Retinal Pigment Epithelium Cells Can Influence Endothelial Cell Plasminogen Activators

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Endothelial cells from both human retinal microvessels (HME) and fetal bovine aortic endothelium (FBAE) were grown in aggregate cultures alone, or with either retinal pigment epithelium (RPE) cells or fibroblasts. The levels of plasminogen activator (PA) and plasminogen activator inhibitor (PAI) in the conditioned media of the various aggregate types were measured. High PA levels were detected in the conditioned medium of pure endothelial cell aggregates (equal to 140% and 124% of urokinase control for HME and FBAE, respectively), and high PAI levels were associated with pure RPE aggregates (inhibiting 93% of the urokinase control). The conditioned medium of pure fibroblast aggregates had very low levels of either PA or PAI. When RPE cells were aggregated with FBAE or HME cells into mixed (heterogenous) aggregates, the PA measured in the conditioned medium was equal to 22% and 30% of the urokinase control, respectively. The PA level in the conditioned medium of mixed fibroblast-FBAE cell aggregates was higher, 104% of the control, and the difference was statistically significant \((P < 0.001)\). Co-incubation of pure RPE aggregates with pure FBAE aggregates or with pure HME aggregates resulted in PA activity in the conditioned medium that was equal to 110% and 96% of the control, respectively. The PA level found when pure FBAE cell aggregates were co-incubated with pure fibroblast aggregates was higher, 134% of the control, and the difference was statistically significant \((P < 0.001)\). Our results indicate that RPE cells can reduce endothelial cell PA, probably through both direct contact between the cells and PAI production. Fibroblasts did not have this influence on endothelial cell PA.

Neovascularization plays an important role in the pathogenesis of a number of ophthalmic disorders, including age-related macular degeneration and proliferative diabetic retinopathy. Neovascularization begins with the invasion of surrounding tissue by vascular sprouts via protease-mediated degradation of adjacent extracellular matrix. Plasminogen activators (PAs) are a group of serine proteases which catalyze the conversion of plasminogen to plasmin. The ability of plasmin to cause destructive proteolysis of many extracellular matrix components, including laminin and fibronectin, makes it well suited as a mediator of basement membrane invasion. It has been shown that endothelial cells produce both urokinase and tissue-type PAs, and urokinase has been identified in migrating endothelial cells and at the tips of vascular sprouts. The factors which control the release of vascular endothelial cell plasminogen activators are not well understood.

Increasing evidence suggests that retinal pigment epithelial (RPE) cells may play a central role in the regulation of intraocular neovascularization. In animal studies it has been shown that choriocapillary invasion of Bruch's membrane, induced by low-intensity laser, ceased after reproliferation and migration of surviving RPE cells to cover the defect. In another study it was shown that neovascularization and leakage stop when the new blood vessels are surrounded by RPE cells. In vitro, RPE-conditioned medium was shown to inhibit neovascularization on the chick embryonic yolk sac, and a specific urokinase inhibitor from media conditioned by RPE cells recently has been purified and characterized.

In the current study we investigated the hypothesis that one of the mechanisms by which RPE cells regulate intraocular neovascularization is through their ability to reduce either the amount of PA released by endothelial cells into the surrounding tissues or to reduce the activity of PA after release. We used the model of aggregate cell culture, a culturing technique in which cells are reggregated into small three dimensional masses. It has been shown that endothelial cells in aggregates form capillary-like structures.
and are generally more differentiated than in monolayer conditions, as has been shown also for other cells. Because different cell types can be aggregated together, this model lends itself to the study of various cell–cell interactions. By mixing endothelial cells with RPE cells in the same aggregate it is possible to create spatial relationships between the two cell types that are closely similar to those occurring in vivo between the endothelial cells of a growing subretinal neovascular membrane and the RPE cells that surround it. The effect of direct contact between endothelial cells and RPE cells on endothelial cell PA release, and on the activity of the released PA, can thus be studied and compared to the effects that other cell types have in the same system. In the current study, endothelial cells from both human retinal microvessels (HMEs) and fetal bovine aortic endothelium (FBAE) were grown in aggregate culture either alone or with RPE cells or fibroblasts. The levels of PA and plasminogen activator inhibitor (PAI) in the conditioned media of the various aggregate types were measured.

