Cytoskeletal Involvement in the Regulation of Aqueous Humor Outflow

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Fluid flow in the anterior chamber maintains intraocular pressure (IOP) and globe shape and supplies oxygen and nutrients to the nonvascularized cornea, lens, and trabecular meshwork (TM). Glaucoma is a progressive optic neuropathy often caused by elevated IOP consequent to abnormally high resistance to aqueous humor drainage via the TM and Schlemm's canal. Compounds with cytoskeletal effects offer therapeutic possibilities for substantial long-term IOP reduction. Most current IOP-reducing agents either suppress aqueous humor production or increase outflow through the ciliary muscle, thus reducing aqueous humor flow through the TM, perhaps compromising an already compromised tissue and potentially stressing the cornea and lens. Glaucoma patients usually receive several of these agents concurrently. Recent studies indicate that the TM cytoskeleton may be involved in the regulation of aqueous humor outflow. A cytoskeletal agent acting directly on the TM/Schlemm's canal to reduce outflow resistance would be more consistent with normal physiological function, and recent developments indicate that this approach is moving toward fruition.

Functional Organization of the TM

The TM consists of arrays of collagen beams covered by endothelium-like cells, with extracellular material/matrix (ECM) occupying the spaces between the beams. The outermost juxtacanalicular or cribriform region has no collagenous beams but rather several cell layers immersed in loose ECM. The adjacent Schlemm's canal is a continuous endothelium-lined channel that drains aqueous humor to the general venous circulation. Trabecular meshwork structure and experimental flow studies suggest that flow resistance is maximal in the outermost region, but the exact location and nature of the major resistance barrier, and the exact flow pathways through the TM and the inner wall of Schlemm's canal, are not clear either in the normal or the glaucomatous eye.

With age, resistance increases, TM cells decrease, and alterations of the ECM in the juxtacanalicular region occur. Glaucomatous eyes exhibit fewer TM cells and abnormal-appearing juxtacanalicular ECM compared with age-matched normals, all suggesting that cells and ECM in the juxtacanalicular region may be critical in resistance regulation. Cell shape, volume, contractility, and adhesion to neighboring cells and to the ECM, and amount and composition of the ECM, could affect resistance by altering the dimensions or direction of flow pathways and could be therapeutic targets to reduce flow resistance.

Cytoskeletal Involvement in the Outflow Pathway

The cytoskeleton is a complex system of cytoplasmic fibers responsible for numerous cellular processes.[1,2] There is no "general cytoskeletal function," because the different filament networks may be responsible for different and even conflicting cellular events.

Microfilaments

Microfilaments, ~7 nm diameter, are involved in multiple cellular processes, from cell adhesion and motility to organelle traffic to adhesion-mediated signal transduction. They assemble within cells into complex bundles or three-dimensional meshworks located subjacent to the plasma membrane, and attach to the plasma membrane at adherens type cell–cell junctions (AJ) and cell–ECM focal contacts (FC) via a network of anchor proteins and additional structural and signaling molecules.[3] This transmembrane association of microfilament bundles, AJ and FC is essential for formation and maintenance of cell adhesion. Filamentous actin is the major molecular component of microfilaments, but other actin-associated proteins modulate their organization.

There are numerous microfilament-based structures in cells along the trabecular outflow pathway (Fig. 1). Endothelial cells on the collagen beams maintain conspicuous FC-like structures and AJ. Bundles of microfilaments are present in juxtacanalicular cells. Schlemm's canal inner wall cells are particularly enriched with microfilaments and intermediate filaments (see below). Inner wall cells maintain prominent AJ, responsible for continuity, and contain numerous vesicles and vacuoles, the formation and transport of which requires intact microfilaments. The juxtacanalicular–Schlemm's canal region is usually contracted, and relaxation by specific inhibitors of

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Supported by Grants EY02698 and EY01894 from the National Eye Institute, National Institutes of Health; the American Health Assistance Foundation; the Glaucoma Research Foundation; Research to Prevent Blindness; the Wisconsin Alumni Research Foundation; and the Ocular Physiology Research and Education Foundation. Benjamin Geiger is the incumbent of the E. Neter Chair in Cell and Tumor Biology.

