Published in final edited form as:

Prog Retin Eye Res. 2015 January ; 0: 86–98. doi:10.1016/j.preteyeres.2014.08.002.

Biomechanics of Schlemm's canal endothelium and intraocular pressure reduction

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Abstract

Ocular hypertension in glaucoma develops due to age-related cellular dysfunction in the conventional outflow tract, resulting in increased resistance to aqueous humor outflow. Two cell types, trabecular meshwork (TM) and Schlemm's canal (SC) endothelia, interact in the juxtacanalicular tissue (JCT) region of the conventional outflow tract to regulate outflow resistance. Unlike endothelial cells lining the systemic vasculature, endothelial cells lining the inner wall of SC support a transcellular pressure gradient in the basal to apical direction, thus acting to push the cells off their basal lamina. The resulting biomechanical strain in SC cells is quite large and is likely to be an important determinant of endothelial barrier function, outflow resistance and intraocular pressure. This review summarizes recent work demonstrating how biomechanical properties of SC cells impact glaucoma. SC cells are highly contractile, and such contraction greatly increases cell stiffness. Elevated cell stiffness in glaucoma may reduce the strain experienced by SC cells, decrease the propensity of SC cells to form pores, and thus impair the egress of aqueous humor from the eye. Furthermore, SC cells are sensitive to the stiffness of their local mechanical microenvironment, increasing their own cell stiffness and modulating gene expression in response. Significantly, glaucomatous SC cells appear to be hyper-responsive to substrate stiffness. Thus, evidence suggests that targeting the material properties of SC cells will have therapeutic benefits for lowering intraocular pressure in glaucoma.

Keywords

Glaucoma; Schlemm's canal; Trabecular meshwork; Ocular hypertension; Conventional outflow; Aqueous humor

1. Introduction

The elevated intraocular pressure (IOP) that is associated with primary open-angle glaucoma (POAG) is caused by an increased resistance to the outflow of aqueous humor from the eye through the conventional² outflow pathway (Grant, 1951). In spite of over 140 years of investigation (Leber, 1873), the precise cause of this increased outflow resistance remains elusive. Interestingly, most treatments for glaucoma focus on altering the rate of aqueous humor formation or altering the outflow path. These treatments lower IOP and thereby slow the progression of ganglion cell damage and associated vision loss, but in most cases do not stop it (Hattenhauer et al., 2000, 1998; Leske et al., 2003; Nouri-Mahdavi et al., 2004). Remarkably, there is currently no drug treatment in clinical use that directly targets the increased flow resistance that is a central characteristic of ocular hypertension in glaucoma, mainly because the mechanism(s) of increased flow resistance remain obscure.

Logically, the primary pathology underlying increased outflow resistance might be cellular, extracellular, or some combination of the two. Cellular contributions might include altered hydraulic conductivity of the endothelial lining of Schlemm's canal (SC), and extracellular contributions might include increased extracellular matrix in the juxtacanalicular tissue (JCT) or altered basement membrane beneath SC cells; however, comparisons of the outflow pathways of glaucomatous and age-matched normal eyes have found only subtle structural differences. Specifically, in glaucoma, there is an accelerated loss of trabecular meshwork (TM) cells that is limited to the inner region of the conventional outflow pathway (Alvarado et al., 1981, 1984) and cytoskeletal changes in the actin architecture of JCT-TM³ and SC cells in the JCT (Read et al., 2007). Additionally, there is an accumulation of a “sheath derived plaque material” in the JCT of glaucomatous eyes (Alvarado et al., 1986; Lutjen-Drecoll et al., 1981), but this accumulation has been shown to have negligible hydrodynamic consequence (Alvarado et al., 1986) (Murphy et al., 1992). There is little other morphological evidence of increased extracellular matrix or altered basement membrane composition in glaucomatous eyes compared to age-matched controls.

At the level of SC, however, several changes in glaucomatous eyes have been observed with the potential to be a significant contributor to the increased outflow resistance. The dimensions of the lumen of SC are smaller in glaucomatous eyes and these changes correlated with outflow resistance (Allingham et al., 1996). Herniations of the inner wall and

²There is also an unconventional outflow pathway that carries a fraction of the total aqueous humor outflow. While this pathway is important in the treatment of glaucoma (as an alternate outflow pathway), there is little evidence that it is involved in the pathogenesis of ocular hypertension characteristic of glaucoma.

³There are, at least, two morphologically and functionally distinct populations of TM cells in the conventional outflow tract. Beam TM cells are endothelial like cells that cover TM cords, lamellae and plates as monolayers, maintaining these structures and also filtering aqueous humor by phagocytosis as it enters the outflow tract. JCT-TM cells are fibroblastic appearing cells in the JCT, functioning to regulate extracellular matrix content and interact with inner wall of SC cells to regulate outflow resistance.

JCT tissue into collector channels are more frequently observed in glaucomatous eyes than age-matched non-glaucomatous eyes (Gong et al., 2007; Hann et al., 2014). There is also a reduced pore density in the inner wall endothelium of SC comparing normal to glaucomatous eyes (Allingham et al., 1992; Johnson et al., 2002) that is potentially quite important. Collectively, these data point to dysfunction at the level of the inner wall of SC in glaucoma.

In this article, we review recent evidence that increased stiffness of SC endothelial cells is responsible for the elevated outflow resistance and IOP characteristic of glaucoma; we also present data showing that drugs that alter cell stiffness also alter outflow resistance. By understanding the coupling between biomechanics and flow through the inner wall endothelium, we outline opportunities to exploit cell biomechanics as a targeted approach to reduce IOP at the site of outflow resistance regulation.

