In Situ Localization of Cytoskeletal Elements in the Human Trabecular Meshwork and Cornea

Robert N. Weinreb* and Mark I. Ryder†

The authors compared cytoskeletal elements of the in situ human trabecular-meshwork cell with in situ human corneal cells using indirect immunofluorescence staining for tubulin and intermediate filaments (vimentin, cytokeratin, and desmin) and NBD-phallacidin staining for f-actin using both fixed frozen and unfixed frozen sections from postmortem eyes. Both f-actin and tubulin were found throughout the cell body of trabecular-meshwork cells, keratocytes, corneal endothelium, and corneal epithelium. The f-actin staining pattern was concentrated at the cell periphery of these four cell types. Vimentin stain was intensely localized in focal areas of the trabecular-meshwork cell, keratocytes, and throughout the corneal endothelium. A general anticytokeratin antibody was intensely localized in corneal epithelium and endothelium. However, PKK-1 anticytokeratin antibody was seen only in superficial layers of corneal epithelium and not in corneal endothelium. The 4.62 anticytokeratin antibody was not observed in either corneal epithelium or endothelium. None of these three cytokeratin antibodies were seen in trabecular-meshwork cells or keratocytes. Desmin stain was not noted in any of these cell types. In general, cytoskeletal staining of unfixed frozen sections showed a similar staining pattern for f-actin and tubulin but a more uniform and intense staining pattern for vimentin and cytokeratin compared with fixed frozen material. The authors conclude that these cytoskeletal stains can differentiate human trabecular-meshwork cells from cells of the cornea in situ.

Materials and Methods

Postmortem eyes were obtained by enucleation within 2 hr after death. A corneoscleral button was excised 2-mm posterior to the limbus to include the...
trabecular meshwork. In preliminary experiments, we found that direct fixation of the corneoscleral buttons resulted in a marked autofluorescence of the sectioned tissue. This autofluorescence was eliminated by storing the buttons in McCoy-Kaufman media for 6–24 hr before fixation and freezing or before freezing alone. Nine nonglaucomatous eyes from nine patients (aged 23–78 yr) were processed for cytoskeletal labeling.

In the fixation and freezing procedure, the corneoscleral tissue from eight eyes were fixed in 2.0% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) with 10 mM of sodium azide at 4°C for 4–6 hr. Washed three times with PBS, and stored in PBS with sodium azide for 3–14 days. A segment of tissue containing cornea, sclera, and trabecular meshwork was excised from each corneoscleral button, mounted in OCT compound (Miles Laboratories, Naperville, IL) and frozen with dry ice and liquid nitrogen. In the freezing-alone procedure, the corneoscleral tissues from five eyes were directly mounted in OCT compound and frozen without prior fixation. The frozen blocks of tissue were then transferred to a Slec HR cryostat (London, England). For each piece of tissue, 5-μm thick sections were cut and then collected on Chrome-Alum-treated slides (Becton-Dickinson, Sunnyvale, CA). They were allowed to air dry for 1–2 hr. One slide from each tissue block was stained with Mayer’s hematoxylin and eosin (H & E) (Roboz Surgical, Washington, DC) to identify historically the different tissues. The remaining slides from each corneoscleral button were immersed in acetone for 20 min at −20°C, air dried, and labeled for tubulin, vimentin, cytokeratin, or desmin. Using an indirect rhodamine-conjugated antibody technique. Some sections were then labeled for α-actin with NBD-phallacidin.

In this labeling technique, the sections were first incubated with one of the following antibodies to selected cytoskeletal elements: (1) mouse monoclonal antialpha tubulin (Amersham, Arlington Heights, IL), (2) mouse monoclonal antivimentin (Amersham), (3) mouse monoclonal antidesmin (Amersham), (4) a general mouse monoclonal anticytokeratin (Amersham) with unknown specific reactivity to particular cytokeratin species, (5) mouse monoclonal PKK-1 anticytokeratin antibody (Labsystems, Helsinki, Finland) which crossreacts with the 44-kilodalton (kD), 46-kD, 52-kD, and 54-kD cytokeratins of HeLa cells, and (6) mouse monoclonal anticytokeratin 4.62 (Miles Laboratories, Naperville, IL) which reacts with the 40-kD (no. 19) cytokeratin seen in differentiated simple epithelium. Each monoclonal antibody was diluted in PBS with 0.1% Triton X-100, 1.0% bovine serum albumin, and 10 mM sodium azide at the following concentrations: antialpha tubulin, 1:50; antivimentin, general anticytokeratin, and antidesmin, 1:7; and PKK-1 and 4.62 anticytokeratins, 1:20.

