Aquaporin Deletion in Mice Reduces Corneal Water Permeability and Delays Restoration of Transparency after Swelling*

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Two aquaporin (AQP)-type water channels are expressed in mammalian cornea, AQP1 in endothelial cells and AQP5 in epithelial cells. To test whether these aquaporins are involved in corneal fluid transport and transparency, we compared corneal thickness, water permeability, and response to experimental swelling in wild type mice and transgenic null mice lacking AQP1 and AQP5. Corneal thickness in fixed sections was remarkably reduced in AQP1 null mice and increased in AQP5 null mice. By z-scanning confocal microscopy, corneal thickness in vivo was (in μm, mean ± S.E., n = 5 mice) 123 ± 1 (wild type), 101 ± 2 (AQP1 null), and 144 ± 2 (AQP5 null). After exposure of the external corneal surface to hypotonic saline (100 mosm), the rate of corneal swelling (3.0 ± 0.3 μm/min, wild type) was reduced by AQP5 deletion (2.7 ± 0.1 μm/min). After exposure of the endothelial surface to hypotonic saline by anterior chamber perfusion, the rate of corneal swelling (7.1 ± 1.0 μm/min, wild type) was reduced by AQP1 deletion (1.6 ± 0.4 μm/min). Base-line corneal transparency was not impaired by AQP1 or AQP5 deletion. However, the recovery of corneal transparency and thickness after hypotonic swelling (10-min exposure of corneal surface to hypotonic saline) was remarkably delayed in AQP1 null mice with ~75% recovery at 7 min in wild type mice compared with 5% recovery in AQP1 null mice. Our data indicate that AQP1 and AQP5 provide the principal routes for corneal water transport across the endothelial and epithelial barriers, respectively. The impaired recovery of corneal transparency in AQP1 null mice provides evidence for the involvement of AQP1 in active extrusion of fluid from the corneal stroma across the corneal endothelium. The up-regulation of AQP1 expression and/or function in corneal endothelium may reduce corneal swelling and opacification following injury.

The cornea consists of a stromal layer that is covered at its external surface by an epithelium in contact with tear fluid and at its inner surface by an endothelium in contact with aqueous fluid in the anterior chamber. Corneal transparency requires precise regulation of water content at ~78 weight %. Changes in corneal water content alter the regular diameter and spacing of collagen fibrils that is believed to be critical for transparency (1). The corneal epithelium carries out active transport of chloride from stroma to tears. Although the transport of sodium from tears to stroma has been demonstrated in experimental models, the electrochemical driving forces in vivo probably favor net NaCl and fluid movement from stroma to tears (2). The corneal endothelium contains transporters (Na+K+ ATPase, Na+K+/2Cl–, HCO3-/Cl–) that pump solutes, primarily sodium and bicarbonate, from the stroma into the aqueous fluid (1, 3–6). Water then moves passively across the endothelium in response to the small osmotic gradients created by salt pumping. Active solute transport across the corneal endothelium is probably critical for the maintenance of corneal transparency to offset the tendency of the stroma to absorb water. The stroma is mildly hyperosmolar relative to aqueous fluid because of the high concentration of negatively charged glycosaminoglycans and the consequent accumulation of monovalent cations, which produces an effective stromal swelling pressure or “imbibition pressure” of ~50 mm Hg (7–10).

Immunolocalization using specific antibodies has shown the expression of aquaporin (AQP) by water channels AQP1 in corneal endothelium and AQP5 in corneal epithelium in human and rat eye (11–13). These aquaporins function as water-selective plasma membrane transporters that facilitate the bidirectional movement of water in response to osmotic gradients or hydrostatic pressure differences. AQP1 and AQP5 are also expressed in many extraocular tissues involved in fluid transport. Mice lacking AQP1 manifest defective urinary-concentrating (14–16), dietary fat-processing (16), lung water permeability (17) and pain perception (18). Mice lacking AQP5 manifest impaired fluid secretion by salivary (19) and airway submucosal (20) glands and have reduced alveolar water permeability in lung (21). It has been postulated without direct evidence that AQP1 and AQP5 are involved in water transport between the corneal stroma and between the tear and aqueous fluids and thus in the maintenance of corneal transparency (13, 22, 23). Studies of water permeability in corneal endothelial cell cultures (23) provide indirect evidence for a role of AQP1 in water transport across the corneal endothelium.

