Aqueous Humor Outflow: Dynamics and Disease

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Eighth annual ARVO/Pfizer meeting attendees are listed on page 2994.

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This review summarizes the goals and outcomes of the eighth annual ARVO/Pfizer Ophthalmic Research Institute conference that was held on May 4th and 5th, 2012, at the Embassy Suites Fort Lauderdale, Fort Lauderdale, Florida. This conference series has been funded by the ARVO Foundation for Eye Research with the help of a generous grant from Pfizer Ophthalmics. Funding from the ARVO/Pfizer Ophthalmics Research Institute has allowed a series of "think tanks" for the leading experts on various subspecialties of ophthalmology research. These meetings have helped to define various challenges and potential solutions to issues relevant to the field of ophthalmology. In 2012, the emphasis of the conference was on the physiological role of the conventional outflow pathway in aqueous humor dynamics in normal and glaucomatous eyes. In particular, clinical observations associated with aqueous humor outflow in health and disease, animal models in the understanding of aqueous outflow, role of mechanosensing in aqueous humor fluid flow, and new paradigms in cell and extracellular matrix crosstalk within the conventional outflow pathway were discussed.

The primary goal of the conference was to bring together a diverse group of experts who are pioneers in conventional outflow biology and related nonocular areas of research with the hope of utilizing the group's knowledge and experience to evaluate the current understanding of aqueous outflow regulation and problems thereof. The invited group consisted of 29 investigators directly involved in conventional outflow research. Also present were three invited outside experts in cellular mechanics and cytoskeleton structures (Jeffrey Fredberg, PhD, Harvard School of Public Health, Boston, MA, USA), molecular mechanisms of cell adhesion and mechanosensing (Benjamin Geiger, PhD, Weizmann Institute of Science, Rehovot, Israel), and murine genetics (Richard Libby, PhD, University of Rochester Medical Center, Rochester, NY, USA). In addition, the conference was attended by 27 observers who participated in discussion of key topics at the end of each presentation. Together, the group was tasked with reviewing the current scientific dogma regarding aqueous outflow diseases and identifying the most pressing research questions and needs in the current funding environment. Above all, the group was asked to think "outside the box" to develop future research directions. The conference generated a list of highly relevant but currently unanswered questions with the hope that solutions to these issues would improve understanding of the role of the conventional outflow pathway in normal and glaucoma eyes.

The meeting was organized into four sessions: (a) clinical insights into conventional outflow dysfunction, (b) the use of mice as model systems for conventional outflow, (c) mechanosensing within the conventional outflow pathway, and (d) the role of the extracellular matrix and signaling in conventional outflow dynamics. Speakers presented their thoughts on the preselected topics and provided key research goals that have been addressed or need to be addressed in the near future. The sessions were followed by discussion aimed at summarizing and interpreting the current knowledge as well as identifying unique research questions that were as yet unanswered.

Anatomy and Physiology of the Conventional Aqueous Humor Outflow Pathway

The conventional outflow pathway is mainly a pressure-driven system. Under homeostatic conditions, this pathway regulates the drainage of aqueous humor from the anterior chamber of the eye, thereby maintaining a constant intraocular pressure (IOP).[1] Relevant tissues of the anterior segment that are anatomically involved in IOP control include the ciliary muscles, trabecular meshwork (TM), Schlemm's canal (SC), collector channels, and aqueous veins. The ciliary muscle is composed of smooth muscle fibers that have a true elastic net of tendons that anchor into the choroid posteriorly and the scleral spur, TM, and inner wall of SC anteriorly.[2,3] It is widely accepted that contraction of the ciliary muscle causes expansion of the TM and opening of SC, which subsequently increases the conductivity of aqueous humor (AH) through the TM. All these, along with reports of nerve innervation in the

TM, indicate that the TM is a self-regulating tissue with both afferent and efferent nervous components responsible for controlling its functions.[2,4]

Session I: Clinical Insights Into Conventional Outflow Dysfunction

Clinical Observations. Glaucoma is defined as a group of disorders that result in death of ganglion cells due to axonal damage at the level of the lamina cribrosa of the optic nerve. In essence, glaucoma is a diagnosis of exclusion in instances in which the cause of the optic nerve damage is unknown. While primary open-angle glaucoma (POAG) is the most common form of glaucoma, there could be 20 to 30 subtypes of the disease, as the panel was told by the late David Epstein, MD (Duke University, Durham, NC, USA), who provided his thoughts on treating glaucoma for nearly four decades. Such diversity in the disease most likely explains why it is so difficult to find a single responsible gene for POAG and why patients with glaucoma have differential responses to medical therapy. For example, 10% to 15% of patients do not respond to latanoprost, one of the most commonly used drugs for treatment of glaucoma.[5] Studying these nonresponders may provide clues for recognizing the multitude of subtypes within glaucoma.

