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Deconstructing aqueous humor outflow – the last 50 years

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Abstract

Herein partially summarizes one scientist-clinician's wanderings through the jungles of primate aqueous humor outflow over the past ~45 years. Totally removing the iris has no effect on outflow facility or its response to pilocarpine, whereas disinserting the ciliary muscle (CM) from the scleral spur/trabecular meshwork (TM) completely abolishes pilocarpine's effect. Epinephrine increases facility in CM disinserted eyes. Cytochalasins and latrunculins increase outflow facility, subthreshold doses of cytochalasins and epinephrine given together increase facility, and phalloidin, which has no effect on facility, partially blocks the effect of both cytochalasins and epinephrine. H-7, ML7, Y27632 and nitric oxide – donating compounds all increase facility, consistent with a mechanosensitive TM/SC. Adenosine A1 agonists increase and angiotensin II decrease facility. OCT and optical imaging techniques now permit visualization and digital recording of the distal outflow pathways in real time. Prostaglandin (PG) F2α analogues increase the synthesis and release of matrix metalloproteinases by the CM cells, causing remodeling and thinning of the interbundle extracellular matrix (ECM), thereby increasing uveoscleral outflow and reducing IOP. Combination molecules (one molecule, two or more effects) and fixed combination products (two molecules in one bottle) simplify drug regimens for patients. Gene and stem cell therapies to enhance aqueous outflow have been successful in laboratory models and may fill an unmet need in terms of patient compliance, taking the patient out of the delivery system. Functional transfer of genes inhibiting the rho cascade or decoupling actin from myosin increase facility, while genes preferentially expressed in the glaucomatous TM decrease facility. In live NHP, reporter genes are expressed for 2+ years in the TM after a single intracameral injection, with no adverse reaction. However, except for one recent report, injection of facility-effective genes in MOCAS have no effect in live NHP. While intracameral injection of an FIV.BOVPGFS-myc.GFP PGF synthase vector construct reproducibly induces an ~2mmHg reduction in IOP, the effect is much less than that of topical $PGF_{2\alpha}$ analogue eyedrops, and dissipates after 5 months. The turnoff mechanism has yet to be defeated, although proteasome inhibition enhances reporter gene expression in monkey organ cultured anterior segments (MOCAS). Intracanalicular injection might minimize off-target effects that activate turn-off mechanisms. An AD-P21 vector injected sub-tenon is effective in 'right-timing' wound healing after trabeculectomy in live laser-induced glaucomatous monkeys. In human (H)OCAS, depletion of TM cells by saponification eliminates

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the aqueous flow response to pressure elevation, which can be restored by either cultured TM cells or by IPSC-derived TM cells.

There were many other steps along the way, but much was accomplished, biologically and therapeutically over the past half century of research and development focused on one very small but complex ocular apparatus. I am deeply grateful for this award, named for a giant in our field that none of us can live up to.

Keywords

Glaucoma; Aqueous humor outflow; Intraocular pressure; Actin cytoskeleton; Gene therapy; Stem cell therapy; Nitric oxide; Mechanosensitivity

1. Introduction

I am honored and grateful to receive the 2018 Endré A. Balázs Prize, and to present the lecture named for him. Endré A. Balázs was a distinguished scientist and entrepreneur, whose outstanding contributions provided far-reaching progress in experimental eye research and in clinical ocular surgery (“Endre Balazs,” 2015). Dr. Balazs was one of a group of scientists who came together in 1968 to establish an international organization to support vision research, which eventually became ISER. Dr. Balázs was the founding editor of Experimental Eye Research, which eventually became the official journal of ISER. He also co-founded the International Society for Hyaluronan Sciences. Dr. Balazs was a world leader in ophthalmic biochemistry and was instrumental in the development of products that revolutionized eye surgery, notably high-molecular weight hyaluronan, commercially known as Healon®. Importantly, he founded several companies and a philanthropic foundation dedicated to exploring and promoting the therapeutic potential of hyaluronic acid. Dr. Balazs’s complete biography is easily found (“Endre-A-Balazs-doctor-who-found-acid-to-treat-arthritic-knees,” 2015) and is an inspiring story well worth reading.

2. Personal History

Mine is a 45-year story filled with amazing people and fascinating and often unexpected science, that redefined how we think about glaucoma, the world’s most common cause of irreversible vision loss. I always knew that I wanted to be both a physician and a scientist, but scientist-physician was a better descriptor for me than was physician-scientist. However, I approached my senior year of medical school with no idea of what my next step should be. Fortunately, the mandatory military service requirement in the US for physicians at that time provided two years of post-internship training and service at the NIH, where I learned a lot about biostatistics, epidemiology and clinical trials design, became engaged and married, and had time for my pin-the-tail-on-the-donkey tentative decision to do my residency training in ophthalmology to jell. By what I can only ascribe to a stroke of good fortune or the hand of a guardian angel, I landed at Washington University in St. Louis, under the tutelage of the great Bernard Becker and the terrific team he had assembled. Although the clinical demands of the residency did not allow much time for research, I was able to design and execute two small studies of the biochemical makeup of subretinal fluid as a predictor of

surgical and visual outcome in patients with rhegmatogenous retinal detachments (Kaufman, 1976, 1975; Kaufman and Podos, 1973; Kaufman PL, 1974, 1973). Small though they were, these studies taught me the joy of asking the “how” and “why” of pathophysiology and therapeutics.

Glaucoma and physiology/pharmacology seemed like the ideal intellectual setting for me. Dr. Becker; his protégé Steven Podos; Carl Kupfer, then Director of the National Eye Institute; and Ernst Bárány, the great ocular pharmacologist/physiologist and the world’s leading authority on aqueous humor dynamics, planned a two-year fellowship for me in Prof. Bárány’s lab at the University of Uppsala in Sweden, where he was the Chair of the Department of Medical Pharmacology. The NEI Study Section enthusiastically voted to support this, but then the application was “administratively withdrawn” when the Nixon Administration suspended all NIH training funds. I managed to secure a Seeing Eye Fellowship of $12,000, and another $2,000 from ALZA, the manufacturer of the pilocarpine Ocusert (Lee et al., 1975) and the Alzet mini pumps (Greenshaw, 1986).

In 1973, this princely sum of $14,000 did not go very far in Sweden. The 1973 Middle East War and oil embargo combined to drive down the value of the US dollar, but with a 6-month old child and minimal resources even for a 1-year fellowship, my wife and I made the final decision to go just three weeks before our scheduled departure shortly after the end of my residency training. Dr. Kupfer provided the security of a 2-year staff fellowship at the NEI when I returned if I had not secured an academic position by that time.

Life overall was good for us in Sweden. Wife Margaret, daughter Alison and I lived in a large if somewhat stark partially university-subsidized apartment. Laundry facilities were an outdoor quarter-mile walk away – lots of fun in the winter - with a drying room rather than automatic dryers, where everything was hung on drying racks and you then waited for magic to happen. We had old bicycles and a hand-me-down baby carriage, a rattletrap ancient car in our second year, and no discretionary money. Indeed, we dipped into savings to make ends meet. Despite the Spartan lifestyle, the apartment complex and the department were both collegial and we made friends - American, Canadian, German, Finnish and Swedish - that we have to this day, 45 years later.

3. Aqueous Humor Dynamics

3.1 Physiology & Pharmacology

The work environment was off-the-charts phenomenal, after getting off to a slow start. It took about six months and a truly great laboratory instrument machine shop to work out the surgical techniques. We worked exclusively in non-human primates (and still do so), partnering with Prof. Anders Bill for certain boutique physiological experiments and Profs. Johannes Rohen and Elke Lütjen-Drecoll for light and electron microscopic studies.

3.1.a Conventional (trabecular meshwork/Schlemm’s canal) outflow pathway

—This pathway encompasses outflow from the anterior chamber through the various regions of the trabecular meshwork (uveal, trabecular, juxtacanalicular (cribriform), then across or through the inner wall endothelium of Schlemm’s canal into the canal lumen, then through

an intrascleral vascular plexus (aqueous collector channels, intrascleral veins) that empty into the episcleral veins and from there eventually into the general venous circulation (Fig. 1).

3.1.a.1 Muscarinic cholinomimetics: We completely removed the iris via a small transcorneal incision in the live monkey eye (Kaufman and Lütjen-Drecoll, 1975). After a short post-surgical recovery, the eyes were quiet and resting perfusion outflow facility and the facility response to intravenous pilocarpine were identical in the surgically aniridic and the contralateral iridic eyes, proving that the iris has no functional role in regulating trabecular meshwork outflow facility or its response to muscarinic cholinergic agonists (Kaufman, 1979). Thus, to say that miotic drugs are a treatment for open angle glaucoma is somewhat a misnomer, as miosis has nothing to do with their facility-increasing effect. There is, however an intimate anatomic relationship between the ciliary muscle and the trabecular meshwork, with some of the muscle's anterior tendons mingling with the elastic network of the trabecular meshwork, which in turn sends fibers that insert into special surface adaptations of the Schlemm's canal inner wall endothelial cells (Suppl Fig. 1) (Rohen et al., 1981, 1967).