Submitted for publication September 27, 1999; revised November 11, 1999; accepted November 12, 1999.

Commercial relationships policy: P (BG, DE, PK; each through their respective academic institutes), C2 (BG), C3 (BG, PK), C5 (BG, PK), C7 (PK), C8 (BG, PK), Cc2 (PK), Cc3 (PK), Cc5-8 (PK).

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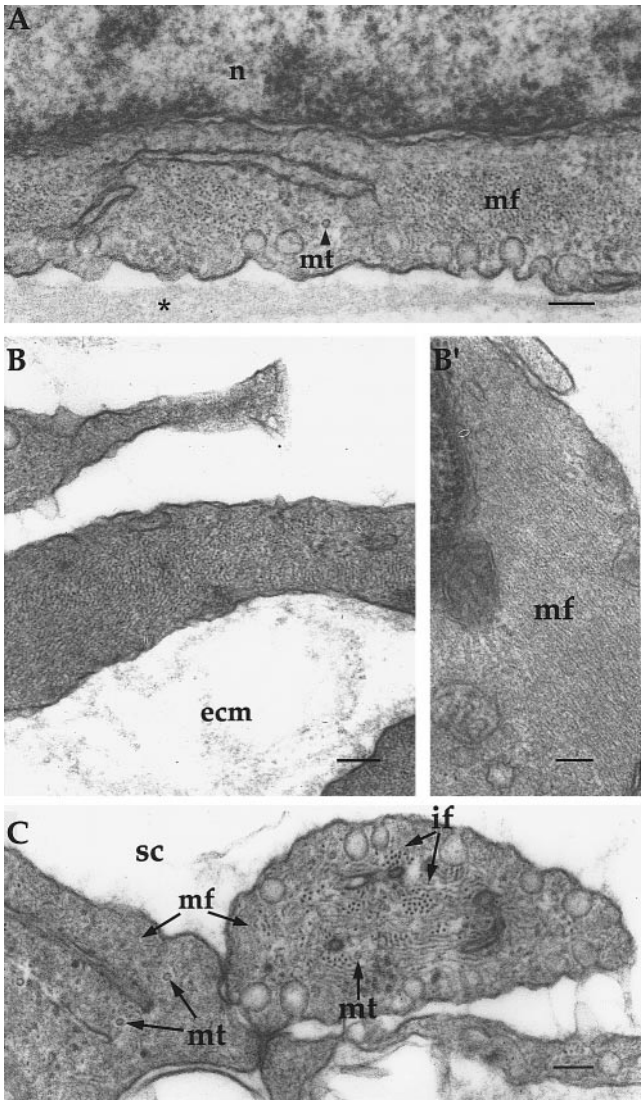

FIGURE 1. Results of transmission electron microscopy showing abundance of cytoskeletal filaments in different cellular compartments of trabecular outflow pathway. (**A**) Ventral aspect of TM cell, which interacts with underlying collagen beam (*) via many focal adhesion-like structures, is highly enriched with cytoskeletal filaments, including actin-rich microfilaments (mf) and microtubules (mt); n, nucleus. (**B**) Juxtacanalicular cells displaying numerous microfilaments (mf), seen in cross section (**B**) or longitudinal section (**B′**); ecm, extracellular matrix. (**C**) Inner wall endothelial cells of Schlemm's canal (sc), displaying numerous intermediate filaments, organized in discrete bundles (if). Microtubules (mt) are also present. Microfilaments (mf) are usually seen in the cortical cytoplasm and near cell–cell junctions. Scale bars, 0.1 μm.