2. The inner wall endothelium of Schlemm's canal unique biomechanical environment

The typical pressure loading on vascular endothelia generates a pressure gradient in the *apical to basal* direction. The basement membrane and other tissues underlying vascular endothelia amply support the transcellular pressure drop generated by this gradient, and thus the vascular endothelial cells themselves do not have to support the associated radial and circumferential stresses. This is not the case for the endothelium of SC, where the SC cells themselves must support a “backwards” *basal to apical* pressure gradient associated with fluid flowing into the SC lumen, which tends to push SC cells off their supporting basement membrane (Fig. 1). While terminal or capillary lymphatics also are exposed to such an adverse pressure gradient, the endothelial linings of these lymphatics are not sealed completely by tight junctions (Raviola and Raviola, 1981; Swartz, 2001), and thus little pressure difference needs be supported by the lymphatic endothelial cells themselves (Ramos et al., 2007; Zweifach and Lipowsky, 1984).

Under physiological conditions, the basal-to-apical pressure drop between intraocular pressure and episcleral venous pressure deforms SC cells to create large dome-like outpouchings into the SC lumen, the so-called “giant vacuoles” (Fig. 1) (Holmberg, 1959). Despite this deformation, the inner wall endothelium remains continuous to preserve the blood-aqueous barrier that prevents plasma entry into the anterior chamber, helping maintain ocular immune privilege (Streilein, 1996). The SC cells appear to be particularly well adapted to function within this biomechanical environment. Forces arising from the pressure gradient are transmitted through the cell to neighboring cells and extracellular matrix via adhesive and elastic tethers (Grierson et al., 1978; Overby et al., 2009; VanderWyst et al., 2011). SC cells must support this transmitted load and do so primarily through the stiffness of their cytoskeleton and contractile machinery.

Importantly, SC cells are also highly contractile, and contraction of these cells causes a significant increase in their stiffness (Zhou et al., 2012). Remarkably, the dynamic range of contractility of an SC cell is similar to that of a smooth muscle cell (Zhou et al., 2012), ideally suited for a lively environment. However, the cytoskeletal architecture of SC cells is

more similar to an endothelial than a smooth muscle cell. Thus, the cytoskeleton of SC cells is enriched in microfilaments and intermediate filaments (Tian et al., 2000), and has a prominent actin-rich cell cortex (Fig. 2). Drugs such as dexamethasone or sphingosine-1-phosphate (S1P) that increase the complexity of the SC cortical actin network also increase outflow resistance (Underwood et al., 1999) (Sumida and Stamer, 2010).

2.1. Pores are reduced in glaucomatous eyes

Aqueous humor passing through the conventional aqueous outflow pathway needs to cross the continuous endothelial monolayer of the inner wall of SC. While the mechanism by which this occurs has been a matter of long-standing debate (Johnson and Erickson, 2000), the conventional wisdom holds that the bulk of this transendothelial flow passes through micron-sized pores, crossing an otherwise continuous endothelial layer in which cells are connected by tight junctions (Bill and Svedbergh, 1972; Ethier et al., 1998; Johnson, 2006).

There appear to be two distinct subtypes of pores, transcellular and paracellular (Ethier et al., 1998). Paracellular pores are circular dilations of the intercellular space (Epstein and Rohen, 1991); transcellular pores occur at locations where the cytoplasm of the endothelial lining of SC becomes sufficiently thinned such that the apical and basal plasma membranes come together (Inomata et al., 1972; Johnson, 2006; Overby, 2011). Theoretically, when apical and basal membranes are closely opposed, there is a high probability of membrane fusion, leading to pore formation such as occurs in other microvascular endothelia (Michel and Neal, 1999). Consistent with this idea, Braakman et al. recently showed that when SC cells in culture are stretched, they form both paracellular and transcellular pores (Braakman et al., 2014). These pores are membrane-lined on their surfaces and are generally similar to transcellular openings in vascular cells. Pore formation in SC cells may be facilitated by vesicle docking/fusion machinery as occurs in other microvascular endothelia (Neal and Michel, 1995, 1996; Palade et al., 1979; Savla et al., 2002). Similar pores are seen in arachnoid villi that resorb cerebrospinal fluid (Tripathi, 1977) in vascular endothelia during leukocyte extravasation (Carman et al., 2007), and in vascular endothelium under special experimental conditions such as treatment of endothelia with a calcium ionophore, vascular endothelial growth factor or with elevated intravascular pressures (Neal and Michel, 1997a, 1995; 1996, 1997b; Overby, 2011).

Previous studies have established that while these pores themselves generate only a small part of the overall flow resistance (10% or less of the total) (Bill and Svedbergh, 1972), the aqueous outflow resistance is greatly amplified by a hydrodynamic inter-action between the pores and their underlying substratum (i.e., the basal lamina of the SC cells and/or the extracellular matrix of the JCT) (Johnson et al., 1992). Specifically, each pore funnels flow through regions of matrix nearest the pores, and thus dramatically decreases the effective area available for flow through these regions (Fig. 3). The consequence of this potent funneling effect is an increased pressure drop near the pore mouth; a corollary of this effect is that any change in pore density will unavoidably alter overall flow resistance (Johnson et al., 2002). The importance of the hydrodynamic interaction of the JCT and SC cells to generate resistance via funneling has been recently appreciated (Overby et al., 2009) and supported by experimental data (Overby et al., 2002; Sabanay et al., 2000). Thus, while

aqueous flow resistance is generated in the extracellular matrix of the JCT, it may be modulated by the pores in the inner wall endothelium of Schlemm's canal.