When the ciliary muscle contracts as in response to muscarinic cholinergic agonist drugs, the meshwork is expanded, relaxed, and less stiff, and Schlemm's canal dilates (Lütjen-Drecoll, 1973), all contributing to increased outflow facility and reduced intraocular pressure (IOP). To prove that ciliary muscle contraction was mediating the facility increase, we used a transcameral goniotomy-type surgical approach to cut the ciliary muscle from the scleral spur over the entire 360-degree circumference at one sitting (Kaufman and Bárány, 1976). This allowed the muscle's elastic posterior attachments to retract posteriorly so that the posteriorly-pulled anterior tips reattached firmly to the inner scleral wall about 2 mm posterior to the scleral spur (Lütjen-Drecoll et al., 1977). In the non-inflamed eyes, now absent the iris and with the ciliary muscle disinserted from the scleral spur and trabecular meshwork/Schlemm's canal (TM/SC) [Fig. 2], resting outflow facility was modestly reduced (Kaufman and Bárány, 1976), as expected if the meshwork became more compact and the canal narrowed absent the tonic pull of the ciliary muscle. Intravenous and intracameral pilocarpine had essentially no effect on outflow facility (Kaufman and Bárány, 1976) [Suppl Fig. 2]. This constellation of results proved that contraction of the ciliary muscle increased outflow facility, that pilocarpine's facility-increasing effect was mediated solely by ciliary muscle contraction affecting TM and SC geometry with no primary drug effect on the TM/SC, and that the iris was completely uninvolved (Kaufman, 1979; Kaufman and Bárány, 1976). It now became feasible to assess the effects of other drugs on outflow facility, free from potential confounding effects on ciliary muscle contraction/relaxation. There was still the hope that the miosis, accommodative and outflow facility effects of cholinomimetics could be decoupled, as the first two were hard-stop side effects in elderly folks with even early cataracts, and younger patients who still retained significant accommodative capacity, respectively. However, it turned out that all three functions were mediated by the same m3 muscarinic receptor subtype, and no way of pharmacologically separating them was found (Gabelt and Kaufman, 1992).

3.1.a.2 Adrenergics: Epinephrine, norepinephrine and 3',5'-cyclic adenosine monophosphate (cAMP) (but not the inactive metabolite 5'AMP) increased facility similarly in iridic and aniridic eyes, and in CM-disinserted and non-CM-disinserted eyes. This proved that the TM/SC was not 'dead', and that epinephrine and its second messenger cAMP were acting directly on the TM/SC (Kaufman, 1987a, 1986a, 1986b; Kaufman et al., 1981; Kaufman and Rentzhog, 1981). We then defined other classes of compounds that provided significant insights into TM biology and regulation, and that might be 'druggable' for glaucoma therapy by upregulating or downregulating an outflow facility – related pathway (Croft and Kaufman, 1995a, 1995b; Hubbard et al., 1997, 1996; Kaufman, 1987b; Kaufman et al., 1982, 1977; Kaufman and Erickson, 1982; Menage et al., 1995; Robinson and Kaufman, 1992; Svedbergh et al., 1978; Tian et al., 1997).

3.1.a.3 Actin cytoskeleton

3.1.a.3.a Cytochalasins: Cytochalasins are fungal metabolites that disrupt actin microfilaments and the actin cytoskeleton. In essence they prevent the addition of actin monomers to one end of the microfilament without affecting the dissolution of actin monomers at the other end. (Kaufman and Erickson, 1982) The process of monomer addition and deletion has been termed the "F-actin treadmill", and keeps the filaments refreshed and stable. With cytochalasins present, the filaments lose but do not replenish monomers, and thus become shorter and weaker. This disrupts the actin microfilament system, impairs acto-myosin contractility, degrades and weakens cell-cell adherens attachments, as well as cell-ECM attachments (focal contacts), and relaxes the cells (Kaufman et al., 1977; Kaufman and Erickson, 1982; Svedbergh et al., 1978; Takano et al., 1975). In cells with a robust actin cytoskeleton such as HTM and Schlemm's canal inner wall cells this may have important functional consequences (Inomata et al., 1972a; Stamer et al., 2015). In live monkeys, the entire meshwork relaxes and expands and Schlemm's canal dilates, allowing fluid to flow through the entire system more easily, i.e., outflow resistance decreases / outflow facility increases (Gabelt and Kaufman, 2012). Unfortunately, given the two compartment – two pressure proximal conventional outflow system that comprises the anterior chamber, trabecular meshwork and Schlemm's canal, the inner canal wall was ruptured and platelets attempted to plug the rupture as in blood vessels (Fig. 3) (Gabelt and Kaufman, 2012; Kaufman, 1987b; Kaufman et al., 1977; Kaufman and Erickson, 1982; Robinson and Kaufman, 1991; Svedbergh et al., 1978; Tian et al., 1999).

While these findings were of great mechanistic interest, this was not a viable therapeutic approach. However, it did provide valuable therapeutic insights that utilized carefully developed epinephrine (Kaufman, 1986a) and cytochalasin B (Kaufman and Erickson, 1982) dose – outflow facility response relationships. Sub-threshold doses of epinephrine and cytochalasin B given together intracamerally induced significant facility increases that were dose-dependent within the subthreshold range for either drug (Robinson and Kaufman, 1991). Furthermore, addition of subthreshold doses of cytochalasin B to a maximal dose of epinephrine gave an additional response (Robinson and Kaufman, 1991). Finally, phalloidin, which stabilizes actin microfilaments and protects them against the disruptive actions of cytochalasins, partially inhibited the facility-increasing effect of epinephrine (Robinson and Kaufman, 1994). Thus, epinephrine was actually the first and the original cytoskeletal drug

for increasing outflow facility, other claims notwithstanding. Indeed, epinephrine predated other cytoskeletal / actomyosin contractility-inhibiting drugs by well over half a century – even though its effector mechanism was completely unknown! Of course, and as is often the case, clues were there from other areas of science. Epinephrine acts as, among other things, a ß$_2$-adrenergic receptor agonist, via the cAMP signaling cascade.(Bazrafkan et al., 2010; Neufeld and Sears, 1974). As noted above, in live monkeys, cAMP, but not its inactive metabolite 5'AMP, increases outflow facility, both in normal and in iridectomized and ciliary muscle inserted eyes (Kaufman, 1986b). Macrophage migration through blood vessel walls and lymphocyte adhesion to vessel walls are altered by epinephrine (Petty and Martin, 1989) and those effects are inhibited by timolol, a non-selective ß$_1$, ß$_2$ adrenergic receptor antagonist (Petty and Martin, 1989). Thus, non-innervated white blood cells have ß-adrenergic receptors mediating cytoskeleton/actomyosin contractility-related functional effects. Welcome to the TM of the 21st century!

3.1.a.3.b Latrunculins: Recognizing that cytochalasin-based 'war' on the TM was not a viable therapeutic strategy, but believing that we had identified an important and potentially therapeutically useful control mechanism, we were drawn to work involving latrunculins, marine macrolides that are metabolites of Red Sea sponges. Latrunculins bind 1:1 with globular free actin monomers in cell cytoplasm, thus 'drying up' the pool of free actin available to add to the F-actin treadmill (Spector et al., 1989, 1983). Both latrunculin A and B, delivered intracamerally or topically to live monkey eyes, dramatically increased outflow facility by ~100–200% in normal (Suppl Fig 3) and aniridic/ciliary muscle disinserted eyes (Peterson et al., 2000, 1999; Sabanay et al., 2006). Latrunculin B was carried forward into a phase 1 human clinical trial, which confirmed its safety and modest efficacy (Rasmussen et al., 2014). It was not developed further because the company, Inspire Pharmaceuticals, was bought by Merck for other reasons, and Merck had no glaucoma drug development program.

3.1.a.4 Actomyosin contractility

3.1.a.4.a Kinase inhibitors: Concurrently we approached the system from the actomyosin contractility aspect. Broad spectrum serine-threonine kinase inhibitors such as H-7, that can inhibit both the Rho kinase (RK) and myosin light-chain kinase (MLCK) pathways, prevent phosphorylation and activation of the myosin light chain and thereby inhibit actomyosin contractility (Fig.4). This leads to degeneration of actin microfilaments and vinculin-rich cell-ECM junctions (focal contacts) (Iizuka et al., 1999; Sabanay et al., 2004; Somlyo and Somlyo, 1994; Traube, 2006), and ultimately to expansion of the JCT and dilation of SC but without SCIWE breaks (Fig 5, Suppl Fig 4). In live monkeys, H-7, Y-27362 (selective RK inhibitor, Suppl Fig 5) and ML-7 (selective MLCK inhibitor) all increase outflow facility by 100–200% in live monkeys (Honjo et al., 2001; Tian et al., 2004, 2000; Tian and Kaufman, 2005; Vasantha Rao et al., 2001). RKIs were selected for development as glaucoma therapeutics perhaps because pharmaceutical companies were more familiar with them, and many companies had them in their libraries. At present, one such agent entered the commercial market in Japan in 2014 (ripasudil) (Honjo and Tanihara, 2018; Inazaki et al., 2017; Inoue and Tanihara, 2017, 2013) and another entered the USA market in 2018 (netarsudil) (Kahook et al., 2019; Serle et al., 2018).

