Immunocytochemical Study of Intermediate Filaments in Cultured Human Trabecular Cells

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In order to study which classes of intermediate filaments (IF) comprise the IF of human trabecular cells, we undertook immunocytochemical investigations on cultured human trabecular cells using anti-keratin, anti-vimentin, anti-desmin and anti-glial fibrillar acidic protein (GFAP) rabbit sera. Immunostaining with the keratin antibody and the GFAP antibody only produced nonspecific staining. Immunostaining with the vimentin antibody and the desmin antibody produced intracytoplasmic filamentous staining. Pretreatment of the cells with colcemid to induce the perinuclear concentration of IF resulted in the demonstration of a characteristic perinuclear immunofluorescence, confirming the existence of vimentin and desmin. Some cells showed particularly strong positivity to desmin. The desmin antibody used in this study only faintly labeled the IF of cultured human skin fibroblasts, known to contain vimentin but not desmin. These results suggest that the IF of human trabecular cells are composed of vimentin and desmin, and that human trabecular cells possess muscle cell-like functions. Invest Ophthalmol Vis Sci 29:244–250, 1988

The cytoplasm of eukaryotic cells contains a filamentous system consisting of so-called intermediate filaments (IF) with a diameter of 10 nm which are distinct from actin filaments, microtubules and myosin filaments. IF contain various proteins according to the types of cells. On the basis of their immunological and biochemical differences, the following five classes of IF are known to exist: (1) the cytokeratins characteristic of true epithelia; (2) neurofilaments characteristic of many but not all neurons; (3) glial fibrillar acidic protein (GFAP) filaments found in various glial cells, such as astrocytes and Bergmann glial cells; (4) desmin filaments characteristic of most myogenic cells; and (5) vimentin filaments typical of various nonepithelial cells. In several cell types, two classes of IF are present together.

On the other hand, trabecular cells are also known to possess IF together with actin filaments and microtubules from electron microscopic investigations of in vivo and cultured cells. The classes of IF which constitute the trabecular IF, however, are not yet known. In order to understand the function of trabecular cells on the aqueous outflow facility, it is important to characterize the IF of trabecular cells.

We have established a culture system for human trabecular cells, and reported that cultured trabecular cells can be distinguished from keratocytes and corneal endothelial cells, the ultrastructure of the cultured cells sharing many common properties with that of in vivo trabecular cells.

In the present study, we undertook immunocytochemical investigations on cultured human trabecular cells using anti-keratin, anti-vimentin, anti-desmin and anti-GFAP rabbit sera.

Materials and Methods

Human Trabecular Cell Culture

Two human eyes, one obtained from cadaver of a 19-year-old female and the other from the cadaver of a 39-year-old male, neither of whom had had a history of glaucoma in life, were used. Cell culture was started within 48 hr after death. Trabecular tissues were resected basically following the method of Tripathi and Tripathi. Resected explants were placed in Falcon (Oxnard, CA) culture dishes (35 mm in diameter), and cover slips were placed over each explant for fixation. Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine and 50 µg/ml gentamicin was used as the culture medium and was changed twice per week. Culture dishes were maintained in a humidified incubator in 5% CO₂ at 37°C. Cells reaching confluence were trypsinized and subjected to further cultivation. Third-passaged cells were used in this study. For transmission electron microscopic (TEM) obser-
Fig. 1. Phase-contrast micrograph of growing cells from the trabecular explant. Each cell appears as a spindle-shaped cell with many cell processes. Bar = 100 μm.

Fig. 2. Phase-contrast micrograph of confluent human trabecular cells at 3rd passage. Cells are grown in monolayer. Bar = 100 μm.

Fig. 3. Transmission electron micrograph of a cultured human trabecular cell at 3rd passage. There are various cytoplasmic organelles, filamentous elements, and villous projections at the upper surface of the cell. Bar = 1 μm.

Specifications, second-passaged cells were cultured on the Falcon dishes. For immunocytochemical studies, trypsinized second-passaged cells were cultured on eight-well slides, and cells at a preconfluent stage were used. A portion of the cells, which were to be immunostained with the vimentin antibody and the desmin antibody, were incubated for 18 hr in medium containing 5 μM colcemid to induce the perinuclear concentration of IF. Cultured cells were observed at various time points using a phase-contrast microscope.

