The Cytoskeleton of the Cultured Human Trabecular Cell

Characterization and Drug Responses

Mark I. Ryder, Robert N. Weinreb, Jorge Alvarado, and Jon Polonsky

To determine the organization of the three major cytoskeletal elements of cultured human trabecular meshwork cells (actin filaments, microtubules and intermediate filaments), we employed fluorescence microscopy and stereo-transmission electron microscopy of extracted, S-1 labeled and critical-point dried cells. Morphologic changes resulting from treatment with cytochalasin B, colchicine, taxol and nocodozole were also characterized. Compared with the cynomolgus monkey trabecular cell, morphologic differences in overall cell shape and orientation of both actin filaments and microtubules were noted. However, the responses to cytoskeletal active drugs were quite similar. Taxol, nocodozole and colchicine had a marked effect on microtubule organization, while nocodozole and colchicine had a marked effect on vimentin filament organization. None of these drugs produced marked changes in human trabecular cell shape. In contrast, treatment with the anti-actin drug cytochalasin B resulted in both a marked change in cell shape associated with organizational changes in all three cytoskeletal elements. These studies suggest a central role of actin filaments in determining overall cell shape and cytoskeletal organization in the cultured human trabecular cell. Invest Ophthalmol Vis Sci 29:251-260, 1988

Trabecular cells perform many of the activities which have been hypothesized to be necessary for maintenance of the structural integrity and normal function of the trabecular meshwork. As in other non-muscle cells, these structural and motile functions probably involve an internal three-dimensional cytoskeletal network of actin filaments, microtubules and intermediate filaments. Using transmission electron microscopic observations of thin-sectioned material, these three cytoskeletal systems have been identified in both cultured and in situ trabecular cells. Also, actin filaments have been observed with immunofluorescent methods. Support for the important role of the trabecular cell cytoskeleton has been obtained by pathophysiologic studies which have demonstrated an increased outflow facility concomitant with an alteration in the morphology of the trabecular meshwork after intracameral infusion of the anti-actin drugs cytochalasin B and D in cynomolgus monkeys. In human subjects, topical administration of the anti-microtubule drug colchicine has reduced intraocular pressure.

Previously, we evaluated cultured cynomolgus monkey trabecular cells using several comprehensive methods to understand structure-function relationships of the trabecular cell cytoskeleton. These methods included combining Nomarski observations with double fluorescent staining for actin and microtubules, as well as extraction, S-1 myosin fragment labeling and critical point drying to simultaneously identify the three major cytoskeletal systems and visualize their three-dimensional nature. While the substrate and extracellular environment of in situ trabecular cells differ from those of cultured trabecular cells, cells under both conditions may share common morphologic and functional properties. Hence, such in vitro methods may enhance our understanding of the role of the trabecular cell cytoskeleton in maintaining outflow facility in vivo. Our morphologic and pharmacologic investigations demonstrated a central role for actin filaments in maintaining cell shape and internal organization in cultured monkey trabecular cells.

However, cultured human trabecular cells are somewhat different from cultured monkey trabecular cells; they are more elongated with several broad pe-
ripheral cell processes.\textsuperscript{5,12,13} Hence, it was of interest to examine the cytoskeletal components and drug responses of these cells and compare them to our prior studies. In addition, we employed a fluorescent probe to examine the relationships of vimentin intermediate filaments to human trabecular meshwork cell shape and cytoskeletal organization.

### Materials and Methods

Human trabecular tissue obtained within 24 hr postmortem from Eye Bank eyes was used for cell culture as we have previously described.\textsuperscript{14} For these studies, we used third passage cells which were verified previously to be authentic trabecular cells based on detailed ultrastructural comparisons of the individual cell cultures with in situ trabecular cells.\textsuperscript{5}

Prior to cell culture, a 1 cm\textsuperscript{2} coverslip was placed at the bottom of each culture well. For light microscopic observations (Nomarski and fluorescence), the cells were grown directly on the coverslip surface. For transmission electron microscopic (TEM) observations of whole cells, 150 or 200 mesh gold grids were attached to the coverslip with an overlying formvar film layer. The trabecular cells were then grown on the film surface. All coverslips were sterilized under ultraviolet light prior to placement in the wells. For TEM observations of sectioned cells, cells were grown directly onto the plastic surface of small petri dishes (3.5 cm diameter).

