Ultrastructural and Immunocytochemical Changes in Retinal Pigment Epithelium, Retinal Glia, and Fibroblasts in Vitreous Culture
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Retinal pigment epithelial (RPE) cells, retinal glia, and fibroblasts, three cell types believed to play a role in the pathogenesis of epiretinal membrane formation, were maintained in vitreous culture to determine the influence of vitreous on their ultrastructure and expression of cytokeratin, glial fibrillary acidic protein (GFAP), vimentin, and glutamine synthetase (GS). Using a highly sensitive, preembedding technique for the immunolocalization of these antigens at the ultrastructural level, most RPE cells were found to lose cytokeratin and vimentin within 1 day after seeding on irradiated vitreous. The percentage of keratin-positive cells then increased with time in culture. If the vitreous was placed on RPE cells cultured in monolayer instead of placing the cells on the vitreous, keratin and vimentin were expressed in a high percentage of cells that migrated into the gel at 2 days, and the percentage of cells expressing these intermediate filament proteins diminished with time. Glutamine synthetase was found in RPE cells grown in monolayer with or without a vitreous overlay, but not in RPE cells grown on the surface of vitreous. Retinal glia grown on vitreous showed a time-dependent decrease in the number of cells expressing GFAP and a corresponding increase in cells expressing vimentin or GS. Some fibroblasts in vitreous culture expressed vimentin but not the other antigens evaluated. A substantial number of cells in each culture did not stain positively for cytokeratin, GFAP, vimentin, or GS. All three cell types showed phenotypic diversity at the ultrastructural level with each cell type being capable of assuming the same morphologic appearance under certain conditions. These results demonstrate the phenotypic plasticity of RPE cells, retinal glia, and fibroblasts when grown in contact with vitreous and provide further evidence that neither ultrastructure, intermediate filament protein expression, nor the presence of GS is sufficient to determine the cell type of origin of cells in epiretinal membranes. Invest Ophthalmol Vis Sci 31:2529-2545, 1990

Epiretinal membranes (ERMs) may form on the surface of the retina in association with various ocular disorders or without other apparent pathologic findings. They are comprised of sheets of cells, that may be dense or sparse, and various amounts of extracellular matrix. The cells proliferate and exert traction on the surface of the retina which may ultimately lead to traction-induced rhegmatogenous retinal detachments. Attempts have been made to determine the cells of origin contributing to ERM formation in various disease processes based on morphologic criteria; however, many of the cells in ERMs undergo striking phenotypic changes so that they no longer resemble the normal cell populations from which they were derived. Other attempts at characterization of the cells in ERMs applied immunohistochemical labeling of intermediate filament (IF) proteins, but the conclusions derived from these studies do not corroborate those derived from the morphologic studies.

To help to reconcile these conflicting data, we studied a series of ERMs with electron immunocytochemistry, simultaneously evaluating the ultrastructure of cells and their expression of IF proteins. This study suggests that neither ultrastructure nor IF protein expression unequivocally reveal the origin of the cells in the ERMs. For example, large undifferentiated cells expressing cytokeratin, suggesting retinal pigment epithelium (RPE) origin, were ultrastructurally indistinguishable from cells expressing glial fibrillary acidic protein (GFAP), which suggests

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a glial origin.\textsuperscript{15} Cells with morphologic features of RPE cells, such as polarization with an underlying basement membrane and microvilli on the free border, were consistently negative for keratin and vimentin, suggesting that they either markedly decreased their expression of these markers or they did not originate from RPE. Fibroblastic cells in ERMs were often negative for vimentin, which is expressed in normal fibroblasts, and some expressed keratin, suggesting that they may be phenotypically altered RPE cells. In addition, many macrophage-like cells were negative for a broad-spectrum macrophage marker (EBM/11),\textsuperscript{16,17} suggesting that they might be morphologically transformed RPE or glia. We designed this study to try and resolve some of these apparent contradictions and further explore the relationship between cell ultrastructure and IF protein expression and the impact of the extracellular environment on each. We also evaluated the usefulness of glutamine synthetase (GS) as a cell-specific marker for Müller cells.\textsuperscript{18,19}

**Materials and Methods**

Human RPE cells were cultured from eyebank eyes (Old Dominion Eye Bank, Richmond, VA) by a previously published technique.\textsuperscript{20} Human retinal glia were cultured by a previously reported modification\textsuperscript{21} of the technique of Oka et al.\textsuperscript{22} Human dermal fibroblasts were obtained from the American Type Culture Collection (Rockville, MD).

