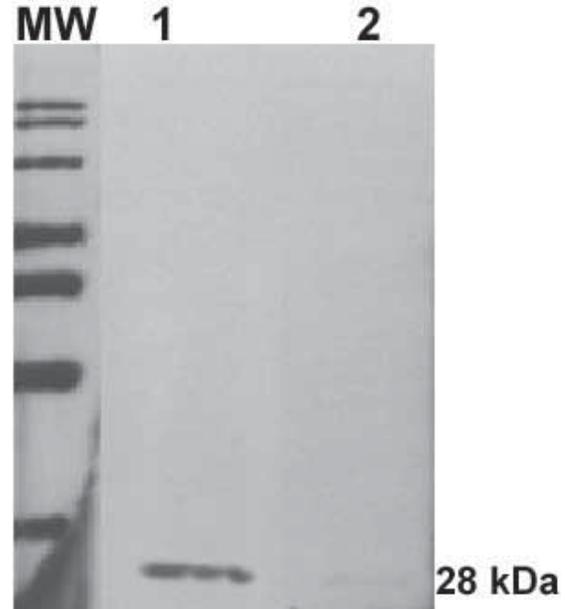


Figure 6. Western analysis of mouse cornea. Western blot analysis (anti-AQP-1; 1/1000) of mouse corneas from normal (lane 1) and from injured (lane 2) tissue. The lane labeled MW contains a standard molecular weight standard.

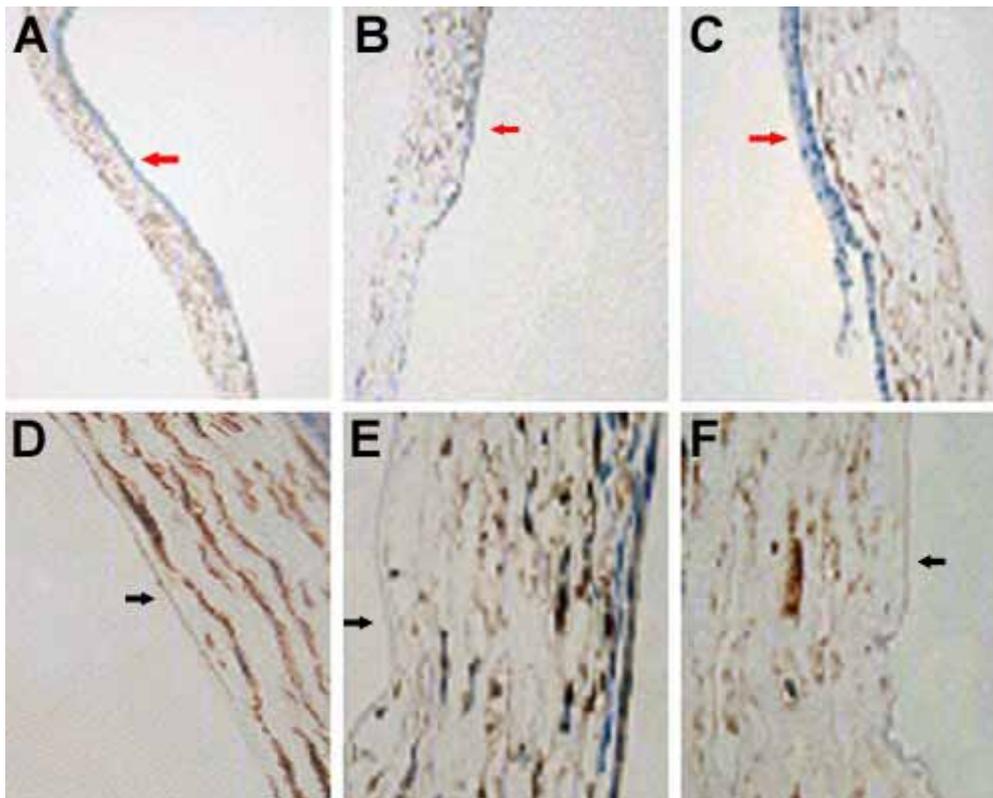


Figure 5. Aquaporin-1 expression in normal versus injured mouse corneas. Representative photographs of aquaporin-1 expression in uninjured mouse corneas (A,D), 1 day after hot sterile water injection (B,E), and 7 days after hot sterile water injection (C,E). The magnification of A, B, and C was 20x and the magnification of D, E, and F was 100x. In A, B, and C the red arrows identify the epithelium. In D, E, and F the black arrows identify the endothelium.

science/Pharmingen, San Diego, CA). The appropriate secondary antibody concentration of 1:5000 (BD Bioscience/Pharmingen) was used to incubate the membrane for 2 h at room temperature, upon which the membrane was washed in PBST and developed onto film using Pierce Chemicals ECL kit (Fisher Scientific).

