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Role of Aquaporin-1 in Trabecular Meshwork Cell Homeostasis during Mechanical Strain

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Abstract

Aquaporin-1 (AQP1) channels are expressed by trabecular meshwork (TM) and Schlemm's canal cells of the conventional outflow pathway where fluid movement is predominantly paracellular, suggesting a non-canonical role for AQP1. We hypothesized that AQP1 functions to protect TM cells during periods of mechanical strain. To test this idea, primary cultures of confluent human TM cells on Bioflex membranes were exposed to static and cyclic stretch for 8 and 24 hours using the Flexcell system. AQP1 expression in TM cells was assessed by SDS-PAGE and western blot using anti-AQP1 IgGs. AQP1 protein bands were analyzed using densitometry and normalized to β -actin expression. Cell damage was monitored by measuring lactate dehydrogenase (LDH) and histone deacetylase appearance in conditioned media. Recombinant expression of AQP1 in TM cell cultures was facilitated by transduction with adenovirus. Results show that AQP1 expression significantly increased by 2 fold with 10% static stretch and 3.5 fold with 20% static stretch at 8 h ($n=4$, $p<0.05$) and 24 h ($n=6$, $p<0.05$). While histone deacetylase levels were unaffected by treatments, release of LDH from TM cells was the most profound at the 20% static stretch level ($n=4$ $p<0.05$). Significantly, cells were refractory to the 20% static stretch level when AQP1 expression was increased to near tissue levels. Analysis of LDH release with respect to AQP1 expression revealed an inverse linear relationship ($r^2 = 0.7780$). Taken together, AQP1 in human TM appears to serve a protective role by facilitating improved cell viability during conditions of mechanical strain.

Keywords

Glaucoma; Schlemm's Canal; Cell Viability; Cell Volume

Introduction

Glaucoma is the second leading cause of blindness and affects approximately 3 million people in the United States alone.(Quigley, 1996) Primary open angle glaucoma is the most common form and is often characterized by an increase in intraocular pressure (IOP). Despite visibly

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unobstructed conventional outflow tissues, an increase in resistance through the conventional outflow pathway is likely responsible for IOP elevation.(Grant, 1963)

The conventional outflow pathway consists of the trabecular meshwork (TM) and Schlemm's Canal (SC), and is part of a dynamic environment subject to multiple forms of environmental stress as well as mechanical strain. Sources of daily mechanical strain in the conventional outflow pathway include intraocular pressure, fluid flow, and contractile activity of surrounding tissues, such as the ciliary muscle. Intraocular pressure and fluid flow are influenced by intraocular processes such as aqueous humor production and drainage (Kaufman, 1984), as well as extraocular processes such as heart beat, eye movement, and blinking. (Coleman and Trokel, 1969) The TM is subject to added sources of strain as mechanical forces stretch it from Schwalbe's line to the Scleral spur, and inward towards the SC lumen.(Johnstone and Grant, 1973) As a result, the TM stretches not only in accordance with changing pressure gradients and fluid movement, but also in conjunction with ciliary muscle contraction. (Wiederholt, et al., 2000)

Previous data shows that the TM responds to mechanical strain through a variety of mechanisms. Studies using whole eyes, anterior segments, and isolated TM cultures have demonstrated a range of response mechanisms to mechanical strain. Experiments in eyes of rhesus monkeys and humans revealed reversible structural changes in TM tissues following increases in pressure.(Johnstone and Grant, 1973) Perfusion studies using anterior segments of human and porcine eyes reported an increase in outflow resistance (Brubaker, 1975) and a decrease in outflow facility following applied cyclic pressure, suggesting pressure oscillations can induce responses in tissue.(Ramos and Stamer, 2008) Investigators have also looked at alterations in cell morphology and actin reorganization following applied mechanical strain to cultured human TM cells.(Brubaker, 1975, Epstein and Rohen, 1991, Mitton, et al., 1997, Tumminia, et al., 1998) Moreover, changes in gene expression have been observed for multiple proteins including myocilin, interleukin factor-6, and matrix metalloproteinases, following applied mechanical strain in TM cultures.(Borras, et al., 2002, Bradley, et al., 2003, Bradley, et al., 2001, Liton, et al., 2005, Tamm, et al., 1999, Vittal, et al., 2005) Regulators of transport mechanisms, commonly associated with cell homeostasis are also influenced by mechanical strain in the TM and other tissues.(Gasull, et al., 2003, Yuan, et al., 2007) Interestingly, AQP4 has been shown to facilitate increased water flux between muscle tissue and the blood during increased physical activity, demonstrating a novel role for aquaporin channels during times of mechanical strain.(Frigeri, et al., 2004, Frigeri, et al., 2001)

