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Recent Developments in Understanding the Role of Aqueous Humor Outflow in Normal and Primary Open Angle Glaucoma

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Abstract

Primary open angle glaucoma (POAG) is the second leading cause of blindness in the world's rapidly aging population. POAG is characterized by progressive degeneration of neural structures in the posterior segment, often associated with a concomitant elevation of intraocular pressure. Changes in IOP are believed to be caused by a disruption in the normal outflow of aqueous humor. This article reviews recent research associated with normal and POAG aqueous humor outflow. Novel findings elucidating biochemical and pathological changes in the ocular tissues affected in POAG are presented. Stem cell research, identification of lymphatic markers, and increased use of mouse models give researchers exciting new tools to understand aqueous humor outflow, changes associated with POAG and identify underlying causes of the disease.

Keywords

Primary open angle glaucoma (POAG); anterior segment; aqueous outflow; trabecular meshwork

Introduction

By 2020, primary open angle glaucoma (POAG), the most common form of glaucoma, will lead to irreversible blindness in approximately 11.1 million people.[1] The most prevalent risk factor for POAG is elevated intraocular pressure (IOP). If left untreated, elevated IOP will result in progressive loss of retinal ganglion cells and axons in the optic nerve leading to irreversible vision loss. Normal IOP is the result of a balance between aqueous humor (AH) secretion by the ciliary body and its removal from the anterior chamber. Based on this, a high IOP may be caused by either excess production or reduced outflow. It is now well

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Compliance with Ethics Guidelines

Conflict of Interest

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accepted that high IOP in POAG is mainly due to decreased outflow and not increased secretion[2]. At present, all treatments for POAG, whether surgical or pharmaceutical are aimed at reducing IOP. Early detection and compliant IOP reduction are proven methods to slow vision loss caused by the disease.

Anterior Segment Structure and Aqueous Outflow Pathways

In the anterior segment of the eye, the ciliary body is responsible for the secretion of AH which nourishes the cornea and other tissues of the anterior segment. AH exits the eye via two pathways – the conventional outflow pathway which is responsible for filtering 75% of the aqueous or the uveoscleral pathway.[3] In the conventional outflow pathway, AH moves from the anterior chamber through the trabecular meshwork (TM) and into Schlemm's Canal (SC) through intracellular pores found in SC endothelial cell giant vacuoles (intracellular or “I” pores) and pores between the endothelial cells (border or “B” pores) [4]. About 25-30 collector channels (CC), which originate from the outer walls of SC, helps drain the AH into the intrascleral venous system[5, 6]. The flow of AH through CCs into aqueous veins has been found to be pulsatile and in sync with cardiac pulse, blinking and eye movement [7-10]. Under elevated IOP conditions, as found in POAG, the juxtacanalicular tissue (JCT) region between the TM and SC, expands while SC giant vacuoles, pores and CC numbers increase [10-12]. The increased number of CCs under elevated IOP suggests a compensatory mechanism for dealing with rapid changes in IOP [13]. One biologic sensor of increasing pressure may be the presence of a glycocalyx (glycoprotein-polysaccharide covering) on SC and CCs in the human conventional outflow pathway. Yang et al identified a glycocalyx structure which senses shear stress in vascular endothelium filling most pores in SC cells with giant vacuoles [14]. It is possible that the glycocalyx in the outflow pathway helps to regulate AH flow through the conventional outflow pathway by detecting shear stress changes in SC and CC endothelial cells [15].

The unconventional or uveoscleral pathway is an alternate pathway of AH egress. AH leaving the anterior chamber by the uveoscleral pathway passes through the face of the ciliary muscle, the iris root through ciliary muscle bundles, the supraciliary and suprachoroidal spaces and ultimately out of the sclera [16]. In humans, different investigations have shown that depending on circumstances, the uveoscleral pathway filters between 12-54% of the total aqueous outflow [16]. Future investigations of this pathway and its modulators could be helpful in identifying therapeutic targets in order to augment the uveoscleral pathway during POAG.

