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Segmental outflow of aqueous humor in mouse and human

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Abstract

The main and only modifiable risk factor in glaucoma, the group of usually late onset progressive and irreversible blinding optic neuropathies, is elevated intraocular pressure (IOP). The increase in IOP is due to impeded aqueous humor (AH) outflow through the conventional pathway. The aberrant increased resistance at the trabecular meshwork (TM), the filter-like region in the anterior eye chamber is the major contributory factor in causing the impeded outflow. In normal as well as in glaucoma eyes the regions of the TM are divided into areas of high and low flow. The collector channels and distal outflow regions are now increasingly being recognized as potential players in contributing to impede AH outflow. Structural and molecular make-up contributing to the segmental blockage to outflow is likely to provide greater insight. Establishing segmental blockage to outflow in model systems of glaucoma such as the mouse in parallel to human eyes will expand our repertoire of tools for investigation. Further study into this area of interest has the potential to ultimately lead to the development of new therapeutics focused on lowering IOP by targeting the various components of segmental blockage of outflow in the TM and in the distal outflow region.

Keywords

Glaucoma; segmental outflow blockage; trabecular meshwork; aqueous humor; intraocular pressure; extracellular matrix; basement membrane

1. Introduction

Glaucoma refers to a group of irreversible blinding optic neuropathies affecting over 70 million individuals worldwide with primary open angle glaucoma (POAG) being the most

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Disclosure

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common form of the disease (Quigley, 1996; Quigley and Broman, 2006; Quigley, 2011; Tham et al., 2014). Elevated intraocular pressure (IOP) is the major and only modifiable risk factor associated with glaucoma (Morrison and Acott, 2003). Increased IOP is caused by impeded aqueous humor outflow. The generation of AH is usually found to undergo only slight variation in most cases of glaucoma.

Aqueous humor (AH) outflow follows two distinct routes, the conventional pathway and the “unconventional” pathway (Figure 1A). Comprised of the trabecular meshwork (TM), a filter-like structure, the conventional pathway is responsible for a vast majority of AH outflow (about 85% outflow) in normal eyes with only 4-27% of outflow (Bill and Phillips, 1971) following the “unconventional” or uveoscleral pathway (Swaminathan et al., 2014). The uveoscleral pathway does not contribute to the increase in outflow resistance in glaucoma, however, the current standard of care medications, prostaglandins, exert a majority of their effect through this pathway (Hejkal and Camras, 1999; Bito, 2001; Camras and Toris, 2008).

The TM is a major component in the conventional outflow pathway. The Schlemm’s canal (SC) is located adjacent to the juxtacanalicular TM (JCT) tissue surrounded by the inner wall of the SC. The location of the SC is often unclear in low magnification view of the anterior chamber (Figure 1A). The sponge-like composition of the TM structure consists of pseudoendothelial cells enveloped by connective tissue. Structurally, it can be divided into three major parts: the uveal meshwork, the corneoscleral meshwork, and the juxtacanalicular tissue (JCT) or meshwork (Figure 1B). Aqueous humor is typically produced by the ciliary processes and filters through the TM into the SC where it travels through collector channels into the episcleral veins (Figure 1B).