Bovine eyes were obtained from a local abattoir and were transported to the laboratory on ice. Under sterile conditions, the eyes were opened just anterior to the equator, and the anterior segment was removed. The vitreous was usually removed from the posterior eye cup with the anterior segment and then was isolated by cutting posterior to the vitreous base. No visible retina or other tissue was included with the vitreous isolates, but to ensure that cultures would not be contaminated with host cells, the vitreous was irradiated with 3000 rads of gamma irradiation in a CS-137 Gammacell Irradiator (Atomic Energy of Canada, Vancouver, BC). The vitreous samples were placed in 35-mm culture dishes (Falcon, Oxnard, CA) and seeded with $1 \times 10^6$ RPE cells, retinal glia, or dermal fibroblasts. In other experiments, RPE cells were grown to confluence in 35-mm culture dishes, and vitreous was overlaid on the monolayer. Cell cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY). At 1, 4, and 10 days, two to four cultures of each cell type seeded on vitreous gels and unseeded vitreous maintained in the same manner were washed in Hank's balanced salt solution (BSS; GIBCO) and bisected, as were vitreous samples derived from monolayer cultures with the vitreous overlaid on the cells for 2, 6, and 15 days. One half of each culture was fixed in buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light-microscopic evaluation of their growth pattern relative to the vitreous gel. The other half of the culture was fixed for electron-microscopic (EM) immunocytochemistry in 2% glutaraldehyde/2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 24 hr at 4°C after which it was transferred to cacodylate buffer and stored at 4°C before immunocytochemical staining. Monolayer cultures of the three cell types were grown in Lab-Tek chambered glass slides (Miles, Naperville, IL) and washed, fixed, and transferred to buffer.

For immunocytochemical staining of vitreous cultures, small pieces of cellular membranes were trimmed under a dissecting microscope so that only a minimal amount of vitreous remained attached, and four to 12 specimens of about 0.5–1 mm X 3–5 mm from different areas of each culture were placed into a small glass vial for each immunocytochemical marker at each time point. Preembedding immunocytochemical staining was done as previously described.\textsuperscript{12} Briefly, the specimens were treated for 30 min with 1% sodium borohydride in phosphate-buffered saline (PBS) containing 1 mM CaCl$_2$ and washed three times for a minimum of 1 hr with PBS and CaCl$_2$ at room temperature. Specimens to be stained using rabbit polyclonal antisera were incubated for 30 min at room temperature with 10% normal goat serum (NGS; Arnel, Brooklyn, NY) in Tris-buffered saline (TBS); those to be stained with mouse monoclonal antibodies were incubated with 10% normal rabbit serum (NRS; Arnel) in TBS. After removing excess normal serum, but without washing, speci...
mens were incubated overnight at 4°C in one of the following solutions: (1) a cocktail of three mouse monoclonal antibodies to keratin consisting of a 1:250 dilution of a 20:1 ratio of antibody from clones AE1 and AE3 (Hybritech, San Diego, CA) and a 1:50 dilution of antibody from clone CAM 5.2 (Becton Dickinson, Mountain View, CA); (2) a 1:1000 dilution of rabbit polyclonal anti-GFAP prepared by Dr. L. F. Eng (Palo Alto, CA); (3) a 1:10 dilution of mouse monoclonal anti-vimentin (V) antibodies (DAKO, Santa Barbara, CA); or (4) a 1:50 dilution of rabbit polyclonal anti-GS prepared by Dr. P. Linser (St. Augustine, FL). Rabbit antibodies were diluted with 1% NGS in TBS, and tissues incubated with them were washed in the same solution; mouse monoclonal antibodies were diluted with 1% NRS in TBS, and the tissues reacted with them were washed in this solution. After washing, specimens incubated with antibody solutions 1 or 3 were placed in a 1:25 dilution of rabbit anti-mouse globulins (RAM; DAKO), washed, and incubated with a 1:400 dilution of a mouse peroxidase-antiperoxidase complex (PAP; Arnel). Tissues covered with diluted antiserum numbers 2 or 4 were placed in a 1:40 dilution of goat anti-rabbit globulins (GAR; Arnel), washed, and incubated with a 1:100 dilution of rabbit PAP (Arnel). After removing the PAP solutions, the specimens were washed twice with 0.05 M Tris buffer, pH 7.6, reacted in the dark for 15 min with freshly made 0.07% 3,3′ diaminobenzidine—4HC1 in 0.05 M Tris buffer, pH 7.6, containing 0.0185% H2O2, and again washed twice in Tris buffer. All wells were incubated with antibodies to keratin, vimentin, or GFAP. Controls were incubated with normal serum. Some were immunoperoxidase stained; others were labeled by immunofluorescence as follows. After warming for 15 min, cultures were washed twice with cold PBS. Wells incubated with antibodies to keratin or vimentin were treated with RAM as above and washed with PBS. All wells were incubated with a 1:10 dilution of GAR conjugated to fluorescein isothiocyanate in PBS for 30 min, washed three times with PBS, and mounted with 90% glycerol in 75 mM Tris, pH 9.5. The slides were viewed with a Leitz Ortholux II epifluorescence microscope (Rockleigh, NJ) and photographed with Kodak Tri-X film developed with Diafine (Accufine, Chicago, IL).