Allingham et al. (1992) reported and Johnson et al. (2002) confirmed that there is a significant reduction in the density of pores in the inner wall endothelium of Schlemm's canal of glaucomatous eyes as compared with normal eyes, with a decrease of both paracellular and intracellular pores (Fig. 4) (Johnson et al., 2002). As this reduction would be expected to have a significant effect on aqueous outflow resistance, via the funneling effect, it is of significant interest to determine the cause of this reduction.

Overby et al. (2014) examined whether this loss of pores in glaucomatous eyes might be due to a fundamental defect in the SC cells themselves. To test this idea, SC cells from normal and glaucomatous human donors were seeded onto and cultured on microporous filters. To mimic the biomechanical and filtration environment of SC endothelium in vivo, monolayers of SC cells were perfused in a custom-made in vitro system (Pedrigi et al., 2011). Results using this in vitro model were consistent with data in situ, showing that, when perfused in the basal-to-apical direction, cultured SC cell monolayers formed both giant vacuole-like structures and pores. Pores passed transcellularly through individual SC cells and paracellularly between neighboring SC cells (Fig. 5A), analogous with the two pore types observed in the SC endothelium in human eyes (Ethier et al., 1998). As expected from results in situ, the total pore density increased significantly with perfusion pressure (Fig. 5B). Apical-to-basal perfusion showed a much lower pore density and no dependence of pore density on perfusion pressure, indicating that these pores act as one-way valves, as previously suggested (Johnstone and Grant, 1973). These data are consistent with two other studies: Alvarado et al. (2004) showed rectified flow through SC endothelium as did Pederson et al. (1978) in live monkeys. This rectified behavior theoretically serves to maintain the blood aqueous barrier, while allowing outflow in vivo.

Compared to normal SC cell strains, Overby et al. (2014) found the pore density during basal-to-apical perfusion of glaucomatous SC cell strains to be markedly reduced to a level comparable to the unperfused normal controls (Fig. 5C). These data supported the notion that the pore forming ability of glaucomatous SC is reduced as compared with SC cells from normal eyes and is consistent with the fact that outflow resistance is increased in people with glaucoma (Grant, 1951). Importantly, this handicap reproduces in a cell culture model for the first time a specific pathology potentially responsible for ocular hypertension in glaucoma.

Based upon these foundational data, it has been hypothesized that pore formation in the inner wall endothelium is driven by cell deformation induced by the transcellular pressure drop across the cell (Braakman et al., 2014; Overby, 2011; Overby et al., 2014). The extent of the cellular deformation is largely determined by the stiffness of these cells, their contractile state, and their attachments to the underlying matrix and to neighboring cells. A conceptual model of this process has been suggested in which pressure-induced deformation of SC cells leads to a thinning of the cytoplasm, and it is this thinning that allows for intracellular pores to form (Overby et al., 2014). Presumably, pores preferentially form in regions where the apical and basal membranes of SC cells come in close proximity to one

another (Johnson, 2006) (Fig. 6). Such cellular tension can also be expected to pull at cell-cell junctions to create paracellular pores. The formation of pores serves both as a release valve and as a physiological mechanism (via funneling) to regulate flow resistance (Overby et al., 2009). Thus, the SC endothelium might be viewed as mechanically-gated one-way valve that becomes disrupted in glaucoma by altered SC cytomechanics, leading to outflow obstruction and IOP elevation.

Furthermore, strain-induced pore formation allows the inner wall of SC to adapt to heterogeneous and dynamic filtration demands created by segmentation of outflow (Chang et al., 2014). In regions where filtration demand and the transendothelial pressure drop is larger, SC cells experience larger deformations on account of giant vacuole formation or ‘ballooning’ of the inner wall. The elevated strain induces pore formation that provides a route for aqueous humor filtration. This mechanism theoretically allows the inner wall to function as a ‘smart’ one-way valve that adjusts its local porosity to accommodate local demands in filtration while simultaneously preserving the blood-aqueous barrier (Braakman et al., 2014). The impaired pore-forming ability in glaucomatous SC cells suggests that this mechanism may become disrupted in glaucoma, leading to impaired filtration, accumulation of extracellular matrix, and elevated outflow resistance.

With this hypothesis as a framework, ocular hypertension in glaucoma can be attributed, at least in part, to elevated stiffness of SC cells themselves. According to this notion, a stiffer SC cell would deform less under the influence of a transcellular pressure drop, inhibiting pore formation, increasing outflow resistance and thereby intraocular pressure. Testing of this idea requires that the mechanical properties of the normal and glaucomatous TM and SC cells be characterized. A variety of methods are available for this characterization that we review below.