3.1.a.4.b Nitric oxide: Nitric oxide (NO) can be both a toxic free radical and an important signaling molecule (Becquet et al., 1997). Acting through soluble guanylyl cyclase (cGMP) as the second messenger (Suzuki et al., 2009), NO relaxes vascular endothelial cells and vascular smooth muscle by inhibiting various aspects of the rho cascade, (Nathanson and McKee, 1995; Stamer et al., 2011) thereby dilating blood vessels. Compounds that contain a nitric oxide-donating moiety relax the TM and dilate SC and increase outflow facility after intracameral injection in non-human primates (Gabelt et al., 2011; Heyne et al., 2013). cGMP itself injected into the anterior chamber also increases outflow facility (Kee et al., 1994). Latanoprostene bunod, a single molecule with a PGF_{2a} backbone and an NO-donating moiety (Garcia et al., 2016; Kaufman, 2017), lowers IOP in ocular hypertensive and POAG human subjects (Kawase et al., 2016; Medeiros et al., 2016; Weinreb et al., 2015) by ~ 1 mmHg more than latanoprost (Weinreb et al., 2015), and entered the US commercial market in 2018 (Weinreb et al., 2018) after FDA approval in December 2017. Since NO inhibits the rho cascade, additivity between RKI and NO-donating compounds is uncertain.

3.1.a.4.c TM/SC mechanosensitivity: Trabecular meshwork/ Schlemm's canal inner wall (TM/SCIW) cells have an intrinsic actomyosin contractility mechanism that may be the efferent arm of an IOP-regulating mechano-sensitivity reflex. IOP, shear stress, various hormones and cytokines may be the afferent arms; endothelial nitric oxide synthase (eNOS) – NO may be a signaling arm (Stamer et al., 2011); and cellular relaxation / contractility and cell-ECM / cell-cell adherens junction formation/degradation may be the efferent arms (Kaufman and Rasmussen, 2012). Carreon and Johnstone have elegantly hypothesized and described the movements of the system at the macro level (Carreon et al., 2017; Johnstone, 2014).

3.1.a.5 Adenosine agonists: Adenosine A_1 receptor agonists injected into the anterior chamber increase outflow facility in monkeys (Tian et al., 1997), perhaps by upregulating the synthesis/release of various metalloproteinases affecting the TM / SCIW (Crosson et al., 2005; Husain et al., 2006; Shearer and Crosson, 2002). A Phase III clinical trial of an adenosine A1R agonist yielded only modest IOP reduction, and its clinical future is uncertain (Liebmann and Lee, 2017; Myers et al., 2016).

3.1.a.6 Conventional outflow facility-decreasing agents: There are several facility-decreasing molecular pathways in addition to glucocorticosteriods (Montecchi-Palmer et al., 2017; Tripathi et al., 1994). For example, $TGFß_2$, found in excess in the aqueous humor and TM of patients with POAG, decreases outflow facility in MOCAS (Bhattacharya et al., 2009; Goel et al., 2012) while ergotamine and angiotensin II decrease facility in live monkeys (Kaufman et al., 1981; Kaufman and Rentzhog, 1981) and angiotensin antagonists have been tested as potential anti-glaucoma agents (Costagliola et al., 2000; Hirooka and Shiraga, 2007; Lotti and Pawlowski, 2009; Shah et al., 2000). These and other strategies discussed below may allow development of experimental molecular models for glaucoma in MOCAS or live monkeys.

3.1.a.7 'Distal' conventional outflow pathway: Decades-old studies and mantra have attributed ~25% of conventional outflow resistance as residing beyond (i.e., downstream

from) the inner wall endothelium of Schlemm's canal, and likely beyond the canal itself (Grant, 1963, 1958). There was awareness of what is now called the distal conventional outflow pathway – the labrynth of intrascleral vessels connecting Schlemm's canal and the episcleral veins (Hamanaka et al., 1992), but their complex anatomy and physiology was poorly understood, and virtually nothing was known about their putative pathophysiology, if any. Recent studies have shown that the collector channels emanating from the outer wall of Schlemm's canal may have contractile and perhaps sphincter-like properties (Rohen and Rentsch, 1968), and even more distal intrascleral venules may have a contractile apparatus, so that resistance in this pathway under some conditions may be higher than previously thought (de Kater et al., 1992; Gonzalez et al., 2017). Recent advances and future developments in optical coherence tomographic imaging e.g. OCT angiography (Huang et al., 2018) may help unravel these mysteries.

3.1.b Unconventional (uveoscleral) outflow pathway—This pathway encompasses the connective tissue-filled spaces between the sheaths surrounding the ciliary muscle bundles (~12 muscle fibers within each bundle) that communicate with the anterior chamber at the anterior face of the ciliary muscle, and with the supraciliary and suprachoroidal spaces all the way back to the macular and optic nerve head region (Inomata et al., 1972b), so that aqueous humor and ciliary process and choroidal stromal fluid and protein can be carried out of the eye (with debate as to how much is carried away trans-sclerally (Anders Bill, 1967; Bill, 1971, 1966; Inomata et al., 1972b, 1972a), by the choroidal vasculature (Pederson et al., 1977), and by recently described ciliary muscle/choroidal putative lymphatics (Yücel et al., 2009). Intense ciliary muscle contraction by cholinimimetic drugs can constrict or even obliterate the spaces between the ciliary muscle bundles (Rohen et al., 1967), impeding uveoscleral outflow dramatically (A Bill, 1967) (Suppl Fig 6), (Crawford and Kaufman, 1987; Nilsson et al., 1989). This was utilized in the late 1980s to help delineate the mechanism by which $PGF_{2\alpha}$ reduced IOP – see section 3.1.b.1 below.

3.1.b.1 Prostaglandin analogues: $PGF_{2\alpha}$ increases uveoscleral outflow (Crawford et al., 1987; Crawford and Kaufman, 1987; Gabelt and Kaufman, 1989; Nilsson et al., 1989)(Table 1) by increasing the synthesis and release of various matrix metalloproteinases and consequently decreasing and remodeling the collagenous extracellular matrix in the ciliary muscle (Gaton et al., 2001). Increased uveoscleral outflow has been reported in monkey eyes with experimental autoimmune uveitis (Mermoud et al., 1994; Toris and Pederson, 1987). Histologic studies have shown enlarged ciliary muscle inter-muscular and suprachoroidal spaces in eyes with experimental uveitis induced by various methods (Liu et al., 1994; Toris and Pederson, 1987). The density of collagen type I in the extracellular matrix (ECM) of monkey ciliary muscle was reduced during anterior segment inflammation (Sagara et al., 1999a),indicating that reduction of ciliary muscle ECM may contribute to enhanced uveoscleral outflow during anterior segment inflammation. Twice daily topical treatment of cynomolgus monkey eyes with 2 μg $PGF_{2\alpha}$-isopropyl ester increased matrix metalloproteinases 1, 2, and 3 in the monkey uveoscleral outflow pathway (Sagara et al., 1999a) and also reduced collagen types I, III and IV immunoreactivity in the ciliary muscle (OCKLIND, 1998; Sagara et al., 1999a, 1999b). $PGF_{2\alpha}$ analogs have become the most widely used anti-glaucoma medications worldwide (Stein et al., 2015, 2007).

3.1.c. Novel products: combinatorial molecules - one molecule, two effects; and combination products - two molecules, two effects, one or more effects for each molecule—The FDA recently approved two novel products. Latanoprostene bunod (Vyzulta®, Bausch & Lomb) is a combinatorial molecule; one molecule with two effects. It has a latanoprost backbone and an NO-donating esterified side chain (Weinreb et al., 2018). Rocklatan®, (Aerie) is a fixed combination product; it contains two separate molecules, and conveys least two different effects, one or more for each molecule. It combines the rho kinase inhibitor Netarsudil (Rhopressa®, Aerie) with the prostaglandin $PGF_{2\alpha}$ analogue latanoprost. (Kahook et al., 2019; Serle et al., 2018).

4. Gene Therapy for Enhancing or Inhibiting Aqueous Outflow

4.1 Background / Unmet Medical Need

Topical self-administered eye drops have become problematic for clinical glaucoma therapy, as they rely on the patient to be an accurate, reliable, reproducible part of the delivery system. Studies have shown that the latter is not the case (Budenz, 2009; McKinnon et al., 2008; Nordstrom et al., 2005; Tsai, 2009). While various sustained release delivery systems are under development (NCT02371746, 2015; Vinod and Gedde, 2017; Walters et al., 2017), we have tried to develop a biological rather than a mechanical approach, namely gene transfer. This has nothing to do with hunting for and replacing a defective gene. Rather, the goal is to have relevant cells in the inflow or outflow pathways make more or less of something that affects an IOP–relevant physiological parameter so as to reduce IOP – whether or not an abnormality in that pathway was present. Thus, we have attempted to transfer genes that interfere with the rho pathway in the JCTM/SCIW to enhance conventional outflow facility, and genes that upregulate the $PGF_{2\alpha}$ – MMP pathway in the ciliary muscle to enhance uveoscleral outflow.