actomyosin contractility substantially alters its structure and flow dynamics. In more apical positions along the intercellular junctional complex of inner wall cells, distinct tight junctions are present and play a central role in sealing the lumen of Schlemm's canal and in maintaining apical–basolateral polarity. The entire junctional complex maintains tight association with the actin-based cytoskeleton. These cytoskeletal interactions can be regulated by a variety of environmental and cytoplasmic factors such as the level of extracellular calcium, activation of specific small G-proteins, mechanical tension and hydrostatic pressure,[4] and particular molecular components of tight junctions may help regulate flow resistance.[5]

Microtubules

Microtubules comprise 25-nm-diameter hollow polar fibers, densely packed near the nucleus, and extending toward the cell periphery. They are not intrinsically contractile but are important for directional cell motility and, driven by specific microtubule motor proteins such as kinesins and dyneins, for cytoplasmic traffic of vesicles and organelles. Their main molecular constituent is the heterodimeric protein tubulin (α and β subunits). In addition, associated proteins bind to microtubules and can affect their stability and potentially attach them to other cellular structures, including other cytoskeletal filaments. Microtubule function could affect outflow pathway events (Fig. 1C) through direct cellular mechanical effects (e.g., tensegrity[6]), influences on ECM or cell membrane turnover (via vesicle movement), or secondary signaling (e.g., leading to activation of the actin cytoskeleton).

Intermediate Filaments

Intermediate filaments are ~10 nm in diameter and are perhaps the most "skeletal" of all cytoskeletal fibers. They form elaborate webs consisting of single filaments and filament bundles without a defined organizing center. Intermediate filaments often run closely along microtubules, suggesting that the two may be linked. At the cell periphery, intermediate filaments of epithelia and cardiac cells attach to desmosomal cell–cell junctions and also interact with hemi-desmosomal cell–basement membrane adhesions. Unlike microfilaments and microtubules, each of which is composed of essentially the same building blocks in all cells (i.e., actin and tubulin, respectively), intermediate filaments are molecularly heterogenous. In many cell types, including all cells throughout the TM, the major intermediate filament protein is vimentin. Intermediate filaments are particularly abundant in inner wall cells (Fig. 1C), with densely packed arrays throughout the cytoplasm, but their role in aqueous humor outflow is unclear.

In maintaining and modulating cell shape, anchorage, and motility, for example, the three cytoskeletal networks function in concert, and changes in each network can induce radical changes in the other two. For example, microtubule disruption can activate actin- and myosin-based contractility, affecting cell adhesion and morphology, or induce collapse of intermediate filaments and formation of large aggregates around the nucleus. Similarly, perturbation of microfilaments by actindisrupting agents (e.g., cytochalasins or latrunculins) or by inhibitors of actomyosin contraction, for example, (1-(5-Isoquinolinesulfonyl)-2-methylpiperazine) [H-7] or (1-(5-iodonaphthalene-1-sulphonyl)-1H-hexahydro-1,4-diazepine hydrochloride) [ML-7], affects cell spreading and polarity and, consequently, other cytoskeletal systems.

CYTOSKELETAL DRUG EFFECTS ON TM STRUCTURE AND OUTFLOW

Much of the current knowledge on the function of different cytoskeletal networks is derived from studies using "cytoskeletal drugs" that have distinct compositions and dynamic properties and destroy or stabilize the microfilament or microtubular systems. Although the drugs' specificities are well es-