Materials and Methods

Cell Lines

Cells were harvested by methods described previously for retinal pigment epithelium cell, FBAE cells, human microvascular endothelial cells, and bovine corneal fibroblasts. Passages between 5 and 12 were used for all experiments.

Cell Aggregates

**Pure cell aggregates:** Cell aggregates were prepared for each of the cell types used, in order to evaluate the PA and PAI levels in their media in this “pure” situation. Cell aggregates were prepared using a gyration-mediated reaggregation system described previously by our laboratory and based on a technique originally described by Moscona. Confluent monolayers of cells were trypsinized by 0.1% Trypsin (Sigma, St. Louis, MO) in Hank’s balanced salt solution (Gibco, Grand Island, NY) and centrifuged at 1000 rpm for 5 min. The cell pellets were resuspended in their prospective growth medium to obtain a density of 5 x 10⁶ cells/ml. The growth medium was medium 199 (Gibco) supplemented with 15% fetal bovine serum (Gibco), 0.2M HEPES buffer solution (Gibco), and 100 units penicillin G and 100 µg streptomycin per ml of medium. A volume of 1.5 ml of the cell suspension was placed in 5-ml Erlenmeyer flasks (Wheaton) and cultured on a rotary shaker (VWR Labline, Bridgeport, NJ) at 100 rpm for 24 hr at 37°C.

**Mixed (heterogenous) cell aggregates:** These were prepared essentially in the same way as above, except that 0.75 ml from the suspension of an endothelial cell line and 0.75 ml of either the fibroblast cell suspension or the RPE cell suspension were mixed in the Erlenmeyer flasks, to obtain a final volume of 1.5 ml mixed cell suspension.

Twenty-four hr after initiation of both pure and mixed aggregates the growth medium was removed, and the aggregates were rinsed with and then placed in 1.5 ml serum-free medium 199 with HEPES and penicillin–streptomycin. Flasks of pure and mixed aggregates were then cultured for 24 hr at 37°C in a rotary shaker bath. At this time, the aggregate-conditioned medium was removed, centrifuged at 2000 rpm, and tested for PA and PAI. Some of the aggregates were removed for morphologic examination. The remaining aggregates were trypsinized, and the cells were counted with a Coulter counter at the conclusion of the experiment.

**Combined aggregates:** In a third series of experiments, beginning 24 hr after initiation of aggregate formation, pure endothelial cell aggregates were combined with either pure RPE aggregates or pure fibroblast aggregates, also in serum-free medium. The combined aggregates were then cultured for 24 hr at 37°C in a rotary shaker bath. At this time the aggregate-conditioned medium was removed, centrifuged at 2000 rpm, and tested for PA and PAI. Some of the aggregates were removed for morphologic examination, and the remaining aggregates were trypsinized and counted as described above.

In all experiments with mixed aggregates and combined aggregates, the RPE cells and fibroblasts were labeled prior to aggregation with an overnight incubation in a 0.01% suspension of 0.29-µm polystyrene beads (Polysciences, Warrington, PA), to allow subsequent identification of the two cell types.

All types of aggregate experiments (pure, mixed, and combined) were repeated separately ten times.

Plasminogen Activator and Activator Inhibitor Assays

Plates coated with 125I-labeled fibrin were prepared as described by Unkeless et al. Briefly, plasminogen-free bovine fibrinogen (Calbiochem, San Diego, CA) was iodinated, diluted with unlabeled fibrinogen, and added to each well of 24-well plates (Nunc, Roskilde, Denmark) at 10 µg/cm² in water. The fibrinogen was dried for 72 hr at 41°C and then converted to fibrin prior to use in the assay, by incubation for 2 hr at 37°C with 1 unit/ml thrombin (Parke-Davis) in minimal essential medium (MEM). Each well was rinsed twice with 2 ml 0.1M Tris-
Control experiment was done on similar dilutions of gates were mixed in ratios from 1:1 to 1:40 of the conditioned media, conditioned media from FBAE aggregates and from RPE aggregates were mixed in ratios from 1:1 to 1:40 (FBAE:RPE), incubated for 20 min at room temperature, and then assayed for PA and PAI. A similar assay was done for mixtures of FBAE-conditioned medium with FIBRO-conditioned medium. The control experiment was done on similar dilutions of FBAE-conditioned medium with MEM. Each experiment was repeated four times.