Aquaporin water channels serve to increase water permeability of cells and facilitate transcellular water movement in the kidney, lung, and the eye; tissues whose proper functioning depends upon efficient water movement. In the conventional outflow pathway, AQP1 has been shown to be expressed in both TM tissue and isolated cultures, though the role of AQP1 in the TM is currently unknown.(Hamann, et al., 1998, Stamer, et al., 2008, Stamer, et al., 1995, Stamer, et al., 1994) Since fluid movement through the TM is primarily paracellular, the presence of AQP1 may fulfill a need other than transcellular water movement, particularly in the uveal meshwork where intertrabecular spaces are large. In fact, recent studies have demonstrated that AQP1 does not appear important for bulk fluid movement through the conventional outflow pathway.(Stamer, et al., 2008) It is currently unclear as to why robust expression of a water channel protein in the TM would be necessary if fluid movement does not require AQP1 expression. Based upon the unique biomechanical environment of the conventional pathway and recent reports of the role of aquaporins during periods of mechanical strain, we hypothesize that AQP1 functions to support TM cell homeostasis during times of mechanical strain.

To test our hypothesis we administered a static and cyclic mechanical stretch to cultured human TM cells and evaluated changes in AQP1 expression and TM cell viability. Our results demonstrate that mechanical strain results in a significant increase in AQP1 expression. To further evaluate the role of AQP1 during mechanical strain we used adenovirus encoding AQP1 to restore AQP1 expression in TM cell cultures to levels closer to those observed in TM tissue. We found that increased expression of AQP1 during static mechanical strain reduced measures of cell damage. Based on the current study, our data suggest that AQP1 in human trabecular meshwork serves a protective role by facilitating improved cell viability during conditions of mechanical strain.

Materials and Methods

Human Trabecular Meshwork Cells

Five human TM cell strains were isolated and characterized by our laboratory as previously described, (Stamer, et al., 1995) and used for experiments (table 1). Figure 1 shows representative expression of AQP1 seen in TM cultures over time and with passaging. Aquaporin-1 is a homotetramer that appears by western blot as two bands indicative of three non-glycosylated (28kD) subunits and one glycosylated (35kD) subunit. These data confirm that TM cells require multiple days at confluence for maximum expression of AQP1 and that cells cultured up to 14 days retain AQP1 expression and that TM cells used at passage 2 to 4 maintain levels of AQP1 expression. For experiments in the present study, cells were used at passage 2–4 after at least 14 days at confluence. For every experimental condition, we used at least 2 different TM cell lines in at least 3 independent experiments.

Stretch Assays

TM cells on Bioflex membrane supports in 6-well format (Product # BF-3001U Flexcell Corporation, Hillsborough, NC) were maintained in 10% fetal bovine serum (FBS, Gemini Bioproducts, Sacramento, CA) low glucose Dulbecco's modified Eagle Media (DMEM, Invitrogen, Carlsbad, CA) supplemented with penicillin (100 units/mL), streptomycin (100mg/mL) and glutamine (0.29mg/mL) for one week after plating at confluence. Cells were then switched to media containing 1% FBS low glucose media for 1 week prior to experiments. Cells were given 2 mL of fresh 0.1% FBS, sodium pyruvate-free media 24 hours prior to and then again during stretch applications. Media was collected before and after stretch to evaluate Lactate Dehydrogenase release. The 6-well plate was placed on a Flexcell stretch frame, housed in an incubator at 37°C and 5% CO₂. Using Flexcell software, mechanical strains capable of stretching the flexible membranes by 10 and 20% static stretch (stretch and hold), or 9 and 13% cyclic (oscillatory) stretch (1 Hz.), were applied by vacuum pump for periods of 8 and 24 hours. The limitations of the equipment allowed only for 9 and 13 % stretch during cyclic stretching at frequency of 1 Hz. Cell lysate samples were collected in 100µL Laemmli buffer at 0, 8, and 24 hours and then boiled for 10 minutes.