All primary incubations were done for 60 min at 20°C. The slides were then washed three times with PBS with sodium azide and incubated for 40 min at 20°C with 50 μl of a 1:50 dilution of rhodamine-conjugated goat anti-mouse antibody (Cappel, Malvern, PA) in the same buffer as used for the primary monoclonal incubation.

Control incubations included incubating several sections with the primary monoclonal antibodies alone and the secondary rhodamine-conjugated antibody alone. After three more washes in PBS with sodium azide, the sections were incubated with 50 μL of 1.5 mg/ml NBD-phallacidin (Molecular Probes, Junction City, OR) in PBS with sodium azide for 30 min at room temperature, washed in PBS with sodium azide, and mounted in a 1:1 mixture of PBS and glycerol.

The H & E- and fluorescent-stained slides were examined and photographed with an Olympus BH microscope (Tokyo, Japan) using a 490-nm excita-
tion filter and a 530-nm barrier filter for NBD and a 545-nm excitation filter and a 610-nm barrier filter for rhodamine. Observations on the intensity of fluorescent staining were made with the 100X objective and graded on a scale of 0 (no discernible fluorescence) to +++ (intense fluorescence) for each cell type.

Results

The H & E-stained sections of representative tissue blocks from each eye enabled each tissue which was fluorescently stained for cytoskeletal elements to be identified (Fig. 1). From these preliminary observations, no major structural differences in tissue integrity were noted between fixed frozen and unfixed frozen sections. The trabecular meshwork could be distinguished clearly from the adjacent corneal endothelium, ciliary body, and overlying sclera. In the trabecular meshwork, cells covered the collagen beams (Fig. 2). Cytoskeletal staining patterns of these trabecular-meshwork cells, and of corneal endothelium, corneal epithelium, and keratocytes, are summarized in Table 1. For f-actin, tubulin, and desmin, the staining patterns were similar between fixed frozen and unfixed frozen tissue. However, with vimentin and the various cytokeratins, a more uniform and intense pattern was observed on unfixed frozen material.

In the trabecular meshwork, both anti-f-actin (Fig. 3) and antitubulin fluorescent staining (Fig. 4) were found around the trabecular-meshwork cell nucleus and within the fine cell processes which cover the collagen beams. Antivimentin staining localized in only a few focal areas of the trabecular-meshwork cells in unfixed frozen sections (Fig. 5), but was seen throughout the trabecular cells in unfixed frozen sections (Fig. 6). Neither cytokeratin staining with any of the three tested antibodies (Fig. 7) nor desmin staining was evident in the trabecular-meshwork cells. Desmin staining, however, was noted in the adjacent ciliary muscle (Fig. 7). Incubation with the secondary rhodamine-conjugated antibody alone revealed a faint nonspecific staining in the collagen beams without specific staining in the trabecular-meshwork cells (Fig. 8).

Corneal endothelium. Descemet’s membrane, and keratocytes could be distinguished readily (Fig. 9). No specific fluorescent staining was noted with either antidesmin or with the secondary rhodamine-conjugated antibody alone (Fig. 10). Anti-f-actin staining was found diffusely throughout the corneal endothelium (with a somewhat more intense staining at the cell periphery) and keratocytes but not in Descemet’s membrane (Fig. 11). Tubulin staining was seen throughout the corneal endothelium (Fig. 12). In fixed frozen sections of keratocytes, the tubulin stain was either seen throughout the cell or just around the nuclear region (Fig. 12), whereas in unfixed frozen sections a more uniform tubulin stain was observed throughout the cells. Antivimentin (Fig. 13) stain was localized throughout the corneal-endothelial cells. An intense stain was noted in the corneal endothelium with the general anticytokeratin antibody (Fig. 14). However, no distinct stain pattern in the corneal endothelium was noted with the PKK-I (Fig. 15) or 4.62 (Fig. 16) anticytokeratin antibodies.