The purpose of this study was to test the hypothesis that aquaporins are involved in corneal fluid transport and transparency. Comparative morphological and in vivo confocal microscopy measurements were made on wild type mice and mice lacking AQP1 or AQP5. Methodology was developed to measure water permeability across the corneal epithelium and endothelium in vivo, and a non-injury experimental model of corneal swelling was used to study the role of aquaporins in the regulation of corneal water content. Our measurements provide the first functional evidence implicating a role of AQP1 and AQP5 as a means of reducing corneal swelling and opacification.

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The abbreviations used are: AQP, aquaporin; Pw, water permeability coefficient; ANOVA, analysis of variance.
in osmotically driven fluid movement in cornea as well as the involvement of AQP1 in the maintenance of corneal water content. The marked impairment of corneal recovery in AQP1 null mice has potentially important implications regarding the mechanisms and treatment of corneal edema.

MATERIALS AND METHODS

Transgenic Mice—Transgenic knock-out mice deficient in AQP1 and AQP5 in a CD1 genetic background were generated by targeted gene disruption as described originally (19, 24). Measurements were done in age-matched wild type and knock-out mice (age 8–10 weeks, weight 25–35 grams). Investigators were blinded to genotype information until the completion of the analysis. Protocols were approved by the University of California San Francisco Committee on Animal Research and are in compliance with the ARVO statement for the use of animals in ophthalmic and vision research.

In Vivo Confocal Microscopy—Mice were anesthetized using ketamine (40 mg/kg intraperitoneal) and xylazine (8 mg/kg). Body temperature was maintained at 37 °C with a heating pad. For microscopy, the head was immobilized using a stereotaxic apparatus (Kopf) with the eye under study facing upward. The center of the cornea was carefully positioned perpendicular to the optical axis of an upright fluorescence microscope (Leitz) equipped with a Nipkow wheel-type co-axial confocal module (Technical Instruments). Reflected white light was collected using a ×20 air objective (Nikon, working distance 20.5 mm, numerical aperture 0.35) and detected by a photomultiplier or cooled CCD camera. The axial resolution of the confocal optics as used for z-scanning measurements was ~3 μm. Axial scanning was carried out at a rate of 100 μm/s by driving the fine focus with a microstepper motor (Compumotor).

Measurement of Corneal Thickness in Vivo—Corneal thickness was determined from z-scans of scattered light intensity by image detection (as in Fig. 2B), and for rapid assessment by integrated signal detection using a photomultiplier (as in Fig. 3A). For image detection, brightfield confocal images were viewed every 5 μm in the z-direction. The external and inner corneal surfaces were identified by characteristic morphometric features (see “Results”). For photomultiplier detection, a 100-μm-diameter circular region of the cornea was illuminated, and total reflected light in the Brightfield confocal mode was recorded during rapid scanning through the corneal tissue. Intensity scans, I(x), were fitted by non-linear least squares fitting to a Lorentzian curve, \[ I(x) = \frac{a}{\Gamma(1/2) + (x - x_0)^2} + b, \] where \( \Gamma \) is the width at half-maximum height (related to corneal thickness \( t_e \) by \( t_e = 0.9\Gamma \)), \( x_0 \) is the position of the intensity maximum, \( a \) is related to intensity, and \( b \) is related to offset.

In Vivo Measurement of Epithelial Osmotic Water Permeability—Mice were anesthetized and prepared for scanning confocal microscopy. The corneal surface was rinsed in phosphate-buffered saline (300 mosm) and bathed by drip perfusion in hypotonic saline (100 mosm). In some experiments, corneas were fixed and harvested for morphology. Corneal swelling rate was determined from the time course of corneal thickness in serial z-scans.