RESULTS

Aquaporin-1 is expressed in normal human cornea: Figure 1 shows representative corneal specimens from normal central (Figure 1A) and limbal (Figure 1B) corneal specimens. Aquaporin-1 expression is demonstrated in the corneal endothelium (Figure 1A,B, arrows) and stroma.

Aquaporin-1 is diminished in human corneas with endothelial disease: Figure 2 displays corneal specimens from patients with endothelial disease (Fuchs' dystrophy, Figure 2A,D and BK, Figure 2B,E) or graft endothelial failure (Figure 2C,F). Endothelial cells, as expected, were dramatically reduced in those with endothelial disease. Aquaporin-1 appeared to be absent in the endothelium of these specimens (Figure 2D,E, red arrows) but was upregulated in the posterior corneal stroma (brown stain) relative to normal specimens and non-endothelial corneal disease specimens. In the low power images (Figure 2A-C) the epithelium is identified by the black arrows.

Aquaporin-1 is unchanged in human corneas with non-endothelial disease: Figure 3 shows corneal specimens from patients with non-endothelial corneal disease (keratoconus, Figure 3A,C) and corneal scarring (Figure 3B,D). Aquaporin-1 expression was present and seemed unchanged in the endothelium of these specimens (Figure 3C,D, arrows).

Figure 4 shows a western blot on representative human tissue from normal central cornea (lane 1) and from a specimen with bullous keratopathy (lane 2). Aquaporin-1 expression was more intense in the normal specimen.

Aquaporin-1 is diminished in an animal model of corneal endothelial injury: Figure 5 shows specimens from both normal (Figure 5A,D) and injured mouse corneas (Figure 5B-F). The corneas of mice subjected to hot water injury showed endothelial attenuation and decreased aquaporin expression (compare Figure 5D, arrow with Figure 5E,F, arrow). In the low power images (Figure 5A-C) the epithelium is identified by the red arrows.

Figure 6 shows western blot analysis on pooled normal and injured mouse corneas (n=8 in each group). Aquaporin expression was significantly more intense in normal than injured corneas.

DISCUSSION

One previous study demonstrated the presence of aquaporin-1 in human corneal endothelium [5]. In addition to confirming this finding, we have found that in patients with corneal endothelial disease, aquaporin-1 expression is reduced in the endothelium and upregulated in the posterior stroma of the cornea. The latter may be a response to the diminution of aquaporin in the endothelium, as Fuchs' dystrophy and bullous keratopathy are not known to affect the stroma primarily.

We have also demonstrated that aquaporin-1 expression is reduced in mice subjected to corneal endothelial injury with sterile hot water. To the best of our knowledge, this is the first clinically significant mouse model for BK, as it can be caused by hypotonic infusions or thermal damage [8,9]. Whether diminished aquaporin-1 expression is a cause or effect of endothelial disease is yet to be resolved. However, it is speculated that upregulation of aquaporin-1 expression may reduce corneal edema. Future treatments relying on this mechanism may constitute a noninvasive alternative to the treatment of corneal edema. The integration of aquaporin-5 into the membranes has been shown to be positively influenced by alpha-1 adrenoceptor agonists and muscarinic acetylcholine receptor agonists [10]. It has also been shown that vasopressin activates aquaporin-2 in the kidney and aquaporin-4 in the brain [11]. However, very little research exists for the regulation of aquaporins in the eye, especially for aquaporin-1 and aquaporin-5, which play an integral part in water balance of the cornea. Future investigation is needed to determine the regulators of aquaporins and to develop corresponding pharmacologic agents. It is thought that upregulation of aquaporin-1 function in the corneal endothelium may facilitate dehydration of edematous corneas, which may have a significant effect on the management of many pathological diseases affecting the eye.

ACKNOWLEDGEMENTS

This work was supported by the Knights Templar Foundation (BKA), a Fight-for-Sight Grant-in-Aid (BKA), a Foundation Fighting Blindness Career Development Award (JA), and University of Kentucky Physician-Scientist Award (JA).

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