Interaction Between Conditioned Media

To evaluate the interaction between components of the different conditioned media, conditioned media from FBAE aggregates and from RPE aggregates were mixed in ratios from 1:1 to 1:40 (FBAE:RPE), incubated for 20 min at room temperature, and then assayed for PA and PAI. A similar assay was done for mixtures of FBAE-conditioned medium with FIBRO-conditioned medium. The control experiment was done on similar dilutions of FBAE-conditioned medium with MEM. Each experiment was repeated four times.

Data Evaluation

The counts of the negative control well (nonspecific counts) were subtracted from each test well count in the same plate, to obtain the actual counts, and these numbers were used for the statistical analysis.

There was some variability among the plates used for the assays and also some decay of the radioactivity over time. Therefore, in order for the data from the various experiments to be comparable, the adjusted counts from each well were converted to percentages of the counts in the urokinase positive control well of the same plate. For the PA assay, the results were expressed as percentage of activity, calculated with the formula: percent activity = sample count × 100/(urokinase control count). Because in some cases the activity of the samples was greater than that of the urokinase control, this formula occasionally yielded percentages higher than 100.

For the PAI assays, the decrease in counts, which indicated the presence of inhibitory activity in the tested conditioned medium, was expressed as a percentage of inhibition, calculated with the formula: percent inhibition = (control count − sample count) × 100/(control count).

Results were analyzed by one-way analysis of variance (ANOVA) and the student t-test. Only differences with $P < 0.01$ were considered significant.

Light Microscopy of the Aggregates

Samples of each of the aggregate preparations were prepared for light microscopic examination. Each sample was fixed in 3% buffered paraformaldehyde for 2 hr at room temperature and then stored in cacodylate buffer at 4°C prior to embedding. After a gradual dehydration, the samples were moved into a 1:1 mixture of ethanol:Epon/Araldite mixture, and then into the pure Epon/Araldite mixture and cured for 4 days at 60°C. Propylene oxide was not used in these experiments because of its detrimental effects on the microspheres used to label cells in some of the aggregates. One-micrometer sections from the embedded aggregates were taken with a Reichert-Jung ultramicrotome, and the sections were toluidine-stained prior to light microscopic examination. Aggregates from each preparation were examined with a Zeiss Axioskop photomicroscope, and the relative numbers of labeled and unlabeled cells were determined in the samples from each of the mixed aggregate preparations. In order to determine the relative frequency of endothelial cells versus labeled fibroblasts or RPE cells, the number of labeled and unlabeled cells in multiple fields of a consistent size were determined using a 40× objective. Depending
upon the size of the aggregate being examined, between 2 and 7 noncontiguous fields were examined. One hundred ten fields in 26 mixed endothelial-RPE aggregates, and 112 fields in 26 mixed endothelial-fibroblast aggregates, were counted. Using this method, the microspheres were easily visualized as homogenous cytoplasmic inclusions. Only cells exhibiting a significant amount of cytoplasm were scored as labeled or unlabeled.

Results

Plasminogen Activator and Plasminogen Activator Inhibitor Assays

Pure aggregates (Figs. 1a, b): The conditioned medium of pure HME aggregates and pure FBAE aggregates had very high amounts of mean PA activity—140 ± 21% and 124 ± 7%, respectively. No PA activity was detected in the conditioned medium of any of the pure RPE aggregates, and a negligible amount was found in pure fibroblast aggregate-conditioned media.