While IOP continues to be the most relied-upon parameter for diagnosis and treatment, it is not always elevated in glaucoma. For example, 90% of Asian populations suffering from POAG have IOP in the normal range in comparison to Caucasians, whose numbers range from 30% to 50%.[6] In any case, effectively lowering IOP is neuroprotective, slowing or stopping vision loss in glaucoma patients, even if they do not have elevated IOP. Moreover, IOP has been shown to be causative, since experimentally induced IOP elevation will result in neuronal glaucomatous damage.

Intraocular pressure is variable, fluctuating widely between visits or between readings obtained at different times of the day. Therefore, it is often difficult to get a true picture of a patient's IOP particularly when it is measured only two or three times every year. Despite this, clinical management of glaucoma is focused on setting a target pressure for each patient and reaching that target through medications, surgery, or both. For patients with advanced glaucoma and considerable damage to the optic neuronal tissues, an even lower target pressure is warranted.

Conventional Drugs Present and Future. At present, the only proven treatment for glaucoma is to lower IOP either by increasing outflow or by reducing AH production. There are currently five classes of drugs commonly used for treating glaucoma—β-blockers, prostaglandin analogues, adrenergic agonists, carbonic anhydrase inhibitor, and cholinergic agents. Of these, only the prostaglandin analogues and cholinergic agents have direct effects on the two outflow pathways and hence will be discussed in more detail. In studies on monkeys and humans, prostaglandin analogues have been shown to lower IOP, primarily by increasing flow via the uveoscleral pathway.[7] However, several studies have shown a secondary effect of prostaglandin analogues on the conventional outflow pathway. Bahler et al.[8] have shown that latanoprost can lower pressure in human anterior segment perfusion culture. Likewise, Stamer et al.[9] and Wan et al.[10] showed that treatment with bimatoprost increased outflow facility in human anterior segment culture, and this effect was blocked with AGN 211334, a prostamide antagonist. Tafluprost, approved as an ocular hypotensive drug in 2012, has also been shown to affect the conventional outflow pathway.[11,12]

Cholinergic agents (e.g., pilocarpine, carbachol) increase conventional outflow by constricting the longitudinal portion of the ciliary muscle, pulling on the elastic net that extends into the inner wall of SC, expanding the TM tissues and preventing collapse of the SC lumen.[13,14] The cholinergic agents are generally used only acutely by glaucoma clinicians due to unfortunate side effects (e.g., miosis, induced myopia, brow ache secondary to ciliary spasm, and even retinal detachment). Side effects are often more pronounced in younger patients.

A novel and emerging way of decreasing resistance in the conventional outflow pathway is through disruption of actin polymerization. Inhibition of the Rho kinase pathway by Rho Kinase (ROCK) inhibitors results in cellular relaxation, increasing the separation distance in the juxtacanalicular region, increasing access of AH to inner wall and improved AH outflow.[15–21] Similarly, latrunculin A and B cause extensive extracellular remodeling through microfilament disruption that

Eighth Annual ARVO/Pfizer Meeting Attendee List

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Investigative Ophthalmology & Visual Science

distends the TM (particularly the juxtacanalicular tissue) and dilates SC.[22,23] Understanding the mode and site of action of these agents will be beneficial in deciphering the biology of the conventional outflow pathway, which in turn would result in development of better therapeutic opportunities.

In addition to cytoskeleton-modifying drugs, vasodilators like nitric oxide have been shown to mediate cytoskeletal relaxation. Ciliary muscles precontracted with carbachol were able to regain their normal state in the presence of various nitric oxide donors, whereas presence of nitric oxide synthase inhibitors exacerbated the carbachol-mediated contraction.[24] Moreover, overexpression of endothelial nitric oxide in SC results in doubling of conventional outflow,[25] emphasizing the importance of this pathway for therapeutic targeting. Several reports have described the ocular hypotensive property of nitric oxide–donating prostaglandin agonists in various preclinical animal models.[26–30]

At the end of the session, the evidence for the TM as a self-sustaining structure versus the existence of an inherent regulatory feedback mechanism was discussed. While this is a controversial topic, research in this area is required. If a feedback mechanism exists, new drug targets can be identified that can help regulate outflow. The group also discussed feasible strategies for subcategorizing glaucoma. These included possible studies on nonresponders to commonly used drugs, linkage analysis, and genome-wide association studies. The group further emphasized the fact that existing and upcoming outflow drugs are probably involved in restructuring already existing mechanisms in a way that is therapeutically useful. In the end, a common theme among the group was that identification of drug targets aimed specifically at conventional outflow is imperative to enhance treatment outcomes for patients with glaucoma.