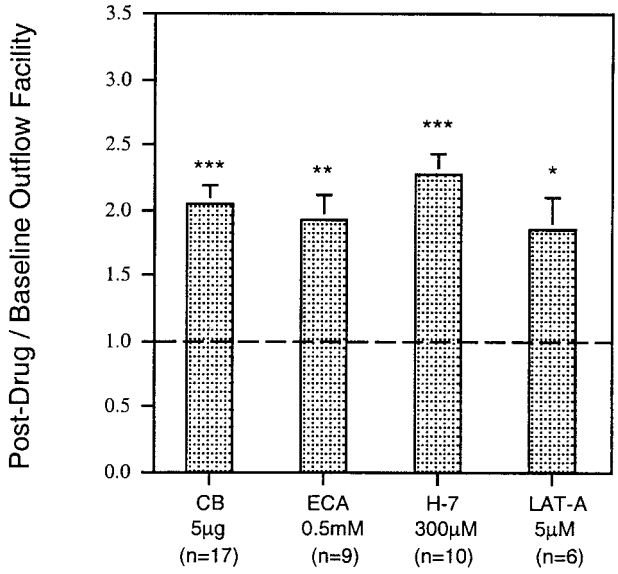

FIGURE 2. Outflow facility (pressure-dependence of fluid drainage from the anterior chamber, AC, in microliters per minute per millimeter of mercury; inverse of resistance) was measured by AC perfusion in living monkeys for ~35 minutes before and after intracameral administration of cytochalasin B (CB), ECA, H-7, or latrunculin A (LAT-A; modified from Refs 7, 9, 11, and 12). Data are mean ± SEM ratio for *n* animals, adjusted for actual (H-7, ECA, and LAT-A) or presumed (CB) control eye vehicle-induced resistance washout. *Dashed line* represents ratio = 1.0 (i.e., no facility increase after drug administration). $^{*}P < 0.02$, $^{**}P < 0.01$, $^{***}P < 0.001$ for ratios different from 1.0 by the two-tailed paired *t*-test.

tablished, cytoskeletal filaments are part of an interactive network so that affecting one system may have considerable indirect effects on the others.

Cytochalasins disrupt the actin cytoskeleton by capping the barbed ends of actin filaments, preventing their elongation. In living monkey and organ-cultured human eyes, cytochalasin B or D decreases outflow resistance (Fig. 2),[7] accompanied by separation of endothelial cells of the beams, the juxtacanalicular region, and the inner wall from their neighboring cells and their surrounding ECM. This leads to distension of the meshwork and ruptures in the inner wall, enhancing fluid flow and washout of ECM.[8]

Ethacrynic acid (ECA) inhibits microtubule assembly in vitro and induces a rapid decrease in phosphotyrosine levels of focal adhesion kinase and a more subtle decrease in paxillin phosphorylation. Enhanced dephosphorylation of focal adhesion kinase and paxillin by ECA blocks signaling pathways normally triggered by integrin-mediated adhesion. In cultured TM cells, ECA causes cellular contraction, reversibly altering cell shape coincident with alteration of the staining pattern of major cytoskeletal components, including actin, α-actinin, vinculin, and vimentin. Ethacrynic acid reduces outflow resistance in enucleated calf and human eyes and in living monkey eyes (Fig. 2)[9] and concomitantly reduces IOP in live rabbit, monkey, and human eyes. In enucleated human eyes, lower resistance–effective ECA doses do not produce morphologic changes in the TM, whereas higher doses induce separations between TM and inner wall cells.

H-7, a serine–threonine kinase inhibitor, inhibits actomyosin-driven contractility and induces general cellular relaxation. Although H-7 does not affect actin polymerization, the inhibition of contractility leads to deterioration of the actin microfilament bundles and perturbation of its membrane anchorage at matrix adhesion sites in human TM and other cultured cells (Fig. 3).[10,11] In living monkeys[11] and enucleated porcine eyes, H-7 dose- and time- dependently decreases outflow resistance (Fig. 2) and IOP. By electron microscopy, H-7 expands the juxtacanalicular intercellular spaces, accompanied by removal of extracellular deposits. The inner wall cells become highly extended, yet cell–cell junctions are well-maintained. H-7 appears to decrease outflow resistance by relaxing and expanding the TM and Schlemm's canal, without significantly changing intercellular adhesion.

Latrunculins alter cell shape and disrupt microfilament organization by sequestering G-actin, leading to disassembly of actin filaments. Cell–cell adhesions and associated microfilaments appear more susceptible than cell–ECM adhesions (Fig. 3). In living monkeys[12] and cultured porcine eyes, latrunculins A and/or B decrease outflow resistance (Fig. 2) and IOP for up to 24 hours. Structural studies of the effects of latrunculin on the aqueous drainage pathways are not yet available, but separation of cells from each other or from the ECM in the TM or Schlemm's canal could be involved.