Early outflow physiologists, based experiments on radioactive tracer (Bill, 1965) and together with other experimental evidence recognized the existence of active as well as inactive AH flow regions in normal eyes and their implications for IOP regulation, (Dannheim and Barany, 1968; Bill, 1975; Bill, 1977). Segmental pigmentation was observed in the trabecular meshwork in human eyes (Johnson, 1989). These observations also led to the recognition of the existence of segmental variability in normal and glaucomatous eyes (Buller and Johnson, 1994). Studies with cationized ferritin (binds to negatively charged surfaces) as a tracer in human and monkey eyes also showed a pattern suggestive of possible segmental changes in aqueous outflow (de Kater et al., 1989; Epstein and Rohen, 1991). The AH outflow, solely based on tracer experiments in the normal eyes thus far have been described as segmental, divided into regions of low and high flow (Bill, 1965; Bill, 1975; Bill, 1977; Vranka et al., 2015a). The tracer experiments introduces an externality of the tracers and therefore there is no ultimate certainty that the findings of these experiments is reflective of natural in vivo outflow patterns. This remains the greatest limitations of current experimental approaches and findings derived from them. The advancement in imaging techniques (such as optical coherence tomography, magnetic resonance imaging, and adaptive optics) are fast emerging, which may enable comparisons of outflow in the natural state and further confirm if the experimental findings with tracers are indeed representative of natural outflow patterns. Although the low flow areas likely results in less fluid passage but direct experimental evidence has not linked them with obstructed flow and therefore low

flow regions are not synonymous with obstructed flow. The local anatomical differences such as iridial synechia, collapsed SC, differences in collector channel (CC) structure, corneal down-growth, or fused TM beams could partially explain differences in tracer without any 'obstruction' per se in the tissues that normally generate resistance.

The blockage to AH outflow in the glaucomatous eyes has been referred to as segmental due to the division of outflow into regions of high and low flow areas (segments) through the trabecular meshwork (Stamer and Acott, 2012; Acott et al., 2014; Swaminathan et al., 2014; Vranka et al., 2015b). With equal probability of similar flow through all TM regions, one interpretation of segmental low flow could be that flow is impeded or blocked in these regions. Very recently nonuniform AH outflow, has been elegantly demonstrated using various combinations of Q-tracker and Q-dot particles with different surface chemistry and charges in a large number of perfused glaucomatous human eyes (Vranka et al., 2015a). Alternative interpretations, for example, absence of segmental flow and anatomical differences elsewhere along the flow route are responsible for these observed flow differences, are possible. This is due to experimental limitations of tracer experiments (as explained above), which has established the concept of segmental outflow. Collector channels and various venous structures extends from the SC. Presence and simultaneously absence of lymphatic markers in the SC has been demonstrated in independent reports (Birke et al., 2010; Kizhatil et al., 2014; Truong et al., 2014; van der Merwe and Kidson, 2014). The SC is a unique vessel that selectively expresses markers of both blood and lymphatic vessels simultaneously (Kizhatil et al., 2014). The SC inner wall consists of a continuous endothelial monolayer that resides on a discontinuous basal lamina (Tripathi, 1968; Hamanaka et al., 1992). The vascular origin of the SC endothelia was controversial for some time (Rodrigues et al., 1980) before a number of new scientific investigations largely settled the questions. It was recognized a while ago (Ramos et al., 2007), much before the recent developmental studies (Kizhatil et al., 2014), that there are unusual features similar to lining of lymphatic capillaries present on the SC inner wall unlike blood capillaries or other vascular endothelia. Recent reports suggest active outflow regions possess more collector channels than inactive regions (Cha et al., 2016). However, molecular characterization of vessels in this region (similar to SC) remains to be done. A significant amount of evidence supports that a majority of outflow resistance is located around the inner wall endothelium of the SC, its basement membrane, and the JCT connective tissue (Stamer and Acott, 2012).

Although the general area of interest contributing to outflow resistance is known, the molecular make-up and exact mechanisms are poorly understood. Establishing parallel findings of segmental blockage to outflow in human eyes and model systems such as the mouse will provide a greater and wider latitude for these investigations. The contributing factors to segmental outflow blockage as well as the differences in model systems will provide greater insight into the potential mechanism surrounding outflow resistance and provide details on future therapeutic avenues.

2. Contributing factors to segmental outflow

There are various contributing factors that affect segmental outflow and result in the maintenance of intraocular pressure (IOP) (Goel et al., 2010; Stamer and Acott, 2012;

Chang et al., 2014). The major outflow resistance in eyes is contributed by the SC as well as the JCT regions. Together, the TM and SC cells are responsible for regulating resistance. Elevated IOP in glaucoma eyes is contributed by increased resistance in all these TM regions (Stamer, 2012; Stamer and Acott, 2012). The TM and SC inner wall cells may work in conjunction to regulate outflow resistance (Stamer, 2012; Stamer and Acott, 2012).