4.2 Reporter gene transfer to the TM and CM

We started with a self-complimentary AAV (scAAV) viral vector carrying the green fluorescent protein (GFP) gene injected into the anterior chamber of live monkeys, and achieved GFP expression in the TM easily visible gonioscopically at the slit lamp for over two years, with no clinically visible ocular inflammation (Buie et al., 2010). A feline immunodeficient (FIV) viral vector carrying the GFP gene achieved the same outcome (Barraza et al., 2009). Immunohistochemically, GFP was localized primarily to the TM and the most anterior part of longitudinal ciliary muscle (Barraza et al., 2009; Buie et al., 2010) (Fig. 6).

4.3 Functional gene transfer to the TM

4.3.a MOCAS—We then progressed to genes that, when overexpressed, would inhibit the rho cascade (the C3 exotoxin of Clostridium) or interfere with the actin/myosin interaction (caldesmon) in both cases inhibiting actomyosin contractility with consequent cellular relaxation and degradation of the actin cytoskeleton and cell-ECM attachments (Gabelt et al., 2006; Grosheva et al., 2006; Liu X, Wang N, 2005; Liu et al., 2005; Slauson et al., 2015). An adenoviral vector carrying either of these transgenes, infused into our MOCAS

system for freshly enucleated monkey eyes, induced a doubling of outflow facility (Gabelt et al., 2006; Liu et al., 2005; Slauson et al., 2015) (Table 2).

Several genes are known to drive outflow facility down in rodents and have also been implicated in the pathophysiology of human POAG. Examples among many are cochlin, SFRP1, TGF$_{ß2}$, and CTGF (Fuchshofer and Tamm, 2012; Goel et al., 2012; Junglas et al., 2012; Lee et al., 2010; Montecchi-Palmer et al., 2017; Pang et al., 2015; Tamm et al., 2015; Webber et al., 2016). This opens the possibility of creating a local genetic molecular model of ocular hypertension/POAG in NHP, absent the outflow tissue disruption, inflammation and scarification that accompanies current laser (Gaasterland and Kupfer, 1974) and trauma models, or the gross mechanical obstruction of microbead models (Chan et al., 2018; Cone et al., 2012; Sappington et al., 2010). Indeed, it is possible that one or more of these genes may underlie human POAG pathophysiology. Locally overexpressing one of more of these genes in the NHP JCT/SCIWE may provide the long sought-after molecular model of NHP POAG (Bhattacharya et al, 2009; (Suppl Fig. 8), and a stable platform for testing new potential ocular hypertensive and glaucomatous optic neuroprotective therapies going forward.

4.3.b Live monkeys

4.3.b.1 Intracameral injection: However, when FIV or scAAV vectors carrying either gene were injected intracamerally into live monkeys there was, with only one exception (high-titer scAAV.C3, that induced corneal endothelium dysfunction and consequent corneal edema, but no IOP or outflow changes (unpublished data), no physiological effect, no inflammation beyond that from the injection procedure itself, or any evidence at all that the eyes had been touched, save for the transcorneal needle tracks (unpublished data). One may hypothesize that in the live animal, where most of the aqueous humor drainage is through the TM/SC and the remainder is via the ciliary muscle, the ‘system’ shrugs its shoulders at GFP, which doesn’t really affect the cellular machinery. However, caldesmon and C3 exoenzyme, which significantly affect the TM cellular machinery, may elicit a strong turn-off response (Aktas et al., 2014). Further, many cell types are exposed to the vector - corneal endothelium, scleral fibroblasts, TM, SC inner/outer wall, ciliary muscle, lens epithelium, iris pigment epithelium, etc. This might mount an ‘all hands-on deck’ turn-off response. Except for one recent report (Tan et al., 2019), injection of facility-effective genes in MOCAS have no effect in live NHP. While intracameral injection of an FIV.BOVPGFS-myc.GFP PGF synthase vector construct reproducibly induces an ~2mmHg reduction in IOP, the effect is much less than that of topical PGF$_{2\alpha}$ analogue eyedrops, and dissipates after 5 months. The turnoff mechanism has yet to be defeated, although proteasome inhibition enhances reporter gene expression in monkey organ cultured anterior segments (MOCAS). In MOCAS, there is of course no vascular circulation and no connection to any systemic ‘assistance’, and there are also only a few cell types – corneal endothelium, scleral fibroblasts, TM and Schlemm’s canal cells - and a vastly reduced (and injured) number of ciliary muscle cells with no posterior attachments.

4.3.b.2 Intracanalicular injection: This difference, as well as a study showing that direct injection of adenoviral vectors into Schlemm’s canal was feasible in rat and human donor

eyes and had potential in glaucoma treatment (Hudde et al., 2005), suggested a live animal strategy of catheterizing Schlemm's canal and passing the catheter 360 degrees around the entire circumference as in ABiC and GATT procedures (Francis et al., 2017; Gallardo et al., 2018; Grover et al., 2018; Rahmatnejad et al., 2017). This has required modifications of the human ABiC catheter design, with generous collaboration from the engineers at Ellex iTrack. Both ab externo and ab interno cannulation of SC over 360 degrees has been achieved, with injection of trypan blue dye visible for up to four clock hours from the catheter tip during insertion (Aktas et al., 2014) (Fig. 7). The entire canal can be decorated blue by passing the catheter 360 degrees and then injecting slowly while withdrawing the catheter (unpublished data), with only a few small leaks/ruptures into the AC. We are currently administering cytoskeleton/contractility-modifying constructs via this route and are also looking at molecular modifications of both the vectors and the transgenes.

4.3.b.3 Proteasome inhibition: We are also exploring proteasome inhibition. Short-term exposure of TM cells to the proteasome inhibitor MG132 increases the transduction efficiency of FIV-mediated reporter gene delivery to TM cells in culture and in the MOCAS model. A more even distribution and intensity of GFP transduction in the MOCAS TM was noted, but no transduction was seen in SCIW endothelium (Aktas et al., 2018).

4.4 Prostaglandin $F_{2\alpha}$ pathway gene transfer to the ciliary muscle

Currently, the most widely prescribed outflow drugs approved for clinical use are prostaglandin $F_{2\alpha}$ analogues. The delivery of genes for the prostaglandin $F_{2\alpha}$ and FP receptor biosynthetic pathways to the anterior chamber angle, and thence to the ciliary muscle, of live cats and monkeys has been achieved using lentiviral vectors, resulting in consistent low single digit IOP reduction, but far less than what is seen following topical administration of $PGF_{2\alpha}$ analogue eyedrops. (Barraza et al., 2010; Lee et al., 2014; Loewen et al., 2004). The IOP lowering effect in NHPs lasted for 5 months (Suppl Fig. 9), (Lee et al., 2014). In cats, the experiments were ended after 5 months, by design.

Although encouraging, these studies have identified a number of challenges to be overcome before prostaglandin gene therapy can be translated into the clinic: the IOP effect needs to be of greater magnitude, possibly by determining the mechanism whereby transgene expression is lost; engineering the vector to resist species-specific restriction factors or using proteasome inhibition to increase the transduction efficiency (Aktas et al., 2018); identifying alternative promoters to better drive gene expression, including more ciliary muscle - and/or trabecular meshwork - specific promoters; and determining if species-specific PGF synthase or codon optimization is effective (Aktas et al., 2018; Brandt et al., 2015; Lee et al., 2014; Slauson et al., 2015).

4.5 Post-glaucoma filtration surgery wound healing/antifibrosis

Glaucoma filtration surgery, commonly used for IOP lowering when medical and laser trabeculoplasty fail, uses a guarded sub conjunctival transscleral incision into the anterior chamber to allow the continuous long-term release of aqueous humor into the subconjunctival space, from whence it is resorbed. Antimetabolites, such as mitomycin C (MMC), are deployed subconjunctivally during surgery to block scarring of the ostomy

during wound healing. Unfortunately, their use can result in undesirable side effects, such as tissue degeneration / cellular destruction leading to wound leaks, hypotony and ocular surface or intraocular infection. The human p21WAF-1/Cip-1 gene (rAd-p21), delivered via a recombinant adenovirus, was developed as a therapeutic alternative, (Heatley et al., 2004; Perkins et al., 2002) causing cell cycle arrest rather than destruction of surrounding cells and invading fibroblasts. In vitro, treatment of Tenon fibroblasts with rAd.p21 resulted in a dose-dependent inhibition of DNA synthesis and cell growth (Perkins et al., 2002). In vivo, in rabbits, rAd.p21 was comparable to mitomycin in inhibition of wound healing and fibroproliferation after filtration surgery without the complications of hypopyon and hyalitis that were associated with mitomycin (Perkins et al., 2002). When delivered to live ocular hypertensive (following laser scarification of the TM) monkey eyes, sub-Tenon / episcleral rAd-p21 treatment resulted in open surgical ostomies by both functional (normalized IOP) and histological criteria for at least 9 months (Heatley et al., 2004), without the tissue destruction seen in control animals treated with MMC (Heatley et al., 2004).