The diverse protein kinase inhibitors staurosporine, chelerythrine, and ML-7 and the protein kinase C (PKC) activator phorbol ester also decrease outflow resistance in monkeys. Their common feature is cytoskeletal perturbation in cultured cells, but some tyrosine kinase inhibitors (tyrphostins) with similar effects on AJ, actin filaments, or both fail to decrease resistance. The $Na^+/K^+/2Cl^-$ cotransport protein is associated with cytoskeletal components, and the cotransport inhibitor bumetanide reduces intracellular volume, increases monolayer TM cell permeability, and decreases outflow resistance in cultured calf eye and some cultured human eye studies,[13] although not in living monkeys.

Cytoskeletal Drugs as Candidates for Glaucoma Therapy

The actomyosin system is present in essentially all cells. Cytoskeletal drugs could, in principle, have detrimental effects on other anterior segment tissues, especially the cornea, which would "see" a higher drug concentration after topical application. However, differences in tissue architecture and physiological milieu between the cornea and TM may allow the cornea to avoid meaningful change at drug concentrations affecting the TM. The TM is a suspended multilayered tissue, juxtacanalicular cells have no real basement membrane, and inner wall cells have only a thin, diaphanous, discontinuous basement membrane. Alteration of cellular contractility or cytoarchitecture can lead to TM/Schlemm's canal distortion and distention by fluid flow down the pressure gradient between the anterior chamber and Schlemm's canal. The corneal endothelium is a single cell layer on a well-defined basement membrane/ECM structure with much less fluid flow across it, and is thus less easily distended or distorted. Supporting such speculation, ECA used both orally and intravenously is systemically safe, intracameral doses of ECA reduce IOP in glaucoma patients without inducing corneal or anterior segment side effects, and a maximal resistance-reducing intracameral dose of H-7 produces general relaxation and expansion in the TM/Schlemm's canal but no visible changes in the corneal endothelium or ciliary epithelium. In theory, a single intracameral