At subconfluent growth (3–5 days) several coverslips were removed from the culture wells and processed for Nomarski observation as described below. The remaining cultured cells were grown to confluence (7–10 days).

The confluent cells grown on the petri dishes for TEM sectioning were then processed without additional incubation, using previously described methods.\textsuperscript{10}

The confluent cells grown on glass coverslips in culture wells were incubated for 1 or 2 hr at 37°C with either: (1) 0.1 M phosphate buffered saline (PBS) (control); (2) 0.1 M PBS with 0.5% DMSO (control); (3) 10\textsuperscript{−5} M cytochalasin B (Sigma, St. Louis, MO) in 0.1 M PBS with 0.5% DMSO; (4) 10\textsuperscript{−6} M cytochalasin B in 0.1 M PBS with 0.5% DMSO; (5) 10\textsuperscript{−5} M taxol in 0.1 M PBS with 0.5% DMSO; (6) 10\textsuperscript{−5} M nocodazole (Sigma) in 0.1 M PBS with 0.5% DMSO; or (7) 10\textsuperscript{−5} M colchicine (Sigma) in 0.1 M PBS.

Following this incubation, the cells attached to the coverslips were processed for either: (1) Nomarski observations (1 and 2 hr incubations); (2) fluorescent labeling for either actin and microtubules or vimentin (1 hr incubation only); or (3) Triton extraction, S-1 labeling, and critical point drying (1 hr incubation only). The light and electron microscopic methods have been described in detail previously with the exception of the fluorescent labeling of vimentin.\textsuperscript{10,11,15}

For fluorescent labeling of vimentin, cells were first fixed and extracted with the same methods used for fluorescent labeling of actin and microtubules.\textsuperscript{10} The cells were then incubated for 20 min with mouse monoclonal antibody to vimentin (Amersham, Arlington Heights, IL), diluted 1:7 in PBS-sodium azide with 0.1% Triton X-100 and bovine serum albumin. This was followed by incubation with rhodamine conjugated goat anti-mouse antibody (Cappel, Malvern, PA) diluted 1:50 in the same buffer for 30 min. The cells were photographed then using previously described methods.\textsuperscript{10}

### Results

Human trabecular cell appeared in a flat monolayer with a flat nucleus (Fig. 1) on thin sectioned material. Numerous electron-dense granules, vacuoles and microvillar-like processes at the free surface were the most prominent cytoplasmic features. When these cells were processed with reduced osmium, the nucleus appeared to contain a fine euchromatic staining pattern and a prominent nuclear envelope (Fig. 2). In contrast, when cells were treated with non-reduced osmium, the chromatin appeared con-

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densed into “spike-like” condensations at the nuclear periphery (Fig. 3). Dense bundles of filaments located primarily in the microvillar-like processes and directly beneath the free surface of the cell (Fig. 4) were the most prominent cytoskeletal features in TEM section.

Nomarski observations of trabecular cell morphology depended upon the extent of their confluence. At subconfluence, the cells appeared flat with several broad cell processes extended outward from the cell body in different directions. The nucleus appeared oval with several prominent nucleoli. As the cells approached confluence, they appeared more elongated with broad processes extending from the long axes of the cells (Fig. 5a). Most adjacent cells oriented with the long axes of the cell bodies parallel to each other. At both subconfluent and confluent stages of growth, the cytoplasm had a marked granular appearance. After a 1 or 2 hr incubation with either PBS alone or PBS with 0.5% DMSO, there were no marked changes in cell shape. However, after treatment with $10^{-5}$ M cytochalasin B several striking changes were noted. The cell body retracted and rounded up leaving fine branching peripheral processes or “microarborizations” (Fig. 5b). Such morphologic changes were not noted in cells treated with $10^{-6}$ M cytochalasin B after a 1 (Fig. 5c) or 2 hr incubation (data not shown), $10^{-5}$ M taxol (Fig. 5d), $10^{-5}$ M nocodazole (Fig. 5e) or $10^{-3}$ M colchicine (Fig. 5f). Rather, the cells in these other four experimental groups appeared similar in shape and orientation to control cells.

