Advances in Glaucoma Treatment and Management: Outflow Drugs

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Intraocular pressure (IOP) rises when the balance between aqueous humor formation and outflow resistance is compromised. In a normal eye, ciliary muscle (CM) and trabecular meshwork (TM) contraction and relaxation function in synchrony to provide fine control of outflow. Recent investigations of the role of endothelial nitric oxide synthase (eNOS)¹ suggest TM mechanosensitivity as a homeostatic mechanism mediated in part by NO to maintain normal outflow facility and IOP. The active role of TM contraction and relaxation in the regulation of IOP is also likely to be mediated in part by the actomyosin contractility/cytoskeleton/cell-cell and cell-matrix adhesion system. The cytoskeleton and contractility mechanisms may be the efferent “execution” arm of the reflexive and regulatory mechanism; their arrangement governs the final facility. The eNOS/NO system appears to be a signal/transduction arm that mediates response to the stressors. It is generally agreed that the greatest resistance to outflow resides in the juxtacanalicular region and inner wall of Schlemm’s canal (SC)—areas that are directly affected by contractile changes in the CM and TM.² The CM plays a major role, but the TM has its own active contractile/relaxant role executed efferently by the system noted above, modulated afferently by the various sensors in the CM tendons, the CM apex, and the TM, mediated by CTGF^{3,4} and NOS/NO,¹ among others, and further modulated by TGF β 2 and consequent downstream mediators and their effects on the ECM and other tissues. The interactions of this system are just now being appreciated. Understanding how this system functions is critical for determining how to effectively and efficiently manipulate elements of the system to therapeutic effect. The molecular pathways that modulate uveoscleral and trabecular outflow are complex. Advances in identifying the most salient therapeutic elements in such complicated systems are best catalyzed in small, focused meetings. These are a valuable complement to the larger research meetings, and more are needed.

CYTOSKELETAL AGENTS

This new class of medical therapeutic compounds for glaucoma is intended to enhance trabecular outflow by directly targeting the extracellular matrix, cell adhesions, and actin cytoskeleton of the TM and CM. The first class of compounds

to target the TM, even if indirectly, was the muscarinic agonists (e.g., pilocarpine). By contracting the CM, traction on the scleral spur expands and relaxes the configuration of the TM, reducing resistance to outflow.⁵ Cytoskeletal agents have a more direct effect on the structural and functional biology of the TM itself. They work by altering the dynamics of cell-cell and cell-matrix adhesions and cellular contractility, thus also relaxing and expanding the TM and reducing resistance. Outflow resistance is reduced in live monkeys (Fig. 1) and in human and monkey organ-cultured perfused anterior segments by treatment with cytochalasins or latrunculins (which alter the actin microfilament system), by treatment with myosin light-chain kinases and rho-associated protein kinase inhibitors, (which alter actomyosin contractility) and by overexpression of caldesmon (which also alters actomyosin contractility).⁶ The effects of several cytoskeletal agents (H-7 and Lat-A) are reversible (Fig. 2), indicating that they are due to transient alterations in cellular contractility and cytoskeletal organization rather than irreversible toxicity. This is an important safety consideration for a potential antiglaucoma medication. Interestingly, there seems to be a lingering “memory” that can last for up to 24 hours, such that IOP elevation or anterior chamber perfusion significantly increases outflow facility, even when anterior chamber drug concentration is declining or absent (Fig. 2).