The mechanism termed “funneling” refers to aqueous humor converging or funneling through the JCT in order to exit through pores present on the inner wall (Johnson et al., 1992). This mechanism is dependent on two factors, the spacing present in the JCT as a result of the interaction between TM cells and SC inner wall cells as well as the number of pores present in the inner wall (Johnson et al., 1992; Overby et al., 2009). The funneling of the aqueous humor to exit the pores thus results in the generation of resistance (Stamer, 2012).

Pore density has also been demonstrated to impact overall resistance to outflow (Johnstone and Grant, 1973; Ethier et al., 1986; Allingham et al., 1992; Johnson et al., 1992). Perfusion studies in living monkey eyes with tracers (Sabanay et al., 2000; Sabanay et al., 2004) and in enucleated bovine eyes (Lu et al., 2008) further supported these ideas. It has been postulated that segmental flow might be a function of regional variations in funneling due to variable tethering of the inner wall to the JCT (Battista et al., 2008; Lu et al., 2008). It has been shown that glaucomatous eyes have lower pore density and impaired pore formation resulting in higher resistance (Johnson et al., 2002; Stamer, 2012; Braakman et al., 2015). Taken together, the generation of outflow resistance specifically through funneling is a major characteristic of segmental outflow. Outflow resistance along with extracellular matrix (ECM) changes, matrix metalloproteinases, cytoskeletal changes, and basement membrane (BM) changes are all potential contributory factors to segmental outflow (Acott et al., 2014). Tracer studies, which can be argued as non-physiological, have limitations. That the tracer patterns are truly representative of outflow patterns as they exist in vivo cannot be said with certainty. There is a possibility that these patterns can also be reflective of ex vivo eye or experimental conditions. However, the fluorescent microspheres used as a tracer with or without the rho kinase inhibitor Y-27632 that affects contractility showed a redistribution of flow patterns from a segmental or punctate distribution localized near collector channel ostia in control eyes to a more uniform pattern following Y-27632 treatment (Lu et al., 2008). The relative decrease in outflow resistance following Y-27632 or sham-treatment was found to correlate significantly with the fraction of inner wall length exhibiting separation from the JCT (Lu et al., 2008). Since these experiments utilized biological contractility with tracer and showed reversal to uniformity, these experimental results partly argue that the patterns do not depend on the properties of the tracer particles but are likely biological. Early radioactive tracer studies (Bill, 1965) will also be consistent with this line of reasoning. However, none of the current experiments offer an ultimate degree of certainty.

3. Extracellular matrix changes attributing to segmental outflow

The extracellular matrix (ECM) is an area of interest in consonance with fluctuations in outflow because of the numerous modifications it continuously undergoes. Various proteins and proteases present in the ECM are responsible for ECM turnover and stabilization during

fluctuations in pressure and resistance (Keller et al., 2008; Keller et al., 2009a; Keller et al., 2009b). Amongst these, some of the most extensively studied are the matrix metalloproteinases (MMPs).

MMPs are proteinases responsible for the degradation of the ECM for maintenance of overall homeostasis. These proteases are commonly produced as zymogens, inactive forms of the enzyme, and later are converted into the active form of the enzyme by various substrates. There are a total of 23 related MMPs as seen in the Degradome Database (Perez-Silva et al., 2016), a tool used to compare proteases as well as identify specific diseases associated with specific proteases. The presence of MMPs in the TM has been well documented and it has been demonstrated that the maintenance of outflow facility depends on ECM turnover conducted by these proteases (Bradley et al., 2001; Keller et al., 2009a). It has been determined that increasing levels of MMPs increases outflow. Inhibiting MMPs decreases outflow, however, a comprehensive investigation into MMPs including multiple enzyme knockouts and their effect on AH outflow system is yet to be performed. Mechanical stretching has also shown to increase the presence of MMPs such as MMP14 and MMP2 but decrease in the presence of MMP inhibitors such as tissue inhibitor of metalloproteinase 2 (TIMP2) (Bradley et al., 2001; Keller et al., 2009a). Taken together, MMPs are components of ECM that contribute to maintenance of outflow homeostasis. Further comprehensive investigation into activity levels of MMPs in the ECM of the TM will provide more insight into their role in ECM changes during changes in outflow.