Fluorescent staining for filamentous actin in control cells demonstrated a concentration of the stain in numerous stress fibers over a diffusely staining background (Fig. 6a). These stress fibers were primarily oriented parallel to the long axes of the elongated confluent cells. With $10^{-5}$ M cytochalasin B treatment, this stress fiber organization was almost completely obliterated (Fig. 6b). The actin appeared to condense into numerous globular condensations. With $10^{-6}$ M cytochalasin B treatment only a portion of the stress fibers appeared to form into these condensations (Fig. 6c). No marked changes in actin filament organization were noted with treatment with taxol, nocodazole or colchicine.

Fluorescent staining for microtubules in control cells demonstrated numerous microtubules radiating outward from the nuclear region in straight lines oriented parallel to the long axis of the cell (Fig. 7a). In cells treated with $10^{-5}$ M cytochalasin B, the microtubule staining pattern condensed down with the cell body leaving microtubule staining in the microarborizations (Fig. 7b). With $10^{-6}$ M cytochalasin B, there was a slight condensation of microtubule staining (Fig. 7c) when compared to control. Treatment with taxol resulted in an increased intensity of microtubule staining around the nuclear region and a reduction of staining at the periphery (Fig. 7d). After treatment with nocodazole the microtubule staining pattern was almost completely obliterated. A few short microtubule lengths were seen to radiate from the residual centriole region (Fig. 7e). A similar obliteration of the microtubule staining pattern was noted in cells treated with colchicine (Fig. 7f).

Fluorescent staining for vimentin in intermediate filaments in control cells demonstrated the vimentin filaments to form a “lacy” meshwork throughout the cell cytoplasm (Fig. 8a). As with microtubules, treatment with $10^{-5}$ M cytochalasin B resulted in a condensation of the vimentin staining into the rounded-up cell body and the microarborizations, while treatment with $10^{-6}$ M cytochalasin B resulted in a slight condensation of the vimentin staining pattern into a coarser lace-like pattern (Fig. 8b). Although no marked changes were noted in vimentin staining with

Fig. 6. Human trabecular cells fluorescently stained for filamentous actin with NBD-phallacidin. (a) The filamentous actin stain localizes primarily in stress fibers (arrow) that orient parallel to the long axis of the human trabecular cell (N-nucleus). (b) After incubation with $10^{-5}$ M cytochalasin B, the stress fiber network is almost completely obliterated leaving globular condensations of actin. (c) After incubation with $10^{-6}$ M cytochalasin B, numerous globular condensations of actin (arrows) are seen among the stress fibers (all micrographs $\times 1400$). Fig. 7. Human trabecular cells fluorescently stained for microtubules using an indirect rhodamine-conjugated antibody technique with mouse monoclonal antibody to alpha-tubulin. (a) Numerous microtubules can be seen to radiate from the nuclear region to the two poles of the elongated human trabecular cell (N-nucleus). (b) After incubation with $10^{-5}$ M cytochalasin B, the microtubule staining pattern condenses down with the contraction of the cell body. The staining can still be seen in the microarborizations of the cell (arrow). (c) After treatment with $10^{-6}$ M cytochalasin B treatment, a slight condensation of microtubule staining can be seen when compared to control. (d) After treatment with $10^{-5}$ M taxol, the microtubule staining is more intense around the nuclear region when compared with controls. (e) After treatment with $10^{-5}$ M nocodazole only a few microtubules are seen to radiate from the microtubule region (arrow) over an amorphous background. (f) After treatment with $10^{-5}$ M colchicine, a similar marked reduction in microtubules is revealed (all micrographs $\times 2500$).
Fig. 9. Low power TEM stereopair of the elongated cell body of a human trabecular cell. The cell has been Triton extracted, S-l labeled to localize filamentous actin, and critical point dried. Amid a fine meshwork of filaments, numerous stress fibers (arrows) are seen to run parallel to the cell body. Numerous granules and vesicles (G) are also seen within the meshwork. Stereo pair taken at ±6° tilt (X4800).