2.2. SC cell isolation and culture

Two cell types populate the human conventional outflow pathway, TM and SC endothelial cells. Due to research focus and the relative ease with which primary TM cells can be cultured, the majority of studies to date have been directed toward the cell biology of the TM [reviewed by Stamer and Acott (2012)]. Isolation of human SC cells has been more challenging due to their comparatively small numbers in the conventional outflow pathway and their anatomical location. Stamer et al. (1998) introduced the cannulation method to collect these cells, in which a suture is placed into SC lumen of organ cultured eyes for three weeks, allowing cells to migrate onto the suture, similar to a endothelial wound healing response. The suture is then removed, and the cells adhering to it are grown in culture. SC cells have also been isolated using explant, immunopanning and puromycin selection methods (Alvarado et al., 2004; Karl et al., 2005; Lei et al., 2010). Each of these methods has limitations such as the number of cells isolated and possible contamination from neighboring tissues. The SC cells isolated by the cannulation procedure are contact inhibited and morphologically similar to cells of the intact inner wall of SC (Stamer et al., 1998). Human SC cells in culture can be distinguished from TM cells based on several criteria including a net transendothelial electrical resistance of $10 \Omega \cdot \text{cm}^2$ or greater (Stamer et al., 1998), the lack of myocilin induction by dexamethasone (Stamer et al., 1998), and the

expression of vascular endothelial cadherin, integrin alpha-6 and fibulin-2 (Perkumas and Stamer, 2012). Important for comparisons between cell strains, measurements of cell stiffness using optical magnetic twisting cytometry (OMTC) showed that SC cells maintain a stable morphological and biomechanical phenotype in culture after five passages (Zhou et al., 2012).

2.3. Characterization of cell stiffness

The tensile stiffness of a soft connective tissue like the conventional outflow tract is largely determined by the mechanical properties of the extracellular fibers, collagen and elastin, within the tissue. In contrast, the ability of a soft connective tissue to resist compression is provided primarily by the glycosaminoglycans (Mow et al., 1984). Cells, for the most part, do not contribute significantly to the bulk stiffness of connective tissues, although tissues with high densities of contractile cells (e.g. muscle) are a notable exception (Bank et al., 1996; Collinsworth et al., 2002). Cell stiffness, along with cell attachment density and strength, is important for limiting cell deformation in response to loading. This is particularly important for SC endothelial cells because of the basal to apical pressure gradient they experience.

Cell stiffness (elastic modulus) and force generating capacity have been measured for a variety of cell types using a wide array of techniques. Early studies that estimated cell modulus were done by measuring the pressure difference necessary to aspirate cells into micropipettes (Hochmuth, 2000), and in such studies, the modulus of bovine aortic endothelial cells was estimated as roughly 0.5–1 kPa (Theret et al., 1988). Later studies employed the use of magnetic microbeads attached to cells that were then either pulled [magnetic pulling cytometry (Overby et al., 2005)] or twisted [magnetic twisting cytometry (Fabry et al., 2001a; Wang et al., 1993)] to make estimates of cell modulus (Fig. 7). The first estimate of the modulus of SC cells was done using magnetic pulling cytometry and yielded a value of approximately 1–3 kPa, similar to, but somewhat larger than other endothelial cells (Zeng et al., 2010).

A modification of magnetic twisting cytometry called allows for rapid and accurate measurements of a large number of cells (Fabry et al., 2001b). Zhou et al. (2012) used OMTC to examine the effect of a variety of pharmacological agents on SC cell stiffness, demonstrating a surprising dynamic range. OMTC has several limitations. First, the value for cell stiffness is a function of bead embedding depth (Mijailovich et al., 2002) that is unknown in most studies, making this technique most valuable for determining relative changes in cell stiffness (e.g. due to pharmacological treatment) rather than determining an absolute value of stiffness. A second possible limitation is that OMTC is thought to be strongly influenced by cortical stiffness, and less so by the underlying cytoplasm (Crocker and Hoffman, 2007; Guo et al., 2013).

Alternatively, atomic force microscopy (AFM) is a method whereby a cell is indented by either a sharp (Fig. 7) or a spherical tip, and the resulting deformation is used to determine cell modulus. Measurements can also depend on the depth of indentation of the probe; for example, if cells are indented too much, artifactual effects from the cell substrate can be introduced. While this method is much more laborious than is OMTC, so that a smaller

number of cells can be queried, the method gives a direct measurement of cell modulus. However, measurements of cell stiffness using AFM give different results in endothelial cells depending on whether a sharp or a rounded AFM tip is used. Vargas-Pinto et al. (2013) showed that sharp tips largely probe the cortex of the cell, while measurements with rounded tips are more significantly affected by the stiffness of the subcortical (cytoplasmic) aspects of the cell. This conclusion was independent of indentation depth over the range investigated (100–400 nm). Thus, the cytoskeletal character of endothelia can be defined, depending upon the AFM tip used.

Cells are not passive mechanical objects but can actively change their stiffness by contracting and/or reorganizing their actin-myosin network. Traction force microscopy is a method that allows a local determination of the traction forces that cells exert. In this technique, cells are grown on top of a gel in which fiducial markers have been embedded. By knowing the modulus of the gel and tracking the motion of the fiducial markers as the cells contract, the distribution of contractile forces in the cell can be determined (Fig. 8) (Butler et al., 2002). Using traction force microscopy, Zhou et al. (2012) demonstrated that SC cells modulate their contractile stress and their stiffness over a significant range, comparable to that of airway smooth muscle cell. As cell contraction is used by the latter cell type to regulate bronchomotor tone, the similarity in contractile behavior of SC cells suggests that cell contraction may be an important physiological function of these cells in the demanding mechanical environment of the conventional outflow tract.

2.4. SC cell stiffness and aqueous humor outflow resistance

Zhou et al. (2012) demonstrated the tenability of the hypothesis that aqueous outflow resistance is modulated by SC cell stiffness by examining the effects on SC cell stiffness of drugs known to influence outflow resistance. These results were striking: the three drugs examined that increased outflow resistance caused SC cells to contract and stiffen; while the four tested drugs that decreased outflow resistance caused the SC cells to relax. This was true for both normal and glaucomatous SC cells (Overby et al., 2014). Table 1 shows a summary of these results and others reported in the literature. Also shown in the table are the effects of these drugs on cell junctional permeability, since an alternate explanation of how these drugs might affect outflow resistance is through junctional tightness, which would hypothetically limit paracellular flow. Remarkably, no such relationship is apparent between outflow resistance and junctional permeability. For example, although bradykinin, histamine and thrombin are associated with increased paracellular permeability, each acts to increase outflow resistance. This strongly supports the hypothesis that aqueous humor outflow resistance is modulated by the stiffness of the SC cell.