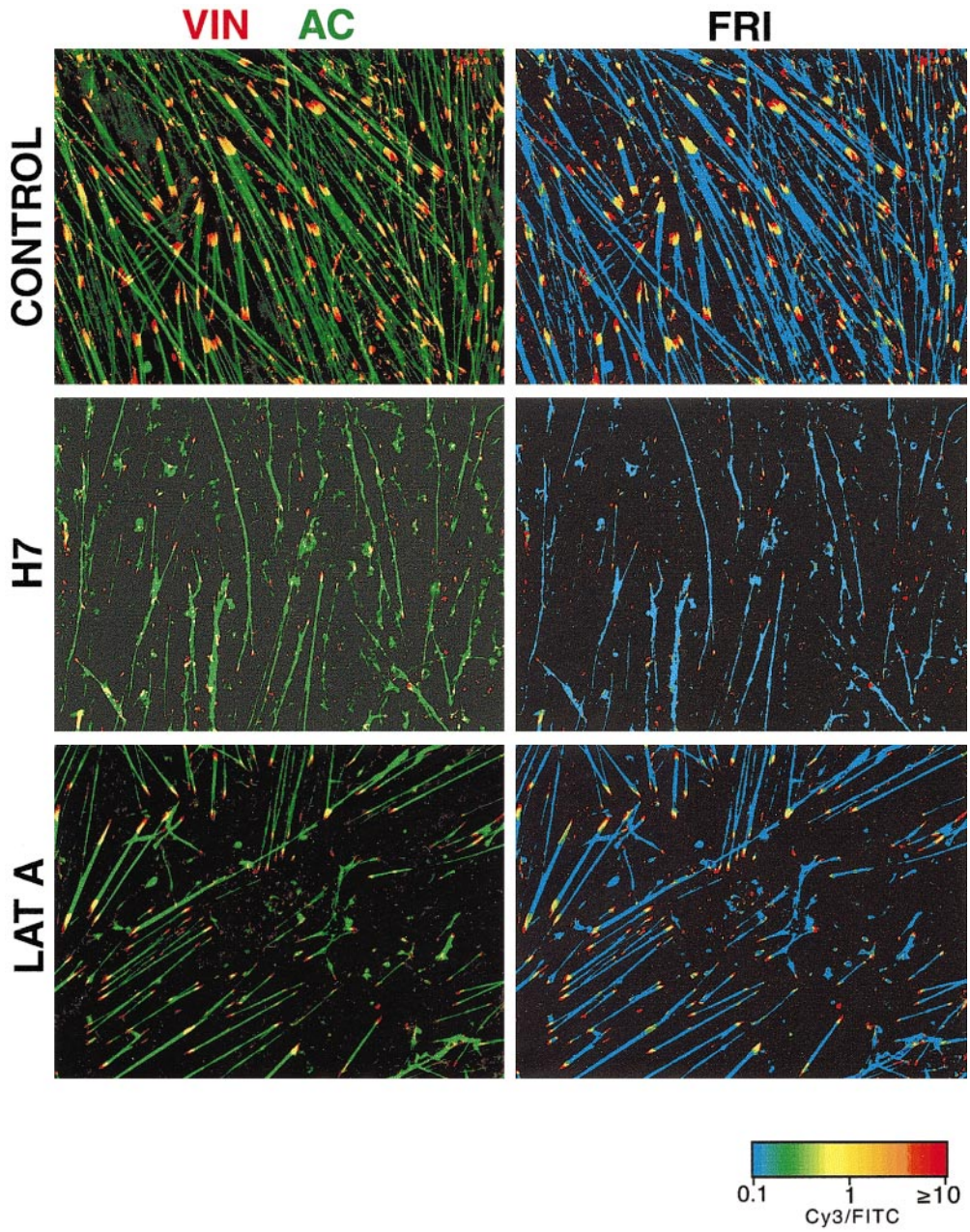

FIGURE 3. Densely plated cultured human TM (HTM) cells, untreated (control), or after 30 minutes' treatment with 100 μM H-7 (H7) or with 2 μM LAT-A (LAT A), double labeled for vinculin (VIN; Cy3-conjugated antibodies) and F-actin (AC; fluorescein isothiocyanate [FITC]–phalloidin), superimposed (*red* + *green*) images (*left column*), or fluorescence ratio images (FRI, *right column*).[14] In FRI, values of Cy3 (*red*) fluorescence intensity in each pixel are divided by FITC fluorescence in corresponding pixel. Ratio values are represented by a color scale: high (≥10), *red*; low (<0.1), *blue*; intermediate, a color spectrum with *yellow* representing a ratio of 1. Untreated HTM cells contain dense arrays of stress fibers, terminating in vinculin-rich focal contacts. H-7 treatment nearly completely abolished stress fiber labeling; residual F-actin was mainly associated with cell–cell junctions. LAT-A disrupted cell–cell junction–associated actin and radically reduced size and number of stress fibers; thin residual stress fibers were still associated with small vinculin-rich focal contacts.

injection of an actin-disrupting drug into glaucomatous eyes, perhaps aided by occasional digital pressure on the eye to elevate the pressure gradient between the anterior chamber and Schlemm's canal, might wash out abnormal ECM and produce long-lasting hypotension.

Lower drug concentrations in larger volumes, as used clinically, rather than high concentrations in small volumes as required experimentally, could minimize corneal toxicity after topical administration. Receptors might be different in different cell types or ECM, so understanding better the biomolecular differences between the cornea and TM, the different molecular targets, or mechanisms for different actin-disrupting agents, and a pro-drug, gene therapy or other site-activated approach, could facilitate development of TM-selecting "drugs" that, by changing TM geometry through cellular relaxation or contraction (e.g., by reorganizing the actin cytoskeleton), reduce outflow resistance without affecting other ocular tissues.

Acknowledgments

Tova Volberg, Ilana Sabany, Suping Cai, and B'Ann Gabelt provided expert assistance in preparation of Figures 1 and 3.

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