5. Stem cell therapy

As with gene therapy, stem cell strategies have the potential to provide patients with one-time long term solutions. With age and glaucoma, trabecular meshwork and JCT cell counts decrease (Alvarado et al., 1984, 1981; Grierson and Howes, 1987). The goal with stem cell approaches is to replace or regenerate lost tissue in the outflow pathways, restoring TM function. TM stem cells have been identified and localized in the Schwalbe's line / insert region of the TM in the primate eye. (Braunger et al., 2014; Yun et al., 2016). Mesenchymal stem cells (MSC) and induced pluripotent stem cells (iPSC) have both been used in TM cell differentiation and regeneration applications. An advantage to these strategies is the ability to use autologous stem cells. Contractility and phagocytosis differ between TM cells and MSCs and can be used as a method to determine the extent to which a functional TM phenotype has been attained (Snider et al., 2018). In a laser-damaged trabecular meshwork mouse model, intracamerally transplanted human trabecular meshwork stem cells (TMSCs) preferentially homed and integrated to the damaged TM region and expressed differentiated cell markers at 2 and 4 weeks. TMSC transplantation resulted in both ultrastructural and functional restoration. (Yun et al., 2018) MSCs isolated from rat bone marrow, injected into the anterior chamber, significantly reduced IOP in rat eyes made hypertensive by episcleral vein cauterization. MSCs were localized in the ciliary processes and the TM (Roubeix et al., 2015). Transplantation of induced pluripotent stem cell (iPSC) derived TM cells (iPSC-TM) restored IOP and outflow facility in young (Zhu et al., 2016) and aged ((Zhu et al., 2017) transgenic mice expressing a pathogenic form of human myocilin (Tg-MYOCY437H) glaucoma. At 12 weeks after transplantation, IOP in iPSC-TM recipients was statistically lower and outflow facility was significantly improved compared to untreated controls (Zhu et al., 2017). An ex vivo HOCAS system was developed to study outflow pathway cell loss effects on IOP homeostatic function. iPSCs were used to repopulate the cell depletion model. The differentiated cells (TM-like iPSCs) became similar to TM cells in both morphology and expression patterns and were able to restore IOP homeostatic function (Abu-Hassan et al., 2015). Collectively, these studies show the potential for stem cell therapeutics targeted to the conventional TM outflow pathway.

6. Conclusion

We have come a long way in understanding and manipulating NHP and human aqueous humor outflow over the past half century. Yet, we still do not really understand the basic pathophysiology of the outflow dysfunction leading to ocular hypertension and POAG, nor even much of the normal physiology much less any putative pathophysiology of the distal conventional outflow pathway.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Cholinomimetics increase outflow facility solely via CM contraction
- Epinephrine, RKIs and NO ↑ facility by inhibiting TM contractility, relaxing TM and dilating SC
- $PGF_{2\alpha}$ ↑ Fu by ↑ CM cell MMP synthesis/release, ↓ and remodeling CM ECM collagen
- Viral vectors (VV) transfer reporter genes expressing for > 2yrs in live monkey TM
- VV transfer genes inhibiting TM contractility and ↑ facility in MOCAS
- VV transfer genes that ↓ facility in MOCAS; molecular models for human POAG?
- iPSC cell-derived TM cells populated TM & rescued outflow in TM cell-depleted HOCAS

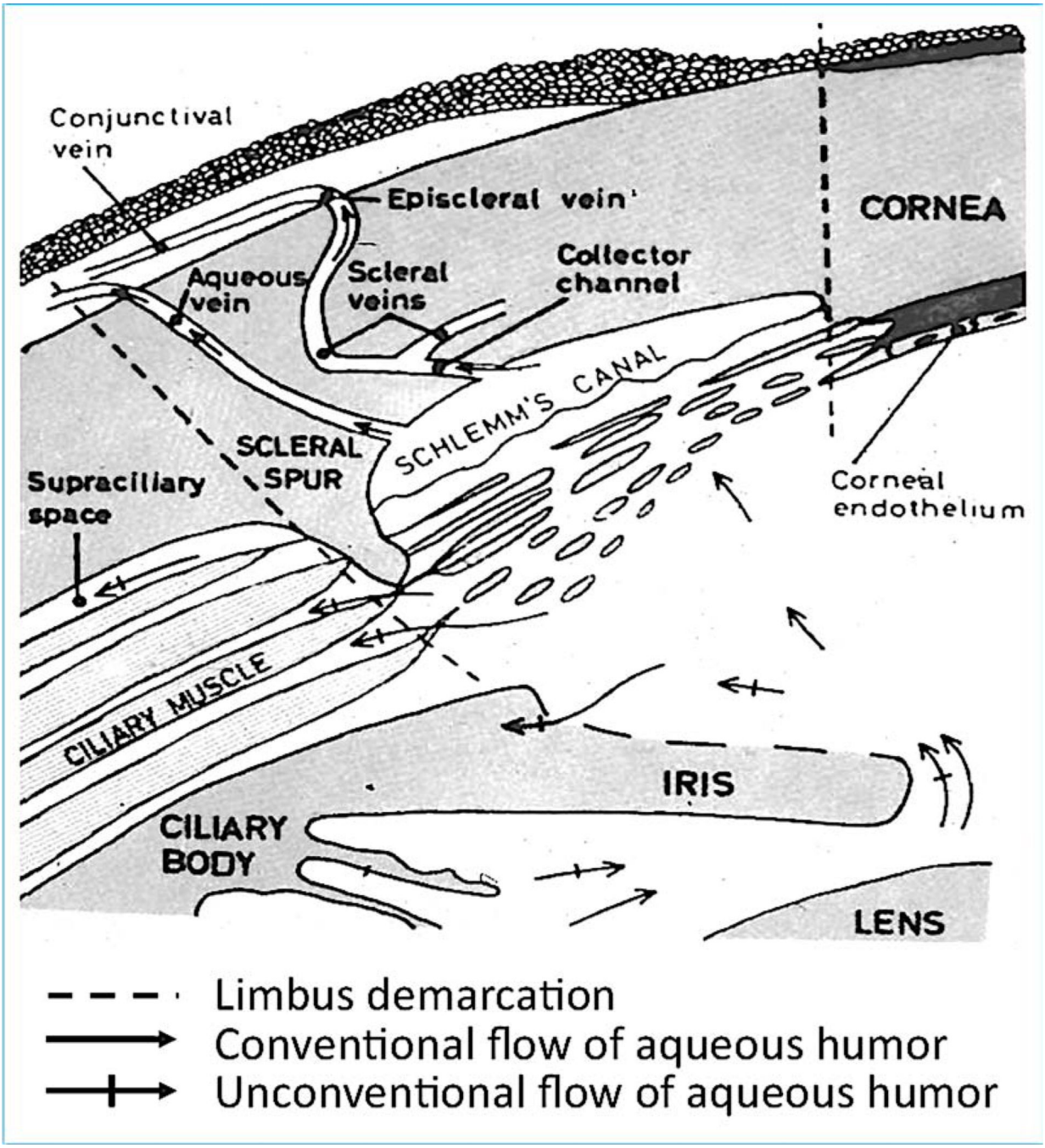

Fig. 1.

Aqueous humor outflow pathways. Aqueous humor, produced by the ciliary body, exits the eye through the conventional trabecular meshwork/ Schlemm's canal pathway (plain arrows), and the unconventional uveoscleral pathway (crossed arrows). Aqueous humor production and outflow are always equal, and the resistance in the trabecular meshwork/ Schlemm's canal determinestheintraocularpressure(IOP). Increasedresistanceresultsinelevation in IOP, a causal risk factor for glaucoma. From Svedbergh, 1976 with permission.