Surgical Manipulation of Aqueous Humor Outflow. All surgical techniques are aimed at increasing aqueous drainage and lowering IOP by creating direct channels for AH drainage into the collecting channels or eye exterior while bypassing the TM. Among surgical modalities, laser trabeculoplasty is widely used to treat POAG patients. Commonly used lasers include argon (wavelength 488–514 nm; also known as argon laser trabeculoplasty or ATL) and Q-switched, frequency-doubled neodymium yttrium aluminum garnet (Nd:YAG; wavelength 532 nm; also known as selective laser trabeculoplasty or SLT). Argon laser trabeculoplasty and SLT are equally effective in lowering IOP in patients with open-angle glaucoma for up to 5 years. However, long-term data have shown that patients undergoing either ALT or SLT will require some form of medical or additional surgical intervention owing to secondary complications.[31] During laser trabeculoplasty, preferential fluid flow has been reported around the "blast" holes.[32] However, the improved facility is not enough to explain the drop in IOP.[33] One possibility is that in addition to the conventional outflow pathway, the uveoscleral pathway is also being affected in the surgery and contributes to the increase in overall outflow. Alternatively, trabeculoplasty has been found to transiently raise phagocytosis in TM, indicating that trauma caused by the laser induces an IL-1α- and TNFα-mediated inflammatory response.[34] Despite this, the success rate for laser trabeculoplasty is around 67% with reference to lowering of IOP to 20% below baseline, although wound healing in the TM renders the surgery unsuccessful with time.

In trabeculectomy surgery, a part of the TM is removed to bypass glaucomatous resistance and increase drainage of AH. However, these surgeries are prone to complications including infection and blood reflux in SC following a rapid drop of IOP caused by the surgery. Like laser trabeculoplasty, trabeculectomy is also successful for a limited amount of time due to wound healing and scar formation. Factors that can lower inflammation, when used in conjunction with this surgery, usually improve its long-term success rate.

New and innovative procedures such as canaloplasty and the use of shunts to bypass the TM are providing additional opportunities to control IOP. In canaloplasty, a microcatheter is placed in SC to enlarge the drainage canal, preventing SC collapse and herniation. The success rate for canaloplasty is approximately 85% over a 3-year period. Insertion of bypass shunts in and through the TM allows AH to drain directly into SC. While survival curves from phase 4 trials have not been reported, several studies have shown successful IOP reduction for more than 1 year without significant side effects.

Episcleral Venous Pressure and IOP. Theoretically, IOP should directly correlate with the episcleral venous pressure (EVP). However, when the entire TM is removed, 25% of total resistance still remains, underscoring the importance of EVP measurement in an accurate and reproducible way. Current methodology assesses the pressure needed to collapse the vein. Unfortunately, this is rather subjective since there is no defined point that will determine that the vein has collapsed enough. Because of this uncertainty of endpoint, a wide range of pressure, from 7.6 to 11.4 mm Hg, has been reported for normal EVP.[35–38]

New devices that record the vein collapse and utilize video frames synchronized to corresponding pressure readings to identify EVP are being developed by Arthur Sit, MD, and colleagues.[39] In inverse profile, the brightness increases as the vein collapses and this can be plotted from the video, giving a characteristic and reproducible curve of vein collapse with distinct transitional zones.[39] Using this system, it is now possible to choose and compare the readings at various points of collapse (e.g., 50% vs. 100%). However, the reading significantly varies across different endpoints, and which endpoint should be chosen is an important consideration. The best answer to this is provided by a study in monkeys in which readings from the venomanometer were compared to readings from a cannula placed inside the vein. According to this study, a 90% to 100% endpoint likely gives the most accurate EVP reading.[40] Using this model, normal EVP is measured to be approximately 7 mm Hg.

An important question is whether glaucoma medications affect EVP. Little or no change in EVP was reported following brimonidine treatment, while a significant reduction in EVP concomitant with an increase in outflow facility was found with latanoprost, betaxolol, and brimonidine treatment.[37,41] While findings from these studies appear to contrast, they can also result from different experimental approaches. Additional research with more standardized experimental conditions will help to determine if EVP can be specifically targeted for control of ocular hypertension.

During panel discussion, experts highlighted some probable limitations that should be considered while measuring EVP, such as the elasticity and/or thickness of the sclera, which can have a significant effect on the amount of pressure required to collapse the veins. Therefore, patients with a stiffer sclera give falsely elevated EVP. One way to counteract this would be to perform the measurements in an area without episcleral veins, which could serve as a control for scleral stiffness. However, this may be technically challenging since episcleral veins are relatively widespread; and the most common noninvasive EVP measurement technique uses a flexible membrane attached to a pressure chamber that is relatively large, making it difficult to pinpoint scleral areas that lack veins. Additionally, distal resistance in episcleral veins may be due to sphincter muscles creating choke points at different parts of the veins. In this respect the episcleral veins are completely different from the conjunctival veins, although they communicate with each other. Although

Investigative Ophthalmology & Visual Science

understanding EVP can help one understand the outflow pathway as a whole, it should be remembered that lowering EVP will need to be specific for ocular hypertension while minimizing systemic complications.