The extracellular matrix of the TM consists of an intricate arrangement of fibronectin, collagen, laminin, proteoglycans, glycosaminoglycans, and matricellular proteins.⁷ Cross-linked actin networks (CLANs) are found in glaucomatous TM cells and in steroid-treated cell and eye organ cultures.⁸ They may contribute to an increase in outflow resistance. There are distinct β 1 and α v β 3 integrin signaling pathways that converge and cooperate to enhance CLAN formation, suggesting that increases in CLAN formation are due to upregulation in one or the other integrin signaling pathway.⁹ A variety of techniques (pharmacologic agents, activating and dominant negative peptides, function-blocking antibodies, and siRNAs) and models (cell and organ culture) were used in these studies in several laboratories, highlighting the importance of interlaboratory collaboration. Long-distance interlaboratory collaboration, making use of different areas of expertise, also played a key part in studies investigating cochlin, an extracellular matrix protein identified by proteomic analysis in human glaucomatous but not in control TM tissue. Cochlin expression in anterior segment organ culture models is increased after transforming growth factor (TGF)- β 2 treatment¹⁰ and is associated with pressure elevation and outflow facility reduction in organ-cultured anterior segments. Further organ culture system experiments demonstrate that cochlin expression alone results in an outflow facility decrease and subsequent pressure increase.¹¹ Understanding the role of cochlin in glaucoma is of considerable value in terms of therapeutics and with regard to the pathophysiology of glaucoma, but perhaps even more so for its potential in the development of an animal model that more closely resembles the actual disease process than anything currently available.

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FIGURE 1. Intracameral infusion of the actin-binding marine macro-lide latrunculin B (A) or the Rho kinase inhibitor Y-27632 (B) increases outflow facility dose-dependently in monkeys. (A) Reprinted from Peterson JA, Tian B, Geiger B, Kaufman PL. Effect of latrunculin-B on outflow facility in monkeys. *Exp Eye Res.* 2000;70:307-313, with permission from Elsevier. (B) Reprinted from Tian B and Kaufman PL. Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys. *Exp Eye Res.* 2005;80:215-225, with permission from Elsevier.

A significant unmet need in this area of research is biological, “tunable” models for TM and CM outflow. Tunable models could function in several ways, including as both on-off and modulatable strategies for enhancing outflow and as models that are capable of responding in a graded manner, as the natural tissue itself must surely be. Both in vitro and in vivo models are needed to characterize the effects of novel compounds, especially early on in the development process, to facilitate translation of findings to the clinic. A systematic review is needed to determine the predictive ability (not predictability) of models. A method of continuous measurement of

outflow facility would be very useful in quantifying physiological effects, understanding their time course, and investigating the additive nature of different classes of compounds. A large body of work in this area was accomplished through long-distance interdisciplinary collaborations among laboratories in Germany, Israel, and the United States, making use of expertise in cell biology, electron and light microscopy, anatomy, and physiology.⁶

PROSTAGLANDIN MOLECULES

Prostaglandin (PG) analogs mimic and amplify the effect of a naturally occurring tissue response pathway, remodeling the extracellular matrix of the CM and sclera and enhancing uveoscleral outflow. Relaxation of the CM plays a secondary role in the process. Commercially available PGs bind and activate the prostanoid FP receptor, a receptor for PGF₂α. Several novel prostanoid FP and EP receptor agonists are in development. BOL-303259-X is a new PGF₂α compound in phase II clinical trials for ocular hypertension. It is thought to contain a unique nitric oxide (NO)-donating element, in addition to latanoprost acid, that increases its efficacy in reducing IOP.¹² A selective prostanoid EP₄ receptor agonist 3,7-dithia-PGE₁ lowered IOP and increased total outflow facility in monkeys. There was no effect on uveoscleral outflow or aqueous flow, suggesting that increased trabecular outflow facility accounted for a substantial proportion of the ocular hypotensive activity.¹³ Further studies in human cell cultures and a whole-eye organ perfusion system showed that human SC and TM cells express PG-EP₄ receptors. Although 3,7-dithia-PGE differentially activates SC and TM PG-EP₄ receptors, their activation in the human conventional pathway results in a significantly increased outflow facility.¹⁴