Western Blot

Proteins collected from TM cell lysates in Laemmli buffer (Laemmli, 1970) were loaded on 10 % SDS-PAGE gels run at 0.3 milliamps for 1 hour. The proteins in gel slabs were transferred to nitrocellulose at 100mV for 90 minutes. Following transfer of the proteins, the nitrocellulose was incubated in Tris/HCl-buffered saline (100mM Tris, 137mM NaCl, 2.7mM KCl, pH 7.4) with 2% Tween-20 (TBS-T) and 5% nonfat dry milk (blocking buffer) for 1 hour. The nitrocellulose blots were incubated for 16 hours at 4°C in blocking buffer containing anti-AQP1 IgGs (300 ng/mL). Antibodies targeted against the carboxy-terminal domain of AQP1 were affinity purified from rabbit serum using column chromatography as previously described. (Stamer, et al., 1995, Stamer, et al., 1996) Blots were rinsed 4 × 15min. in TBST, and goat anti-rabbit IgGs conjugated to horseradish peroxidase (Santa Cruz, CA) were incubated with

blots in blocking buffer at a dilution of 1:5000 for 1 hour. The solutions containing secondary antibodies were removed and blots were rinsed 4×15 min. with TBS-T. The blots were then incubated with HyGlo (Denville Scientific, Metuchen, NJ) or Amersham (Buckinghamshire, UK) chemiluminescence western blotting detection solutions (CAT # RPN2106 and RPN2135). Following incubation with chemiluminescence reagents, the protein antibody complexes were visualized by timed exposures to X-ray film (Genesee, CA). Following detection of AQP1, the blot was re-probed with solution containing β -actin IgGs (Sigma, St. Louis, MO CAT # A5441) raised in mouse and peroxidase-conjugated secondary antibody raised in goat against mouse Immunoglobulin G (Santa Cruz, CA). The non-glycosylated (28kD) band was used for analysis of changes in AQP1 expression in all experiments. Signals in the linear range of the film were digitized, and densitometry was performed using Labworks 4 software (UVP, Inc., CA).

Cell Viability Assays

Cell damage and/or death, as determined by release of lactate dehydrogenase (LDH) into conditioned media, was used as an indication of stress levels imposed on TM cells during mechanical stretch. Conditioned media collected from each well was used in analysis of LDH content prior to and following periods of mechanical stretch. Briefly, media were collected, centrifuged at $10,000 \times g$ for 1 hr at 4°C , and a $250\mu\text{L}$ sample was tested for LDH activity. Samples were analyzed using a Roche Cytotoxicity Detection Kit LDH cat. no. 11644793001, Indianapolis, IN). Media samples of $250\mu\text{L}$ were mixed with $250\mu\text{L}$ of the dye/catalyst solution. The dye/catalyst solution consists of catalyst (diaphorase and NAD^{+}) and dye (Iodotetrazolium chloride and sodium lactate) mixed in a 1:46 ratio and was incubated for 30 minutes in the dark. Reduction of NAD^{+} to NADH/H^{+} and subsequent reaction with catalyst allows for the conversion of a yellow tetrazolium salt to a red formazan salt. Each sample is loaded in triplicate into a 96 well plate at $150\mu\text{L}$ per well. Samples were analyzed using a 490nm filter of Emax precision microplate reader from Molecular Devices (Sunnyvale, CA). Cell death was also assessed by analyzing conditioned media for the presence of a nuclear enzyme, histone deacetylase (HDA). Conditioned media were subjected to centrifugation at $10,000g$ at 4°C for 1 hr. and pellets were solubilized in $100\mu\text{L}$ of Laemmli buffer. Histone deacetylase content was analyzed using SDS-PAGE and western blot with commercially available antibodies from Sigma (St. Louis, MO) and Santa Cruz (Santa Cruz, CA) as described above.