In Vivo Measurement of Endothelial Osmotic Water Permeability—To establish an osmotic gradient across the corneal endothelial surface, glass micropipettes were inserted into the anterior chamber on opposite sides of the eye for fluid inflow and outflow (Fig. 4A, inset). Micropipettes (tip diameter = 40–50 μm) were pulled from borosilicate glass using a vertical pipette puller followed by breaking to produce a very sharp tip. Micropipettes were introduced into the mouse cornea using 4-axis micromanipulators at an angle of ~20° from the corneal surface at locations of ~0.1 mm from opposite edges of the cornea. The anterior chamber was perfused initially with saline and then with hypotonic saline (100 mosm) at a rate of 30 μl/min using a syringe pump. The external corneal surface was covered with mineral oil to prevent fluid transport across the epithelial surface. Corneal swelling rate was determined from the time course of corneal thickness in serial z-scans.

Recovery of Corneal Thickness after Osmotic Swelling—The hypotonic solution challenge was used as a non-injury model of corneal swelling. Corneas were hypotonically swollen by exposure of the external corneal surface to hypotonic saline (100 mosm) for 10 min. The hypotonic solution was removed and replaced with mineral oil. Scanning confocal microscopy was done at different times to assess the recovery of corneal thickness. In some experiments, the external corneal surface was bathed in saline containing ouabain (100 μM) for 15 min before hypotonic swelling.

Immunocytochemistry and Morphology—Mice were perfused through the aorta with 4% paraformaldehyde in phosphate-buffered saline and then perfused with freshly prepared 4% paraformaldehyde in phosphate-buffered saline for immunocytochemistry and 4% paraformaldehyde and Bouin’s buffer for light microscopy. After fixation, eyes were dehydrated and embedded in Tissue-Tek OCT compound for 3–4-μm cryostat sections and in glycol methacrylate for plastic sections. Immunocytochemistry was done using affinity-purified polyclonal anti-AQP1 and AQP5 antibodies. For light microscopy, eyes were infiltrated with JB-4 monomer (Polyscience) and sectioned on a microtome (Sorvall) and stained with toluidine blue.

RESULTS

Fig. 1A shows immunofluorescence localization of AQP1 protein in plasma membranes of mouse corneal endothelial cells (left upper panel) and AQP5 protein in the plasma membranes of corneal epithelial cells (left lower panel). Immunostaining of corneas from AQP1 and AQP5 null mice was negative using their respective antibodies (right panels). These results are in agreement with the expression patterns of AQP1 and AQP5 in the corneal tissue. Reverse transcriptase-PCR analysis using primers specific for aquaporins 1–9 revealed AQP1 and AQP5 transcript in the corneas of wild type mice, AQP5 transcript in AQP1 null mice, and AQP1 transcript in AQP5 null mice as expected (data not shown).

Corneal morphology was evaluated in tissue sections. Fig. 1B
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Fig. 2. Corneal thickness and transparency measured by z-scanning brightfield confocal microscopy. A, summary of corneal thickness measured from tissue sections for mice of indicated genotypes. Data for individual mice are shown as circles with the mean ± S.E. shown as squares. *, p < 0.05 compared with wild type (ANOVA). B, Brightfield confocal images of cornea of wild type mouse taken at indicated depths showing epithelial and endothelial surfaces. C, corneal thickness for left and right eyes (data for different mice are shown as circles and averaged data are shown as squares) measured by in vivo confocal microscopy. ***, p < 0.01 (ANOVA).

shows micrographs of corneal sections taken from wild type, AQP1 null and AQP5 null mice. Compared with wild type mice, corneal thickness was consistently lower in AQP1 null mice and greater in AQP5 null mice. The increased corneal thickness in AQP5 null mice appeared to involve both the epithelial and stromal layers. The decreased corneal thickness in AQP1 null mice was predominantly stromal.

Fig. 2A summarizes corneal thickness measured on micrographs of stained plastic sections as in Fig. 1B. Corneal thickness was significantly lower in AQP1 null mice and greater in AQP5 null mice than in wild type mice. Corneal thickness was also measured in vivo to avoid potential fixation artifacts. A scanning confocal microscopy approach was used based on the work of Jester and colleagues (25–27) in mice and other laboratories in larger mammals (28) and humans (29). After anesthesia and immobilization of the mouse head to position the center of the cornea perpendicular to the optical axis, brightfield (reflective) confocal images were viewed every 5 μm through the cornea using a computer-controlled z axis microstepper motor. Fig. 2B shows representative images at four z-positions showing the locations of the external (left) and inner (right) corneal surfaces. The distinctive ring patterns produced by the curved cornea and permit the determination of corneal thickness in living mice to better than 5-μm accuracy.