Of particular relevance to discussions of outflow is the identification of lymphatic channels in the ciliary body stroma and in the CM between muscle bundles.¹⁵ These lymphatic channels, with a central lumen formed by endothelial cells, are distinct from blood vessels, in that they lack a collagen IV-positive basement membrane. Nonspecific staining was absent in all negative controls. Green fluorescent nanoparticles were injected intracamerally into sheep eyes and subsequently observed within a central lymphatic channel lumen 15 to 45 minutes after injection. Iodine-125 radio-labeled human serum albumin was also injected intracamerally into sheep eyes. Four hours after injection, the tracer was drained preferentially into lymph nodes in the head and neck region compared to reference popliteal lymph nodes in the hind leg. These experiments demonstrate that the lymphatic channels are involved in the egress of particles injected into the anterior chamber and the movement of particles into head and neck lymph nodes. Most likely, they work to clear proteins and interstitial fluid from the eye. This system may function as a backup outflow system, when the TM is injured or inflamed, rerouting aqueous drainage from the TM to the CM pathway to both maintain physiologic IOP and remove the excess interstitial proteins that accumulate in the uvea during inflammation. Lymphatic vessels respond to a variety of biochemical and pharmacologic agents, including PGs, which may be important signaling molecules in the regulation of lymphatic channel contractility. Uveoscleral outflow increases fourfold in experimental inflammation of the ciliary body in monkeys. It is possible that the uveolymphatic pathway plays an important role in extracellular matrix remodeling consequent to ocular inflammation. Targeting lymphatic drainage may be a new approach for modulation of outflow.

Increasing age is associated with decreased uveoscleral flow in humans and also in monkeys. It is assumed that the conventional outflow pathway compensates for the effect of aging, preventing an increase in IOP in normal eyes. Identification

FIGURE 2. Reversibility and memory of H-7 (myosin light chain kinase & Rho kinase inhibitor) (A) and latrunculin A (B, C) effects on outflow facility in monkeys. (A) Reprinted from Sabanay I, Tian B, Gabelt BT, Geiger B, Kaufman PL. Functional and structural reversibility of H-7 effects on the conventional aqueous outflow pathway in monkeys. *Exp Eye Res*. 2004;78:137–150, with permission from Elsevier. (B, C) Reprinted from Peterson JA, Tian B, Bershadsky AD, et al. *Invest Ophthalmol Vis Sci*. 1999;40:931–941, with permission from ARVO.

and quantification of specific changes that occur in the uveoscleral pathway with age is needed to explain the reduction in uveoscleral flow and distinguish that from the effects of therapeutics. At present, there is no direct, noninvasive method for determining uveoscleral outflow. Calculations based on the modified Goldmann equation are the only way to estimate uveoscleral outflow in clinical studies. Better clinical techniques for the measurement of uveoscleral flow would be a high priority on any unmet needs list.

ALTERNATIVE TARGETS

The Wnt signaling pathway is another example of a target with therapeutic potential as well as potential for development of a glaucoma model. This pathway is a network of proteins involved in several physiological processes in adult animals (regulation of hippocampal neural stem cell behavior, cell growth, and apoptosis).¹⁶ Components of the Wnt signaling pathway are found in the ciliary body, TM, cornea, and retina of the adult eye.¹⁶ Secreted frizzled-related protein-1 (sFRP-1) is an antagonist of the Wnt signaling pathway that is differentially expressed in glaucomatous human TM cells compared with normal human TM cells. Studies in rodent models and perfusion-cultured human anterior segments¹⁷ suggest that Wnt signaling plays a role in regulating IOP, that increased expression of sFRP-1 in the TM is associated with elevated IOP and decreased outflow

facility (concurrent with reduced levels of β -catenin, a Wnt-signaling mediator), and that restoring Wnt signaling in the TM would be an intervention strategy for treating glaucoma.