Heterologous AQP1 Expression

Human TM cell monolayers cultured to confluence for 2 weeks were exposed to 1mL of media containing adenovirus (Multiplicity of Infection (MOI) = 1) encoding AQP1 or GFP (green fluorescent protein) 48 hours prior to the start of the stretch for a period of 3 hrs with rocking every $\frac{1}{2}$ hour. (Stamer, et al., 2001) Viral media was removed and cells were given 2mL of fresh media containing 1% FBS overnight. After 16 hours, the media was removed and 0.1% FBS sodium pyruvate-free media was added for 24 hours. Media was collected as a “pre-stretch” sample for LDH analysis. Each 6-well plate had two wells that were treated with media alone (uninfected control), two wells infected with GFP adenovirus (virus control), and two wells infected with AQP1 adenovirus. MOI for each virus were obtained by screening virus transduction efficiency using immunofluorescence microscopy of transduced COS cells (lacking endogenous AQP1) as previously described. (Stamer, et al., 2001) After collection of initial pre-stretch media samples, cells were subjected to 20% static stretch for 24 hours, as previously described. Cell lysates and media samples were collected and analyzed for AQP1 expression and LDH release.

Statistical analysis

Values for aquaporin-1 expression acquired through densitometry were normalized to β -actin expression from experimental and control samples and were analyzed by a two tailed, paired student's t-test. Values for LDH release following stretch were normalized to prestretch control values and analyzed by a two-tailed, paired student's t-test. Differences were considered significant at $p < 0.05$.

Results

Our first study was designed to evaluate the effect of a cyclic mechanical stretch on AQP1 expression by cultured human TM monolayers. After subjecting TM monolayers to cyclic stretch of either 9% or 13% at a frequency of 1 Hz for a period of 8 and 24 hours, cell lysates were collected and analyzed for AQP1 expression. Application of 1 Hz cyclic stretch reduced the magnitude of stretch achievable on the Flexcell instrument from 10% to 9% and from 20% to 13%. Figure 2A shows representative western blots of AQP1 expression prior to (0) and following either 8 or 24 hours of cyclic stretch. We analyzed the non-glycosylated AQP1 band (28kD) and used β -actin expression as a reference for normalization purposes. Analyses that included both the non-glycosylated and glycosylated AQP1 bands (35kD smear) yielded similar results (data not shown). Figure 2B shows summary data (mean $\pm$ SD) indicating that we observed no significant change in expression at either 9% or 13% cyclic stretch for 8 or 24 hours compared to pre-stretch controls.

We also tested whether a static mechanical stretch of cultured human TM monolayers affected AQP1 expression. Cell lysates from TM monolayers subjected to 10% or 20% static stretch for periods of 8 and 24 hours were analyzed for AQP1 protein expression. Figure 3A shows a representative western blot of AQP1 expression from TM cell lysates prior to (0) and following 8 and 24 hours of static stretch. Normalized data (mean $\pm$ SD) of AQP1 expression for 10% and 20% static stretch is shown in the summary graph in figure 3B. A significant 2-fold increase was observed in AQP1 expression following the 10% stretch for 8 and 24 hours ($p < 0.05$). Similarly, we observed a significant 3.5-fold increase in AQP1 expression following the 20% stretch for 8 and 24 hours ($p < 0.05$).

The presence of lactate dehydrogenase (LDH) in media was used as an indirect measure of stress experienced by TM cells during mechanical stretch. Media collected before and after stretch were compared to negative control (no stretch) and positive control (1% Triton lysis) samples. Significant amounts of LDH were detected in conditioned media following 24 hours 13% cyclic ($p = 0.044$) and 20% static ($p = 0.002$) stretch. With both of these two types of stretch we observed a ~2 fold increase in LDH compared to control. Viability was also tested by probing western blots with primary antibodies against histone deacetylase (HDA). Interestingly, we observed no difference in the appearance of HDA between any of the control and experimental groups (data not shown). Taken together, results indicate that the levels of mechanical strain applied to cells in the present study induced some degree of cell damage, but not global cell death.

