The Cytoskeleton of the Cynomolgus Monkey Trabecular Cell

I. General Considerations

Mark I. Ryder* and Robert N. Weinreb†

Many cellular functions involve the complex network of actin filaments, microtubules, and intermediate filaments collectively known as the cytoskeleton. Stereo transmission electron microscopic observations of whole cynomolgus monkey trabecular cells, which were extracted, S-1 labeled, and critical-point dried, were employed to simultaneously identify these three major cytoskeletal systems and visualize their three-dimensional nature. A double fluorescence technique for actin and microtubules was used to provide a broad view of cytoskeletal relationships within the cell. Actin microfilaments were the most prominent elements of the cytoskeleton. They appeared as bundles in stress fibers. Between stress fiber bundles, a continuous meshwork of microfilaments and intermediate filaments could be seen. Numerous microtubules radiated from the centriole region to the cell periphery. This comprehensive overview of the cytoskeleton of the cynomolgus monkey trabecular cell can be used to understand structure-function relationships of the trabecular cell cytoskeleton and its influence on outflow facility. Invest Ophthalmol Vis Sci 27:1305-1311, 1986

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.1-3 As a lining of the aqueous channels in the outflow pathway, they must remain attached to the trabecular beams, possess the ability to spread to cover beams that become denuded, and maintain a flat appearance to prevent aqueous flow turbulence. Trabecular cells also have phagocytic capabilities which allow them to remove materials, such as pigment or inflammatory debris, which could obstruct the outflow of aqueous humor.4-6 Further, these cells regulate the deposition and degradation of the extracellular matrix in the trabecular meshwork,2,7-11 as well as functioning as secretory12 and target cells for drugs1 and hormones.13 As in other cells,14-16 these cellular functions most likely involve the complex, three-dimensional network of actin filaments, microtubules, and intermediate filaments collectively known as the cytoskeleton. To understand these functions of the trabecular cells and their influence on outflow facility, therefore, it is important to characterize the structure-function relationships of the trabecular cell cytoskeleton.

Two morphologic techniques, transmission electron microscopic (TEM) observation of thin-sectioned material and immunofluorescence, previously have been employed to study the cytoskeleton of trabecular cells. Both Ringvold17 and Gipson18 used S-1 labeling in glycerinated cells to localize filamentous actin in TEM sections. In unlabeled thin sections, Grierson19 observed both 6 nm filaments (probably actin) and 10 nm (intermediate filaments), while Alvarado20,21 noted microfilaments and microtubules. However, thin sections can only provide a limited view of the cytoskeleton, and glycerination distorts the cell shape. Grierson19 and Tripathi22 used antibody techniques to localize actin in trabecular cells. These immunofluorescent methods can be used to localize other cytoskeletal elements as well.

In the current investigation, we employed stereo pairs of whole cells which were extracted, S-1 labeled,
and critical-point dried to simultaneously identify all three major cytoskeletal systems (actin filaments, microtubules, and intermediate filaments) and visualize the three-dimensional nature of the cytoskeleton. Further, we used a double fluorescence technique for actin and microtubules to provide a broad view of cytoskeletal relationships within the same cell. With these techniques, we have obtained a comprehensive overview of the cytoskeleton of the cynomolgus monkey trabecular cell. These data can be used as a basis for comparison with pharmacologic and physiologic investigations of outflow facility in the cynomolgus monkey eye.

Materials and Methods

Monkey Trabecular Cell Culture

Trabecular tissue was obtained for cell culture from one eye of a 3.2 kg cynomolgus monkey immediately postmortem and propagated as we have previously described. We used third passage cells.

Prior to cell culture, a 1 cm² coverslip was placed at the bottom of each culture well. Cells were then grown on the coverslip for 7–10 days. 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, they were grown directly onto the plastic surface of small petri dishes (3.5 cm diameter).

Light Microscopy

For Nomarski observations, coverslips with attached cells were rinsed briefly in 0.1 M phosphate buffered saline (PBS) with 10 mM sodium azide, and then fixed for 20 min in a mixture of 1.0% gluteraldehyde and 0.5% Osmium tetroxide in the same buffer. The coverslips were then stored in the buffer. Fixed cells were photographed with a Nomarski optics system on an inverted microscope.

For fluorescent labeling of actin and microtubules, the cells were double-labeled. First, they were labeled for tubulin with an indirect rhodamine-conjugated antibody technique, and then for actin with a direct NBD-phallacidin technique. With these techniques, cells were washed once in the PBS-sodium azide buffer, and then fixed and extracted simultaneously for 20 min in 2.0% formaldehyde, 0.2% Triton X-100 and PHEM buffer. After two rinses in PBS-sodium azide, the cells were incubated with mouse monoclonal antibody to alpha-tubulin for 20 min. The mouse monoclonal antibody was previously diluted 1:100 in PBS-sodium azide with 0.1% Triton X-100 and 1.0% bovine serum albumin. All incubations were performed at room temperature. The cells were then rinsed twice in PBS-sodium azide and incubated with rhodamine-conjugated goat antimouse antibody for 30 min. This secondary antibody was diluted 1:50 in the same buffer as the mouse monoclonal. After another rinse in PBS-sodium azide, the cells were incubated with NBD-phallacidin (Molecular Probes, Inc., Junction City, OR) diluted 1:20 in PBS-sodium azide for 20 min. The coverslips were then rinsed and mounted in 50% glycerol-50% PBS onto a glass slide. Fluorescent micrographs were obtained with the appropriate excitation and barrier filters to screen only NBD-fluorescence for actin (green) or rhodamine fluorescence for microtubules (red). Color slides were converted to black and white prints with an intermediate negative using Tech Pan film (Kodak, Rochester, NY).