Results

RPE Cells

In monolayer cultures fixed with methanol, virtually all of the RPE cells were positive for cytokeratin and vimentin and negative for GFAP by light microscopic immunoperoxidase staining or by immunofluorescence microscopy. Using EM fixation and processing the monolayer cultures in the same manner as the vitreous cultures prepared for EM, but viewing them by light microscopy, all cells were positive for vimentin (Fig. 1B) and GS (Fig. 1C) using either polyclonal or monoclonal antibodies (Fig. 1A). Nearly all RPE cells were positive for cytokeratin (Fig. 1A), but the relative intensity of stain was not uniform. About 15–25% of the cells stained intensely for keratin, but the rest of the cells showed weak positivity. Occasional RPE cells treated in this manner were positive for GFAP (Fig. 1D). Since this was a surprising finding, two other RPE cell lines were examined for GFAP, and identical results were obtained.

One day after seeding RPE cells on bovine vitreous, the number of cells positive for cytokeratin decreased to 5.0% (Table 1), and there were only 7.7% of the cells positive for vimentin by electron immunocytochemical staining using the same fixative and monoclonal antibodies to compare with the results obtained with polyclonal antiserum. Controls for light microscopy and EM were processed in the same manner, substituting normal serum for the primary antibodies.

Some monolayer cultures of RPE cells were grown in the chambered slides, washed in Hank's BSS, and placed in a vacuum desiccator at 4°C for 2 days. They were fixed for 20 min in cold methanol with 0.5% H2O2 added to inhibit endogenous peroxidase activity and then washed three times with cold PBS and once with distilled water. They were then incubated with 10% NRS or NGS and one of the primary antibody solutions for detecting keratin, vimentin, or GFAP. Controls were incubated with normal serum. Some were immunoperoxidase stained; others were labeled by immunofluorescence as follows. After warming for 15 min, cultures were washed twice with cold PBS. Wells incubated with antibodies to keratin or vimentin were treated with RAM as above and washed with PBS. All wells were incubated with a 1:10 dilution of GAR conjugated to fluorescein isothiocyanate in PBS for 30 min, washed three times with PBS, and mounted with 90% glycerol in 75 mM Tris, pH 9.5. The slides were viewed with a Leitz Ortholux II epifluorescence microscope (Rockleigh, NJ) and photographed with Kodak Tri-X film developed with Diafine (Accufine, Chicago, IL).
reagents with which all the cells were labeled in monolayer cultures. This cannot be interpreted as a problem of antibody penetration into the vitreous gel since most cells were found in clusters on the surface of the vitreous gel (Fig. 2A) with only occasional cells in the matrix of the vitreous. Most cells were very...