PAI activity was not detectable in the conditioned medium of both large and small vessel endothelial cell aggregates. The RPE aggregates, however, produced very high amounts of PAI, and were able to inhibit 93 ± 11% of the urokinase positive control. Low but measurable PAI activity (14 ± 7%) was found in the fibroblast aggregate-conditioned medium.

Mixed aggregates (Figs. 1c, d): Conditioned media from mixed FBAE-RPE aggregates and mixed HME-RPE aggregates had a much lower mean level of PA activity (22 ± 19% and 30 ± 14%, respectively) than conditioned medium from mixed FBAE-FIBRO aggregates (104 ± 17%). The difference was statistically significant (P < 0.001).

The conditioned medium of both mixed FBAE-RPE aggregates and mixed HME-RPE aggregates had high mean levels of PAI activity—72 ± 21% and 65 ± 16%, respectively. However, the conditioned medium from mixed FBAE-FIBRO aggregates had a much lower mean PAI level, only 15 ± 10%. The difference was statistically significant (P < 0.001).

Combined aggregates (Figs. 1e, f): When endothelial cell (FBAE or HME) aggregates were placed together with RPE cell aggregates for 24 hr, the conditioned medium contained less PA activity than the conditioned medium of FBAE aggregates conditioned with fibroblast aggregates under the same experimental conditions. The mean PA levels for combined FBAE-RPE aggregates and combined HME-RPE aggregates were 110 ± 19% and 96 ± 15% of the control, respectively, whereas the mean PA level for FBAE aggregates incubated with fibroblast aggregates was 134 ± 5%. The difference was statistically significant (P < 0.001).

The conditioned medium from endothelial cell aggregates (HME or FBAE) incubated with RPE aggregates had only trace PAI activity. The conditioned medium of combined FBAE aggregates incubated with fibroblast aggregates had no PAI.

Interaction Between Conditioned Media

Except for the 1:1 mixture, the mixture of conditioned media from pure FBAE aggregates and pure RPE aggregates had a markedly lower PA activity than the equivalent dilutions of pure FBAE aggregate conditioned medium with MEM (Fig. 2). The difference is 23% at the 1:4 mixture, 69% at 1:9 mixture, and 85% at 1:19 mixture. While a marked activity (46%) could still be detected at the 1:39 dilution with MEM 0, none was detected at the 1:39 mixture with RPE-conditioned medium. The difference for each dilution (except the 1:1 dilution) was statistically significant (P < 0.01). The experiment was performed in the same way for mixtures of FBAE aggregate-conditioned medium and FIBRO aggregate-conditioned medium. The PA activity in the mixtures was exactly the same as in the equivalent dilutions of FBAE-conditioned medium with MEM.

Inhibitory activity (PAI) was not detectable in any of the mixtures.

Cell Count Data

All aggregate experiments were begun with 7.5 × 10⁶ cells/flask. After 24 hr, the serum-containing medium was removed, and the aggregates were rinsed with and then incubated in serum-free medium for an additional 24 hr. This procedure ensured that all cells not incorporated into the aggregates were removed. Therefore, the PA and PAI activities found in the various media originated from cells in the aggregates and not from loose cells in the media. The cell counts of the trypsinized aggregates, 48 hr later, are shown in Table 1. Generally, all of the pure cell aggregate cultures showed a decrease in cell counts relative to the original cell suspension. The endothelial cell aggregates showed the largest decrease—to 37.3% and 28.0% of initial count for FBAE and HME, respectively. The mixed cell aggregates also demonstrated some loss of cells relative to the initial concentration, to 63–76% of the initial count (Table 1). The differences in cell loss among the three mixed aggregates were not statistically significant (P = 0.104). The cell counts in the experiments where two types of pure aggregates were co-cultured give the total number of cells in the flask, without differentiating between the two cell types used. In order to
evaluate if the co-culturing was associated with cell loss, we compared these cell counts with the sum of the separate counts of the two pure aggregates in that experiment. For example, the HME count was 2.1 × 10^6 cells, and the RPE count was 4.6 × 10^6 cells (Table 1); together these add up to 6.7 × 10^6 cells, a figure very close to the 6.9 × 10^6 cells found for the combined HME–RPE aggregates experiment. Gener-
Fig. 2. Interaction between conditioned media. PA activity is shown, as a percentage of the urokinase control, for three mixtures of media: pure endothelial cell aggregate-conditioned medium with pure RPE aggregate-conditioned medium (ENDO CM: RPE CM); pure endothelial cell aggregate-conditioned medium with pure fibroblast aggregate-conditioned medium (ENDO CM: FIBRO CM); and pure endothelial cell aggregate-conditioned medium with serum-free medium (ENDO CM: SFM). The endothelial cell aggregate-conditioned medium was increasingly diluted with the other three media. Each point shows the average of four assays.