Changes in specific proteins in the ECM seem to influence outflow as a whole. As stated before, blockage to AH outflow is segmental. A number of ECM proteins for example, cochlin, matrix gla protein, type V collagen, MMP-1, and MMP-10 have been found to have an altered pattern of gene expression in glaucoma (Vranka et al., 2015a). Parallel to segmental outflow blockage, segmental cochlin deposits have been found in human POAG (Bhattacharya et al., 2005b; Goel et al., 2012) as well as the DBA/2J mouse model of glaucoma (Bhattacharya et al., 2005a). The observed cochlin deposit regions are also associated with segmental mucopolysaccharide deposits (Bhattacharya et al., 2005b). Cochlin mechanosensing transduced via mechanosensitive TREK-1 (Goel et al., 2011) and potentially via other TM mechanosensitive channels (Tran et al., 2014) are involved in homeostasis of AH outflow. The TM mechanosensitive channels activities (Grant et al., 2013) and their modulation, regulates cell shape and motility leading to AH outflow regulation (Goel et al., 2011; Goel et al., 2012). Large diurnal fluctuations have been found in glaucomatous eyes (Asrani et al., 2000). Thus homeostatic mechanisms exist in the normal eye that regulate the IOP fluctuations, which undergoes a dysregulation in the pathologic state. Mechanosensing in the TM has been conjectured to detect shear stress as a result of changes in AH outflow (Goel et al., 2010; Goel et al., 2011; Goel et al., 2012). Mechanosensing is common to other fluid flow regimes in the body including the blood and kidneys. Although the eye has a substantially lower fluid flow rate than either of these systems, similar mechanotransducing molecules are present in these different systems. An important factor for mechanosensing in the hemodynamic system is von Willebrand factor A (vWFA), a mechanosensor that senses shear stress allowing for various protein interactions to initiate or take place (Stockschlaeder et al., 2014). Cochlin has two distinct von Willebrand Factor A-like (vWFA) domains (Bhattacharya et al., 2005b; Bhattacharya, 2006).

The distinct multimerization of vWFA in response to different degrees of shear stress (termed mechanosensing) has been well characterized (Shankaran et al., 2003; Singh et al., 2009; Dayananda et al., 2010; Madabhushi et al., 2012; Gogia et al., 2015; Gogia and Neelamegham, 2015). Different multimers of vWFA interact with different protein partners and brings about different biological changes and/or consequences for hemodynamic systems. In parallel, cochlin undergoes multimerization under shear stress rendering it a potential mechanosensor in the TM similar to vWFA in the hemodynamic system. Soluble phase mechanosensing by cochlin is conjectured to be transmitted to cell surface-bound mechanotransducers (Bhattacharya et al., 2005b; Goel et al., 2011; Goel et al., 2012), which is consistent with the observation that cells of the JCT region are able to detect an elevation in IOP as mechanical stretch (Johnstone and Grant, 1973). TM cells in conjunction with SC cells are able to respond to shear stress by changes in cell signaling, gene expression, ECM turnover, and cytoskeletal reorganization in order to correct the increase in IOP (Stamer and Acott, 2012). Further investigation into segmental deposits of cochlin, mucopolysaccharides and their co-relation with flow patterns using different modalities will provide new insights into their potential role in regulation of outflow.

