Modulation of Growth in Retina-Derived Cells by Extracellular Matrices

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Intravitreal membranes from patients with proliferative vitreoretinopathy (PVR) consist partly of retinal glial (RG) and retinal pigment epithelial (RPE) cells surrounded by varying amounts of extracellular matrix (ECM). The contribution of the ECM to the growth of PVR membranes is unknown. This study was undertaken to determine if proliferation in cultured RPE and RG cells is affected by different substrates, including some ECM materials which have been identified in PVR membranes. Substrates tested included type I collagen, basement membrane Matrigel, and poly-D-lysine, as well as uncharacterized cell type-specific matrices deposited by cultured RPE and RG cells. Proliferation was quantified by \(^{3}H\)-thymidine incorporation and radioautography 24 hours after plating and by cell counts after 14 days in the presence of serum. Relative to uncoated culture plastic, growth of RPE cells was inhibited by Matrigel, enhanced by poly-D-lysine, and unaffected by type I collagen. In contrast, growth in RG cells was inhibited by type I collagen and unaffected by the other substrates. Analysis of the timing of DNA synthesis after plating suggested that the substrates which affected RPE growth did so by altering the fraction of cycling cells rather than the cell cycle time. For the cell-derived matrices, heterotypic matrix (matrix produced by the other retinal cell type) enhanced the growth of both RPE and RG. The results suggest that the ECM may modify the growth of cells contributing to PVR membranes. Of note is that the cell-derived matrices reciprocally stimulated growth of RG and RPE cells, cell types which may interact in PVR membranes. Invest Ophthalmol Vis Sci 31:1717-1723, 1990

Proliferative vitreoretinopathy (PVR) is a complication of rhegmatogenous retinal detachment in which tissue membranes composed of proliferating cells form in the vitreous and on the retina. These membranes may complicate the surgical repair of a primary retinal detachment and/or cause recurrent traction retinal detachment. Morphologic examination of PVR membranes indicates that they are composed of cells surrounded by varying amounts of extracellular matrix (ECM). The cells appear to have originated primarily from retinal glial and retinal pigment epithelial cells. The ECM of PVR membranes may contain incarcerated vitreous, but much of the matrix appears to have been produced by the cells themselves. Some matrix components have been identified; these include laminin, fibronectin, collagen types I and IV, and basement membrane proteoglycan.

The contribution of the ECM to the development of PVR membranes is unclear. Interactions of cells with specific ECM components in vitro have been shown to modify cell functions such as migration and proliferation—cellular processes likely to be involved in PVR membrane formation. Our purpose was to determine if proliferation in cultured retinal pigment epithelial (RPE) and retinal glial (RG) cells is affected by different culture substrates. Materials tested included some ECM macromolecules which have been identified in PVR membranes and uncharacterized ECMs deposited by cultured retina-derived cells.

Materials and Methods

Cell Cultures

Bovine RPE cells and rabbit RG cells were propagated in vitro as previously described. The RPE cells were isolated by trypsin incubation of eyecups after removal of the vitreous and retina. The RG cells were grown from explants of rabbit retina after discarding the medullary ray; the cells are predominantly Müller glia. Cells of both types were routinely maintained in Eagle's Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS). Cultures were fed biweekly and passaged
weekly by trypsin treatment. Cells from the first to the fourth passage were used for growth experiments which were conducted on at least three populations of each cell type.

Three assays, which required different types of culture dishes, were used to evaluate growth on various substrates including: two-chamber glass chamberslides (Lab Tek, Naperville, IL) for 3H-thymidine radioautography, 24-well plates for 3H-thymidine incorporation, and six-well plates for cell counts. For all growth experiments, cells were plated at a density of 1.5 x 10^4 cells/cm² in MEM containing 10% FBS. Although early attachment and spreading of cells was affected by type of substrate (unpublished observations), cell numbers were equivalent at the start of the growth assay as confirmed in each experiment by cell counts taken 24 hr after plating. Cultures were examined and photographed by phase-contrast microscopy to evaluate cell morphology on the various substrates.