ally, the cell counts of combined aggregates indicate that cell loss was minimal, if at all, during the 24-hr incubation period.

Light Microscopy

Light microscopic examination showed that the aggregates from these preparations appeared to be viable at the time of fixation, with no evidence of interior necrosis (Figs. 3a–c), except in the occasional very large aggregate. Mitotic figures were seen on rare occasions, but there was no other evidence to suggest that the aggregates grew in size after their initial formation.

Examination of the mixed aggregates revealed a mixing of unlabeled endothelial cells with either labeled fibroblasts or labeled RPE cells throughout the aggregates. There was no clear evidence of cell clustering or sorting in any of the mixed aggregates (Fig. 3d), although often slightly more of the labeled cells were found at the periphery in both types of mixed aggregates. Endothelial cells accounted for 55.2 ± 5.7% of the cells in the mixed endothelial–RPE aggregates, and 52.5 ± 2.6% of the cells in the mixed endothelial–fibroblast aggregates. The difference was not statistically significant.

Discussion

During the formation of pure cell aggregates, there was substantial cell loss, more so for the endothelial cells, but also for RPE cells and fibroblasts (Table 1). However, all cell types formed sizable populations of viable aggregates. Cells that were not incorporated into the aggregates were removed during the rinsing of the aggregates. Significantly more endothelial cells than RPE cells or fibroblasts were lost, suggesting that either the experimental conditions are harder on the endothelial cells or that they aggregate less readily with each other. All three types of mixed aggregates had fairly similar cell counts at the conclusion of the experiment. Also, we know from the microscopic analysis that in all types of mixed aggregates the endothelial cells accounted for about 50% of the cells in the aggregate. These findings allowed us to make comparisons of PA production by the different mixed aggregate types. The counts for the combined aggregates indicate that the co-incubation of two different cell aggregates was associated with an insignificant cell loss in the ensuing 24 hr.

Endothelial cells from various sources have been shown to secrete variable amounts of PA. The amount of PA secreted depends on the species and source of endothelium, and the exact culture conditions. Our results indicate that the conditioned medium of endothelial cells in gyration-mediated aggregate cultures has high PA activity. This was true for both FBAE (124%) and HME cells (140%). The results were strikingly similar for the small vessel endothelial cells (HME) and the large vessel endothelial cells (FBAE). The high levels of PA activity probably reflect both a concentration effect (a large number of cells secreted into a small volume of conditioned medium) and the existence of favorable conditions for PA production in these experiments. At this stage we have not addressed the question of identifying the type of PA produced by the endothelial cells. It could be urokinase-type PA, as found in vascular sprouts.