To test the importance of AQP1 expression in TM cell homeostasis under conditions of strain, we used adenovirus encoding recombinant AQP1 to bring total AQP1 levels closer to physiological levels (Figure 5a). Because we observed that static stretch had the most consistent effect on TM cell viability, we expressed recombinant AQP1 in TM cells and administered a 20% static stretch for 24 hours. Media was collected prior to and following 24 hours of static stretch and analyzed for LDH activity. Figure 5b shows that LDH from AQP1 infected cells was 50% lower in comparison to GFP control cells ($p = 0.03$). Interestingly, when analyzed together with uninfected cells, these data demonstrate an inverse linear relationship between AQP1 expression and LDH in conditioned media in response to stretch. ($r^2 = 0.7780$)

Discussion

The purpose of our study was to examine the role of AQP1 in TM cells during mechanical strain. TM cells responded to static mechanical stretch by increasing AQP1 expression. Interestingly, cyclic mechanical stretch did not significantly impact AQP1 expression. Consistent with a role for AQP1 in cell homeostasis, the most significant impact on cell viability was found during static mechanical stretch. We observed that LDH release from TM cells was dependent upon the level of AQP1 expression. Taken together, our data support a protective role for AQP1 in human TM cells during static mechanical strain.

Our studies were designed to approximate physiological levels of static strain in vivo by applying a stimulus of 10 and 20% to cultured human TM cells. With static stretch we attempted to model two physiological stressors experienced by the TM, ciliary muscle contraction and/or elevated IOP. Earlier studies have shown that IOP elevations in rhesus monkeys result in as much as a 50% stretch of TM and SC cells in the JCT region.(Ethier, 2002, Grierson and Lee, 1977) Qualitative studies of sectioned human conventional outflow tissue however, demonstrate less distention in the TM in comparison to the SC (Grierson and Lee, 1974), in part due to the lack of giant vacuoles which allow for larger increases in surface area.(Grierson and Lee, 1975) We note that 10% stretch is commonly used by other groups and estimated to be within the physiological range experienced by TM cells.(Chow, et al., 2007, Mitton, et al., 1997, Tamm, et al., 1999, Tumminia, et al., 1998, WuDunn, 2001) Consistent with this idea, we observed elevations in LDH release from cells exposed to higher stretch magnitudes for longer times, but did not see global cell death as measured by histone deacetylase. These data further suggest that though the stretch magnitudes used in the current study are estimated to approximate the mechanical strain in vivo, the TM is likely subjected to much greater levels of strain in vivo. It is important to note that the TM in vivo is also subject to a variety of other stresses associated with phagocytosis of cellular debris and exposure to oxidative byproducts in the anterior chamber which may induce cell damage or death that would not be achievable with the stretch system alone.(Liton and Gonzalez, 2008)

The cyclic mechanical stretch in the current studies was intended to simulate changes associated with ocular pulse. Previous studies, particularly in TM cultures have used a 10% cyclic stretch and estimated this level of stretch to be within a physiological range.(Chow, et al., 2007, Mitton, et al., 1997, Tamm, et al., 1999, Tumminia, et al., 1998, WuDunn, 2001) To our knowledge, studies have not looked the level of strain experienced by the TM in response to ocular pulse. However, studies in other tissues such as pulmonary capillaries have suggested that 5% stretching of endothelial cells is within a physiological range.(Birukov, et al., 2003, Birukova, et al., 2006) We were not able to detect a change in AQP1 expression at either the 9 or 13% levels, yet we did see an increase in LDH release during 13% cyclic mechanical stretch; indicating that the cells were experiencing physical strain. The reason that TM cells increased AQP1 expression in response to both 10 and 20% static stretch, but not cyclic stretch at either the 9 or 13% levels is unclear. The difference in response of TM cells to cyclic versus static stretch may be explained in part by the amount of stress incurred by the cells with strain. The model system employed was limited in terms of the magnitude of strain achievable when applying cyclic stretch (13% maximum).