In POAG, the anterior segment anatomy and AH flow is altered. As IOP increases, a decrease in TM cellularity is observed along with trabecular beam fusion [17, 18]. Both “I” and “B” pore densities decrease in POAG eyes [19]. SC cross-sectional area and length decrease and adhesion areas may be present between the inner wall and outer wall at or near CCs, inhibiting or occluding AH flow [5, 18, 20,21]. POAG eyes perfused at high pressures are less responsive than normal eyes showing decreased CC orifice area, SC volume, number of CCs and increased number of CC occlusions [13]. The uveoscleral outflow pathway also loses filtration area due to the increase in ECM between the ciliary muscle bundles [22]. Pulsatile flow abnormalities are also present in glaucoma resulting in slower

flow and a lack of normal pulsatile response when pressure is placed on the eye [8]. All these changes in the conventional and unconventional pathways significantly restrict normal AH outflow.

Extracellular Matrix (ECM) and Outflow

The ECM of the conventional outflow pathway is a dynamic substrate for cell attachment and signaling. It is responsive to changes in pressure, flow and shear stretch in the conventional outflow pathway. The ECM is continually exposed to multiple stresses arising mainly from fluid flow. The ECM responds to these forces by remodeling, up-regulating and/or down-regulating its respective components such as fibronectin, collagens, elastin, laminins and fibrillin. In addition, the ECM of the TM is also comprised of proteoglycans and glycosaminoglycans which appear to be important for modulating outflow resistance [23]. Areas in the TM that contain high levels of the proteoglycan versican correspond to low flow regions while areas with reduced versican levels exhibit higher flow [24]. Experimental destruction of GAG components (through inhibition of biosynthesis, sulfation or by using GAG-degrading enzymes) result in reduced outflow resistance and increased outflow facility [23, 25]. In POAG, the distribution of glycosaminoglycans is altered. For example, in the JCT region of the TM, hyaluronic acid is decreased while chondroitin sulfate levels are increased, providing higher resistance and thus increasing the potential for developing an elevated IOP [26]. Therefore, current studies underscore the importance of GAGs by suggesting a strong link between GAG expression and outflow regulation.

Role of ECM turnover in regulation of outflow

The ECM is under constant remodeling, through regulation of biosynthesis as well as degradation. Matrix metalloproteases (MMPs) which are responsible for ECM remodeling have been shown to have a role in outflow resistance. Eyes when perfused with MMPs showed increased outflow, whereas inhibition of MMP activity by tissue inhibitor of metalloprotease 2 (TIMP-2), caused a decrease in outflow [27]. Caveolins are also involved in matrix turnover and recycling. By reducing CAV1 and CAV2 protein levels, an increase in fibronectin along with actin stress fiber formation was reported, indicating that disruption of normal caveolin expression influences ECM content. [28] Genetically, CAV1 and CAV 2 have been linked to POAG through genome-wide association studies (GWAS) [29].

Matricellular proteins, which are non-structural regulatory proteins associated with the ECM, facilitate ECM deposition in the conventional outflow pathway. Overexpression of the matricellular protein SPARC (secreted protein acidic and rich in cysteine) increases IOP while increasing ECM deposition in the JCT of the TM [30]. The ECM remodeling role of SPARC has been shown to be reversed in SPARC-null mice. When these mice are stimulated with transforming growth factor-beta 2 (TGF- β 2), a potent modulator of ECM proteins, expression of collagen IV and fibronectin is markedly reduced when compared to the wild type mice. [31] Matricellular protein thrombospondin-1 activates TGF- β 2, a major player in modulating the outflow facility [32]. But not all matricellular proteins have an effect on outflow. Osteopontin is expressed in both normal and POAG AH, with reduced levels found in POAG. However, change in osteopontin expression did not have any

observable effect on IOP[33, 34]. Further investigation into individual extracellular matrix proteins and their role in AH outflow regulation is warranted.