Fig. 10. High power TEM stereopair of the peripheral region of a human trabecular cell processed as in Figure 9. The major elements of the cytoskeleton are readily distinguishable. Actin filaments (AF) appear with their S-l myosin arrowheads attached along their entire length. Also, they are seen to concentrate into stress fibers (SF). Intermediate filaments (IF) appear as 10–12 nm smooth, straight lines. Microtubules (MT) are readily distinguishable as two parallel electron-dense lines. Stereo pair ± 3° tilt (X65,000).

taxol (Fig. 8c), dramatic changes were seen with nocodazole and colchicine. In these two latter experimental groups, the vimentin stain was localized around the nucleus and there was little discernable vimentin stain at the periphery (Fig. 8d, e).

Stereoscopic observations on whole, extracted, S-l labeled, critical-point dried cells revealed the detailed relationships between the actin filaments, intermediate filaments and microtubules in both control and drug treated cells. In control cells at low power, the most predominant cytoskeletal features were the numerous stress fibers within a continuous meshwork of filaments (Fig. 9). Higher power views of this meshwork revealed numerous actins filament with their S-l arrowhead labels (Fig. 10). The actin filaments appeared both as a component of the meshwork of filaments and in parallel bundles in the stress fibers. Between stress fibers, both intermediate filaments and microtubules were prominent. Intermediate filaments could be distinguished by their smooth and straight appearance and 10–12 nm diameter. Microtubules were readily identified by their parallel dense lines and 20–25 nm thickness (Fig. 10).

Stereoscopic observations of cells treated with 10⁻⁵ M cytochalasin B showed a marked disruption of the actin meshwork into dense globular structures (Fig. 11). With 10⁻⁶ M cytochalasin B, only portions of the actin condensed into these structures while the actin meshwork in other areas appeared relatively intact (Fig. 12). Treatment with taxol, nocodazole or col-
chicine had little effect on the structural integrity of the actin or intermediate filaments. However, with taxol the number of microtubules was especially prominent (Fig. 13) in all areas of the cell, while with nocodazole and colchicine (Fig. 14) microtubules were generally absent.

**Discussion**

In previous studies, we examined the organization of the complex network of actin filaments, microtubules and intermediate filaments in the cultured cynomolgus monkey trabecular cell and evaluated its relationship to overall cell shape and internal structure. These cells were selected because of their morphologic similarity to human trabecular cells. In the current investigation, we compared these two different species of trabecular cells and demonstrated both important similarities and differences between them.

The cultured human and monkey trabecular cells have a similar appearance on routine TEM thin sections. Among their common characteristics are flat cell profile with a flat euchromatic nucleus, numerous electron dense granules and microvillar-like projections at the free surface. Most strikingly, when both the monkey and human trabecular cells were processed with non-reduced osmium, there was condensation of euchromatin into a spiked band at the nuclear periphery with a disappearance of the nuclear...
envelope. Such morphological characteristics have been described previously by Alvarado in other cultured human trabecular cells.5

In contrast to these similarities with routine TEM thin sections, both Nomarski observations and fluorescent staining for actin filaments and microtubules revealed several morphological differences between the cynomolgus monkey and human trabecular cells. From Nomarski observations of confluent cells, the monkey trabecular cell appeared flat and polygonal in shape10 while the human trabecular cell was more elongated in shape. Fluorescent staining revealed differences in the overall organization of actin filaments. In the monkey trabecular cell, the actin stain localized in short stress fiber lengths at the periphery, oriented parallel to the polygonal cell borders.10,11 In the human cell, these stress fibers were oriented parallel to the long axis of the elongated cell. Fluorescent staining of microtubules in the monkey trabecular cell demonstrated that they radiate from the nuclear region in all directions towards the periphery in overlapping arcs.10,11 In contrast, the microtubules of the human trabecular cell radiated in straight lines from the nuclear region, parallel to the long axis of the cell.