We structured the present study to test our novel hypothesis that AQP1 accommodates rapid changes in TM cell shape (and possible associated volume) during mechanical strain to maintain TM homeostasis. A precedent for the role of aquaporins in cell volume regulation has already been demonstrated in the TM as well as other tissues.(Chan, et al., 2004, Krane, et al., 2001, Liu, et al., 2006, Mitchell, et al., 2002, Stamer, et al., 2001) It would be interesting to determine in future studies the connection between cell volume regulation and cell viability during mechanical strain. Moreover, it would be of interest to determine if in some glaucomas,

AQP1 levels are unusually low, making TM cells more vulnerable to mechanical insults such as elevated IOP. (Alvarado, et al., 1984) Characterizing the mechanisms responsible for TM maintenance and protection is critical for improving our general understanding of TM physiology as well as providing potential targets for treatment of TM cells that are subject to mechanical strain as well as other environmental stressors.

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Figure 1. Aquaporin-1 expression by TM cell monolayers over time and with passaging
 Left panel shows changes in AQP1 expression over time (passage 2) and with passaging (passages 1–6, right panel) compared to AQP1 expression in TM Tissue=T. Displayed are representative western blots for a single cell line (TM90) of three lines total that were tested (table 1).

Figure 2. Effect of cyclic stretch and Aquaporin-1 expression by TM cell monolayers

TM cells plated at confluence and maintained for two weeks on Bioflex membranes were subjected to cyclic stretch (9% or 13%) at 1 Hz for 8 or 24 hours. Cell lysates at 0, 8, and 24 hours were collected and analyzed by SDS-PAGE/western blotting using affinity purified antibodies specific for aquaporin-1 (panels A). Panel B shows a histogram of AQP1 expression levels over time of exposure to stretch that were normalized to β -actin prior to stretching. Data represent combined densitometry data (mean $\pm$ SD) from at least 3 independent experiments.

Figure 3. Effect of static stretch on Aquaporin-1 expression by TM cell monolayers

TM cells plated at confluence and maintained for two weeks on Bioflex membranes were subjected to static stretch (10 or 20%) for 8 or 24 hours. Cell lysates at 0, 8, and 24 hours were collected and analyzed by SDS-PAGE/western blotting using affinity purified antibodies specific for AQP1 (panel A). Panel B shows a histogram of AQP1 expression levels over time of exposure to stretch that were normalized to β -actin prior to stretching. Data represent combined densitometry data (mean $\pm$ SD) from at least 3 independent experiments. (*p < 0.05)

Figure 4. Viability of TM cells exposed to cyclic or static stretch

TM cells plated at confluence and maintained for two weeks on Bioflex membranes were then subjected to no stretch (control), static stretch (10 or 20%) or cyclic stretch (9 or 13% at 1Hz). Conditioned media was collected after 8 or 24 hours of stretch and analyzed for lactate dehydrogenase (LDH) content. Shown are combined data (mean $\pm$ SD) from at least 3 independent experiments that were normalized to optical density (OD) values for total lysis controls. (* $p < 0.05$)

Figure 5. Viability of TM cells exposed to 20% static stretch following expression of recombinant AQP1

TM cells plated at confluence and maintained for two weeks on Bioflex membranes were either not transduced (control) or were transduced with GFP or AQP1 adenovirus and subjected to 20% static stretch for 24 hours. Conditioned media was collected after 24 hours of stretch and analyzed for lactate dehydrogenase (LDH) activity. Cell lysates were collected and analyzed by SDS-PAGE/western blot using affinity purified antibodies for AQP1, GFP and β -actin (Panel A). Shown in panel B is a histogram of combined data (mean + SD) from at least 3 independent experiments that were normalized to optical density (OD) values for total lysis controls. (* $p < 0.05$)

Table 1

TM Cell Lines used for each Experimental Paradigm

HTM Strain	Donor age	Passaging	Time course	10% static	20% static	8% cyclic	13% cyclic
TM26	15yrs			X	X		
TM86	3mon					X	X
TM88	25yrs	X	X	X		X	X
TM90	4mon	X	X	X	X	X	X
TM92	44yrs	X	X			X	X