**Preparation of Culture Substrates**

Rat tail type I collagen, poly-D-lysine hydrobromide, and Matrigel (Collaborative Research, Bedford, MA) were used as substrates in assay wells. For type I collagen (4.0 mg/ml in 0.02 N acetic acid), the stock solution was pipetted at a volume of 0.2 ml/cm² into culture wells and spread evenly. After 5 min, the excess was removed, and the coated substrate was maintained under sterile distilled water at 4°C for 24 hr before cell plating. For poly-D-lysine hydrobromide (approximate molecular weight, 540,000; diluted to 0.1 mg/ml in distilled H²O), the diluted solution was pipetted at a volume of 0.1 ml/cm² into culture wells. After 5 min, the excess was removed, the treated surface was rinsed twice with sterile distilled water, and the substrate was used immediately for cell plating. Matrigel, which is supplied as a frozen stock solution, was thawed at 4°C overnight before use. To coat assay wells, 3.6 μl/cm² was pipetted into wells of a precooled culture dish. A sterile wooden applicator was used to spread the solution thinly over the surface which was then held at 4°C for 30 min to allow the solution to spread evenly. The Matrigel was gelled at 37°C for 30 min and used immediately for plating.

Growth was also analyzed in assay wells containing matrices derived from RPE or RG cells. To prepare these cell type-specific matrix substrates, cells were plated at a density of 3 x 10^4 cells/cm², grown to confluency in MEM containing 10% FBS, then maintained at confluency for 10–14 days. Cells were removed from underlying matrices by lysis with 0.5% Triton-X-100 in phosphate-buffered saline (PBS) for 30 min at 37°C, followed by three rinses with PBS and one with distilled water. Removal of cells was verified by phase-contrast microscopy. It is possible that membrane fragments remained which were not detected. If present, they could be growth inhibitors.

The cell-derived matrices were stored under sterile distilled water containing antibiotics at 4°C for up to 1 month before use. All matrices were rinsed with serum-free MEM before cell plating.

**Growth Assays**

**Radioautography:** Cells plated on uncoated (control) and other substrates in chamber slides were labeled with 3H-thymidine (3.5 μCi/ml) using a 6-hr incubation beginning 18 hr after plating. Incubations were terminated by removing the culture medium and rinsing the slides three times with warm (37°C) PBS. Radioautographs were prepared by dipping slides in Kodak NTB2 emulsion (Rochester, NY) followed by an exposure period of 5 days. Tritiated-thymidine labeling was quantitated by counts of 500 contiguous nuclei/well taken from a region in which cells had two to three cell–cell contacts. Data were expressed as percent labeled and are reported as means of two replicate wells.

**3H-Thymidine incorporation:** Cells on culture plastic and other substrates in 24-well plates were labeled from 18–24 hr after plating as described for radioautography. At the end of the labeling period, cells were rinsed once with warm (37°C) PBS, then lysed with 200 μl of 0.1 N NaOH. The DNA was precipitated by the addition of 1 ml cold 12% trichloroacetic acid (TCA) for 10 min, and the TCA precipitates together with two rinses of the well were collected on methylcellulose filters (Gelman supor 200, Gelman Sciences, Inc., Ann Arbor, Michigan, 0.2 μm) in a filter manifold under suction. The filters were rinsed once with 100% ethanol and then placed in scintillation vials containing 7.5 ml Budget-Solve (Research Products International, Mount Prospect, IL) for scintillation counting. Results of 3H-thymidine incorporation are the mean dpm per well of four replicate wells.

In some experiments, cells in 24-well plates were labeled with a different protocol to determine the timing of the DNA synthetic response after plating on the various substrates. For these experiments, quadruplicate wells were labeled for 4 hr with 3H-thymidine and harvested at 4-hr intervals 10–54 hr after plating.

**Cell counts:** Cells on culture plastic and other substrates in six-well plates were maintained for up to 14 days in MEM containing 10% FBS with three medium changes per week. Coulter cell counts of trypsin-detached cells were made at 1, 7, and 14 days after
plating. The results are reported as the mean cell numbers per well of three replicate wells. The cell counts reflect the net change in cell number over time which can be affected by both proliferation and loss of cells due to detachment from the substrate.

Results

Phase-Contrast Microscopy

Twenty-four hours after plating on Matrigel both RPE and RG cells were less spread than on culture plastic. As culture density increased, cells of both types on Matrigel tended to form clusters. Figure 1 shows paired cultures of RPE cells on culture plastic (Fig. 1A) and on Matrigel (Fig. 1B).

Sparse cultures of RG cells 24 hours after plating were also less spread on type I collagen, with narrower cell processes than on culture plastic (Fig. 2). As the density of RG cultures increased on type I collagen, the cells retained a more elongate morphology than on all other substrates (not shown).

The morphology of RPE cultures was unaffected by type I collagen at any density. Similarly, no shape changes were observed for either retinal cell type on poly-D-lysine or on the cell-derived matrices.