Fig. 3. Osmotic water transport across the corneal epithelium in AQP5 null mice. A, in vivo z-scans of scattered light intensity for corneas from mice of indicated genotype at 0 and 10 min after exposure of the corneal surface to hypotonic saline. Dotted lines show measured intensity with non-linear least squares Lorentzian fits as solid curves. Left, stained plastic sections of corneas from wild type mice showing swelling at 10 min; right, z-scans of cornea from AQP1 null mouse shown for comparison. B, left, time course of corneal thickness of individual eyes in response to exposure of the external surface to hypotonic saline. Thickness was measured in vivo by z-scanning confocal microscopy. Right, summary of swelling rates for individual eyes (circles) with the mean ± S.E. (squares). **, p < 0.01 (Student’s t test).

Fig. 2C summarizes corneal thickness measured on the left and right eyes from a series of mice. The averaged corneal thickness was 123 ± 1 μm (mean ± S.E.) in wild type mice, significantly thinner in AQP1 null mice (101 ± 2 μm, p < 0.01), and thicker in AQP5 null mice (144 ± 2 μm, p < 0.01).

The roles of AQP5 and AQP1 in corneal epithelial and endothelial water permeability, respectively, were determined from the kinetics of corneal swelling in response to exposure of the corneal surfaces to hypotonic saline (100 mosM). To study AQP5 water permeability in corneal epithelia, corneal thickness was measured by z-scanning confocal microscopy at different times during continuous topical application of hypotonic saline. The corneal sections in Fig. 3A, left, summarize the kinetics of swelling for a series of corneas. Initial swelling rates (in μm/min) were reduced ~2-fold in corneas of AQP5 null mice compared with wild type mice (p < 0.01) (Fig. 3B, right). Osmotic water permeability coefficients (P) computed for a smooth corneal surface were (2.3 ± 0.1) × 10⁻³ cm/s in wild type mice and (1.2 ± 0.03) × 10⁻³ cm/s in AQP5 null mice. Therefore, AQP5 provides an important route for water movement across the corneal epithelial surface.

Similar experiments were done to measure the role of AQP1 in osmotically induced water permeability in corneal endothelium, with the exception that the inward-facing surface of the cornea was exposed to hypotonic saline. These studies required perfusion of fluid into the anterior chamber using glass mi-
cropipettes (Fig. 4A, inset). To ensure that the measurements of corneal endothelial water permeability were not affected by water movement across the corneal epithelium, the external corneal surface was covered with mineral oil throughout the measurements. Control studies showed that the mineral oil did not interfere with the confocal microscopy measurements. Exposure of the corneal endothelium to hypotonic saline in wild type mice caused rapid corneal swelling followed by spontaneous recovery (Fig. 4A). AQ1 deletion produced significant slowing of the initial swelling rate as well as the recovery rate. Fig. 4B summarizes initial swelling rates for a series of mice. P<sub>f</sub> computed for a smooth corneal endothelial surface were (3.3 ± 0.4) × 10⁻³ cm/s in wild type mice and (0.7 ± 0.2) × 10⁻³ cm/s in AQ1 null mice (p < 0.01). Thus, AQ1 provides the major route for osmotic water movement across the corneal endothelial surface.