Despite these differences in cell shape, and actin filament and microtubule organization, the morphologic responses to the various anti-cytoskeletal drugs were similar in both the cynomolgus monkey and human trabecular cells. In order to make valid comparisons, the drug concentrations and incubation...
times employed for the human trabecular cells in this investigation were the same as those we used previously to evaluate the cynomolgus monkey trabecular cell. As we previously observed in the cynomolgus monkey trabecular cell, the most dramatic changes in human trabecular cell shape were observed after incubation with $10^{-5} \text{ M cytochalasin B}$. The change in shape was accompanied by a near complete alteration of the stress fiber organization into globular condensations. After treatment with $10^{-6} \text{ M cytochalasin B}$, both fluorescence and stereo TEM observations on whole extracted cells revealed a partial dissolution of the actin filaments without marked changes in cell shape. These observations suggest changes in the cytoskeleton, particularly actin filaments, may precede actual changes in overall cell shape. Further, after incubation with either concentration of cytochalasin B, there were marked effects on the overall organization of microtubules. In this study, we also observed the effects of cytochalasin B on vimentin intermediate filaments. The condensation of microtubules and vimentin intermediate filaments at $10^{-5} \text{ M cytochalasin B}$ is probably due to the contraction of the entire cell body. Even after incubation with $10^{-6} \text{ M cytochalasin B}$, however, there was a subtle condensation of microtubules and vimentin into a coarser staining pattern without a marked change in cell shape. This supports the important role of actin filaments in maintaining the spatial relationships of these other two cytoskeletal elements, the microtubules and vimentin intermediate filaments.

Although treatment with the microtubule promoter drug taxol, or the microtubule depolymerization drugs nocodazole or colchicine, resulted in marked changes in microtubule organization and structure, as shown by fluorescence and stereo TEM, there were no marked changes in cell shape or actin organization. This was similar to what we observed previously in our investigations of the cynomolgus monkey trabecular cell. However, the free surface blebbing seen in monkey trabecular cells treated with nocodazole or colchicine, and to a lesser degree with cytochalasin B, was not seen in human trabecular cells. Previously, we hypothesized that this blebbing may be a degranulation process stemming from the depolymerization of microtubules or the disruption of the actin filament meshwork. The absence of blebbing in the human trabecular cells may be due to the differences in cell shape, cell thickness or cytoskeletal organization. The functional significance of these differences is unknown, but should be considered when extrapolating other data from the monkey trabecular cells to the human trabecular cells.

Intermediate filaments have been proposed as serving as mechanical integrators in the cytoplasmic space and, hence, as having a structural role in non-muscle cells for maintaining cell shape and internal organization. Also, they have been implicated as an anchor for the nucleus and in protein synthesis. Since no drug is known to directly alter vimentin filaments, their role in maintaining cell shape cannot be unequivocally determined. In this study, however, a dramatic perinuclear condensation of vimentin was achieved through incubation with colchicine and nocodazole. Although these two microtubule depolymerization drugs have similar effects on vimentin distribution in other cells, the perinuclear condensation of vimentin filaments seen in cultured fibroblasts treated with taxol was not observed in cultured human trabecular cells. These observations suggest that microtubules play an important role in the distribution of the vimentin intermediate filament in the cultured human trabecular cell. Since the redistribution of vimentin is not accompanied by marked changes in cell shape, however, this type of intermediate filament probably does not play as pivotal a role in maintaining cultured human trabecular cell shape as the actin filament.

Hence, the actin filament appears to be a central cytoskeletal element responsible for maintaining cell shape and the internal organization of other cytoskeletal elements and organelles in the human trabecular cell. Although microtubules and vimentin intermediate filaments may not markedly affect the shape of the trabecular cell, their influence on its phagocytic and secretory functions should be evaluated.

**Key words:** trabecular cells, cytoskeleton, cytochalasin B, taxol, colchicine

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**References**