Session II: The Mouse as an Animal Model for Conventional Outflow

Mouse-based ocular research has gained impetus in the last decade due to the well-characterized genetic variability, ability to genetically manipulate, ease in use for drug studies, and inexpensive cost. However, it has not been until the last couple of years that mouse models have gained a lot of traction as viable experimental systems for studying glaucoma. Unlike many nonprimate models, mice have a lamellar TM and SC with both conventional and nonconventional outflow pathways.[42,43] Differences from primate outflow pathways include a narrower anterior angle and a posteriorly placed TM with fewer beams. Unlike primate model systems, mice do not have any washout effects, which makes mice a unique model for studying the outflow pathway.[43] Also, age-dependent glaucomatous damage in ocular tissues of mice is similar in pattern to that of humans.[44] This, along with the fact that anatomy of the outflow drainage pathways in mice is remarkably similar to that of humans, makes the mouse an ideal model to study glaucomatous diseases.[44]

Intraocular pressure varies widely across mouse strains. Diurnal fluctuations in mice are not due to posture but to higher rates of AH formation at night, as they are nocturnal animals.[45] Savinova et al.[46] performed an extensive survey of basal IOP in various mouse strains and found that normal IOP varied from 11 to 20 mm Hg. The difference in baseline IOP between strains is due in part to changes in conventional outflow facility.[47]

There are a number of models of elevated IOP in mice. All these models have their share of pros and cons, and ultimately the specific requirements of individual research aims dictate the choice of the particular model. Several models were highlighted that may have a significant impact on future studies.

Spontaneous Mouse Models of Elevated IOP. The DBA/2J mouse is used as one of the most common models of glaucoma and has given a number of insights into the disease. This is a spontaneous pigmentary glaucoma model that leads to angle closure and eventually asynchronous IOP elevation due to two mutations causing iris stromal atrophy (*Tryp 1* gene) and pigment dispersion (*Gpnmb* gene).[48] Intraocular pressure change starts at 6 months, and females seem to be affected earlier than males. However, because the IOP elevation is asymmetrical, the same mouse can have high IOP in one eye and normal IOP in the contralateral eye. Also there is a wide variation in the time when the animals start getting high IOP.[49]

Induced Models of Glaucoma. Models that provide sustained IOP elevation are the best-characterized models for development of glaucoma. Methods involving laser treatment,[50] cautery of vortex veins,[51] and injection of hypertonic saline into episcleral veins[52] have proved successful in elevating IOP and causing retinal changes consistent with glaucoma. Viral vector deliveries of secreted frizzled-related protein 1 (SFRP1) and mutated myocilin have all shown the ability to elevate IOP and cause glaucoma.[53,54] Models like these are useful in understanding the physiology behind elevated IOP through manipulation of the relevant intracellular signaling mechanisms.

More recently, newer methods such as injection of microbeads into the anterior chamber where they accumulate and block the outflow pathway, causing sustained IOP elevation, have been reported.[55] However, this model can be user dependent, and the specifics of IOP elevation vary even within the same lab. Surgical implantation of osmotic minipumps that release dexamethasone have been successful in elevating IOP, essentially creating a steroid-induced glaucoma model.[56] This method was found to elevate IOP in some but not all mouse strains tested. This mimics what happens clinically, with some individuals susceptible to steroids and others not. This kind of inducible model could be a nice way of genetically dissecting out which genes are responsible for steroid responsiveness.

Transgenic Models. Transforming growth factor-beta 2 (TGF-β2) levels are elevated in AH in greater than 50% of POAG patients.[57] Additionally, downstream mediators of TGF-β2 such as connective tissue growth factor (CTGF) when overexpressed in mice using a lens-specific chicken βB1-crystalin promoter cause a progressive elevation of IOP that leads to a glaucomatous phenotype similar to that in patients with POAG.[58] Both TGF-β2 and CTGF mouse models can provide exciting new opportunities to study and understand the biology and pathogenesis of POAG.

Mice undergoing insertion of the Tyr437His point mutation in the *MYOC* gene develop glaucomatous eye damage.[59] Intracellular accumulation of overexpressed, misfolded myocilin protein that activates the endoplasmic reticulum stress response is believed to be the underlying mechanism associated with the phenotype. Interestingly, this phenotype can be reversed with a chemical chaperone that promotes the secretion of mutant myocilin in AH and decreases intracellular accumulation in the endoplasmic reticulum.[60]

The Col1a1$^{(r/r)}$ transgenic mouse has multiple mutations in the collagen 1a gene that inhibit proteolysis and increase expression of collagen 1. Eventually, there is a breakdown in extracellular matrix turnover and increased immunoreactivity to collagen in various ocular tissues, which over time is thought to develop elevated IOP and decreased outflow facility along with glaucomatous damage.[61,62]

Another mouse model of interest is the transgenic mouse overexpressing mutated optineurin. Aged, optineurin mutation–carrying mice show distinct glaucomatous changes in the retina without an increase in IOP.[63] As mentioned previously, not all glaucoma is characterized by elevated IOP. Normal-tension glaucoma makes up to 90% of POAG in some ethnicities. Therefore, the optineurin transgenic mouse could be an important model to study normal-tension glaucoma and compare the associated molecular and cellular changes between normal and elevated mouse models of IOP.