Fig. 2. (A) A cluster of RPE cells grown 1 day on the surface of bovine vitreous. Some cells (right and bottom) appear undifferentiated with a moderately large-sized nucleus. Other cells have abundant mitochondria and microvilli predominantly on one side, suggestive of polarized, metabolically active cells. All of these cells were immunostained for, but do not express keratin (original magnification X3750). (B) Keratin positivity, as demonstrated by the granular reaction product, in the cytoplasm of an RPE cell with a convoluted morphology grown 4 days on the surface of vitreous (original magnification X10,000). (C) An RPE cell with a convoluted morphology that was completely surrounded by extracellular matrix from a 4-day vitreous culture. Unlike the cell in (B), this cell is negative for keratin (original magnification X7500). Cells are counterstained with uranyl acetate.
undifferentiated with a prominent nucleolus. Some cells had microvilli, and 2% of the cells had large nuclei with sparse cytoplasm and no microvilli. Cells with a highly convoluted morphology were also present.

By 4 days in vitreous culture, most of the cells retained the same morphologic appearance, but some of the cells invaded the matrix, both individually and in cords (Fig. 3A). Most of the undifferentiated cells with or without microvilli were in clusters. Highly convoluted cells were usually found alone, either on the surface (Fig. 2B) or deep in the matrix (Fig. 2C). Keratin and vimentin positivity did not increase appreciably from day 1 (Table 1). The number of cells with large nuclei and sparse cytoplasm increased to 4%.

By 10 days in vitreous culture, most of the cells were found in clusters often forming cord-like structures on the surface or in the matrix. Between the clusters, the cells formed a monolayer on the vitreous surface (Figs. 3B–C). Most cells had a prominent nucleolus, and some had microvilli. The percentage of keratin-positive cells increased to 26% (Figs. 3B–D) without an increase in vimentin-positive cells. The percentage of cells with large nuclei and sparse cytoplasm, many of which were keratin or vimentin positive, increased to 16%. Some cells, particularly those that had migrated deep into the matrix, began to reacquire pigment granules, which had been lost in monolayer culture. None of the cells from any of the RPE vitreous cultures at these times showed GFAP or GS by EM immunocytochemistry.

To determine whether contact with vitreous or the position of the cells with respect to the vitreous matrix accounted for the rapid loss of cytokeratin and vimentin from RPE cells, confluent RPE cells were overlaid with irradiated vitreous rather than being seeded on the surface of the vitreous. The cells migrated onto the vitreous, and all of the reported results refer to cells that remained adherent to the vitreous when it was removed from the plate. After 2 days, 48.7% of the cells remained positive for cytokeratin, and 42.7% expressed vimentin (Table 1, Fig. 4A). In addition, 30.0% of the RPE cells showed GS (Fig. 4B), which was not evident when the RPE cells were grown on the surface of the vitreous, but was expressed in monolayer cultures. Morphologic heterogeneity was observed when vitreous was placed on monolayers similar to that seen when cells were seeded on vitreous. Large cells with large nuclei and sparse cytoplasm were prominent in areas where a monolayer of cells was attached to the vitreous. In areas where cells piled up to form cords, the predominant cell type had a smaller nucleus and more cytoplasm and did not express keratin or vimentin. Cells with numerous microvilli and highly convoluted cells were not seen.

By 6 days, the percentage of cells with cytokeratin had diminished to 31.0% (Table 1). Vimentin and GS positivity persisted. All three antigens were primarily expressed by cells on the surface and not by those that invaded the matrix.

By 15 days, only 19.0% of the cells were keratin positive, and 26.0% were vimentin positive (Fig. 5A). Still evident was GS positivity in a relatively high percentage of cells, particularly those on the surface (Fig. 5B). In some areas of the culture, the cells were loosely packed, and in others, they were dense. In vitreous cultures of RPE cells, GFAP was not expressed at any time.

Retinal Glia

In monolayer cultures using EM fixation, all retinal glial cells were positive for vimentin and GS (monoclonal and polyclonal antibodies) (Figs. 1E–F); weak positivity for GFAP was sometimes seen. Occasional cells stained positively for cytokeratin in a filamentous configuration.

After 1 day in vitreous culture, 21.7% of the cells expressed GFAP, and the percentage of vimentin-positive cells decreased to 16.3% (Table 1). Occasional keratin-positive cells were also seen. Only 3.2% of the cells had GS.