Another major protein in the ECM of the TM is secreted protein acidic and rich in cysteine (SPARC), a matricellular glycoprotein that is important in mediating ECM organization. This protein has been identified as the most highly expressed gene product in the TM (Haddadin et al., 2009; Swaminathan et al., 2013). It has been demonstrated that SPARC knockout mice exhibit a 15-20% decrease in IOP (Haddadin et al., 2009) and also maintained a more uniformed outflow pattern as mentioned previously (Swaminathan et al., 2013). Both these proteins are significant contributors to the maintenance of the outflow pathway in one form or another.

Interestingly, another protein found to have segmental distribution is versican, a large proteoglycan consisting of various glycosaminoglycans (GAGs) side chains. This distribution correlates inversely with outflow rates (Keller et al., 2008; Keller et al., 2009a; Vranka et al., 2015b). GAGs themselves have been suggested to play a role in outflow resistance as far back as the 1950s. Hyaluronan, another protein in the ECM, has been shown to form long GAG chains and also exert influence on outflow resistance (Keller et al., 2009a; Keller et al., 2012). It is also important to note that simultaneous hypo and hyper glycosylation is found in glaucomatous TM compared to controls (Sienkiewicz et al., 2014). However, a comprehensive analyses of glycosylation states of proteoglycans of the TM remains to be done. The alteration of glycosylation states will influence both biomaterial properties and mechanosensing experienced by the cells surrounded by the ECM harboring significant amounts of proteoglycans. TM stiffness (elastic modulus) measurements have demonstrated normal TM to possesses lower stiffness than glaucomatous TM (Last et al., 2011). Recent investigations suggest low flow regions to have increased TM stiffness compared to high flow regions (Russell and Johnson, 2012; Morgan et al., 2015; Vranka et al., 2015a). The molecular changes underlie the increased resistance of TM (Morgan et al., 2015).

The specific ECM of high and low flow areas is yet to be understood. Polycystin-1 (PC1), an integral membrane protein activated by fluid flow in the kidney (Low et al., 2006) generates

a truncated part, which enables the entry of the degraded part in the cells and in the nucleus resulting in transcriptional regulation of cytoskeleton and cell dynamics. Thus PC1 in kidney represents a shear sensing mechanism which links external cellular environmental changes (ECM degradation) as a consequence of shear stress with transcriptional regulation. The PC1 system in kidneys demonstrates the complexity associated with the ECM changes in different fluid flow regimes and their involvement in a complex and dynamic regulatory network. No such regulation has been identified in the TM as yet, however, finding existence of such a regulatory network cannot be ruled out.

4. Basement membranes and their components

The basement membrane (BM) is a thin but dense structure outside the cells and is visible under electron microscopes. Isolation and characterization of the BM has found that it is composed of various protein fibers and GAGs that function to separate epithelium from underlying tissue. The term BM was coined in 1938 by electron microscopists. However, the BM is not a membrane in the classical definition as it lacks a lipid bilayer (that defines classical membranes) and resides outside cellular layers. Although the BM of all organs or tissues looks similar under the electron microscope, their composition is vastly different (Kalluri, 2003). Recent rigorous proteomic studies have revealed the BM of other ocular regions such as the inner plexiform layer (IPL) undergoes a substantial thickening during aging (Candiello et al., 2010). Parallel changes have been found in a mouse model presenting retinal abnormalities (Hu et al., 2010). Retinal BM has been found to undergo substantial changes in disease states such as diabetes and retinal ectopias (Hu et al., 2010; To et al., 2013). The BM of the TM has yet to be subjected to such systematic rigorous studies. Our initial imaging and unbiased proteomic studies suggest significant changes in the BM of the TM in glaucoma which is beyond age-related changes (Bhattacharya, 2015). The BM environment affects the isolated TM cell stiffness (Garland et al., 2014). Changes in the BM have been also found in retinal vasculature, vitreous, and lens in other ocular diseases (Uechi et al., 2014; Halfter et al., 2015).