Following description of mouse models of glaucoma that affect the outflow pathway, the panel members acknowledged that the mouse has become an important model system for defining the pathophysiology of glaucoma in two respects: (1) the effect of IOP on axonal health and the chain of events that leads to retinal ganglion cell death and (2) molecular and cellular dysfunction in the conventional outflow tract that increases resistance while having an open angle and normal-appearing TM. With technological advances in measuring AH dynamics in mice, new knowledge will be obtained from the different model systems. How these different models relate to different forms of the disease will have to be investigated. All in all, further development and characterization of mouse models for glaucoma are necessary to provide important clues to understanding the biology of the disease and the role of the outflow pathway in IOP elevation.

Session III: Mechanosensing Biology in the Conventional Outflow Pathway

Mechanosensing refers to ways in which cells transfer mechanical changes into biologic signals. Cellular mechano-

sensing in biologic systems is accomplished through mechanoreceptors that may be either specialized molecules known as integrins, membrane stretch receptors, or nonspecific cell surface protein receptors. The mechanosensory cells and their organ systems may form feedback loops that comprise both fast and slow response elements. Many physiologic systems such as the lung and cardiac system, which require fastidious control of flow, pressure, and shear stress, have feedback loops to monitor ongoing changes within the body. For example, blood pressure and heartbeat are sensed by baroreceptors in the carotid and aortic arch. Activation of the baroreceptors (primarily stretch receptors) sends signals to the brain stem, which in turn uses the parasympathetic and sympathetic systems to adjust blood pressure and heart rhythm, collectively known as the baroreflex.[64] Like blood pressure and heart rate, IOP is regulated by fluid flow, pressure, and shear stress. Aqueous humor produced in the ciliary body flows from the posterior to the anterior chamber through the pupil and the TM into SC and aqueous veins. The fluid movement through the conventional outflow pathway is directed by pressure change. In the eye, normal IOP is 15 mm Hg, but the pressure drops to 9 mm Hg in SC and further to 7 to 8 mm Hg in aqueous veins.[65]

Mechanosensing in the TM and SC. While there is no direct evidence of baroreceptor activity in the eye, partial evidence for an "ocular baroreflex" may be found in the facts that eye pressure is tightly regulated over a normal life span[66] and that eyes exposed to stretching and increased fluid flow return to starting IOP levels.[67] Additionally, eyes perfused at high pressures return close to the pressure of the contralateral control eye over time.[68] The most likely site for baroreceptor activity within the conventional outflow pathway appears to be at the interface between the juxtacanalicular region of the TM and SC.[69] In this region, SC cells are attached to an attenuated basement membrane,[70,71] an elastic fiber system,[72] and the juxtacanalicular cells.[73] The cells and extracellular matrix within this region are believed to be the site of outflow resistance in glaucoma. Human, bovine, and porcine TM cell cultures along with human anterior segment cultures showed changes in extracellular matrix turnover and extracellular matrix reorganization following stimulation with various forms of cyclic or static stretch.[74] Schlemm's canal endothelium stretches and expands to pressure in both size and contractile ability.[75,76] Schlemm's canal endothelial cells form giant vacuoles and pores that increase in size and number with pressure.[75,77] Additionally, SC cells undergo shear stress comparable to that in large arteries and respond by aligning in the direction of flow.[78] The pressure in the outflow system is also known to vary rapidly with pulse, blinking, and head movement,[79] indicating that fast and slow adaptation mechanisms may be present to control rapid pressure change. Pulsation has been found to be important, as treatment of cultured human and porcine segments with pulsation results in a decrease in outflow facility.[80]

TM Tissue and Cell Stiffness. Tissue and cell stiffness are factors that may alter the responsiveness of the TM and SC to normal fluid flow, pressure, and shear stress. Trabecular meshwork cells respond to changing substrate stiffness with changes to cytoskeleton, morphology, protein and gene expression, signal transduction, and fibronectin deposition.[81–83] It is known that TM stiffness increases in POAG eyes with significant regional variability.[84] Additionally, SC stiffness was found to vary widely with drug treatments that are known to influence outflow. If the drug treatment stiffened SC cells, resistance increased. If drug treatment relaxed the cells, resistance decreased.[76] In SC cells, vacuole and pore formation is dependent on pressure and most likely is related to stiffness. Aqueous humor passing through the inner wall pores is therefore affected by SC cell stiffness. Interestingly, SC pore numbers are reduced in glaucoma tissue.[77,85] The moduli of SC cells in culture are measured at 0.5 to 3 kPa, but under load the cells stiffen by an order of magnitude.[86] By atomic force microscopy (AFM), the cortex of the cell is found to be 20 times stiffer than the cytoplasm. To support IOP, finite element modeling suggests that cells either must have extensive attachments or must have a large increase in modulus under load.[87] Increased SC and TM cell stiffness with age or POAG could reduce sensor function, compliance, and pore formation, likely increasing outflow resistance. Thus, it makes sense that drugs that modify the cytoskeleton either directly or indirectly, such as Rho kinase inhibitors and latrunculins, decrease cell stiffness and reduce outflow resistance.[20,22,23] Interestingly, these drugs do not always affect cell junctions but control actin stress fiber formation, focal adhesions, and cellular contraction.[18]