Ultrastructurally, the glial cells were difficult if not impossible to distinguish from comparably cultured RPE cells. The glia often had prominent nuclei and numerous mitochondria, and many had microvilli. Highly convoluted cells and cells with large nuclei and sparse cytoplasm, like those seen in RPE cultures, were also present (3%). One apparent difference from RPE cultures was that the glial cell clusters were smaller and more loosely packed than those in RPE cultures.

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Fig. 3. (A) RPE cells invading the extracellular matrix as a cord from a 10 day vitreous culture. These cells were negative for keratin (×3750). (B) A monolayer of RPE cells on the vitreal surface from a 10-day culture. Some cells expressed keratin positivity (arrowheads); others were negative (asterisk) (×3750). (C) RPE cells on the surface of vitreous from 10-day cultures. Some cells had an epithelioid-like morphology (top). One cell with a large, lightly stained nucleus and sparse cytoplasm demonstrated limited cytokeratin positivity in a portion of the cytoplasm (arrowhead) (×3750). (D) Cytoplasmic positivity for keratin (arrowheads) in RPE cells with large, lightly stained nuclei and sparse cytoplasm grown on the surface of vitreous for 10 days (×5000). Inset: The filamentous configuration of keratin positivity is apparent in an RPE cell from a 10-day vitreous culture (×6250). All cells are counterstained with uranyl acetate.
By 4 days in vitreous culture, some of the glial cells had formed a monolayer on the surface of the vitreous between the cell clusters (Fig. 5C). Few cells invaded the vitreous. Microvilli were still evident on some cells, and 4% of the cells had large nuclei with sparse cytoplasm. Vimentin was expressed in 19.2% of the cells, GFAP expression decreased to 10.3% of the cells (Fig. 6A), and no cells stained for cytokeratin. Expression of GS increased to 12.7% of the cells.

After 10 days in vitreous culture, the entire surface of the vitreous appeared to be covered with a monolayer or small loosely packed clusters of glial cells (Fig. 7A). Unlike the RPE and fibroblast cultures, very little invasion of the extracellular matrix occurred, but when it did occur, the cells often had a fibroblast-like morphology (Fig. 6B). The cells with large nuclei and sparse cytoplasm increased to 13%. Vimentin positivity increased (Fig. 4C), and GFAP was no longer evident (Table 1). A fairly high percentage of cells (45.3%), regardless of morphology, expressed GS (Figs. 7B–C).

**Fibroblasts**

In monolayer culture, fibroblasts appeared bipolar and with EM fixation, all expressed vimentin and GS (Figs. 1G–H) but were negative for cytokeratin and GFAP.

After 1 day in vitreous culture, clusters of fibroblasts were seen. In some, the cells appeared to be tightly adherent to each other, but in others they seemed loosely packed. Fibroblasts invaded the extracellular matrix of the vitreous in greater numbers than RPE or glia. No prominent nucleoli were evident. Many of the cells had a fusiform appearance, but 5% had large nuclei with sparse cytoplasm like those seen in RPE and glial cultures; 9.6% of the cells expressed vimentin (Fig. 8A, insert), but none of them stained for cytokeratin, GFAP, or GS.

By 4 days in culture, there were fewer cell clusters since most of the fibroblasts appeared to have spread and invaded the vitreous. Many of these cells were surrounded completely by matrix. Ultrastructurally, some of the fibroblasts appeared undifferentiated, some were convoluted (Fig. 8A), and others appeared fusiform.

After 10 days in vitreous culture, nearly all of the fibroblasts had invaded the vitreous. Many of the cells accumulated a lipid-like material (Figs. 6C and 8B). The percentage of cells with large nuclei and sparse cytoplasm increased to 9%, and there was a moderate increase in the vimentin-positive cells to 15.9% (Fig. 8C).

**Controls**

Vitreous, unseeded by cells, but otherwise processed in an identical manner to the vitreous cultures at the same time points, did not show positivity for any of the antigenic markers tested and did not appear to contain any viable cells. Occasional cell debris was found, particularly at day 1, possibly representing host cells destroyed by irradiation. Cultures incubated with normal serum in place of primary antibodies were consistently negative (Fig. II).