Further confirmation of this hypothesis can be found in the work of Rao et al. examining the effects of cytoskeletal-active agents on outflow resistance, particularly those affecting rho-kinase. Rao found that cell contraction was associated with increased outflow resistance, while cell “relaxation” was associated with decreased outflow resistance (Mettu et al., 2004; Rao et al., 2001; Rao and Epstein, 2007). Contraction of cells cause their stiffness to increase (Nagayama et al., 2004), and thus Rao's results are consistent with a central importance of cell stiffness in the regulation of conventional outflow. Rho kinase inhibitors

decreased stress fibers in both TM and SC cells, and increased the permeability of SC monolayers to tracers by ~80% (Fig. 9). These findings were confirmed by Kumar and Epstein (2011).

Additional support for the role of SC stiffness in outflow regulation is found in studies examining the effects of latrunculin on the outflow pathway. Latrunculin, an actin-depolymerizing agent, decreases SC cell stiffness (Vargas-Pinto et al., 2013). In line with this finding, Ethier et al. showed that latrunculin-B decreased outflow resistance in enucleated human eyes, and that this decrease was associated with an increased density of inner wall pores (Ethier et al., 2006). Likewise, Peterson et al. found that both latrunculin-A and latrunculin-B decrease outflow resistance in live monkeys (Okka et al., 2004; Peterson et al., 1999). Other drugs that relax SC cells such as H-7 and BDM also decreased outflow resistance, however pore counts of the inner wall were not performed in these studies (Epstein et al., 1999; Sabanay et al., 2000). Thus, it appears that drugs that specifically target cell stiffness have therapeutic value.

2.5. SC cell stiffness is altered in glaucoma

Several studies have explored the biomechanical behavior of SC cells, finding them to be highly contractile, to have a stiffness similar to other endothelial cells, to increase their stiffness in response to stretch and to have elevated stiffness in their cortex (Vargas-Pinto et al., 2013; Zeng et al., 2010; Zhou et al., 2012). These studies established for the first time the mechanical properties of SC cells isolated from non-glaucomatous donor eyes. Overby et al. (2014) recently reported altered biomechanical behavior of glaucomatous SC cells as compared to SC cells isolated from normal eyes. While, the cortical stiffness of glaucomatous SC cells was similar to that of normal SC cells (Fig. 10A), the subcortical stiffness of glaucomatous SC cells was significantly elevated (50%).

To demonstrate a link between cell stiffness and pore formation, Overby et al. examined 2 normal and 3 glaucomatous cell strains in which both cell stiffness and pore density were measured, finding that increasing subcortical stiffness was correlated with decreasing pore density (Fig. 10B). These data support the idea that impaired pore formation in glaucomatous SC cells is attributable to increased subcortical cell stiffness.

2.6. The role of substrate stiffness and the extracellular matrix

The physiological substrate of the SC cell is the JCT, the most external aspect of the TM. The JCT is rich in extracellular matrix, the biochemical character and morphological appearance of which are altered in glaucoma (Gong et al., 1996; Lutjen-Drecoll et al., 1981, 1986; Rohen et al., 1981). Interestingly, Camras et al. (2014) reported that the tensile stiffness of the TM was significantly *reduced* in glaucoma; note that this type of measurement probes the stiffness of the TM as an interconnected structure in the circumferential direction. In contrast, Last et al. found a significantly *increased* compressive elastic modulus of glaucomatous TM tissue compared to normal TM tissue (Last et al., 2011); we note that these experiments measured a different physical property than those of Camras et al. In fact, the elastic modulus of TM tissue from glaucomatous eyes was 20 times stiffer than that from age-matched normal eyes (Fig. 11). Thus, the biomechanical

microenvironment of the TM, with which SC cells are in direct contact, appears to be mechanically altered in glaucoma.

The mechanisms by which TM tissue stiffness is altered in glaucoma are unknown. Data suggest that TGF β 2 is elevated in the aqueous humor of glaucoma patients (Prendes et al., 2013; Tripathi et al., 1994). Exposure of conventional outflow tissues to TGF β results in increased outflow resistance and increased accumulation of fibrillar material in the JCT, reminiscent of glaucoma (Fleenor et al., 2006; Gottanka et al., 2004; Shepard et al., 2010). The molecular mechanisms that underlie increased resistance appear in part to be mediated by bone morphogenic protein-1, which activates cross-linking enzymes (LOX and LOXL1), resulting in resistance to matrix degradation and increased tissue stiffness (Wordinger et al., 2014).