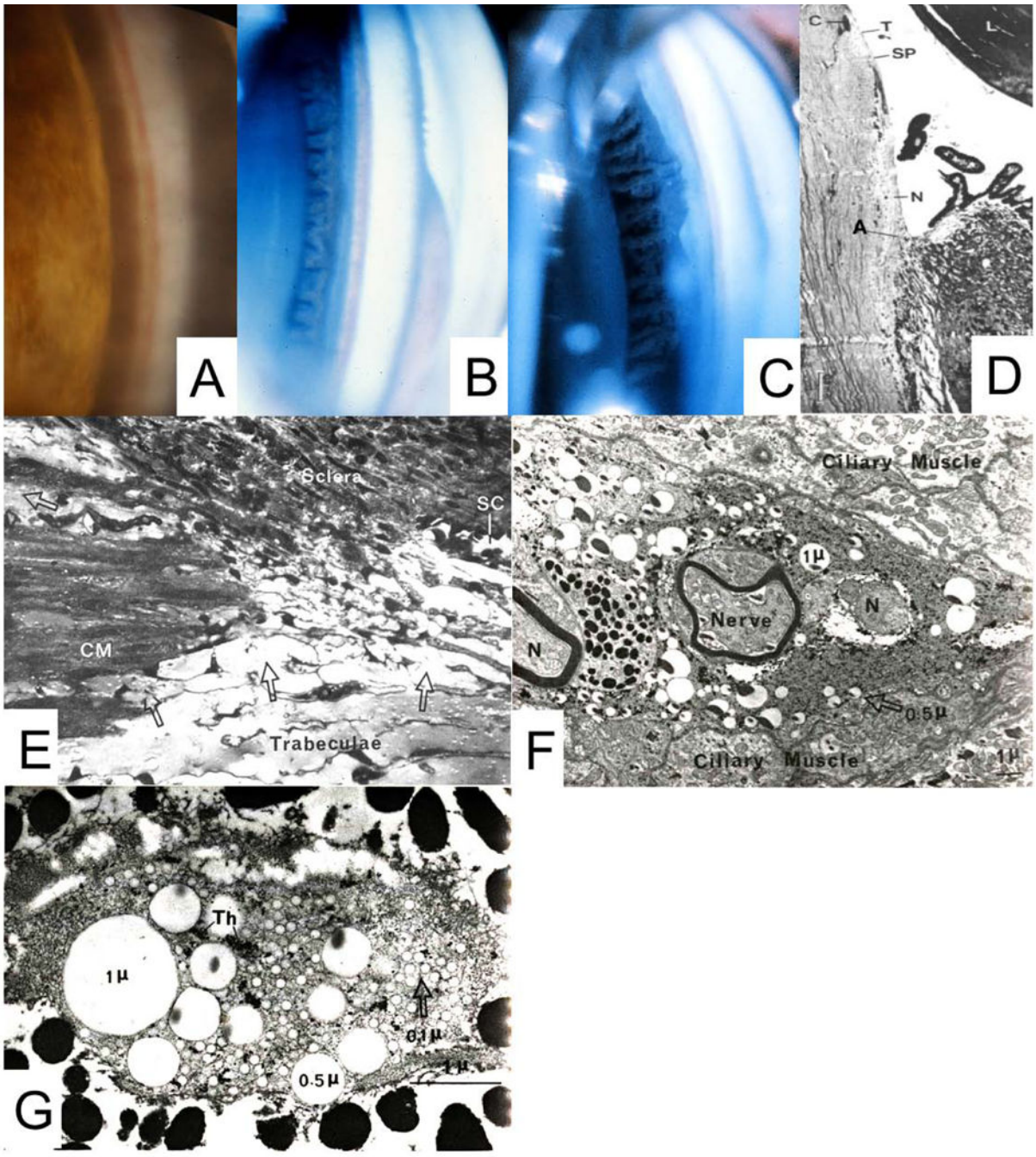

Fig. 2.

A: Normal cynomolgus monkey anterior chamber angle. B: Gonioscopic appearance of surgically aniridic eye. C: Surgical aniridia and ciliary muscle disinsertion and retrodisplacement. Deep, fairly uniform retrodisplacement of ciliary body. Thinbandofmuscleremainsattachedtoscleralspur. D: Typical histologic appearance ofanterior chamber angle following ciliary muscle disinsertion; C = canal of Schlemm, filled with blood in lower half of the image; A = adhesion of retrodisplaced muscle to sclera; L = lens; N = naked sclera; SP=scleralspur;T=trabecularmeshwork.E:Light micrograph of the

posterior portion of the trabecular meshwork/anterior portion of the ciliary muscle after perfusion with three sizes of latex spheres and thorotrast in gelatin. Streams of homogeneous gelatin, containing tracer particles (arrows), enter the ciliary body from the posterior portion of the trabecular meshwork and pass through the connective tissue between bundles of the ciliary muscle. CM: Ciliary muscle; SC: Schlemm's canal (x 460). F: Monkey ciliary muscle intermuscular space showing latex spheres that had been injected into the anterior chamber. N: Nerve fiber (x 5500). G: Monkey suprachoroid at the posterior pole, showing 3 sizes of latex spheres that were injected into the anterior chamber. Th: Thorotrast. (X22,000). From: B - Kaufman PL, Lütjen-Drecoll 1975; C - Kaufman PL, Bárány EH 1976; D - Lütjen-Drecoll E, et al 1977; E, F, G - Inomata H, et al 1972; all with permission.

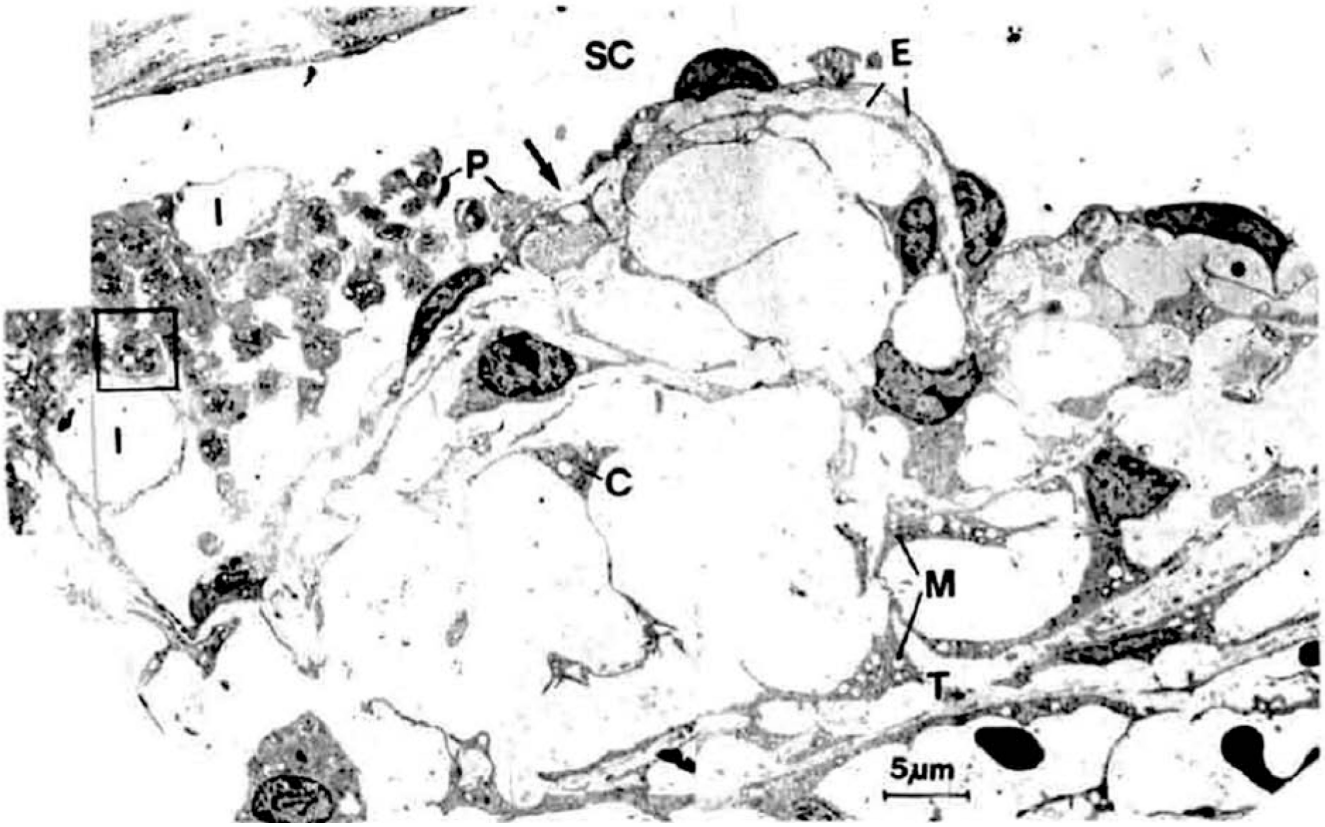

Fig. 3.

Schlemm's canal (SC) and cribriform meshwork approximately 30 min after intracameral infusion of 5 μg of cytochalasin B. Inner wall endothelium demonstrates ruptures (arrow) and abnormally large invaginations (I). Extracellular material (E) has been lost from some areas between the inner wall endothelium and the first subendothelial cell layer (and replaced by plasma (asterisk)) and is completely absent from most parts of the cribriform meshwork. The cribriform (juxtacanalicular) meshwork is greatly expanded. P, degranulated platelets; C, cellsof cribriform meshwork; M, swollen mitochondria; T, first corneoscleral trabeculum. From Svedbergh et al., 1978, with permission.