Jeffrey Fredberg, PhD, gave an overview of some of the common responses to mechanosensation that exist across the biologic spectrum of cells and organisms. In particular, he focused his presentation on what pulmonary cells can reveal about SC endothelial cells. For example, dichotomies are normal in a cell's response to stress. Cells exist in a state of global tension and can either reinforce or fluidize. How the cell responds ultimately is mechanoprotective for the organ. Routinely, SC cells deform readily with changes in pressure. Fredberg's recent work with Zhou et al.[76] demonstrated that SC endothelial cells have a greater contractile scope than smooth muscle cells, likely due to their dynamic biomechanical environment. Commonalities across biologic disciplines can be instructive when one is searching for modulators of outflow resistance. While the panel discussion focused on the possible roles for a baroreceptor process, it was apparent from the enthusiasm of the group that a better understanding of this area of research will be important in the future. Defining the mechanosensing activity of the conventional outflow pathway may provide a novel and selective opportunity for therapeutic development. A proposed model for the ocular baroreflex would include the combination of meshwork stiffness, shear stress on SC cells, stretch of SC cells, and stretch of TM cells acting as the reference sensor to feed back to the matrix in the juxtacanalicular region acting in conjunction with SC inner wall pores as the controller. Lack of an understanding regarding the innervation of the outflow pathway and the effect of this on IOP control was also discussed with reference to an 1890 publication by Boucheron[88] and more recent work by Stone and Laties.[89] Further investigation in this area may lend more understanding to possible underlying mechanisms of neural control of IOP.

Session IV: Extracellular Matrix, Signaling, and Conventional Outflow Dynamics

The extracellular matrix is no longer thought of as a passive substrate to which cells are attached. The extracellular matrix is known to be composed of numerous proteins, enzymes, and signaling molecules that are dynamic, interactive, and linked to the cells that reside in and on its surfaces. The extracellular matrix interacts in large part with integrins, molecules that link the cell actin cytoskeleton with the extracellular matrix. Composed of α and β subunits, integrins bind to different extracellular matrix molecules[90] and regulate cell migration, morphogenesis, differentiation, and survival.[91] Integrins in combination with the actin cytoskeleton and interconnecting adaptor proteins are found in focal adhesion complexes. Focal adhesion complexes are now known as adhesomes due to the >180 molecules of which they are composed.[92] The adhesome responds to different

Investigative Ophthalmology & Visual Science

compositions of the extracellular matrix, different ligands, substrate rigidity, and mechanical stimuli,[93] suggesting that adhesomes signal and that they are regulated.[92]

Extracellular Matrix in the Conventional Outflow Pathway. The extracellular matrix in the TM juxtacanalicular region is thought to be responsible for a large part of the outflow resistance in the anterior segment of the eye. Composed of elastin, collagens, laminin, fibronectin, and fibrillin, the extracellular matrix in the juxtacanalicular region increases with age and in POAG.[94] This increase is seen primarily in the sheath that has been termed "sheath-derived plaque material." The axonal damage in the optic nerve correlates to the plaque material increase seen in POAG.[95] Additionally, matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMP), and other inhibitors of MMPs are responsible for remodeling and maintaining the extracellular matrix.[96] By increasing IOP, which involves mechanically stretching the TM, MMPs increase and outflow resistance decreases.[67] Faster extracellular matrix turnover occurs if cultured anterior segments are treated with tumor necrosis factor (TNF) and interleukin-1 (IL-1), resulting in increased MMP activity and reduced resistance.[97]

Another key component of the TM extracellular matrix is the presence of various glycosaminoglycans (GAGs) that reside in the intertrabecular spaces.[66] Glycosaminoglycans have been shown to be a key factor in modulating outflow resistance. Outflow resistance is reduced in both pigs and humans through inhibition of GAG biosynthesis and sulfation.[98] Additionally, versican appears to be an important GAG in modulating outflow resistance, since low versican levels were found in high-flow regions of the TM.[99]