Corneal epithelium was distinguished readily from the underlying stroma (Fig. 17). Filamentous actin was seen to localize in all cell layers (Fig. 18) with the most intense staining at the periphery of the cell. In some sections, the actin stain was slightly more intense in the basal layer of cells (Fig. 18). Antitubulin stain was diffusely distributed throughout these cells. Fluorescent stain for antivimentin was very faint and nonspecific in the epithelium (Figs. 19, 20). In fixed frozen sections of underlying keratocytes, the antivimentin stain varied from an intense stain of some cells to a general faint stain. By contrast, in unfixed frozen sections the vimentin stain of keratocytes was more uniform and intense (Fig. 20). In fixed frozen sections, the general anticytokeratin staining was seen as a diffuse stain in the basal layers of corneal epithelium.

Table 1. Fluorescence staining of trabecular meshwork and corneal cells*

<table>
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<tr>
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<th>Trabecular meshwork</th>
<th>Corneal endothelium</th>
<th>Keratocytes</th>
<th>Corneal epithelium</th>
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<tr>
<td>NBD (f-actin)</td>
<td>+++/++++</td>
<td>+++/++++</td>
<td>++++/+++</td>
<td>+++/++</td>
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<tr>
<td>Tubulin</td>
<td>++++/+++</td>
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<td>++++/+++</td>
<td>+/+</td>
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<td>Vimentin</td>
<td>0, ++/+</td>
<td>++/+/++</td>
<td>++++/++</td>
<td>0/0</td>
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<tr>
<td>Desmin</td>
<td>0/0</td>
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<td>0/0</td>
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<tr>
<td>Cytokeratin (Amersham)</td>
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<td>4.62</td>
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<td>Secondary antibody alone</td>
<td>0/0</td>
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* Values represent fixed/unixed staining patterns. Fluorescence intensity is graded as follows: (0) no discernible stain; (+) faint staining; (++) moderate staining; (+++) intense staining. A comma between intensity gradings indicates intercellular variation.
Figs. 9-16. Fig. 9. H & E stained fixed frozen section of the area just anterior to the trabecular meshwork. A single flat layer of corneal endothelial cells (CN) covers Descemet's membrane (DM). Beneath the membrane, keratocytes (F) can be seen. ×900. Fig. 10. High power fixed frozen section of the corneal endothelium region incubated with the secondary rhodamine conjugated antibody alone. A faint background stain is noted in the corneal endothelial cells and underlying stroma. ×900. Fig. 11. High power fixed frozen section of the corneal endothelium region stained for filamentous actin with NBD phallacidin. Actin is seen to localize both in the corneal endothelium (CN), especially at the periphery, and in the keratocytes (F) beneath Descemet's membrane (DM). ×800. Fig. 12. The same area as in Figure 11 fluorescently stained for microtubules. The microtubules staining is found throughout the corneal endothelium (CN) and in focal areas of the keratocytes (F) (DM—Descemet's membrane). ×800. Fig. 13. Fixed frozen corneal endothelium fluorescently stained for vimentin. The vimentin stain is localized throughout the corneal endothelium. Fig. 14. Fixed frozen corneal endothelium fluorescently stained with the general anticytokeratin antibody. As with vimentin, the stain is seen throughout the corneal endothelium (DM—Descemet's membrane). ×900. Fig. 15. Fixed frozen corneal endothelium fluorescently stained with the PKK-1 anti-cytokeratin antibody. There is no discernable stain in the corneal endothelium (DM—Descemet's membrane). ×900. Fig. 16. Fixed frozen corneal endothelium stained with the 4.62 anti-cytokeratin antibody. Again, there is no discernable stain in the corneal endothelium (DM—Descemet's membrane). ×900.
Figs. 17–22. Fig. 17. H & E stained fixed frozen section of the corneal epithelial region. The corneal epithelium appears in a stratified layer of cells over the connective tissue stroma. ×560. Fig. 18. High power fixed frozen section of the corneal epithelial region stained for filamentous actin using NBD phallacidin. The actin stain is most intense at the periphery of each cell. The staining is slightly more intense in the basal layer of cells overlying Bowman’s membrane (BM) than in the more superficial layer of cells (arrow). ×900. Fig. 19. High power section of the corneal epithelium fluorescently stained for vimentin. A faint nonspecific stain is seen in the epithelial cells (BM—Bowman’s membrane). ×900. Fig. 20. Unfixed frozen section of the nasal region of the corneal epithelium and underlying stroma stained for vimentin. A faint, nonspecific stain is seen in the corneal epithelium, while an intense stain is observed within the underlying keratocytes (arrows). ×900. Fig. 21. High power fixed frozen section of corneal epithelium stained with the general anti-cytokeratin antibody (Amersham). A diffuse stain is seen throughout the cytoplasm of the epithelial cells. This stain is markedly more intense at the superficial layer of epithelial cells (arrow) (BM—Bowman’s membrane). ×900. Fig. 22. Unfixed frozen section of corneal epithelium stained with the general anti-cytokeratin antibody (Amersham). Note a more uniform intense staining throughout all layers of epithelium when compared to Figure 21. ×900.