Electron Microscopy

Cells grown directly onto small petri dishes for TEM sectioning were fixed in 0.5% paraformaldehyde and 1.0% gluteraldehyde in 0.1 M sodium cacodylate with 0.2 mM CaCl₂, pH 7.4. Cells were then post-fixed in either a reduced (with 1.5% potassium ferrocyanide) or non-reduced (no potassium ferrocyanide) 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer. The cells were then stained with 1.0% uranyl acetate for 2 hr, dehydrated in ethanol, and embedded in the dish with Epon-Araldite. The embedded cells were then separated from the petri dish, re-embedded in Epon-Araldite, sectioned, and stained with uranyl acetate and lead citrate.

For TEM observations on the cytoskeletons of whole unsectioned cells, the cells were extracted, labeled with the S-1 myosin subfragment to localize filamentous actin, fixed, and critical-point dried. These methods have been previously described. The only modifications we employed in this study were: 1) 0.1% formaldehyde was added to the Triton X-100 extraction solution; and 2) all procedures, up to the critical point drying of the cells, were done with the TEM grids attached to the coverslip.

Grids with either thin sections of cells or whole critical-point dried cells were scanned and photographed on a Philips 300 TEM operated at 60 KV (for sections) or 100 KV (for whole cells). Stereo pairs were obtained by maneuvering the tilting stage on this microscope.

Results

Cultured monkey trabecular cells grown either directly on glass coverslips or formvar film appeared at...
confluence as a continuous sheet of broad, flat polygonal cells when viewed under Nomarski optics (Fig. 1). Each cell had a “granular” cytoplasm and a round or oval nucleus with two prominent nucleoli. When viewed in TEM cross sections, the cells appeared in flat monolayers (Fig. 2). The most prominent features of the cytoplasm were the numerous electron dense inclusions and microvillar-like projections at the upper surface of the cell (Fig. 2). With reduced osmium, the nucleus of the cells appeared to contain a fine euchromatin pattern with a prominent nuclear envelope (Fig. 3A). On the other hand, with non-reduced osmium, the chromatin condensed into “spike-like” condensations at the nuclear periphery (Fig. 3B). In addition, the nuclear envelope was not prominent. Intercellular junctions between overlapping cell processes were not a prominent feature. However, gap junctions, coated pits, and coated vesicles were observed (Figs. 4A, B). With conventional TEM, the most prominent cytoskeletal features were short lengths of filaments which were oriented parallel to the length of the cell (Figs. 4A, C). These filaments were especially prominent at the upper and lower surfaces of the cell.

A more complete view of the cytoskeleton could be seen with the fluorescence micrographs and with the whole critical-point dried cells. Under fluorescence, the
actin filaments appeared in bright stress fibers over a darker fluorescent background (Fig. 5). These stress fibers appeared in groups of dense parallel lines that traversed all or part of the cell diameter, and concentrated along the periphery. Numerous microtubules were seen to radiate from the nucleus to the periphery in overlapping arcs (Fig. 6).

TEM observations on extracted, S-1 labeled, and critical-point dried cells revealed the organization of all three major cytoskeletal elements (actin filaments, microtubules, and intermediate filaments). Low power views of the cells revealed the density of the total filament organization within the cell as well as the stress fiber organization (Fig. 7). In addition, numerous granules and vesicles were seen to concentrate around the nuclear region. High power stereo image pairs revealed the complexity of the organization of the three major cytoskeletal elements. Around the nucleus, this organization was extremely dense due to the thickness of the cell in this area (Fig. 8). However, larger elements, such as microtubules and stress fibers, could be distinguished. At the thinner periphery, all three cytoskeletal elements could be distinguished. Actin filaments appeared in bundles of stress fibers as well as in a fine meshwork of single filaments between the stress fibers (Fig. 9). At the periphery of cells, individual actin filaments were observed with the “barbed” end of the S-1 label directed outwards (Fig. 10). Between these dense stress fibers, numerous intermediate filaments were observed (Fig. 9). They appeared as smooth and straight filaments with a thickness of 10–12 nm. Microtubules, with their parallel dense lines and 20–25
Fig. 8. High power TEM stereo pair of the perinuclear region of a whole, Triton-extracted and S-labeled critical-point dried cell. Due to the thickness of the cell in this region, the cytoskeleton appears very dense and complex. However, numerous microtubules (MT) can be distinguished by their parallel dense lines, and a stress fiber is seen (SF) (N-nucleus). Stereo pair taken at ±3° tilt (×40,000). Fig. 9. High power TEM stereo pair of the peripheral region of a trabecular cell processed as in Figure 8. At the thinner periphery, the major elements of the cytoskeleton are more readily distinguishable. Intermediate filaments (IF) appear as 10-12 nm smooth, straight lines. Actin filaments (AF) appear with their S-l myosin arrowheads (arrows—barbed end) attached along their entire length. They are also seen to concentrate into stress fibers (SF). Microtubules (MT) are also readily distinguishable. Stereo pair taken at ±3° tilt (×68,000). Fig. 10. High power TEM of the periphery of a cell processed as in Figure 8. Note how individual actin filaments (AF) radiate outwards from the periphery. Along these peripheral filaments, the barbed ends of the S-1 arrowheads (arrows) are directed outward from the cell body (×62,000).