Signaling molecules and their associated pathways play critical roles in modulating outflow resistance and IOP by regulating ECM synthesis. Known modulators of ECM components are TGF- β 1, TGF- β 2, bone morphogenetic proteins (BMP)-7, and Smad 7 which inhibits TGF- β 2 signaling [35]. The Wnt signaling pathway has been investigated as a key pathway in IOP regulation. Induction of miR-29 family via Wnt signaling regulates extracellular matrix protein and ECM synthesis in the TM [36]. Adenosine monophosphate-activated protein kinase (AMPK), a serine/threonine protein kinase, interacts with SPARC. When activated, AMPK inhibits RhoA interaction with RhoA-associated protein kinase ROCK resulting in a decrease of ECM and actin stress fibers. In addition, AMPK α 2-null mice display higher IOP than their WT controls thereby linking the ECM modulating role of AMPK to IOP[37]. Targeting the molecules involved in regulating ECM homeostasis may be an important therapeutic approach in the future.

Mechanotransduction and tissue stiffness

The cells that reside on and within the ECM matrix in the aqueous outflow pathways are critical to outflow regulation. The cells, which detect shear stress and pressure resulting mainly from fluid flow, respond to mechanical stimuli by mechanotransduction, a process that converts mechanical stimuli into biological signals. SC cells are highly sensitive to pressure change with giant vacuoles increasing in number and volume with rising IOP. In SC monolayer culture, biomechanical stress has been found to trigger the formation of "I" and "B" pores, with "B" pores being the most prevalent [38]. Giant vacuole and pore formation in SC cells are important fluid flow regulators, acting together with the ECM in the JCT as the primary locus of increased resistance to AH outflow in POAG[39].

Another set of molecules active in the regulation of the ECM are the integrins. Integrins are a group of transmembrane proteins that link the actin cytoskeleton with the ECM. Integrins act in concert with the cell to detect biomechanical stretch. Integrins have important roles in cell differentiation, migration, development and survival [40]. Integrins are composed of various combinations of α and β subunits [41]. Integrins are now known to be part of a signaling complex known as the "integrin adhesome" which currently is comprised of approximately 180 components [42]. In the TM eleven integrins have been found [43, 44]. The α 4 β 1 integrin is heavily involved in regulating outflow facility. In anterior segments from monkeys perfused with the Heparin II (Hep II) domain of fibronectin, the outflow facility was increased. The α 4 β 1 integrin and collagen were found to be important to outflow changes along with the disruption of the actin cytoskeleton caused by Hep II[45]. β 1/ β 3 integrins are involved in a stiffening response as displayed by cross-linked actin networks (CLANS) found in human TM cells treated with dexamethasone[46]. If stiffening could be regulated through integrin cross-talk, these molecules may be targets for treatments to regulate abnormal aqueous outflow. Integrins also detect differences in substrates. TM monolayers cultured on fibronectin and collagen IV use α 5 β 1 to detect fibronectin and α 1/ α 2/ β 1 to detect collagen IV. If Hep II is added to TM cells cultured on fibronectin, the actin cytoskeleton remained normal whereas TM cells cultured on collagen IV showed

disorganized actin. The ability to specifically enhance the integrins which interact with actin, can influence outflow facility since actin and RhoA signaling pathways play important roles in regulating outflow facility [47].

In POAG, changes in the biochemical structure of the TM ECM along with reduced pore formation in the SC inner wall coincide with the finding that the TM and SC in POAG eyes are significantly stiffer than normal eyes [48]. With increased substrate stiffness, primary cultures of POAG SC cells had a much greater stiffening response than normal SC cells. POAG cells were found to have two genes modulated by substrate stiffness that were more highly expressed than in normal cells: connective tissue growth factor (CTGF) and decorin (DCN) [49]. CTGF in a mouse model has been linked to modification of the cytoskeleton resulting in glaucoma[50, 51]. Additionally, SC cells isolated from POAG eyes were treated with agents that change outflow and studied using optical magnetic twisting cytometry and traction force microscopy. SC cell responses were found to vary in contractile scope and in cell stiffness which suggested that SC cells may be a responsive modulator in the outflow pathway [52]. Modulators of the biomechanical properties of SC cells are thus important in regulation of IOP. Previous work with eNOS/NO has shown it to be a modulator of IOP in mice [53]. Changes in endothelial nitric oxide synthase (eNOS) in aged porcine angular plexus cells have shown a diminished mechanotransduction response when exposed to shear stress which may enhance the risk for the development of POAG [54]. Understanding the roles of various molecules that modify the biomechanical properties of SC cells and the ECM are important since they may assist in restoring the reduced mechanotransduction responses caused by POAG.