In the current study, the staining pattern for tubulin and α-actin in human trabecular-meshwork cells

Discussion

In the current study, the staining pattern for tubulin and α-actin in human trabecular-meshwork cells
in situ was somewhat different from that which we found previously in cultured human and monkey trabecular-meshwork cells.\textsuperscript{12-14} Cultured human cells and cynomolgus monkey cells had a well-defined stress-fiber network of f-actin and microtubules radiating from the nuclear region. Such fine resolution of structures was not observed in human trabecular-meshwork cells in situ. This is probably due to the fact that our observations of cultured cells were made on broad flat cells approximately 0.25-0.5 \textmu m in thickness, and observations of trabecular-meshwork cells in situ were made on 5-\textmu m thick sections. In these latter observations, only the nuclear profile and a cross-sectional area of thin cell processes enveloping the collagen beams were normally seen. Nevertheless, both f-actin and tubulin were demonstrated around the nucleus and extending into the fine cell processes surrounding the collagen beams. Previous electron microscopic observations on S-l labeled, thin-sectioned in situ trabecular-meshwork cells noted bundles of actin filaments.\textsuperscript{17} As in other cell types,\textsuperscript{18,19} actin in these trabecular-meshwork cell processes may play a role in the attachment of the cell to the collagen beam, maintaining the flat cell profile against the beam, and providing the motile force for trabecular-meshwork cell process migration over the beams. Tubulin, which localized in the fine cell processes, may enhance the structural integrity of the cell and be involved in phagocytic and secretory functions.

Tubulin and f-actin also were found throughout the corneal endothelium and corneal epithelium. The f-actin stain was especially prominent in the cortical regions of these two cell types. A similar concentration of f-actin in the cortical region of other cell types has been reported.\textsuperscript{20,21} This cortical actin may play a role in maintaining the shape of the corneal endothelium and epithelium and may form part of the attachment apparatus for cell-cell or cell-substrate interactions. The cortical staining pattern for actin was particularly intense in the basal cells of the corneal epithelium. Similar observations have been made in migrating corneal-epithelial cells during wound healing.\textsuperscript{22}

One striking finding of this study was the differential staining patterns seen with desmin, vimentin, and cytokeratin intermediate-filament proteins. Staining for desmin intermediate-filament protein, found normally in muscle cells,\textsuperscript{21} was not seen in the examined cell types, but was observed in the adjacent ciliary body. By contrast, in a recent in vitro study of cultured trabecular-meshwork cells, desmin staining was observed.\textsuperscript{24} This may be due to a different expression of intermediate filaments in these cultured cells or to an in situ masking of the desmin protein in the present study. In the trabecular-meshwork cells and keratocytes in situ, the vimentin stain was seen only in a few focal areas of the cell processes on fixed frozen tissue, but it was seen more uniformly throughout the cells in unfixed frozen tissue. Such observations suggest the possibility of a structural and/or functional heterogeneity of the trabecular-meshwork cell population. However, this focal staining pattern of trabecular-meshwork cells and keratocytes may also be due to the processing method used in this study. Although the fixation step we used may help to maintain the structural integrity of the tissue, it may also alter the antigenicity of certain cytoskeletal proteins. Such a mechanism is supported by our observations on keratocytes and trabecular-meshwork cells in unfixed frozen tissue and in a recent study using unfixed frozen corneal tissues which showed vimentin staining in keratocytes.\textsuperscript{25} Using fixed tissue, we may have detected only higher concentrations of the vimentin protein in the trabecular-meshwork cells and keratocytes. By comparison, in corneal endothelium an intense vimentin stain was seen throughout each cell. Although the biologic processes mediated by vimentin are not understood clearly, it has been implicated as playing a role in maintaining cell shape and internal organization\textsuperscript{26} and mediating cell-substrate and cell-cell attachment.\textsuperscript{26} Hence, it is likely that vimentin has similar functions in situ in trabecular-meshwork cells, corneal endothelium, and keratocytes.