The BM is continuously undergoing modifications and changes in order to maintain a healthy environment. In the eye, AH passes through the BM of the SC endothelial cells before entering the SC itself. It is emerging that the BM is a dynamic component as well as an integral component for maintenance of vascular homeostasis. The state of degradation of the BM renders it into two states: (1) the provisional matrix which is supportive of vessel growth or (2) the assembled matrix, which is vessel growth arresting for the surrounding vicinity (Kalluri, 2003). As stated above, the BM is greatly affected by aging and tends to increase in thickness (Candiello et al., 2010). Depending on the state of degradation of the BM, the proteins it may release are, pro-angiogenic or anti-angiogenic peptides for example canstatin or tumstatin of type IV collagen (a major BM component), factors necessary for vascular homeostasis. BM proteins such as collagens and actins have been determined to be important regulators in the BM as well as the ECM space. Within the ECM, several collagen genes, COL1A1, COL4A2, COL6A1, COL6A2, and COL16A1 were found at a 1.5-fold higher in high flow regions compared to low flow regions (Vranka et al., 2015a). Type IV Collagen expression has also been demonstrated to increase in the TM of human cadaveric eyes as a result of SPARC overexpression (Oh et al., 2013). Other genes have been found to

increase expression after application of stretch or pressure such as Collagen 14, NELL2, and Endoglin. NELL2 is a glycoprotein containing a vWFC domain as well as epidermal growth factor (EGF) domains and has been shown to play roles in vascular homeostasis. All of these factors play important roles in the maintenance of the trabecular meshwork BM and ECM (Acott et al., 2014) as well as the distal outflow region.

Our recent quantitative proteomic and Western analyses have also found differences in degraded BM proteins with angiogenic properties such as fragments of type IV collagen (endostatin, tumstatin) between high and low flow TM regions (Bhattacharya, 2015). In general we find a lack of degradation of BM proteins such as type IV collagen in glaucoma. A lack of BM degradation is associated with reduced or absent vessel sprouting (Kalluri, 2003). Podosomes are unique structures composed of actin filaments among other structural molecules that control degradation of the ECM and promote cell migration (Warren and Iruela-Arispe, 2014). Interestingly, a reduction of podosome/invadopodia like structures (PILS) has been identified in low outflow areas in TM cells and glaucomatous mice (Keller et al., 2009b) (Aga et al., 2008). The new vessel sprouting correlates with podosome rosette formation (Warren and Iruela-Arispe, 2014) in other organs and tissue. Glaucomatous TM (Keller et al., 2009b) and glaucomatous mice (Sh3pxd2b mutant) (Mao et al., 2011) lack podosome formation, consistent with our hypothesis that this pathology arises in part from altered vessel formation.

5. Collector channels and distal outflow

Topical steroids elevated IOP even after a 360° trabeculectomy, which presumably removed all of the TM resistance. Even this drastic technique is not able to completely abolish resistance to AH outflow altogether. The reduction or lack of collector channels in regions across 360° has recurrently emerged repetitively from observations in the clinical setting (Johnstone and Grant, 1973; Grover et al., 2014; Grover et al., 2015). From clinical imaging studies regional variability in ostia and collector channels have been appreciated, which has led to the call for advancement in OCT imaging software (Kagemann et al., 2010; Kagemann et al., 2012). In a recent report, greater frequency of collector channels has been found associated with areas of active outflow. These channels have been demonstrated to reside primarily in the nasal or inferior quadrants making them an ideal route to assist in lowering outflow resistance (Cha et al., 2016). Areas of high flow have also been observed to concentrate more around collector channels (Battista et al., 2008; Lu et al., 2011). Pigmented regions as well as giant vacuoles have been demonstrated to be located around areas with collector channels potentially signaling to a larger pressure gradient (Parc et al., 2000). Labeling experiments have shown that areas of human TM located under collector channels have preference towards increased fluid flow possibly due to an expanded JCT that may enable less resistance to fluid flow (Hann and Fautsch, 2009). The expansion of the JCT may result in the loss of the “funneling” effect due to the lack of interaction between the SC and BM as well as ECM (Johnson et al., 1992). Future research will provide insight to whether altered ECM protein expression, BM degradation, turnover, stability, altered dimension, and frequency of collector channels in the pathologic state are related to each other as part of a general mechanism that remains to be understood.