Integrin Signaling. Changes in the extracellular matrix are detected by integrins and can affect the outflow in the TM. Eleven integrins are expressed by TM cells.[100] As integrins interact with the actin cytoskeleton their function becomes closely tied to the contractility of the TM. Changes in the extracellular matrix (e.g., stretch) bring the extracellular matrix molecules into contact with the integrins, which then change conformation to unfold, bind, and "activate" integrin signaling.[101] This outside activation of integrin signaling can affect outflow facility by regulation of cell contractility. In monkey anterior segment cultures, perfused with the Heparin II (Hep II) domain of fibronectin, outflow facility was increased and the juxtacanalicular region was expanded.[102] This response was determined to be in conjunction with the $\alpha4\beta1$ integrin and collagen. Hep II causes disruption of the actin cytoskeleton. The $\alpha4\beta1$ integrin is involved in Rho activation. Cross-linked actin network (CLAN) formation, which is thought to cause stiffness in the actin cytoskeleton in TM cells, has been found to be influenced by $\beta1/\beta3$ crosstalk.[103] Trabecular meshwork cell monolayers cultured on fibronectin ($\alpha5\beta1$) and collagen IV ($\alpha1/\alpha2/\beta1$) use different integrins to detect the difference in extracellular matrix. When Hep II was added to both cultures, the actin cytoskeleton of cells cultured on fibronectin was unaffected whereas in cells on collagen, actin was disorganized.[104]

In addition to outside-in signaling, changes that occur within the cell also affect the external environment. Inside-out signaling can be initiated by growth factor receptor pathways or by G-protein–coupled receptors that change the integrin from an "inactive" to an "active" or extended state.[105] The effect of dexamethasone (DEX) on the formation of CLANs in TM cells is thought to occur via the $\beta3$ integrin through inside-out signaling.[106] Transforming growth factor $\beta2$ is a cytokine that is involved in regulation of proliferation and cellular differentiation of many different types of cells. Transforming growth factor $\beta2$ is increased in the AH of patients with POAG.[107–110] If TM cells are cultured and treated with TGF-$\beta2$, this causes an increase in collagen types I, III, IV, and VI, laminin, elastin, fibronectin, fibulin, versican, thrombospondin-1, and myocilin,[111–118] which are key components or associated proteins of the TM extracellular matrix. Additionally, TGF-$\beta2$ increases cochlin, a protein that normally is not expressed in the TM but is found in the extracellular matrix of glaucomatous TM tissue.[119–121] Transforming growth factor $\beta2$ also activates CTGF. Together, TGF-$\beta2$ and CTGF expression can exacerbate extracellular matrix production. Interestingly, overexpression of CTGF in mice leads to elevated IOP with associated increases in fibronectin levels in the TM.[58]

Benjamin Geiger, PhD, discussed how the function of integrins as substrate adhesions has expanded to include their function as mechanoreceptors. Investigation of integrins and the associated adhesome proteins and their intricate regulation is important to the basic understanding of a cell's response to its environment. In panel discussion, versican, a GAG with multiple binding partners, was examined in relationship to segmental outflow. Questions regarding its reactions in other species were discussed, as well as its presence as revealed with Q-dots. Transforming growth factor $\beta2$ and its role in POAG provoked many questions, starting with the following: Where does the TGF-$\beta2$ in the anterior chamber originate? What are the initial stimuli for its production? Are oxidative stress and/or mechanical overload involved? While significant strides have been made in understanding the role of the cells and extracellular matrix in outflow regulation, it is clear that considerable research is required to further our knowledge base regarding the molecular and cellular activities associated with normal and abnormal conditions.

Summary

After extensive overviews and discussion, this meeting provided a chance to review current knowledge and discuss future directions that are essential if more effective treatments are to be developed for glaucoma patients. Future understanding of glaucoma not only will come from studies at the bench, but also will come from clinical clues from the patients with the disease. A top research priority should be characterizing the different subtypes of glaucoma. Understanding the pathogenesis between the different subtypes will enable more individualized treatment that will be more effective in slowing the disease progression in a targeted fashion. Until this occurs, research efforts should focus on identifying cellular pathways that are directly affected in glaucoma pathogenesis. Developing drugs that reduce outflow resistance and increase outflow facility and that specifically target the TM and SC interface would be a significant advance.

Advances in glaucoma detection at earlier stages of disease pathogenesis are important for slowing disease progression. Due to this treatment paradigm, patients may suffer severe nerve damage of up to half of the neuronal cells before observable changes are detected and treatment is initiated. Identifying trophic factors that communicate between the anterior and posterior chambers of the eye, as well as TM mechanosensors and baroreceptors, may provide future targets for IOP regulation. In vivo models, such as those provided by genetically engineered mice, will be important to advance the science due to similarities to human outflow. In the end, significant research efforts have furthered our grasp of glaucoma pathogenesis. As our understanding has grown, so has the list of questions that need to be addressed (Table). Finding the answers to these questions will focus efforts, provide better understanding of the disease mechanism, and more importantly improve the future outcome for patients with glaucoma.