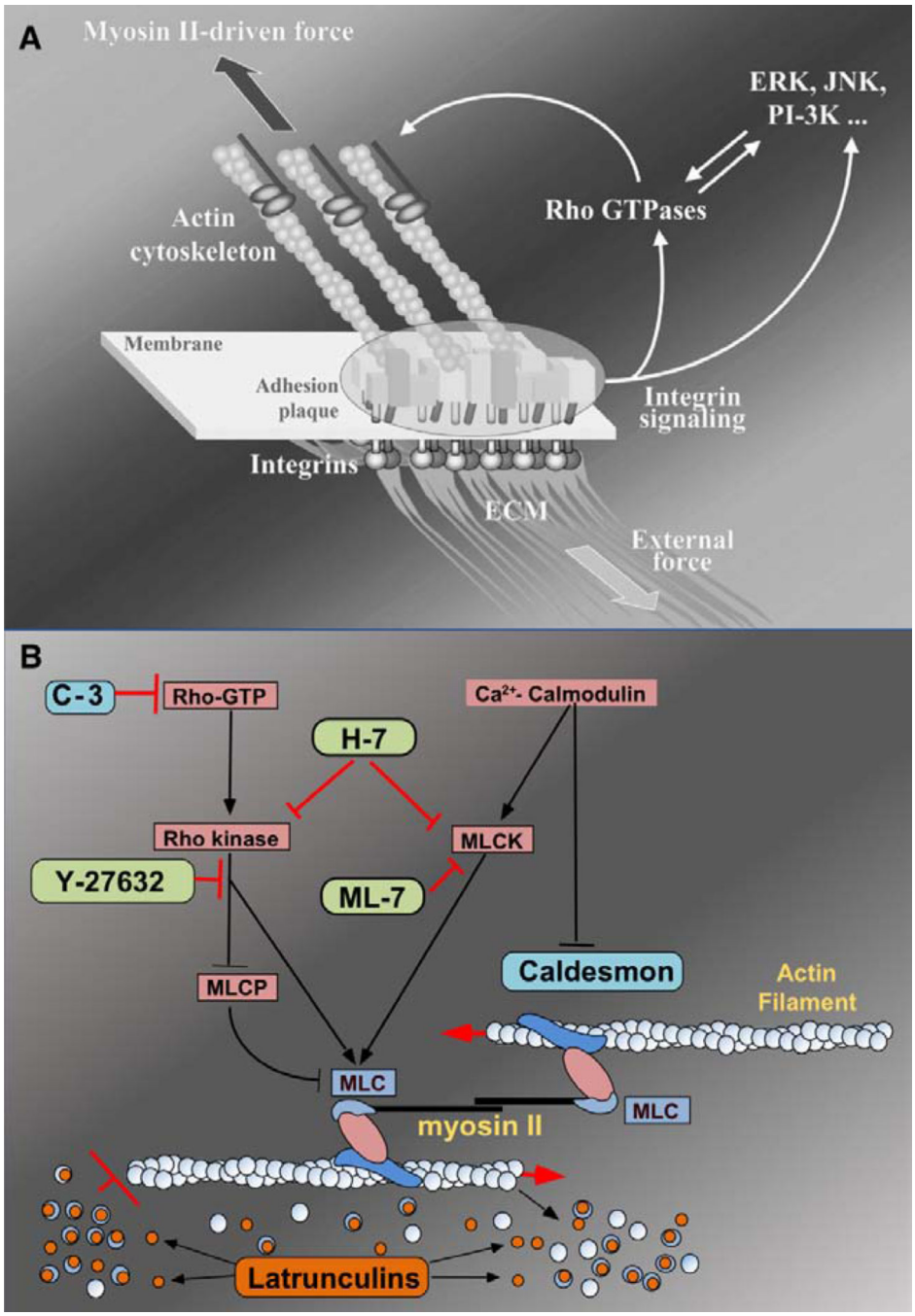

Fig. 4.

A: Focal adhesions (FAs) as a mechanosensors. FAs are multi-molecular complexes connectingthe extracellular matrix with the actin cytoskeleton. Heterodimeric trans membrane integrin receptors (pink) bind matrix proteins via their extracellular domains,while theircytoplasmicdomains are associated with a dense submembrane plaque containing more than 50 different proteins (“boxes” enclosed in the oval area) including structural elements as well as signal transduction proteins such as FAK, Src, ILK, etc. The plaque, in turn, is connected to the termini of actin filament bundles. The assembly and

maintenance of FAs depend on local mechanical forces. These forces may be generated by myosin II-driven isometric contraction of the actin cytoskeleton, or by extracellular perturbations such as matrix stretching or fluid shear stress. Force-induced assembly of the adhesion plaque leads to the activation of a variety of signaling pathways that control cell proliferation, differentiation, and survival (e.g., MAP kinase and PI 3- kinase pathways) as well as the organization of the cytoskeleton (e.g., Rho family GTPase pathways). Rho, in particular, is an indispensable regulator of FA assembly affecting, via its immediate targets Dia1 and ROCK, actin polymerization and myosin II-driven contractility. Geiger and Bershadsky, 2002 with permission. B: Schematic drawing illustrating targets for agents known to disrupt the actin cytoskeleton to enhance outflow facility. C-3, Y-27632 and H-7 block the Rho cascade, inhibiting actomyosin contraction and disrupting actin stress fibers; H-7 and ML-7 block myosin light chain kinase phosphorylation of the myosin light chain to interfere with actinmyosin interactions; latrunculin sequesters monomeric G actin leading to microfilament disassembly; caldesmon negatively regulates actin-myosin interactions. Modified from original by Alexander Bershadsky, with permission.

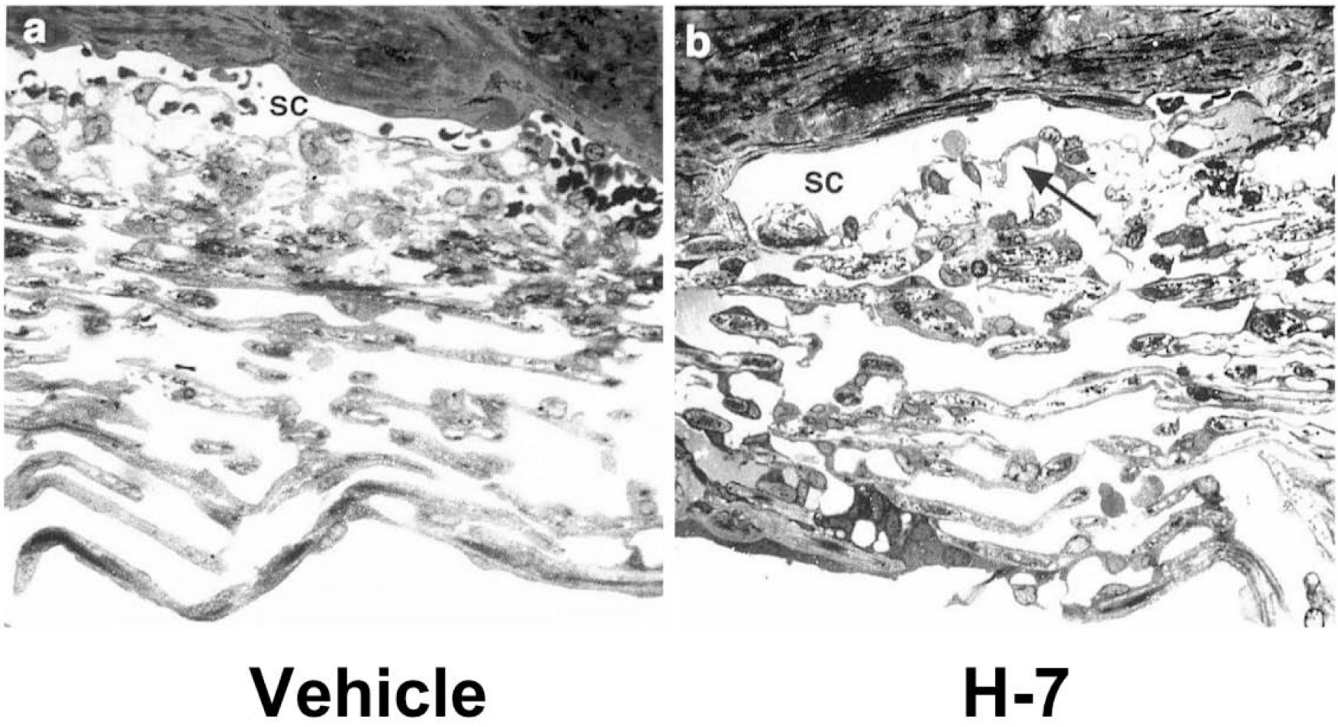

Fig. 5.

Light micrographs of trabecular meshwork (TM) and Schlemm's canal (SC) in monkey eyes treated with vehicle (a) or H-7 (1-[5-isoquinoline sulfonyl]-2-methyl piperazine), 300 μmol/L (b). The juxtacanalicular area (arrow in b) and intercellular spaces are extended. Inner wall endothelial cells in H-7–treated eyes were thinner than in controls, and cell-cell junctions were intact (not shown). Inner uveal TM were not significantly affected (b). From Sabanay et al., 2000 with permission.