Cells sense and respond to the mechanical properties of, and cues from their microenvironment (Byfield et al., 2009; Solon et al., 2007). For example, this “mechanosensing” allows vascular endothelial cells to alter their morphology, function, and gene expression in response to shear stress (Ando and Yamamoto, 2009; Birukova et al., 2013). Moreover, cells adapt their stiffness to match that of their substrate (Solon et al., 2007). Consistent with this principle, several investigators have reported that SC and TM cells in culture alter their behavior and stiffness in response to changes in the stiffness of the substrate on which they are grown (McKee et al., 2011; Overby et al., 2014; Raghunathan et al., 2013; Schlunck et al., 2008). Since SC cells interact with the JCT, it is intriguing to postulate that SC cells become stiffer in glaucoma because they grow on a pathologically stiff substrate (Russell and Johnson, 2012), or because they are more sensitive than normal cells to biochemical or biomechanical changes in the substrate. Along these lines, a recent study has shown that glaucomatous SC cells have altered cytoskeletal structure and significantly increased stiffness in response to increased substrate stiffness as compared to the response of normal SC cells (Overby et al., 2014).

SC and TM cells also modify their gene expression in response to changes in substrate stiffness or architecture. In response to increasing substrate stiffness, expression levels of genes for extracellular matrix proteins increased in SC cells (collagen 1, SPARC, CTGF, TGM2 and decorin) and TM cells (CTGF, SPARC, TGM2 and fibronectin); myocilin expression was also reported to increase in TM cells with increasing substrate stiffness (Overby et al., 2014; Thomasy et al., 2012). Schlunck et al. (2008) reported that the structure of fibronectin deposits from TM cells differed when cultured on stiff as opposed to soft matrices, while McKee et al. (2011) found that TM cytoskeletal dynamics, and particularly responses to latrunculin-B, were affected by substrate stiffness.

Importantly, glaucomatous SC cells have an exaggerated and possibly pathological response to increased substrate stiffness as compared to normal SC cells. CTGF and decorin are strongly upregulated by elevated substrate stiffness in glaucomatous SC cells (Fig. 12). Significantly, CTGF has already been associated with increased stress fiber formation, IOP elevation and glaucomatous optic neuropathy in a mouse model of glaucoma (Junglas et al., 2012). Taken together, these data demonstrate that SC cells behave similarly to other

endothelial cells and stiffen in response to increasing substrate stiffness, but glaucomatous SC cells appear more sensitive to substrate stiffness and have a larger stiffening response.

2.7. Targeting the inner wall of Schlemm's canal

In the current treatment regimen for glaucoma, no drugs are used that directly target the conventional outflow pathway, the diseased tissue responsible for ocular hypertension. Rather, the drugs currently used primarily act either by inhibiting aqueous inflow or increasing unconventional outflow, both of which result in a consequential decrease in flow through the conventional outflow pathway. Such deprivation of aqueous humor and its nutrients likely causes further damage to the already diseased TM tissue (Johnson, 1996; Kopczynski and Epstein, 2014). Development of new drugs that act to reduce conventional aqueous outflow resistance is expected to be synergistic to existing drugs, improving perfusion to conventional outflow tissues and thus giving promise of better IOP lowering and clinical outcomes in glaucoma patients. Because of the significant clinical benefit, development of such drugs is important (Heijl et al., 2002; Kopczynski and Epstein, 2014). The finding that the stiffness of SC cells is closely linked to outflow resistance (Zhou et al., 2012), and that SC cell stiffness can be specifically targeted (Wang et al., 2013; Zhou et al., 2013), suggests that agents can be identified to achieve this goal.

Although no currently marketed drugs target the inner wall of SC, four classes of drugs are currently in clinical trials that improve conventional outflow, and directly or indirectly impact cell stiffness: adenosine A1 agonists (clinical trials identifier: NCT01917383), nitric oxide donors (Krauss et al., 2011), Rho kinase inhibitors (Williams et al., 2011) and actin depolymerizers (Chen et al., 2011). These drugs have been shown to interact and physiologically impact both TM and SC cells and to decrease outflow resistance (Karl et al., 2005; Rao et al., 2001; Stamer et al., 2011; Vittitow et al., 2002); however, there appears to be potential with some for undesirable side effects, including corneal disorders and conjunctival hyperemia (Chen et al., 2011; Williams et al., 2011). New, safer, ocular hypotensive drugs specifically targeting the SC cells are needed, since they offer the promise of fewer off-target effects. Identification of compounds that interact specifically with SC cells and decrease outflow resistance will also give important insights into cellular control of aqueous outflow and aid in the understanding of its dysfunction in glaucoma.

To identify such ocular hypotensive drugs, the use of high-throughput functional screening has been recently described (Zhou et al., 2013). Traction force microscopy on SC cell plated hydrogel substrates was recently used to screen the Prestwick library of 1200 FDA-approved drugs. The most efficacious of these drug was the endoprostanoid alprostadil (PGE1), a potent vasodilator used clinically to treat erectile dysfunction. Alprostadil significantly relaxed both TM and SC cells, with apparent preference for SC cell relaxation. Importantly, when perfused into enucleated mouse eyes, Alprostadil significantly decreased outflow resistance by 25% (Zhou et al., 2013).

In separate studies, using a technique called cell dielectric spectroscopy, which measures cell impedance (an indirect indicator of cell relaxation state in the context of responses from controls, thrombin and Y27632), it was observed that the prostaglandin EP4 receptor selective agonist L-902688 had opposite effects on TM versus SC cells, decreasing

impedance in TM cells while increasing it in SC cells (Wang et al., 2013). Importantly, selective activation of EP4 receptors decreases outflow resistance in live monkeys plus in enucleated mouse and human eyes, consistent with the relaxation response of SC cells and a critical role for SC in regulating outflow resistance (Boussommier-Calleja et al., 2012; Millard et al., 2011; Woodward et al., 2009). Taken together, these studies demonstrate that drugs can be identified that specifically target SC cells and increase outflow facility.