To investigate the role of AQ1 in the maintenance of corneal water content, corneal swelling and recovery were measured in a non-injury model of corneal edema. The corneal epithelium was exposed to hypotonic saline for 10 min to produce corneal swelling (Fig. 5A, left). At 10 min, the corneal surface was covered with mineral oil to prevent trans-epithelial fluid movement, and the time course of corneal thickness was measured by z-scanning confocal microscopy. Gross corneal opacification was observed for >30 min in AQ1 null mice (Fig. 5A, right), whereas corneas remained grossly transparent in wild type mice after hypotonically induced swelling. Fig. 5B shows the kinetics of corneal swelling and recovery for wild type and AQ1 null mice as well as wild type mice in which the cornea was exposed to the Na⁺/K⁺ pump inhibitor ouabain. Ouabain exposure or AQ1 deletion produced a remarkable slowing of recovery (fluid extrusion) after swelling as well as greater maximal swelling. Fig. 5C shows that AQ1 deletion did not affect the initial rate of corneal swelling (top), which results from osmotic water transport across the AQ5-containing epithelial surface. However, AQ1 deletion caused significant slowing of recovery after swelling resulting from water movement from the stroma through the corneal endothelium (bottom, p < 0.01).

**DISCUSSION**

The purpose of this study was to investigate the role of aquaporin water channels in important aspects of corneal physiology that depend on water/fluid transport. Water channels AQ1 and AQ5 are expressed in corneal endothelial and epithelial cells, respectively. Because these proteins function as water pores that facilitate osmotically driven water transport, it has been postulated that these proteins play a role in fluid movement across corneal epithelial and endothelial cell barriers. In addition, aquaporins may have other non-water-transporting roles. AQ1 is an early response gene that may play a role in wound healing (30). AQ1 has been proposed to transport CO₂ and possibly other gases (31), although subsequent reports have refuted this idea (32, 33) including measurements of CO₂ transport in corneal endothelial cells in culture (34). We used transgenic knock-out mice deficient in AQ1 and AQ5 to investigate the role of these proteins in corneal functions that can be reasonably postulated to involve aquaporins including corneal thickness, epithelial and endothelial barrier water permeabilities, and corneal recovery after hypotonic swelling.

The involvement of aquaporins in water permeability across epithelial and endothelial barriers in cornea is probably a prerequisite for their involvement in corneal physiology. The measurement of apparent osmotic water permeability from the kinetics of corneal swelling in response to osmotic gradients indicated that AQ5 and AQ1 provide the principle routes for osmotically driven water transport across corneal epithelial and endothelial barriers, respectively. Epithelial water transport was reduced ~2-fold in AQ5 null mice, and endothelial water transport was reduced ~5-fold in AQ1 null mice. These results provide evidence against the functional expression of a different aquaporin in the null mice that compensates for the reduced water permeability. However, as concluded in several studies in aquaporin null mice (35), the cell-specific expression of a functional aquaporin does not ensure its physiological significance. For example, a 30-fold reduction in osmotic water permeability across the airspace-blood barrier in lung had no physiological consequences in active alveolar fluid absorption (21) or response to experimental injury (36). The relatively modest reduction in apparent water permeability of the corneal epithelium in AQ5 null mice might be because of its relatively low abundance compared with AQ1 in corneal endothelium. Additionally, unstriped layer effects in the multi-layered epithelium might limit maximum water flow and hence blunt the reduction in apparent P<sub>f</sub> in AQ5 null mice.

Morphological analysis and in vivo confocal microscopy showed remarkably increased corneal thickness in AQ5 null mice and reduced thickness in AQ1 null mice. Our results do not establish the mechanistic basis for the differences in corneal thickness, but we speculate that reduced water permeabilities of the corneal epithelium and endothelium produce adaptive changes in stromal properties. The corneal epithelium is chronically exposed to a mildly hyperosmolar layer of tear...
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Fig. 5. Role of AQP1 in corneal recovery after osmotic swelling. A, left, schematic showing procedures for recovery experiments; right, photographs of wild type (top) and AQP1 null (bottom) mice showing corneal transparency before and after corneal swelling. B, representative time courses of corneal thickness after exposure of the external surface to hypotonic saline in wild type (open circles) and AQP1 null (filled circles) mice and ouabain-treated wild type mouse (triangles). Thickness was measured in vivo by z-scanning confocal microscopy. C, top, initial swelling rates for wild type and AQP1 null mice (mean ± S.E., n = 4); bottom, recovery rates calculated from linear fits of decrease in thickness from the time of maximum swelling for wild type and AQP1 null mice (mean ± S.E., n = 4). **, p < 0.01 (Student’s t-test).