Equally striking was the distribution of the cyto-keratin intermediate-filament proteins as revealed by three different anticytokeratin antibodies. The general anticytokeratin stain was prominent in corneal epithelium. As with the vimentin staining, the cytokeratin staining was variable in the corneal epithelium from fixed frozen sections and more uniform in unfixed frozen sections. It is possible that in fixed material the cytokeratins are antigenically altered with less labeling affinity for the antibody. Furthermore, this general anticytokeratin staining pattern was seen also in the corneal endothelium. Although the presence of cytokeratin in endothelial cells is not encountered normally, these observations of general cytokeratin concur with those of a recent report in which keratin staining in normal human corneal-endothelial cells was shown on fixed sections using a general cytokeratin derived from epidermal cells.\textsuperscript{29} In that study, the corneal endothelium also lacked several histochemical and ultrastructural markers characteristic of endothelium in other areas. From those observations, it was concluded that the corneal endothelium may not be a true endothelium and may have several epithelial-cell characteristics. However, transmission electron-microscopic studies of corneal...
endothelium have not demonstrated keratin tonofilaments or tonofibrils. It is possible that the cyto-
keratins in these cells exist in a more globular form. In fact, it is possible that morphologic features of
corneal endothelium may be similar to simple epithelial cells in other tissues such as Bowman’s capsule in
the kidney and the rete testis. Both vimentin and cytokeratins are seen routinely in the flat monolayer of
simple epithelial cells which lines fluid chambers in these tissues. As in these other tissues, both cytoker-
atin and vimentin may contribute to the regulation of the shape and mechanical resistance of the corneal
endothelium. However, no marked cytokeratin-staining pattern in corneal-endothelial cells was
noted with either the PKK-I or the 4.62 anticytokeratin antibodies. The absence of stain with the 4.62
antibody is of interest in that it specifically stains for the 40-kD cytokeratin species seen in differ-
entiated simple epithelium. Furthermore, the PKK-1 antibody has been shown to be reactive to the
46-kD, 52-kD, and 54-kD cytokeratins in HeLa cells which may be similar to the 46-kD, 52-kD, and
54-kD cytokeratins seen in most simple epithelia. It is possible that the general anticytokeratin used in
this study and in the previous study may stain for one of the 19 cytokeratin species not detected by
the PKK-1 or 4.62 antibodies.

Various fluorescent cytoskeletal stains to actin, tubulin, and especially intermediate filaments are now
used widely to type both normal and neoplastic cells in various tissues. In our study, the use of unfixed
frozen material did not appear to alter structural integrity markedly compared with fixed material. How-
ever unfixed frozen tissues had a more uniform staining pattern. Therefore, it appears that using un-
fixed frozen material is preferable to fixed material when studying in situ cytoskeletal localization on this
type of tissue. Also, we demonstrated that trabecular-meshwork cells display a staining pattern to f-actin,
microtubules, and especially vimentin and cytokeratin intermediate-filament proteins which is clearly
distinct from neighboring corneal endothelium, corneal epithelium, and keratocytes. Such labeling tech-
niques may be valuable adjuncts in monitoring phenotypic cell expression in glaucoma and other oc-
cular diseases.

Key words: cornea, trabecular-meshwork cell, glaucoma, actin, intermediate filaments

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