3. Conclusions

SC cells are surprisingly contractile, capable of changing their contractile state to employ forces that are comparable to those exerted by smooth muscle cells in the lung (Zhou et al., 2012). While the source of resistance to the flow of aqueous humor through its primary outflow pathway from the eye is still a topic of active research (Keller and Acott, 2013; Overby et al., 2009; Swaminathan et al., 2013), a number of laboratories have now converged on the hypothesis that the contractile state of cells in the aqueous outflow pathway can regulate outflow resistance (Junglas et al., 2012; Peterson et al., 1999; Rao et al., 2005, 2001; Sabanay et al., 2000; Tian et al., 2009; Tian and Kaufman, 2012; Zhou et al., 2012). Changes in the contractile state of the cell are associated with changes in cell stiffness (Zhou et al., 2012). Drugs that increase cell stiffness also increase outflow resistance, and vice versa (Table 1) (Zhou et al., 2012). These findings emphasize the importance of cell stiffness and cell contractile state to the regulation of aqueous humor outflow resistance and control of IOP.

The influence of the level of the inner wall of SC on IOP can be explained mechanistically through the mechanosensitivity of the SC cells and how this impacts on the process of paracellular and transcellular pore formation. Studies suggest that stretch induced-pore formation acts as a mechanically-gated one-way valve, rectifying and modulating the outflow of aqueous humor. The elevated outflow resistance associated with primary open angle glaucoma appears to be, at least in part, the result of increased SC cell stiffness that impedes the formation of these pores. Specifically, inner wall pores are thought to modulate aqueous outflow resistance through a hydrodynamic interaction with the flow of aqueous humor passing through the JCT known as 'funneling' (Johnson et al., 1992; Overby et al., 2009). Thus, decreased pore density is expected to increase the resistance (in an amplified manner) to the outflow of aqueous humor from the eye and thereby increase intraocular pressure, a characteristic feature of glaucoma.

In glaucomatous eyes, the ultrastructure and material properties, particularly stiffness, appear to be altered in the TM that supports the SC cells (Camras et al., 2014; Last et al., 2011; Lutjen-Drecoll et al., 1981). Glaucomatous SC cells are more sensitive to substrate stiffness than normal SC cells, and they alter their expression of genes implicated in outflow obstruction and glaucoma in response to changes in substrate stiffness, particularly CTGF, an agent which has been shown to cause ocular hypertension and glaucomatous optic neuropathy in mice (Junglas et al., 2012). The interdependence between substrate stiffness, cell stiffness and pore formation reveals a mechanism by which extracellular matrix stiffness might increase outflow resistance, and thereby may explain why glaucomatous SC cells in

culture (grown on hard substrates) show increased cell stiffness and decreased pore formation (Russell and Johnson, 2012).

Targeting SC cell stiffness thus seems a promising anti-glaucoma therapy to decrease outflow resistance at the location responsible for regulating the majority of outflow resistance. The efficacy of therapies that decrease cell stiffness has been demonstrated by the use of nitric oxide donors (Krauss et al., 2011), Rho kinase inhibitors (Williams et al., 2011) and actin depolymerizers (Chen et al., 2011) that are currently in clinical trials for use in lowering IOP in glaucoma. Additionally, adenosine A1 receptor agonists are thought to increase extracellular matrix turnover (Shearer and Crosson, 2002), and thus potentially soften JCT-TM cells and thus the substrate for SC cells. However, such compounds lack specificity toward the SC cells, potentially decreasing their potency and increasing the possibility of side effects.

4. Future directions

To develop new therapeutic strategies that specifically target SC cells, progress in several areas is needed. First, better mechanistic understanding of the pore forming machinery in SC cells will provide novel targets for intervention. For example, it may be helpful to apply knowledge from mechanistic studies of fenestrae formation or transcellular diapedesis to intracellular pore formation in SC cells (Michel and Neal, 1999). Second, a better understanding of the molecular changes in glaucomatous SC cells that underlie greater-than-normal responses to substrate stiffness will provide insight into pathways that are critical for SC mechanobiology. For instance, it will be important to understand whether genetic or epigenetic changes underlie altered mechanobiology of glaucomatous SC cells. Third, robust high throughput assays that key into unique SC-cell physiology (e.g.: pore formation, contractility, etc) are necessary to screen for novel small molecules or biologics that can be used as leads for further development. Fourth, promoters that selectively target SC cells are needed for development of potential gene therapeutics to modify SC responses to their substrates, gene editing or altering overall SC stiffness. Fortunately, SC cells express an array of unique proteins not found in neighboring cells that can be exploited for this purpose. Progress in these key areas will likely catalyze the development of new agents specifically targeted to decrease SC cell stiffness or SC cell responses to stiff substrate at the level of the inner wall, giving promise for a better IOP control in this debilitating disease.

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Fig. 1.

Aqueous humor flow pathway. Left panel: schematic of anterior segment of eye showing the direction of aqueous humor flow in red. Center panel: an enlargement of the angle region of the eye (boxed region in left panel) showing the conventional outflow pathway; SC: Schlemm's canal. Right panel: Transmission electron micrograph of the inner wall of Schlemm's canal showing a giant vacuole through which the aqueous humor passes; V: giant vacuoles. Panel C is reproduced with permission (Overby et al., 2014) [Permission needed].

Fig. 2.

Structured Illumination Microscopy images of normal and glaucomatous SC cells labeled with actin filament marker, rAV-LifeAct-TagGFP2 (IBIDI, Verona, WI) (Riedl et al., 2008) before and after application of the actin-depolymerizing agent Latrunculin-A (1 μ M) for 30 min (Overby et al., 2014). Large arrows point to cortical cytoskeleton, while small arrows indicated subcortical cytoskeletal structures. [Permission needed].