**Discussion**

The pathogenesis of ERM formation is poorly understood. Although the cellular nature of the membranes has been recognized for several years, the origin of the cells involved and their relative importance in the initiation of ERM formation has been controversial. Based on ultrastructural characteristics, several studies suggest participation of RPE, retinal glia, fibroblasts, and macrophages. Most authors are hesitant to suggest a predominant cell type, although it has been implied that RPE cells play a central role in proliferative vitreoretinopathy (PVR) membranes as do glial cells in idiopathic membranes. However, one recent study assigned a predominant role in idiopathic membranes to RPE. The problem with using ultrastructural criteria as the sole indicator of cellular origin is that the morphologic features of cells may change when they are removed from their normal microenvironment. Basement membrane synthesis, cell junction formation, and polarization are characteristics commonly associated with RPE, but can, under certain circumstances, be exhibited by glia and fibroblasts. Conversely, RPE and glia can adopt a fibrocytic morphology and RPE can take on the appearance of macrophages.

When IF protein expression was identified as a useful marker for certain cell types, several investigators using immunohistochemical staining for IF proteins suggested that glial cells predominate in ERMs associated with minor degrees of traction and fibroblasts are the major cell type in membranes associated with significant traction. Another study using cytokeratin staining suggested that RPE cells predominate in PVR membranes, which are generally associated with considerable traction. A study using simultaneous evaluation of ultrastructure and IF expression did not determine definitively the origins of cells in ERMs of varied etiology and suggested that both cell morphology and IF protein expression may be altered by the microenvironment of the cell.

We confirmed that each of three cell types implicated in ERM formation, RPE, glia, and fibroblasts, when cultured on vitreous, undergoes striking changes in morphology and IF protein expression. The morphologic changes are very similar to those previously reported by Forrester et al. Early after seeding, most RPE cells have a very undifferentiated
Fig. 4. (A) Vimentin positivity in the cytoplasm of large, undifferentiated RPE cells with large nuclei and little cytoplasm that have been grown under a vitreous overlay for 2 days (original magnification $\times5000$). (B) Immunocytochemical demonstration of glutamine synthetase positivity in the cytoplasm of an RPE cell grown for 2 days under a vitreous overlay. The nucleus and mitochondria are negative (original magnification $\times6250$). (C) Vimentin positivity in the cytoplasm of a large cell with a large, lightly stained nucleus and sparse cytoplasm from the surface of a 10-day vitreous culture of retinal glia (original magnification $\times3750$). Nuclei and mitochondria are consistently negative for intermediate filament proteins. All cells are counterstained with uranyl acetate.
Table 1. Percentage of cells in vitreous cultures expressing various antigens by electron immunocytochemistry

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Days in culture</th>
<th>Keratin-positive cells (%)</th>
<th>GFAP-positive cells (%)</th>
<th>Vimentin-positive cells (%)</th>
<th>Glutamine synthetase-positive cells (%)</th>
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<tbody>
<tr>
<td>RPE on vitreous</td>
<td>1</td>
<td>5.0</td>
<td>0</td>
<td>7.7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5.8</td>
<td>0</td>
<td>8.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>26.0</td>
<td>0</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td>RPE under vitreous</td>
<td>2</td>
<td>48.7</td>
<td>0</td>
<td>42.7</td>
<td>30.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>31.0</td>
<td>0</td>
<td>60.9</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>19.0</td>
<td>0</td>
<td>26.0</td>
<td>34.4</td>
</tr>
<tr>
<td>Retinal glia on vitreous</td>
<td>1</td>
<td>4.7</td>
<td>21.7</td>
<td>16.3</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>10.3</td>
<td>19.2</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>45.8</td>
<td>45.3</td>
</tr>
<tr>
<td>Fibroblasts on vitreous</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>9.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
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<td>10</td>
<td>0</td>
<td>0</td>
<td>15.9</td>
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</tbody>
</table>

The individual cells show the same variety of phenotypes (including polarized monolayers with microvilli) and are essentially indistinguishable from RPE by ultrastructural criteria. Fibroblasts also assume various undifferentiated morphologies similar to those seen in RPE and glial cultures, but over time, they are more prone to invade the gel and do not form polarized monolayers on the surface.