Growth on Matrigel, Collagen, and Poly-D-Lysine

Relative to culture plastic, growth of RPE cells was inhibited by Matrigel. At 24 hours after plating, RPE cell cultures showed lower $^3$H-thymidine incorporation (Fig. 3) and fewer labeled cells in radioautographs on Matrigel (27%) than on culture plastic (81%). At saturation density after 14 days on Matrigel, the RPE cell number was 28% lower than on culture plastic (Table 1). On poly-D-lysine, $^3$H-thymidine uptake at 24 hours was slightly enhanced (Fig. 3), and RPE cell numbers on this substrate after 14 days were 26% greater than on culture plastic. Growth of RPE appeared unaffected by a type I collagen substrate.

In contrast to RPE, growth of RG cells was approximately equivalent on all substrates when analyzed 24 hours after plating (not shown). After 14 days, however, there were fewer RG cells on type I collagen than on other substrates (Table 1).

Analysis of the first entry into DNA synthesis after plating cells on different substrates indicated that for both RPE and RG, and for all substrates, the first peak of $^3$H-thymidine incorporation occurred at 20–24 hours (Figs. 4, 5). For RPE, cells on all sub-
strates appeared to undergo two additional synchronized peaks of DNA synthesis at approximately 38 and 52 hours after plating (Fig. 4). For RG cells, synchronized peaks of $^3$H-thymidine incorporation were not observed after the first peak (not shown).

**Growth on Cell Type-Specific Matrices**

The RPE cells plated on an RPE-derived matrix (homotypic matrix) showed a slightly lower $^3$H-thymidine incorporation level after 24 hours than RPE cells which were on a heterotypic matrix (deposited by RG cells) or on control culture plastic (Table 2). By day 14, there were significantly fewer RPE cells on homotypic matrix and significantly more cells on heterotypic matrix than on the control substrate (Table 3).

For RG cells, both homo- and heterotypic matrices enhanced growth relative to culture plastic. Elevated growth on the cell-derived matrices was reflected in both greater $^3$H-thymidine incorporation 24 hours after plating (Table 2) and larger cell numbers at 14 days (Table 3).

**Discussion**

The data in this study demonstrate that the in vitro growth of RPE and RG cells is differentially affected by culture substrates. For the substrates which were tested, the growth of RPE cells was enhanced by poly-D-lysine, inhibited by Matrigel, and unaffected by type I collagen. In contrast, the growth of RG cells was inhibited by type I collagen and unaffected by poly-D-lysine and Matrigel.

The effects of ECM on the growth of RG cells have not previously been studied to our knowledge, but some studies of matrix modulation of RPE proliferation have been reported. Similar to our data for growth on a two-dimensional collagen substrate, RPE cell number was also not affected by plating on the surface of a three-dimensional type I collagen gel. In another study, confluent monolayers of RPE cells overlaid with a collagen gel showed increased $^3$H-thymidine uptake, partly due to the expanded surface area available for growth of cells which migrated into the gel. A preliminary report indicated that laminin has no effect on RPE cell proliferation. Since laminin is a major component of Matrigel, the inhibition of RPE growth by Matrigel shown here may be due to some other component of this basement membrane material. However, since each component of Matrigel was not tested separately in this study, it is not clear if laminin contributes to growth inhibition of RPE when complexed with other materials.

For RPE on Matrigel, $^3$H-thymidine incorporation was inhibited, relative to other substrates, within the first 24 hours after cell plating. Changes in DNA synthesis did not appear to be due to shifts in the time of entry into the first S-phase (which was the same for both cell types for all substrates). Since three coincident peaks of DNA synthesis were observed within the first 52 hours on all substrates, it appears that the

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cell number/well (×10^4)</th>
<th>Cell type</th>
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<tbody>
<tr>
<td>Culture plastic</td>
<td></td>
<td>RPE</td>
</tr>
<tr>
<td>Type I collagen</td>
<td>11.4 ± 0.5</td>
<td>4.2 ± 0.5†</td>
</tr>
<tr>
<td>Matrigel</td>
<td>7.8 ± 1.1†</td>
<td>4.2 ± 0.5†</td>
</tr>
<tr>
<td>Poly-D-lysine</td>
<td>13.6 ± 0.6†</td>
<td>6.9 ± 0.1</td>
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* Data are means ± SD of three replicate wells in one experiment.
† Significantly different from culture plastic ($P < 0.05$, t-test).
substrate also did not alter RPE cell cycle time. However, peak height for RPE on Matrigel (Fig. 3) was lower, and the percent of labeled nuclei on radioautographs was also lower, suggesting that Matrigel suppresses growth in RPE cells by altering the fraction of cycling cells. For RG cells, no differences were observed in DNA synthesis within the first 24 hours after plating, although there were fewer glial cells after 14 days on type I collagen. It is unknown why the final density was lower on this matrix, but it is possible that changes in cell cycle time contributed because, unlike RPE, RG cells did not show multiple synchronous peaks of DNA synthesis.