Adenosine receptors may serve as endogenous modulators of IOP, and adenosine agonists may have potential as IOP-lowering therapies. Aqueous adenosine concentrations are higher in ocular hypertensive individuals than in normal, and there is a linear correlation between IOP and adenosine levels.¹⁸ Topical application of adenosine A1 agonists decreased IOP and increased outflow facility in monkeys.¹⁹ The adenosine A1 agonist N6-cyclohexyladenosine increased outflow facility and was associated with matrix metalloproteinase (MMP) activation in bovine organ-cultured anterior segments.²⁰ The selective adenosine A1 agonist INO-8875 significantly reduced IOP in glaucoma patients in a phase I/II single ocular dose clinical trial, reportedly by increasing outflow of aqueous humor through the TM. Recruitment of patients is under way for a phase II randomized, double-masked, placebo-controlled, dose-escalation trial. The adenosine A2a receptor agonist OPA-6566 is currently in development for the treatment of glaucoma. This compound is also thought to lower IOP by enhancing aqueous humor outflow via the TM.

Angiotensin 1 (AT1) receptors are found in human and rabbit ocular tissues. AT1 antagonists increase vasodilation and extracellular matrix formation, which can in turn affect aqueous humor dynamics. Intracameral infusion of AT2 resulted in

a decrease in outflow facility in monkeys that was not mediated by the CM.²¹ Topical AT1 receptor antagonist CS-088 decreased IOP and increased uveoscleral outflow in monkeys²² and appeared to lower IOP in ocular hypertensive rabbits²³ by increasing uveoscleral outflow (with no effects on aqueous flow and outflow facility), although the effects were small.²⁴ The AT-converting enzyme inhibitor enalaprilat lowered IOP in monkeys by promoting the formation of endogenous PGs, which in turn modified the outflow pathway and caused an increase in outflow facility.²⁵

NO is a multifunctional signaling molecule implicated in a variety of physiological processes, including the regulation of vascular tone, neuronal communication, and inflammatory response. Endogenous production of NO is a complex process requiring multiple substrates and cofactors. Both over- and underexpression of NO may contribute to pathologic conditions in the eye. There can be decreased bioavailability or production of NO or, conversely, a sustained overproduction that results in toxicity. In perfused anterior segments from human donor eyes, elevating the pressure from 10 to 25 mm Hg caused a significant increase in NO levels in the perfusate, accompanied by an upregulation of iNOS gene expression determined by quantitative PCR.²⁶ Previous studies from the same group demonstrated that elevated NO levels increase outflow; taken together, these findings suggest the existence of a regulatory feedback mechanism involving NO in the TM that contributes to the regulation of IOP. In such a system, an increase in IOP enhances NO production, which in turn increases outflow facility, leading to a normalization of IOP (Ethier CR, et al. *IOVS* 2011;52:ARVO E-Abstract 6618). Similarly, overexpression of eNOS combined with increased production of NO lowered IOP and increased conventional outflow facility in transgenic C57BL/6 mice overexpressing a fusion protein of active eNOS. The NOS inhibitor L-NAME eliminated the outflow increase in the transgenic animals. When pressure was elevated to >35 mm Hg in control animals, outflow increased to match that of transgenic mice at low IOP, suggesting eNOS induction at high IOP. Theoretical calculations of the expected shear stress at high IOPs due to aqueous humor flow in SC was comparable to that of humans and sufficient in range to induce eNOS expression. These findings tie in with recently discovered polymorphisms in the NOS3 gene associated with ocular hypertension in glaucoma.¹ NO induction is a potential therapeutic strategy for increasing TM outflow facility as glaucoma therapy.

Kappa opioid receptors (KORs) are present on cell membranes in human nonpigmented ciliary epithelial and TM (HTM-3) cells. Activation of these KORs by the selective KOR agonist spiradoline resulted in increases in NO production in both cell types that were inhibited by KOR antagonists. It is possible that previously demonstrated KOR-mediated reduction in IOP occurred partly because of NO production in both the ciliary body and the TM.²⁷