Transmission Electron Microscopy

Cultured trabecular cells grown directly onto small petri dishes for TEM sectioning were fixed in 1.0% glutaraldehyde in phosphate-buffered solution (PBS) for 1 hr. The cells were then post-fixed in 1% osmium tetroxide and dehydrated through graded alcohols. The cells were then embedded in the dish with Epon 812. The embedded cells were separated from the dish, re-embedded in Epon 812, sectioned, and stained with uranyl acetate and lead citrate. A JEM-100CX electron microscope (Nihondenshi, Japan) was used to examine the specimens.

Immunocytochemical Study

The following antibodies were used: anti-keratin rabbit serum (Transformation Research, Framingham, MA), anti-desmin rabbit serum (Dako, Santa Babara, CA), anti-vimentin rabbit serum (Transformation Research), anti-cow GFAP rabbit serum (Dako, Glostrup, Denmark), and fluorescein-conju-
gated goat anti-rabbit IgG (Cooper Biochemical, Malvern, PA). An indirect immunofluorescence method was employed.

Cultured trabecular cells on glass slides were rinsed with PBS (0.1 M, pH 7.4) and fixed in 3.5% formaldehyde for 20 min at room temperature or cold acetone for 10 min. Fixed cells were rinsed with PBS, and formaldehyde-fixed cells were further incubated with 0.05 M NH₄Cl in PBS for 10 min. All of the fixed cells were then washed with PBS and incubated with 0.2% Triton X-100 in PBS for 5 min. After washing with PBS, the cells were incubated with normal goat serum (Cooper Biochemical, Malvern, PA) diluted 1:10 in PBS (0.01 M, pH 7.2) for 20 min at room temperature. The formaldehyde-fixed cells were then reacted with PBS or each primary antibody (anti-keratin, anti-GFAP, anti-vimentin, anti-desmin) diluted 1:10 in PBS for 30 min at room temperature. The acetone-fixed cells were reacted with the vimentin antibody or the desmin antibody diluted 1:10 in PBS for 30 min at room temperature. After thorough washing with PBS, the cells were reacted with fluorescein-conjugated goat anti-rabbit IgG di-

Fig. 4. Phase-contrast micrograph of cultured human skin fibroblasts at confluency. Multiple cell layers are characteristically observed. Bar = 25 μm.

Fig. 5. Fluorescence micrographs of cultured human trabecular cells stained with the secondary antibody alone (A), the keratin antibody (B), and the GFAP antibody (C); 3.5% formaldehyde fixation. A, The cells only show weak and ill-defined staining. B and C, no filamentous staining is observed. Bar = 25 μm.
Fig. 6. Fluorescence micrographs of cultured human trabecular cells stained with the vimentin antibody (A) and the desmin antibody (B, C). Cold acetone fixation. The cells were stained in filamentous patterns around the nucleus and within the cytoplasm (A, B). Some cells were stained intensely by the desmin antibody (C). Bar = 25 μm.

luted 1:30 in PBS for 30 min at room temperature. After thorough washing with PBS, the glass slides were mounted in Gelto (Immunon, Chicago, IL), and viewed in a Nikon immunofluorescence microscope.

To examine cross-reactivity between the vimentin antibody and the desmin antibody, we immunostained cultured human skin fibroblasts with the vimentin antibody and the desmin antibody. The cells were fixed in cold acetone for 10 min. Other procedures were identical to those described for cultured trabecular cells.

Results

Morphology

Under phase-contrast microscopy, cell growth from the trabecular explants occurred within 2 weeks (Fig. 1). Propagated cells were observed as spindle-shaped cells with many cell processes, and appeared in monolayer at confluency (Fig. 2). When viewed in TEM cross sections, the cells appeared in flat monolayer. The most prominent features of the cytoplasm were the numerous electron-dense inclusions and villous projections at the apical surface of the cells. Numerous organelles and filamentous elements were also observed in the cytoplasm (Fig. 3). These morphological features are the characteristics of cultured human trabecular cells previously reported by Alvarado et al. and by us. Cultured human skin fibroblasts showed a typical arrangement after confluence (Fig. 4).