Surprisingly, we found that in addition to morphologic changes, each of the three cell types in vitreous culture undergo striking changes in their expression of IF proteins and GS, as judged by electron immunocytochemistry. These changes cannot be explained solely by limitations in antibody penetration or other technical variables as suggested by the following: (1) although the percentage of cells staining for each of the markers was greater for cells on the surface of the vitreous than for those that had invaded the matrix, in almost all fields, positive cells could be found deep in the matrix, suggesting that inadequate antibody penetration was not sufficient to explain the relatively high number of unstained cells and (2) a decrease in expression of IF proteins and GS did not occur in association with matrix invasion, eg, fibroblast invasion of the vitreous increased with time in culture, as did the percentage of vimentin positive cells. Therefore, technical variables such as the fixative used, the use of sodium borohydride, and potential differences in antigen accessibility cannot explain the differences in immunocytochemical staining over time in our study.

The correlation of IF staining with ultrastructural characteristics of the cells in this culture system and their comparison to cells in surgical specimens of epiretinal membranes was particularly interesting. Large cells with sparse cytoplasm and large, lightly stained nuclei were observed in cultures of all three cell types, and as in clinical specimens, some cells expressed cytokeratin, some GFAP, and some vimentin. Spindle-shaped fibroblast-like cells were also found in cultures of each of these cell types, although in glial cell cultures, unlike fibroblast and RPE cultures, they were rarely found surrounded by matrix, as was the common location of this morphologic cell type in epiretinal membranes. Cells of this morphologic type in vitreous cultures were usually unlabeled for any of the three IF proteins under investigation as was found in clinical specimens. These findings suggest that the two morphologic cell types described could be derived from RPE cells, fibroblasts, or glia as previously postulated. Some cells of each type in vitreous cultures contain microvilli, particularly at day 1, and some appear polarized, especially RPE cells, but none are as highly polarized or have microvilli as abundant as in ERM specimens, in which this was often the predominant morphologic type. Microvilli-containing cells from vitreous cultures also do not align themselves in an epithelioid or acinar configuration as in some clinical cases. The RPE cells that assumed a polarized morphology in vitreous culture never expressed keratin or vimentin, suggesting that this phenotypic change did not represent a redifferentiation phenomenon or that keratin and vimentin expression are not markers of differentiation. Pigment-laden cells, also frequently seen in human specimens, were not found in this culture system.

The manner in which RPE cells interact with vitreous seems to be important in regulating their cytokeratin expression. Virtually all RPE cells grown in monolayer culture are positive for keratin. When vitreous is placed on top of these cells and they migrate...
into the vitreous, the percentage of keratin-positive cells remains relatively high at 2 days and then slowly diminishes with time. A significant number of these cells stained for GS at all times. In contrast, when RPE cells are seeded on the surface of vitreous, the cells promptly attach to it, but the percentage of keratin-positive cells drastically decreases during the first day and then gradually increases with time. The RPE cells seeded on top of the vitreous did not show positive staining for GS at any time. The reasons for these
Fig. 6. (A) Positive staining for GFAP in the cytoplasm of a retinal glial cell from a 4-day vitreous culture (x25,000). (B) Fibroblast-like retinal glial cells found deep in the extracellular matrix in 10-day vitreous cultures. These cells are negative for vimentin (x3750). (C) Vimentin-negative fibroblasts that have migrated deep into the vitreous in 10-day cultures. The cells have accumulated an electron-dense, lipid-like material (x5000). All cells are counterstained with uranyl acetate.
Fig. 7. (A) A loosely packed cluster of retinal glia on the surface of a 10-day vitreous culture. Two cells (arrowheads) demonstrate cytoplasmic positivity for vimentin, but the others are negative (original magnification ×3750). (B) Glutamine synthetase positivity in the cytoplasm of a large retinal glial cell with a large nucleus and sparse cytoplasm on the surface of a 10-day vitreous culture (original magnification ×5000). (C) Intense cytoplasmic staining for glutamine synthetase in a retinal glial cell with numerous mitochondria and Golgi bodies grown on the surface of vitreous for 10 days (original magnification ×8750). (A) is counterstained with uranyl acetate; (B) and (C) have no counterstain.
Fig. 8. (A) A convoluted, vimentin-negative fibroblast completely surrounded by extracellular matrix from a 4-day vitreous culture (original magnification x6250). Inset: Vimentin staining is associated with filaments in a representative fibroblast from a 1-day vitreous culture (original magnification x25,000). (B) A fibroblast from the surface of a 10-day vitreous culture that expresses vimentin only in the periphery. Electron-dense, lipid-like material is contained within the cell (original magnification x8750). (C) Immunocytochemical staining for vimentin in the cytoplasm of a fibroblast found deep in the extracellular matrix of a 10-day vitreous culture (original magnification x15,000). The inset of (A) is without a counterstain; other cells are counterstained with uranyl acetate.