In some (but not all) cell-substrate combinations in which growth was affected, changes in culture morphology were also observed. For example, growth of RPE was suppressed by Matrigel, and the cells also showed morphologic evidence of reduced matrix association as indicated by poor spreading in sparse cultures and cell clustering in dense cultures. Similarly, RG cells, which showed reduced growth on type I collagen, also demonstrated morphologic changes on this substrate. However, when RG were plated on Matrigel, cell morphology was altered, but growth was not. This latter observation indicates that, although Matrigel does not affect RG growth, this sub-
strate does alter other properties of RG cells which determine cell shape.

The cell-derived matrices also differentially affected the growth of RPE and RG cells. The RPE-derived matrix enhanced the growth of RG, but inhibited the growth of RPE; RG-derived matrix enhanced the growth of both RPE and RG.

The cell type-specific matrices used in these experiments were uncharacterized, but they are likely to contain some of the ECM materials which were tested. In vitro RPE has previously been shown to produce fibronectin, laminin, and collagen types I, III, and IV. \(^ {17-20} \) The basement membrane components laminin and type IV collagen are also the major macromolecules in Matrigel. \(^ {16} \) Since both Matrigel and RPE-derived matrices inhibited RPE growth, it is possible that basement membrane materials produced by RPE contributed to the growth suppression. However, both Matrigel and the cell-derived substrates are complex secretory products which may contain multiple growth modulators.

The RG cells have been shown to secrete fibronectin and types I and V collagen in vitro and to produce similar matrix materials when injected into the rabbit vitreous in an experimental model of PVR. \(^ {21} \) The concept that the composition of matrix produced by cells within the vitreous is cell type specific is also supported by morphologic data, \(^ {22} \) suggesting that different matrices may be found in PVR membranes depending on the cellular components. In this study matrices produced by the retina-derived cells in vitro appeared to differ as evidenced by their different growth effects. Of note is the observation that heterotypic matrix was growth stimulatory; that is, RG-derived matrix promoted RPE growth and RPE-derived matrix promoted RG growth. It has previously been observed that more severe PVR membranes often contain both RPE and RG cells, \(^ {1,23} \) which led to the suggestion that interactions between these cell types may potentiate cellular processes, such as growth, which are important in membrane development. \(^ {24} \)

Cells may interact in several ways, including by means of secretory products which are diffusible and/or deposited in matrices. In a previous study a reciprocal induction of DNA synthesis was observed in short-term co-cultures of RPE and RG in which part of the growth stimulation was due to humoral factors found in the culture medium. \(^ {24} \) This study suggests there is also a reciprocal stimulation of growth in RG and RPE mediated by matrices deposited in culture. The formation of complex PVR membranes, containing both RG and RPE cells may, therefore, be potentiated by many types of interactions between these cell types.

**Key words:** retinal pigment epithelium, retinal glia, basement membranes, collagen, culture substrate

### References

10. Burke JM and Foster SJ: Injured vitreous stimulates DNA

### Table 2. \(^ {3} \)H-thymidine uptake by RPE and RG cultures 24 hr after plating on cell-derived matrices

<table>
<thead>
<tr>
<th>Substrate</th>
<th>RPE</th>
<th>RG</th>
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<tbody>
<tr>
<td>Culture plastic</td>
<td>117.8 ± 10.8</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>RPE-derived matrix</td>
<td>89.9 ± 13.0†</td>
<td>4.1 ± 0.2†</td>
</tr>
<tr>
<td>RG-derived matrix</td>
<td>113.0 ± 10.0</td>
<td>3.9 ± 0.3†</td>
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\* Data are means ± SD of four replicate wells in one experiment.

† Significantly different from culture plastic (\( P < 0.05 \), t-test).

### Table 3. Numbers of RPE and RG cells observed 14 days after plating on cell-derived matrices

<table>
<thead>
<tr>
<th>Substrate</th>
<th>RPE</th>
<th>RG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture plastic</td>
<td>32.0 ± 1.1</td>
<td>12.4 ± 1.2</td>
</tr>
<tr>
<td>RPE-derived matrix</td>
<td>26.9 ± 1.8†</td>
<td>16.3 ± 1.5†</td>
</tr>
<tr>
<td>RG-derived matrix</td>
<td>39.1 ± 4.6†</td>
<td>15.2 ± 1.1†</td>
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</table>

\* Data are means ± SD of three replicate wells in one experiment.

† Significantly different from culture plastic (\( P < 0.05 \), t-test).