Stem Cells, Mesenchymal Cells and the TM

Stem cells are precursor cells which act as a biological reserve which can differentiate along pluripotent lines of cell lineage. TM cells normally do not proliferate and TM cell loss has been reported with age and in POAG [55]. When stem cell-like cells were isolated from human TM, they were found to express stem cell markers, differentiate into various cell types as well as being able to differentiate into phagocytic TM cells[56].Adult stem cells have also been identified in the region of Schwalbe's line adjacent to Descemet's membrane in primate eyes. These cells retained BrdU and displayed OCT 4 immunoreactivity [57]. A similar region was observed in human TM by Acott et al who found 4-fold replication of cells in the anterior TM adjacent to Schwalbe's line after treatment with laser trabeculoplasty [58]. Additionally, induced murine pluripotent stem cells (iPSCs) co-cultured with human TM cells developed a TM cell phenotype [59]. These exciting new findings open the door for new studies to further characterize TM stem cell based properties.

The epithelial to mesenchymal transition (EMT)-like transformation is a stem cell like process that is associated with other pathological conditions such as cancer metastasis, fibrosis and inflammation [60]. An investigation using primate TM cells found mesenchymal markers and showed increased motility with ECM changes [61]. When null mice for the focal adhesion-associated protein paxillin were examined, the ECM-JNK-paxillin pathway was inactivated and cells did not display EMT-like changes, increased levels of fibronectin or TM cell motility [61]. This suggests that if an EMT-like

transformation occurs in the TM, increased levels of fibronectin and TM cell motility may contribute to changes in the outflow pathway in POAG [61]. Further characterization of TM EMT and stem cell properties will be important in furthering our understanding of the disease process as well as providing new opportunities to explore cell-based treatment strategies for POAG.

The Mouse and Its Role in POAG Research

Use of mice as models to study normal and diseased states has increased exponentially in the last ten years in all areas of biomedical research. Mice are inexpensive to maintain and most importantly relatively easy to genetically manipulate. Anatomically the mouse conventional outflow pathway is similar to humans containing a lamellated TM and continuous SC. The outer wall of mouse SC also contains CCs that drain fluid into the intrascleral vasculature. In mice, the uveoscleral pathway has been reported to vary from 20.5% in BALB/cJ mice to 66% in C57BL/6 mice with washout not being present [62]. Recent work in mice has also demonstrated ciliary muscle connections with a 3D elastic net tethering it to the TM and SC. Treatment with pilocarpine resulted in a significant increase in outflow facility similar to the response in non-human primates and humans [51]. Pilocarpine treated mice also caused SC lumen dilation and prevented SC collapse suggesting a primary role of the ciliary muscle is to prevent the collapse of SC to maintain patency of the outflow pathway [63]. Additionally, variation in IOP between mouse strains appears to be due to changes in conventional outflow facility [64]. Investigators have also developed new techniques to accurately measure outflow in mouse models, thus providing an opportunity to define outflow parameters under experimental conditions [62, 65]. Together, these new studies have established the mouse as a viable model of choice for studying the aqueous outflow pathways.