differences may be related to differences in metabolism and cell motility in response to changes in the microenvironment.

Retinal glia showed a decrease in GFAP-positive cells and a concurrent increase in vimentin-positive cells with time in vitreous culture. Some cytokeratin positivity was observed in 1-day glial cultures but was not seen at later times. It is possible that a few nonviable host cells containing keratin were present at day 1, but such cells were not seen in control vitreous in
which no cells were seeded. An alternative explanation, which is more likely since some keratin-positive cells were seen in monolayer culture, is that under these conditions, there was a transient expression of cytokeratin by a subpopulation of retinal glia. Using the AEI/AE3 monoclonal antibody solution, which is one of the components of our antikeratin cocktail, cytokeratin positivity has been reported in neoplastic glial cells, showing that glial cells under the appropriate conditions can express cytokeratin.

In the retina, GS has been reported to be restricted to Müller cells; however, the enzyme has been found in brain astrocytes which have the same origin as retinal astrocytes. In addition, GS has been reported in other cells and tissues such as oligodendrocytes, liver cells, skeletal muscle, heart, adipose tissue, and intestines, cells from the kidney and lung of some species; and presumably in endothelial cells. In light of these previous findings, it is perhaps not unexpected that we found GS in RPE cells and fibroblasts in monolayer cultures and in some RPE cells cultured with a vitreous overlay. It can be induced by hormones or growth factors, which may be present in culture medium or vitreous. Under appropriate attachment conditions, these molecules may cause an induction of the enzyme in retinal glia, RPE cells, and fibroblasts. These findings limit the usefulness of GS as a cell-specific marker due to its enhanced expression in these culture conditions, in which the cells are presumably in an elevated metabolic state, as suggested by the abundant mitochondria. Mitochondria are also quite prevalent in many cells in ERMs. The expression of GS is likely to differ from that of the normal cell populations from which these pathologic cells are derived. Enhanced expression of other metabolic enzymes that had been thought to be cell-specific, such as neuron-specific enolase and carbonic anhydrase, also were reported in cells not normally expressing the enzyme in question but which achieved an elevated metabolic state.

Many conflicting reports regarding the expression of the antigens we investigated by normal or pathologic retinal cells have been presented in studies in which different fixatives and processing techniques were used. Providing the appropriate controls were done, the immunocytochemical demonstration of an antigen indicates its presence. The absence of stain may not necessarily indicate its absence but may be a reflection of the limitation of the technique used. Therefore, previous studies in which normal Müller cells were reported to be negative for GFAP or RPE cells negative for cytokeratin may have resulted from inaccessibility of the antigen to the antibody due to the technique used. Similarly, in our study, it is conceivable that a higher percentage of cells than that listed in Table 1 may contain each marker, but possibly due to inadequate penetration of some cells by the antibodies or by masking of the antigens by the fixative, the antigen of interest may be undetected in some cells.

Our results make it increasingly clear that the expression not only of metabolic enzymes, but even of structural proteins, may be inconclusive in determining cell origin. Phenotypic plasticity by RPE cells, retinal glia, and fibroblasts has been demonstrated on contact with vitreous, but all of the cell morphologies seen in ERMs have not been duplicated in this culture system, suggesting that either other factors are necessary to initiate further phenotypic diversity, or other cell populations are involved in epiretinal membrane formation.

**Key words:** retinal pigment epithelium, glia, intermediate filament proteins, vitreous culture, glutamine synthetase

## References