6. Mouse models for glaucoma pertaining to segmental outflow

Segmental outflow and blockage to outflow has been investigated using various model systems including human, mouse, monkey, and bovine eyes (Sabanay et al., 2000; Ethier and Chan, 2001; Hann et al., 2005; Battista et al., 2008; Swaminathan et al., 2013). While these studies have been advantageous in furthering the view of segmental outflow and segmental blockage, there are still questions left unanswered. Further investigation using various species of these models are needed to further elucidate all factors that lead to segmental outflow.

As stated above, one of the earliest investigations for uniformity in outflow was in the secreted protein acidic and rich in cysteine (SPARC) knockout mice and their wild type counterparts. The morphology of high flow and low flow regions was extensively studied in these mice (SPARC-null mice and wild type mice) demonstrating a greater separation in TM cells in high flow regions (Haddadin et al., 2009; Oh et al., 2013; Swaminathan et al., 2013).

The DBA/2J mouse develops elevated IOP in a spontaneous manner similar to the increase in IOP in human glaucoma (John et al., 1997; John et al., 1998; John et al., 1999; Libby et al., 2005). Non-invasive anterior segment imaging (Phoenix Research Laboratory, Pleasanton, CA), optical coherence tomography (Wang et al., 2015b), slit lamp ophthalmoscopic examination, and IOP measurement has found about 5.3 percent of total mice (about 18-24 percent of hypertensive mice between 8-9 months) have none to very little pigment dispersion as well as an open angle with elevated IOP (Wang et al., 2015a). For the time span of about 20 days around 8-9 months of age, we have termed the DBA/2J mice with elevated IOP but little or no pigmentary dispersion as “pure ocular hypertensive”. This subset of DBA/2J may provide a valuable tool to investigate regions of high and low flow in the TM along with details of turnover and stability of ECM, BM, collector channels, and distal outflow system. An added benefit of DBA/2J mice is the availability of a genetic matched control, the DBA/2-Gpnmb⁺-Sj/J mouse, which lacks the feature of spontaneous IOP elevation or characteristic neuropathy of the DBA/2J mouse (Howell et al., 2007). The analyses of PERG (Porciatti et al., 2010) and lipids (Wang et al., 2015a) clearly point out functional and compositional differences between the DBA/2J and the DBA/2-Gpnmb⁺-Sj/J mouse maintaining that they are genetically matched but not genetically identical, nevertheless they provide an additional control that closely resemble each other (Chou et al., 2015; Wang et al., 2015a).

Investigation into the murine outflow pathways has been performed using various techniques including, histological cross sections, wholemount immunostaining, and vascular corrosion casts. Although all of these techniques have provided valuable information, the spherical shape of the eye, causes issues in visualizing a true segmental outflow pattern. To overcome this barrier, individuals have focused on visualizing the entire outflow pathway by wholemount immunostaining and three-dimensional imaging. This type of imaging allows for the visualization of the outflow pathway in its entirety from perilimbal vasculature, collector channels, aqueous veins, and Schlemm’s canal (van der Merwe and Kidson, 2010; van der Merwe and Kidson, 2014).