Mice used most often in POAG research include but are not limited to the DBA-2J, a spontaneous model of pigmentary glaucoma [66]; transgenic models Colla1(r/r) with mutations in the Collagen 1a gene leading to increased expression of collagen 1 [67], a Tyr437His point mutation to the MYOC gene causing misfolding of the myocilin protein resulting in stress to the endoplasmic reticulum [68] and a CTGF-overexpressing mouse model that leads to elevation of IOP [50]. Manipulation of the outflow system using lasers, beads, hypertonic saline injected into the episcleral veins and viral vector delivery of various proteins have been shown to induce glaucoma and are used in mice and rats. Recent work with ocular hypertensive mice overexpressing bone morphogenic protein 2 (BMP2) documented changes in the aqueous outflow pathway over time using SD-OCT imaging. In these mice, changes in the patency of the angle, TM thickening, retinal ganglion cell, and axon loss were observed [69].

Lymphatic Markers in the Anterior Segment

Recent discoveries of lymphatic markers in the eye have opened a new avenue of research in the anterior segment. Lymphatic regulators of development and function modulate normal SC endothelial functions but become dysregulated during POAG. Similarities between SC endothelial cells and lymphatic endothelial cells have been known to exist for some time [70]. In 2009, a uveal lymphatic system was discovered by injecting radiolabeled albumin

into sheep eyes and tracking its presence to head and neck lymph nodes [71]. With the advent of numerous specific lymphatic markers, SC in Prox1-GFP mice was found to acquire lymphatic identity by upregulation of prospero/homeobox protein 1 (PROX1). PROX1 is a known regulator of lymphatic development. In addition to PROX1, SC was found to express lymphatic valve markers forkhead box protein C2 (FOXC2) and integrin $\alpha 9$, and displayed continuous vascular endothelial-cadherin junctions with a basement membrane – a characteristic of collecting lymphatics. This is an interesting finding as a small population of collector channels have been reported to have “flap-like” structures in both humans and primates [6, 7]. The authors also found that PROX1 expression was decreased in pathologic conditions and in dysfunctional SCs [72]. In a separate study, lymphatic endothelial markers were tested in zebrafish, mice and human SC. SC expressed platelet-endothelial cell adhesion molecule-1 (PECAM-1), PROX1, and lymphangiogenic receptor tyrosine kinase VEGFR-3. Mice with VEGF-3 deletion which were treated with function blocking antibodies demonstrated lymphangiogenic growth factor VEGF-C and its receptor VEGF-3 are necessary for the development of SC. These factors have been used to treat buildup of lymph or lymphedema in other diseases raising the intriguing possibility of using them to decrease buildup of AH in SC [73].

Future Work in POAG

The IOP in normal eyes remains at a fairly constant rate over a lifetime. When challenged under experimental conditions by using increased inflow to elevate IOP, the TM is able to “reset” and return to its former IOP or “homeostatic” state [74, 75]. The complete molecular events that are involved in homeostatic regulation of outflow is not fully understood. However, recent studies have revealed new information regarding the composition and function of the various functional compartments of the outflow pathway. The use of mouse models to investigate outflow pathways and define phenotypic variations through genetic manipulations has led to a better understanding of outflow pathway regulation in normal and POAG patients. The ECM molecules are now known to be major players contributing to turn-over, re-modeling, regulation and signaling in the aqueous outflow pathway. Alteration of ECM components, their modulators, or changes in ECM deposition decreases outflow. The role of cell and substrate stiffness inhibits cell mechanosensation, thus impairing pore formation, as well as reducing pliability of SC cells and their attachment to the ECM. Findings in POAG indicate commonality with molecules found in other disease processes such as fibrosis and atherosclerosis: both of which are involved in abnormal deposition of ECM. The discovery of cell relaxation by lantrunculins and Rho kinase inhibitors with resultant increase in outflow have set the path for development of new drugs that influence the cell cytoskeleton and ultimately cellular tone. Future work on induced pluripotent stem cells (iPSCs) may help outline strategies for TM cell replacement during POAG. This method of self-cell replacement would be extremely beneficial since there would be no harmful side effects of drugs or surgery. The discovery of lymphatic markers in the TM is indeed intriguing as similarities in SC and lymphatics have been noted for some time. Further work will be instructive as it casts the TM-tissue functions in a new light. The investigation of many of these newly reported areas hold promise to further our understanding of the role of AH outflow in development of POAG.

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