Fluid that is produced by balanced lacrimal gland secretion and evaporative water loss. We showed previously that AQP1 or AQP5 deletion in mice does not impair tear secretion (37). The corneal endothelium is exposed to aqueous fluid in the anterior chamber at a greater pressure (intraocular pressure) than that of the surface tear layer (atmospheric pressure). Aqueous fluid production involves near-isosmolar fluid secretion by the ciliary epithelium in which AQP1 and AQP4 are expressed. We showed previously that AQP1 deletion in mice produced a mild reduction in intraocular pressure (from 16.0 to 14.2 mm Hg) as a result of decreased aqueous fluid secretion (38). The forces acting on the cornea are established by the solute activities and osmolalities of the tear and aqueous fluids and the hydrostatic forces imposed by intraocular pressure. The responses of the cornea to these forces depend on active and passive solute-transporting properties of the epithelial and endothelial barriers as well as their osmotic water permeabilities.

If driving forces and permeability properties do not change in AQP5 null mice with the exception of reduced osmotic water permeability of the corneal epithelium, there would be a reduced rate of osmotically driven water efflux from the stroma to the tear layer. Chronically, the impaired exit of corneal water may produce an increase in corneal thickness. The reduction of water permeability of the corneal endothelium by AQP1 deletion would similarly reduce osmotic water transport from the aqueous fluid to the corneal stroma. The continued osmotic water transport from the stroma to the tear layer might produce a chronically dehydrated and thinned cornea in AQP1 null mice. Although there may be complex solute movements among the tear, stromal, and aqueous fluid compartments, the reasoning above applies specifically to osmotically driven transport of solute-free water driven by the higher osmolality of the tear fluid than the aqueous fluid. However, we cannot rule out alternative explanations for altered corneal thickness in aquaporin null mice, such as developmental changes or altered expression of solute transporters in epithelial or endothelial cells. Studies using specific non-toxic aquaporin inhibitors, when available, are needed.

Perhaps the most interesting observation in these experiments was the significant impairment in corneal recovery in AQP1 null mice following swelling produced by exposure of the corneal surface to hypotonic saline. The prompt recovery of corneal transparency and thickness after swelling in wild type mice supports previous findings that the endothelium is very important in active fluid transport out of the stroma (3, 5, 8, 39). Indeed, we found that exposure of the corneal endothelium to hypotonic saline resulted in prompt swelling followed by recovery even in the continued presence of a hypotonic perfusate. Recovery was blocked by the Na⁺/K⁺ pump inhibitor ouabain, confirming the involvement of active solute transport across the corneal endothelium. AQP1 deletion produced a remarkable delay in the reduction of corneal thickness after swelling, which was observed as a grossly opaque cornea in the first hour after swelling. The delay was seen when the corneal epithelial surface was covered with oil, implicating an abnormality of the corneal endothelium. The simplest explanation for the delayed recovery in AQP1 null mice is the impairment of osmotically driven water transport out of the stroma across the corneal endothelium in response to active salt extrusion out of the stroma. However, the adequacy of this explanation is uncertain because of the modest water permeability of the corneal endothelium even after AQP1 deletion. Improved understanding is needed of the mechanism by which solute and water flow are coupled in the corneal endothelium. Alternative explanations that cannot be excluded at this time include down-regulation of active solute transport across the corneal endothelium in AQP1 null mice or changes in stromal properties of the thinned cornea, such as increased imbibition pressure. Also, the possibility cannot be excluded that other as yet unidentified non-water-transporting functions of AQP1 are re-
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sponsible for the impaired recovery after corneal swelling.

In summary, the deletion of AQP5 in mice increases corneal thickness and reduces osmotic water permeability across the corneal epithelium. AQP1 deletion reduces corneal thickness and osmotic water permeability across the corneal endothelium and impairs the restoration of corneal transparency after experimental swelling. As discussed above, these phenomena might be explained by acute and chronic effects of reduced water permeability across the corneal epithelium for AQP5 deletion and the corneal endothelium for AQP1 deletion. The inhibition of AQP5 or AQP1 by non-toxic blockers may thus alter corneal structure and water content. The up-regulation of AQP1 in corneal endothelium may be particularly useful in reducing corneal edema and improving transparency after injury.

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