Fig. 3. Schematic of the “funneling” of aqueous humor through the JCT caused by inner wall pores. If the inner wall were not present, the flow would pass uniformly through the upstream matrix of the JCT and generate a lower flow resistance (Overby et al., 2002; Overby et al., 2009) [Permission needed].

Fig. 4. The density of pores in the inner wall endothelium of Schlemm's canal in post-mortem normal (blue) and glaucomatous (red) human eyes. Fixation was done under conditions of constant flow (2 ml/min) (Johnson et al., 2002).

Fig. 5.

(A) Representative image of transcellular and paracellular pores in normal and glaucomatous cultured SC cells; (B) Pore density increases in 3 non-glaucomatous SC cell strains as a function of pressure drop in the basal-to-apical direction measured following monolayer perfusion at 0, 2 or 6 mmHg; perfused in the apical-to-basal direction (“6 mmHg AB”), pore densities are similar to the unperfused control at 0 mmHg; (C) Pore density is lower in glaucomatous compared to normal SC cells following perfusion at 6 mmHg in the basal-to-apical direction (Overby et al., 2014) [Permission needed].

Fig. 6. Conceptual model of the sequential process (A to D) of formation of a giant vacuole and pore in an SC cell in the face of a pressure gradient in the basal (intraocular pressure) to apical (episcleral venous pressure) direction.

Fig. 7.

Methods utilized to examine mechanical properties of SC cells: (A) Magnetic pulling cytometry: a magnetic bead is bound to the surface of a cell while a magnetic force (arrow) is applied to the bead using an electromagnetic microneedle or a microneedle attached to a permanent magnet. The resulting bead displacement is optically recorded using a microscope and used to determine cell modulus (Overby et al., 2005)[Permission needed; image modified]. (B) Magnetic twisting cytometry: A bead is bound to the surface of a cell and initially magnetized in the horizontal direction. It is then subjected to a vertical oscillatory magnetic field and the resulting torque, T , causes the bead to pivot back and forth. This motion is resisted by the cell, and the resultant bead motion is recorded using a microscope and used to determine cell modulus (Smith et al., 2003) [Permission needed; image modified]. (C) AFM: a rounded (not shown) or sharp tip is used to indent the surface of a cell. The relationship between indenting force and magnitude of indentation is used to deduce cell stiffness [Permission needed; image modified].

Fig. 8. SC cell exerts traction when plated on a deformable substrate. Phase-contrast image is shown in (A) and traction stress intensity map in (B) (Zhou et al., 2012) [Permission needed].

Fig. 9.

Y-27632 induces changes in actin stress fibers in cultured TM (A) and SC (B) cells. Cells were treated with 10 μ M Y27632 for 1 h at 37 °C under serum-free conditions and subsequently stained for actin (with rhodamine-conjugated phalloidin). In both TM and SC cells, Y-27632 caused significant decreases in staining for actin stress fibers. Magnification, $\times 400$ (Rao et al., 2001) [Permission needed].

Fig. 10.

Comparison of cell stiffness of different SC cell strains isolated from normal and glaucomatous donor eyes: (A) Mean and standard errors of Young's modulus (E , a measure of stiffness) of 6 normal and 5 glaucomatous non-confluent SC cell strains as measured with three different AFM tips. Modulus is determined from force–deformation curves using a modified Hertzian analysis; note the log scale on the vertical axis (Vargas-Pinto et al., 2013) (B) Results show a significant correlation between pore density and Young's modulus of the subcortical cytoskeleton, as measured by atomic force microscopy using a 10 μ m spherical tip. Bars represent 95% confidence intervals on pore density and Young's modulus. Light curves in panel B represent 95% confidence intervals on the slope of the general linear model regression (dark curve) (Overby et al., 2014) [Permission needed].

Fig. 11.

Elastic modulus of the TM as a function of donor age and disease status (glaucoma versus normal) as measured using a 2 μ m spherical AFM tip on (A) normal (*inset*: data plotted on a smaller y scale) and (B) glaucomatous samples (Last et al., 2011) [Permission needed].

Fig. 12.

Increases in substrate stiffness differentially modulated SC cell gene expression differentially between normal SC cells strains (blue) and glaucomatous SC cell strains (red). All expression levels were normalized to that on the softest gel. Mean $\pm$ SEM (Overby et al., 2014) [Permission needed].

Table 1

Relationship between cell stiffness, junctional permeability and aqueous humor outflow resistance (Zhou et al., 2012).

Agent	Outflow Resistance ¹	stiffness	Junctional Resistance ²
PGE1		↓	
colchicine		↓	?
cytochalasin D		↓	↓
dibutyl-AMP			↑
forskolin		↓	↑↓
isoproterenol			↑↓
latrunculin			?
Y-27632			
butanedione monoxime		↓	?
bradykinin		↑	↓
histamine			↓
thrombin			↓
lysophosphatidic acid			↑↓
sphingosine 1-phosphate			↑
dexamethasone		↑	
triamcinolone acetonide		↑	

Red arrows are measurements made on outflow pathway cells/tissues; blue arrows are measurements on vascular endothelial cells; black arrows are measurement on other cell types. Dash Indicates no effect.

¹ Outflow resistance Is the Inverse of outflow facility

² Junctional resistance as determined from electrical resistance, hydraulic conductivity or permeability to solutes