Collectively, investigators conducting these NO studies had backgrounds in pharmacology, bioengineering, ophthalmology, mechanical engineering, and neuroscience. More opportunities for interdisciplinary collaboration are needed. Pairing selected investigators from different disciplines with incentives to collaborate—a variant on arranged marriages—would be likely to result in more innovative approaches. More involvement of physicians in basic research that extends beyond their clinical residency/fellowship is essential because the clinical perspective is unique and necessary for addressing real-world clinical needs and developing realistic therapeutics. Discussions of novel targets for glaucoma therapy highlight the need for more willingness on the part of pharma (large and small) to go after higher branch fruit. Pharma is spending less on in-house research, relying on start-ups to do the discovery work—

and to struggle for early funding. The mantra used to be that the pharmaceutical industry would not look at anything that did not originate in the United States and would spend a great deal of money trying to produce it here. The current trend is to not spend money on producing it here, to close their research units, and to partner with or buy small companies when their development of a novel idea has reached a certain stage, usually after some initial validation in early preclinical or clinical trials. In tandem with this is an increase in the number of small start-up companies with an early exit strategy geared toward a buyout by a larger company. Products are developed to make the company an attractive target. The model has changed, but there may be less money in the research aspect of the drug discovery process overall, whereas costs of clinical trials and postmarket surveillance have increased.

DRUG DELIVERY

Topical eye drops have poor bioavailability, with only 1% to 7% of the active ingredient absorbed into the eye. Corneal permeability is often hindered by solution drainage, lacrimation and tear turnover, tear evaporation, and conjunctival absorption. Nasolacrimal drainage and conjunctival absorption can result in systemic side effects from topical drops. Patient adherence is critical in chronic diseases such as glaucoma, where estimates of nonadherence range from approximately 25% to 60%. Over time, most patients will require more than a single class of topical drop to control their disease, but increased complexity and frequency of dosing regimens often result in decreased adherence. Removing the patient from the drug delivery system is a key goal in glaucoma therapeutics. Novel methods for delivering drugs to target tissues are in development, including encapsulated cell technology, gene transfer, nanoparticles, microspheres, sustained release technologies, and coated microneedles.

The strategy for several of these methods is similar. Target cells are reprogrammed to increase or decrease expression of a gene product resulting in up- or downregulation of a biochemical/physiological process.

Gene expression activation or inhibition can be manipulated by increasing the amount of the construct (e.g., introducing greater numbers of gene-expressing viral particles or siRNA), using regulation strategies like Tet off/on or inducible vectors that “turn on” in the presence of a physiological event such as elevated IOP.

Encapsulated cell therapy (ECT) is a novel sustained delivery strategy for ciliary neurotrophic factor. ECT implants are anchored to the sclera within the posterior segment. They contain cells that are genetically modified to produce the desired therapeutic factor, which is released over time. The cells are encapsulated within a semipermeable, hollow-fiber membrane, thus avoiding immune reaction. Currently, ECT is in phase II and III clinical trials for the treatment of geographic atrophy associated with dry age-related macular degeneration and retinitis pigmentosa.

RNA interference (RNAi) is a useful strategy in situations in which suppressing the expression of a single protein will address the symptoms or pathology of the disease. RNAi therapies can be effective at lower concentrations than small molecules, which could potentially mean lower doses and fewer adverse effects.

In vivo delivery is a challenging aspect of RNAi therapeutic development. Effects can be short-lived, as endo- and exonucleases and RNases that are present in many tissue microenvironments quickly degrade unmodified, naked siRNAs.²⁸ It is not clear whether RNases are present in human aqueous humor or that of other species. Off-target effects are difficult to

predict, and careful consideration of species differences during preclinical testing is critical. It remains to be seen whether species-specific surrogate sequences will have to be designed so that preclinical toxicity testing can be performed in parallel with human-specific sequences. As with some novel strategies, RNAi may be as valuable in modeling diseases, studying the effects of silencing-specific genes in vitro and in vivo, as it is in treating them.