nm thickness, were also observed at the periphery (Fig. 9).

Discussion
In this study, we have demonstrated the presence and organization of the three major cytoskeletal elements (filamentous actin, microtubules, and intermediate filaments) in the cultured cynomolgus monkey trabecular cells. These cells were selected because of their morphologic similarity to human trabecular cells and the opportunity they provide for comparing our in vitro data with in vivo physiologic studies. 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, structure-function investigations of cultured cynomolgus monkey trabecular cells may provide insights regarding these same relationships in in situ cynomolgus monkey trabecular cells.
Although Nomarski observations of the cynomolgus monkey trabecular cells revealed a more polygonal cell than the spindle-like trabecular cells seen in human culture, the conventional TEM features were identical to those reported in human trabecular cells. These identifying features included the growth of cells in thin monolayers, the presence of microvillar-like projections at the free cell surface, phagocytic inclusions, the presence of gap junctions, coated vesicles, coated pits at the cell-to-cell interface, and, most characteristically, the condensation of euchromatin into a spiked band at the nuclear periphery with disappearance of the nuclear envelope when processed with non-reduced osmium. As with previous conventional TEM studies, filamentous structures could be observed in short lengths. However, the absence of such plaques at the basal surface. However, observations did not reveal such actin plaques at the basal surface.

Stress fibers were a prominent feature of cultured cells in these studies, and were composed primarily of bundles of actin filaments (as revealed by S-1 labeling and fluorescence). In the in situ situation, these actin stress fibers may maintain the flat shape and longitudinal orientation of the trabecular cells upon the collagen beams. Actin has also been implicated in the attachment of cells to substrate. In other non-muscle cells, actin has been observed to form dense attachment plaques along its basal surface at sites of adhesion. In the cultured cynomolgus monkey cell, stereoscopic observations did not reveal such actin plaques at the basal surface. However, the absence of such plaques may be due to the type of attachment substrate used in this study (glass or formvar). In the in situ situation, such plaques may form along the basement membrane and collagen complex to which the trabecular cells are adherent.

Mediation of cell motility is another common role for actin in non-muscle cells. Although myosin has been localized in several non-muscle cells, an organized actin-myosin complex has yet to be demonstrated. Thus, the sliding filament model for motility associated with muscle cells has not yet been applied to the non-muscle cell. As a result, several other models have been proposed for actin participation in cell motility. In other cells, for example, the three-dimensional lattice of actin filaments has been observed to contract and relax. Such a lattice of actin has been observed between stress fibers and may be a viable mechanism for movement of the trabecular cell. Actin polymerization at the periphery of the cell, which can possibly push the overlying cytoplasmic membrane in the direction of movement, has also been offered as a means of motility in non-muscle cells. This hypothesis is derived from two important observations. First, S-1 labeled actin filaments orient with their barbed ends directed outwards and, second, actin polymerizes preferentially at this barbed end. In the current study, this type of peripheral orientation of actin was also observed. Thus, the trabecular cell may extend its processes using a similar actin polymerization mechanism.

Both microtubules and intermediate filaments were also prominent in the monkey trabecular cells. In other non-muscle cells, these two cytoskeletal elements have been implicated in the maintenance of overall cell shape, as well as the organization and intracellular transport of various organelles. The possible role of intermediate filaments and microtubules in the organization and transport of secretory granules (involved in extracellular matrix synthesis), lysosomal granules, and phagosomes (involved in both phagocytosis and degradation of extracellular material) is of particular interest in trabecular cells. Although these organelles are intimately associated with microtubules and intermediate filaments in other non-muscle cells, such an organelle-cytoskeleton relationship was difficult to ascertain in the current study. This was most likely related to the density of the cytoskeleton around the organelle-rich nucleus and the effects on organelle shape of the Triton extraction. However, further insights into the structure-function roles of microtubules and intermediate filaments, as well as actin filaments, can be obtained by treating the trabecular cells with drugs known to have effects on the cytoskeleton.

Key words: cytoskeleton, actin, intermediate filaments, microtubules, trabecular cells

Acknowledgments

The authors gratefully acknowledge Dr. Mark Kirschner for providing the antitubulin antibodies and Dr. Richard Niedermaier for providing the S-1 label. We also appreciate the assistance of Donna Chesnut and Carol Fiuren in preparing the manuscript.

References