Immunocytochemical Study

Control experiments using the secondary antibody alone gave negative results (Fig. 5A). In the case of immunostaining with the keratin antibody and the GFAP antibody, cultured human trabecular cells only showed weak and ill-defined staining of the nucleus and cytoplasm (Fig. 5B, C). Thus, the reactions with the two antibodies were judged to be negative. Almost all cells were stained in filamentous patterns with the vimentin antibody, regardless of the fixation conditions.
Fig. 7. Fluorescence micrographs of cultured human trabecular cells stained with the vimentin antibody (A) and the desmin antibody (B) after incubation with 5 nM colcemid for 18 hr. A, fixed with 3.5% formaldehyde. B, fixed with cold acetone. Note the characteristic perinuclear immunofluorescence. Bar = 25 μm.

methods used (Fig. 6A). In the case of immunostaining with the desmin antibody, most of the acetone-fixed cells were stained in filamentous patterns (Fig. 6B). Some cells were stained more intensely than those reacted with the vimentin antibody (Fig. 6C). Formaldehyde-fixed cells were stained in granular patterns around the nucleus. Colcemid-treated cells showed characteristic perinuclear immunofluorescence by both the vimentin antibody (Fig. 7A) and the desmin antibody (Fig. 7B). The IF of cultured human skin fibroblasts were labeled intensely with the vimentin antibody (Fig. 8A), but only faintly labeled with the desmin antibody (Fig. 8B).

Discussion

Use of cultured cells and fluorescent antibodies is the standard technique for studying IF, and the result can be evaluated not only on the basis of fluorescence intensity but also from fluorescence distributions. Since normal rabbit serum is known to contain a high proportion of auto-antibody against IF, this type of serum is not appropriate as a control for the study of IF in cultured cells. On the other hand, the antisera against each IF class used in this study were thought to serve as good controls for each other.

We demonstrated in the present study that the IF of cultured human trabecular cells contained vimentin and desmin. It is known that both vimentin and desmin IF show the characteristic concentration around the cell nucleus when they are properly treated with colcemid. A similar phenomenon was also observed in the present study and thus confirmed the existence of vimentin and desmin in cultured human trabecular cells. It may be argued, however,
that desmin antibodies with a low but significant affinity for vimentin would produce false-positive labeling of vimentin. To examine this possibility, we performed indirect labeling on cultured human skin fibroblasts IF presently known to contain vimentin but no detectable amount of desmin.\(^3,26\) As a result, the IF of cultured skin fibroblasts were labeled intensely with the vimentin antibody, but only faintly labeled with the desmin antibody. On the other hand, cultured human trabecular cells were stained in clearly filamentous patterns by the desmin antibody (Fig. 6B). In addition, some cells stained more intensely than those reacted with the vimentin antibody (Fig. 6C). The combination of these results cannot be explained by only the cross-reactivity of the desmin antibody to vimentin, and strongly suggests that the IF of cultured human trabecular cells contain not only vimentin but also desmin.

Co-existence of two different IF proteins occurs in several cell types.\(^5-11\) All currently known examples involve vimentin together with one of the other systems. Co-existence of vimentin and desmin is known in vascular smooth muscle cells in the adult animal (rat, cow, human and chicken),\(^10\) chick embryo fibroblasts derived from myogenic cultures,\(^8,9\) and baby hamster (BHK-21) cells.\(^11\) Therefore, the result of the present study, that vimentin and desmin co-exist in cultured human trabecular cells, is not surprising. We consider the existence of desmin noteworthy. Since most previous studies of desmin have been carried out using skeletal muscle, little is known about the function of desmin in non-muscle cells at present. It is possible, however, that desmin IF affect the contraction and extension of trabecular cells through interaction with other components of the cytoskeleton, and consequently play an important role in regulation of the aqueous outflow facility.

The present study also revealed the sporadically distributed cells in which cytoplasm showed intensely positive staining with the desmin antibody. Gipson and Anderson\(^14\) have reported the presence of actin-rich cells in all regions of the trabecular meshwork by electron microscopic study using subfragment-one of myosin as the histochemical marker. They also suggested that these cells may represent extensions of the ciliary muscle into the meshwork. Although the significance of these actin-rich and desmin-rich cells is unknown, these cells are of particular interest in relation to development and differentiation of the trabecular cells.

In the present study, we found that vimentin and desmin are the major constituents of the IF in human trabecular cells. Since these results were obtained in culture conditions, investigations in vivo will be required to confirm these observations. In addition, it is not possible to state from the present study whether vimentin and desmin co-exist in the same cell.

Finally, together with investigations on other cytoskeletal elements, studies on the function of intermediate filaments in trabecular cells may provide novel information for clarifying the various pathogeneses of glaucoma.

**Key words:** cytoskeleton, intermediate filaments, vimentin, desmin, human trabecular cells

**References**

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