Both nonviral and viral gene transfer methods have proponents. Nonviral gene delivery methods (mechanical, physical, and chemical) are advantageous because of their low immunogenicity, a large capacity for DNA size, ease of manipulation, and low-cost production and production ramp-up. Generally, these methods are somewhat less efficient in gene transfer, requiring a larger amount of vector to achieve a response comparable to that achieved with viral vectors. Nonviral methods have a relatively short therapeutic duration (e.g., naked DNA), and not all ocular cell types can be easily transfected by these methods (e.g., cultured human trabecular meshwork [HTM] cells); work is ongoing to overcome these weaknesses. Viral vectors tend to have higher transfection efficiencies and smaller loading capacities, and to be more difficult to produce at a large scale. Although advances have been made to greatly reduce the risk of inflammatory and immunogenic responses and insertional mutagenesis, it cannot be said that the risks are nil. Successful viral vector-mediated gene therapy for ocular diseases such as Leber's congenital amaurosis are encouraging the development of gene therapy strategies for glaucoma, where targets include cytoskeletal-modulating proteins that enhance outflow through the TM, PG pathway elements that increase uveoscleral outflow, and neurotrophic factors (brain-derived neurotrophic factor, ciliary neurotrophic factor, glial cell-derived neurotrophic factor) that have been used in laboratory studies of neuroprotection. Recent work demonstrates that long-term (>2 years thus far) expression of reporter genes in the primate outflow pathway is possible in vivo with self-complementary AAV²⁹ and FIV³⁰ vectors, with low immunogenicity and clinically quiet anterior segments.

Viral vectors that have been investigated for ocular delivery of genes include adenovirus, herpes simplex virus, AAV, and lentivirus (FIV, EIAV). Each has strengths and weaknesses that are being addressed. The National Eye Institute's Ocular Gene Therapy unit is studying and developing the therapeutic potential of AAV vectors, which have different serotypes with different tropisms as well as novel hybrid "pseudotyped" recombinant AAV vectors.

Nanotechnology applications are being developed for several ocular diseases using a variety of nanosuspensions, liposomes, dendrimers, nanoparticles, ocular inserts, implants, and hydrogels. For glaucoma, at this stage, most are topical drop formulations that it is hoped will achieve improved corneal permeability, increased bioavailability, reduced dosages, and extended release. Surface-modified nanoparticulate carriers may be used to accommodate a wide variety of active compounds, including poorly water-soluble drugs. Several types of biodegradable polymers can be used in a single formulation to create a release profile consisting of an initial burst followed by sustained release or to facilitate penetration across different tissue layers. Drugs can also be coupled to nanocarriers that are specific for cells and/or organs. No standardized procedure for the formulation of drug-loaded nanoparticles has been developed that addresses formulation stability, particle size uniformity, consistent control of drug release rate, and large-scale manufacture of sterile preparations. As with all new treatment options, the risks and benefits and the impact of these therapies on patterns of clinical practice remain to be seen.

Delivery strategies, such as viral and nonviral gene transfer, sustained release, microneedles, canaloplasty, and others to

deliver compounds directly to the relevant tissue, and encapsulated cell therapy, all offer improvements over topical drop therapeutics. However, the need for better biodelivery strategies for outflow modulations remains and includes the need for improved vectors for gene transfer in vivo. Suppression of inflow is a proven method of IOP reduction. As the molecular pathways are now known, they could be good targets for gene or siRNA approaches. More focused studies of genetic manipulation of aqueous inflow would be complementary and offer further opportunities for identification of therapeutic targets and development of research models.

IOP MONITORING

Much of the data in the literature about IOP are conflicting—how, when, and where to measure. It is possible that more comprehensive monitoring of IOP would have predictive value in disease progression. IOP range studies indicate that office visit IOP measurements do not reflect the true extent of a patient's IOP fluctuation and risk of disease progression, with peak IOP and a larger fluctuation in IOP values found outside of office hours.³¹ Current home measurement of IOP with handheld devices is variable and not reliable across patients. Measurement devices in development use sensors in contact lenses, intraocular lenses, or other intraocular-implanted devices to provide continuous IOP measurement that is transmitted to a nearby external recording device (eye to iPhone?) and then transmitted to and analyzed by a computer. Until reproducible, accurate, continuous measurement of intraocular pressure can be achieved, it will not be known what this information will contribute to our understanding of glaucoma pathophysiology or to the treatment decision-making process.

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