Investigative Ophthalmology & Visual Science

Table. Questions About Conventional Outflow That Require Answers

Clinical Questions

General.

1. How many subtypes of POAG are there? How can they be characterized?
2. What can we learn about glaucoma subtypes from patients who are nonresponders to traditional glaucoma drugs?
3. Why do half of pigmentary dispersion and exfoliation patients develop severe glaucoma while half do not?
4. What is the one characteristic of the conventional outflow pathway that clinicians wish they could measure? Tonography? TM movement? Diurnal IOP?
5. What is the most reliable and most user-friendly method to measure outflow function, both conventional and nonconventional?
6. Is episcleral venous pressure affected in POAG? How is it modulated? Is it uniform across the episcleral region? Can it be used as a drug target to regulate IOP?
7. Where is distal resistance coming from in the conventional outflow pathway and how is it regulated?
8. Is induced inflammation beneficial for the TM?

Pharmaceutical and Surgical Treatment

1. How and where do conventional drugs work? Can we restore function of conventional tissues with pharmaceuticals?
2. Are current conventional drugs in development harmful to the TM? How will long-term treatment affect TM function?
3. Does prostaglandin analogue treatment affect laser trabeculoplasty success? Is it due to prostaglandin analogue involvement in mode of action of laser trabeculoplasty?
4. What do differential responses to laser tell us about conventional outflow in POAG? Does laser trabeculoplasty induce progenitor cell response? Does argon laser trabeculoplasty and selective laser trabeculoplasty spot size matter?
5. What does device failure (shunts, trabectome, and so on) tell us about functionality of TM? What is evidence of immune involvement in surgical failure?

Models of Outflow

General.

1. What are the relevant endpoints for a good glaucoma model?
2. Why do some animals have washout while others don't? What does washout tell us about IOP regulation?

Mouse Models of Outflow.

1. Are aging mouse eyes a good model for aging human eyes? What are typical age-related ocular changes in normal mice?
2. What underlies differences in mouse strain–variant IOP? Can breeding strategies of mice with elevated IOP (3 standard deviations from mean) uncover a POAG gene? How do current outflow drugs in development affect outflow facility in mouse?
3. Can steroid-induced ocular hypertension in mice tell us something about human responders and nonresponders?
4. Why do different strains of mice respond differently to adenovirus expressing MYOC mutants? Some have elevated IOP, some don't?
5. How do we develop better in vivo imaging technologies for the mouse?
6. What is the best method to measure outflow? How do we overcome technical challenges of measurements in mice? Do we need to establish guidelines or gold standards?

Understanding IOP Modulation

General.

1. How is IOP modulated in humans?
2. Is the TM a responsive self-aware, self-regulating tissue? Is it innervated? Does it participate in a feedback loop?
3. What is the role of ciliary muscle–tendon anchors in glaucomatous development? Is there a protective mechanism for ciliary contraction?
4. How do SC cells support pressure drop?
5. Since the SC is frequently collapsed in POAG, is there something wrong with the valve function?
6. Does the presence of abnormal elements in AH cause TM dysfunction?

Mechanosensation, Mechanotransduction, and Tissue Stiffness

1. How does the eye know that a pressure of 15 mm Hg is 15 mm Hg? What is the mechanosensing system in the TM? Is there mechanosensing in the SC and distal outflow region? Are there defects in the mechanosensing system that affect conventional outflow in ocular hypertension and POAG?
2. How can we target mechanosensation for glaucoma therapy?
3. Are there stretch receptors in the TM? Do conventional outflow cells fluidize after stretch?
4. Do humans have different types of baroreceptors in the eye for different time scales?
5. Does tension balance (plithotaxis) exist between cells within the conventional outflow pathway or does it fluctuate?
6. What causes TM stiffness to increase in glaucoma? Are there drugs that can be used to reduce TM stiffness? What are possible effects of altered TM stiffness in glaucoma?
7. Do current or new pharmaceuticals to treat elevated IOP alter tissue stiffness?
8. Can we increase stiffness in an animal model?
9. Why do SC cells have such a wide range of stiffness?

Cell and Extracellular Matrix Involvement in Outflow

1. How do cell–cell junctions respond to changes in pressure?
2. What role do focal adhesion dynamics play in conventional outflow?
3. What are the levels of flow segmentation (macro versus micro) in outflow pathway? Do preferential flow pathways change with time?
4. Can changes in the extracellular matrix microenvironment affect cell behavior that impacts outflow facility? Are extracellular matrix components different in high- versus low-flow areas? How are MMPs controlled?
5. Are there different cell types in the TM? What distinguishing characteristics or protein expressions do they have?
6. What induces TGF-β2 in POAG eyes?
7. What effect does lens removal have on TGF-β2 or other trophic factors?
8. Do TM progenitor cells exist? What is their relative contribution to the function of the TM? How do they change with age?

Investigative Ophthalmology & Visual Science

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Investigative Ophthalmology & Visual Science

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