Our recent investigation into the segmental outflow pathways have shown profound differences in the distribution of high flow and low flow pathways in 8 month old (IOP 18 mm of Hg) DBA/2J mice compared to that of DBA/2-Gpnmb+Sj/J mice (Figure 1C). The en face images with tracers were obtained following established protocols in a previously published study (Swaminathan et al., 2013). We would like to emphasize the use of “pure ocular hypertensive” DBA/2J mice for these experiments, as noted above. The reduction in high flow regions observed in 8 months old DBA/2J mice (up to about 11-12 months) is expected as this is the common age in which an increase in IOP is observed. In DBA/2-Gpnmb+Sj/J mice TM regions are much more uniform with respect to regional differences in flow distribution throughout the ages: 3-12 months that we have investigated thus far. These findings render DBA/2J and DBA/2-Gpnmb+Sj/J mice as an additional tool to further investigate segmental outflow and distal flow in these model systems for comparison and gaining insight into these aspects (Wang et al., 2015a). The availability of model systems enables greater control of environment, food, age, gender, and treatment with medications opening up greater possibilities in investigation which are not possible to achieve with human anterior eye segments.

7. Conclusion

Segmental pattern of outflow through the conventional pathway remains an area of interest as impeded outflow leads to an increase in IOP, a major risk factor in glaucoma. Could segmental blockage to outflow result in an overall impeded outflow in glaucoma, future research may provide insight into this question. Although there has been substantial work leading up to identification of the molecular components responsible for outflow in various models, a great deal remains to be elucidated. Further avenues of investigation may open up through further investigation of distinct segmental flow patterns and segmental blockage to outflow characterization in the murine model, specifically in glaucoma models. The segmental outflow has thus far been studied using tracers. These methods have fundamental limitations to address the question of segmental outflow. Science is always the present verification of natural facts without ultimate certainty. There is no ultimate certainty that tracer patterns are truly representative of outflow patterns as they exist in vivo. They could also be reflective of circumstances that exist in the ex vivo eye or under experimental conditions. Current methods do not elucidate whether the observed segmental patterns depend on the properties of the tracer particles. A future development would be non-invasive use of optical coherence tomography and adaptive optics to study in living eyes to validate these findings. These noninvasive approaches are currently under advancement at a rapid pace and may be ready for such interrogation in future.

The overall mechanism of segmental outflow is a dynamic event and the various contributing factors from the ECM to the BM make it a perpetual occurrence. Taken together, there are various areas that can be further elucidated as potential therapeutic avenues to overcome impeded aqueous humor outflow.

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- Elevated intraocular pressure is a characteristic of glaucoma.
- Segmental outflow consists of high and low outflow areas.
- There are several factors that contribute to segmental outflow.
- Segmental outflow has been found in the DBA/2J mouse model.

Figure 1.

Schematic depiction of segmental outflow in the anterior segment **A)** The ciliary processes (CB) produces aqueous humor that flows into the anterior chamber and passes through two distinct routes. The convention pathway consists of the aqueous humor passing through the trabecular meshwork (TM) into the Schlemm's canal (SC). The unconventional pathway consists of the aqueous humor bypassing either structure. **B)** The trabecular meshwork (TM) consists of layers of cells in a filter-like conformation. The aqueous humor passes through the TM into the juxtacanalicular connective tissue (JCT) region then passes the inner wall (IW) of the Schlemm's canal (SC) into collector channels (CC) and finally into episcleral veins (EV). Red arrows and their relative thickness signify the area of increasing resistance. **C)** Q-tracker 655 injected into the anterior chamber of 8 month old DBA/2J mice and their genetic counterpart DBA/2J-Gpnmb+/SjJ mice to depict the high flow as well as low flow areas associated with segmental outflow. At the time of these measurements IOP in DBA/2J and DBA/2-Gpnmb+/SjJ mice eyes were 18 ± 1 and 15 ± 1 mg Hg respectively. Experiments were performed on $n=30$ ($n=15$ male and $n=15$ females) animals for each mice strain and representative images have been presented. A notable difference is seen between the distributions of these regions in these mouse models. Blockage to outflow in the DBA/2J mice appear to be segmental consistent with findings in human glaucoma eyes.