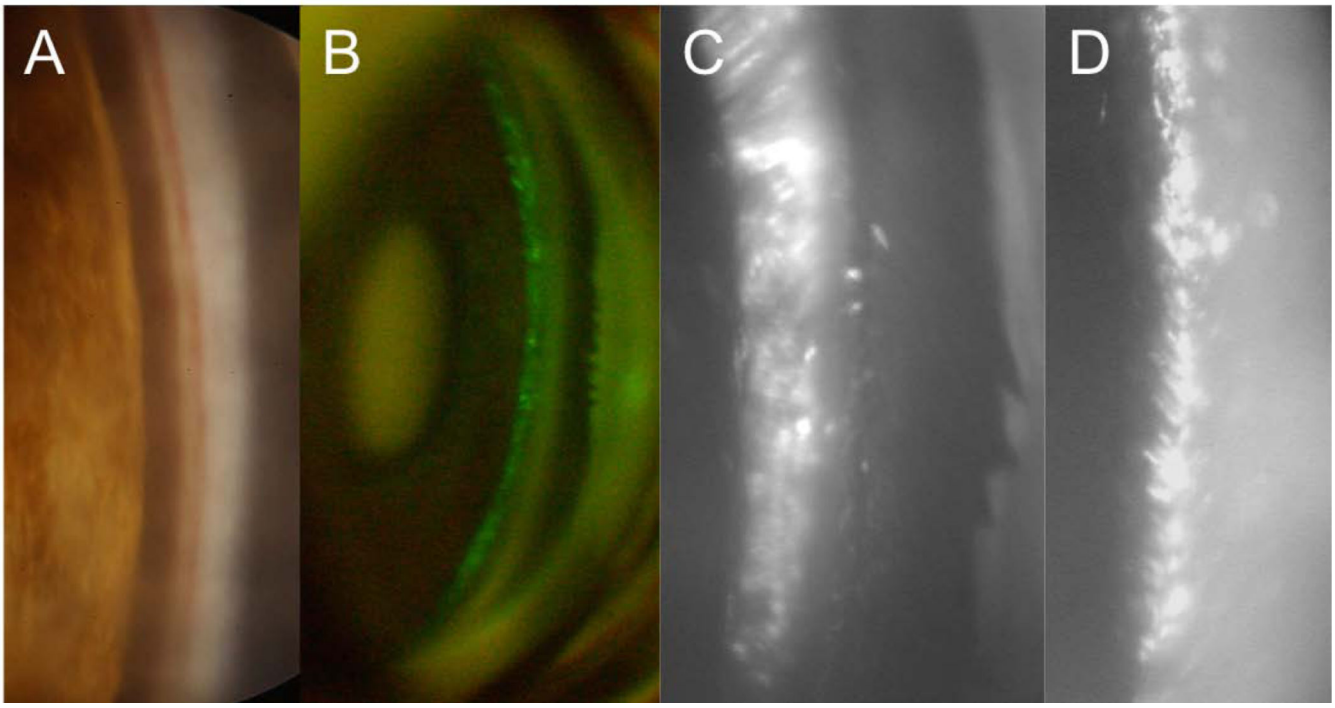

Fig. 6.

A, B, C: Monkey chamber angle after intracameral administration of scAAV.GFP vector, day 641 post injection, SLE - quiet, IOP normal. Panel A: A digital camera with a 175-W xenon nova light source and a 3-mm-6-cm 0° teleotoscope probe (Hopkins II; Karl Storz Endoscopy-America, Inc., Culver City, CA) captured the image. Panel B: A retinal camera (50EX; Topcon, Tokyo, Japan), fitted with a digital SLR color camera body (D1X (Nikon Instruments, Inc., Melville, NY); with standard clinical fluorescein exciter and barrier filters, and a gonioprism, captured the image. Panel C: A customized microscope (Nikon) with a 12-bit, monochromatic, cooled-CCD camera (Retiga, 2000RV; QImaging Burnaby, BC, Canada), a specific GFP exciter and barrier filter set and a gonioprism captured the image. D: Monkey anterior chamber angle after intracameral administration of FIV. GFP vector, day 515 post injection, SLE - quiet, IOP normal.

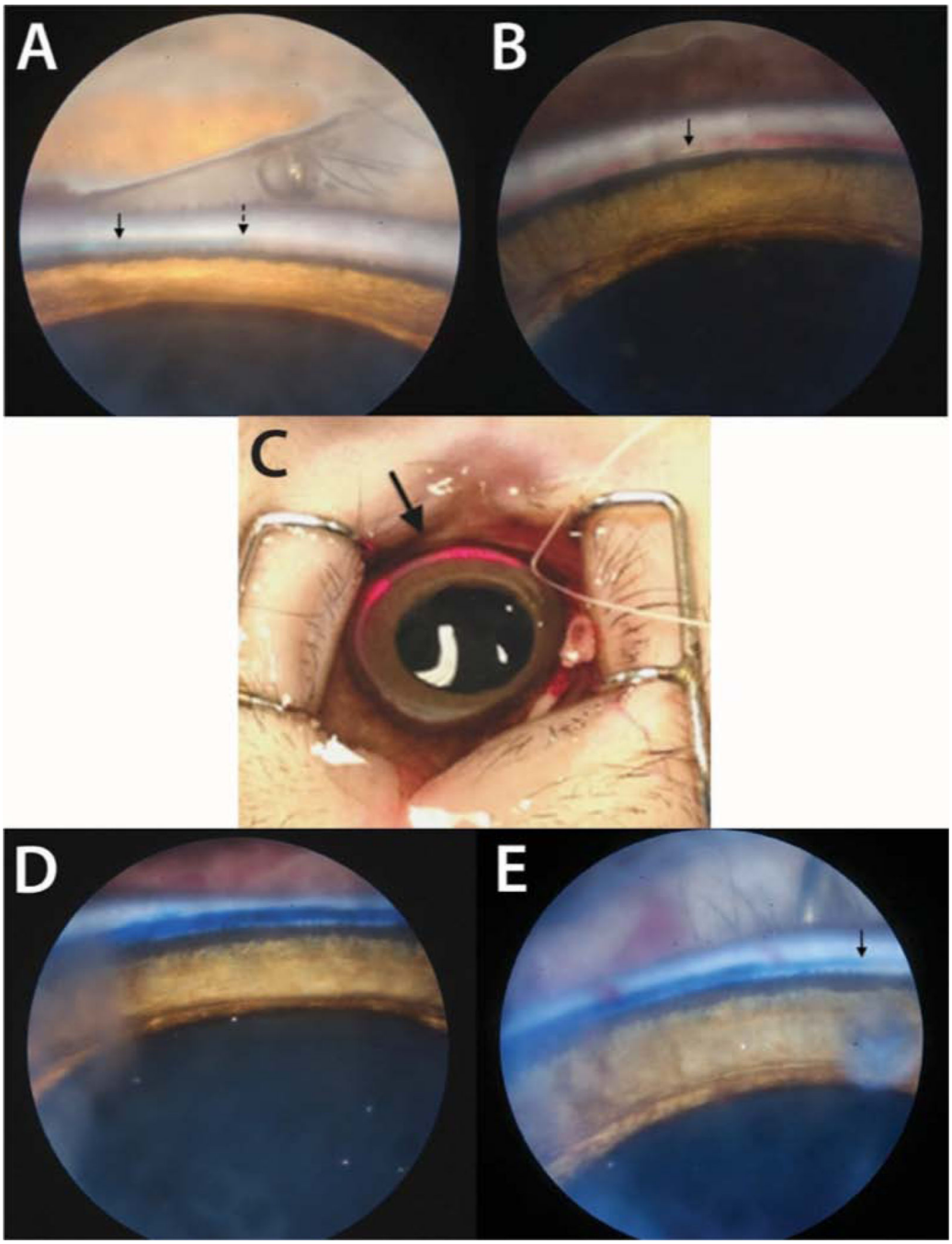

Fig. 7.

Drug delivery via catheterization of Schlemm's Canal in monkeys A: Endoscopic camera image showing 6–0 prolene suture (solid arrow) and its end (dotted arrow) in Schlemm's canal. B: Microcatheter in Schlemm's canal (arrow). Note the blood in the canal blocking the gonioscopic view of thecatheterattherightofthepicture.C:LEDlightof the microcatheter in Schlemm's canal. D: Endoscopic camera image of the temporal side of left eye at 3:00–4:00 o'clock showing trypan blue in Schlemm's canalafterinjectionbycatheter(ostiumwasat12:00

o’clock, catheter tip at 1:00 o’clock). E: Infero-temporal site at 3:00–4:00 o’clock and the point where the visible dye column ended (arrow) at 5 o’clock.

Table 1

Effect of topical $PGF_{2\alpha}$ on uveoscleral outflow in monkeys 5th day of treatment bid with 2 μg $PGF_{2\alpha}$ – IE

$PGF_{2\alpha}$	Control	PG/Cont
1.101	0.489	2.329 *
±	±	±
0.141	0.07	0.235

Data are mean ± s.e.m. uveoscleral outflow (μl/min) for 9 animals, each contributing one treated and one control eye, following the ninth unilateral dose of $PGF_{2\alpha}$ on day 5.

* P<0.00001. Ratio significantly different from 1.0 by the two-tailed paired t-test. From Gabelt, 1989, with permission.

Table 2

The effect of adenoviral vectors on outflow facility in monkey organ cultured anterior segment.

A				B			
N=8	**Treated**	**Control**	**T/C**	**N=4**	**Treated**	**Control**	**T/C**
Baseline	0.35±0.04	0.48±0.07	0.78±0.08 *	Baseline	0.31±0.09	0.41±0.10	0.82±0.16
AdGFPCald or AdGFP	1.03±0.30	0.67±0.17	1.47±0.17 *	AdGFPC3 or AdGFP	0.66±0.14	0.39±0.06	1.79±0.38
Ad/BL	2.63±0.47 *	1.34±0.20	2.01±0.19 *	Ad/BL	2.44±0.54	1.09±0.20	2.25±0.28 *
Caldesmon				C3			

A: Caldesmon ($1.5v10^7$ plaque forming units, 20 µl); B: C3 (1.2×10^8 viral particles, 80 µl). Ad, Adenoviral vector; GFP, green fluorescent protein; Cald, caldesmon; Data are mean ± s.e.m. outflow facility; ratios are unitless. Ratio significantly different from 1.0 by the two-tailed paired t-test;

* $P<0.05$. From Gabelt et al., 2006: B: From Liu et al., 2005, with permission.