Table 1. Cell counts of aggregates 48 hr after initiation

<table>
<thead>
<tr>
<th>Aggregates</th>
<th>Cells (x10⁶)/flask (mean ± SD)</th>
<th>Percent of initial count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBAE</td>
<td>2.8 ± 0.8</td>
<td>37.3</td>
</tr>
<tr>
<td>HME</td>
<td>2.1 ± 0.5</td>
<td>28.0</td>
</tr>
<tr>
<td>RPE</td>
<td>4.6 ± 1.4</td>
<td>61.3</td>
</tr>
<tr>
<td>FIBRO</td>
<td>4.1 ± 0.4</td>
<td>54.6</td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBAE–RPE</td>
<td>4.9 ± 1.6</td>
<td>65.3</td>
</tr>
<tr>
<td>FBAE–FIBRO</td>
<td>4.7 ± 0.6</td>
<td>62.6</td>
</tr>
<tr>
<td>HME–RPE</td>
<td>5.7 ± 0.7</td>
<td>76.0</td>
</tr>
<tr>
<td>Combined*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FBAE–RPE</td>
<td>6.5 ± 1.5</td>
<td>87.8</td>
</tr>
<tr>
<td>FBAE–FIBRO</td>
<td>6.3 ± 1.0</td>
<td>91.3</td>
</tr>
<tr>
<td>HME–RPE</td>
<td>6.9 ± 0.6</td>
<td>102.9</td>
</tr>
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</table>

* The percentage for the combined aggregates was calculated with the formula: combined aggregates count × 100/(pure aggregate A count + pure aggregate B count).
Fig. 3. One-micrometer sections through typical aggregates of endothelial cells (a), RPE cells (b), and fibroblasts (c). The aggregates are composed of large numbers of apparently viable cells, with a relatively smooth perimeter formed by elongated cells, and no evidence of interior necrosis. In (d), the microsphere-labeled RPE cells (arrowheads) of a mixed FBAE-RPE aggregate can be seen interspersed with the unlabeled endothelial cell population. (a–c, ×850; d, ×2500).
or tissue-type PA, or both. The assay used in this study only measures the net PA activity present in the conditioned media.

Endothelial cells have also been shown to produce PAI, but our assay did not detect it. This may be due to one or more of several reasons: the endothelial cells either did not produce or did not release PAI into the aggregate culture medium; the PAI was present but in an inactive form; or the PAI could not be detected by our assay in the presence of high PA activity. This might be a limitation of the PA assay we used, but because we were interested in comparing results of different aggregates, and not in absolute values, the simplicity of the assay made it the method of choice.

High levels of PAI activity (93%) but no PA activity were detected in the conditioned media of pure human RPE cell aggregates. This reconfirms previous studies in which RPE cell-conditioned media were shown to have protease inhibiting activity.

The conditioned medium of fibroblast aggregates, on the other hand, had only trace PA activity and low PAI activity. Fibroblasts have been shown previously to have urokinase concentrated in fibers that are located beneath the cells, and to have PAI-1 homogeneously distributed in their substratum. Our inability to detect PA and PAI activities in the conditioned medium of the fibroblast aggregates may be due either to the actual absence of these molecules under our test conditions, to their sequestration within the aggregates, or to their release in inactive forms.

The experiments in which pure aggregates of two different types were incubated together allowed us to evaluate the total effect of diffusible factors on the endothelial cell PA production. When pure endothelial cell (HME or FBAE) aggregates were incubated with pure RPE aggregates, the conditioned medium had significantly lower levels (96% and 110%, respectively) than when they were incubated with pure fibroblast aggregates (134%). PAI was not detected in any of these conditioned media. It is possible that the PAI released by the RPE aggregates neutralized part of the PA produced by the endothelial cell aggregates, thus lowering the net measurable PA levels, and becoming undetectable. The results of the assay that evaluated the interaction of endothelial aggregate-conditioned medium with RPE aggregate-conditioned medium (Fig. 2) support this possibility. In the interaction assays it was found that the conditioned medium of RPE aggregates was capable of partly neutralizing the PA activity in the conditioned medium of endothelial cell aggregates. However, volume for volume, the endothelial aggregate conditioned medium had much more PA activity than the RPE aggregate-conditioned medium had PAI activity. Fibroblast aggregates did not affect the PA levels in the conditioned medium when incubated with endothelial cell aggregates. This is in agreement with other findings in our study: the fibroblast aggregates have been found to produce only low PAI levels and their conditioned medium did not neutralize the endothelial PA when the two media were incubated together (Fig. 2). Therefore, there is a clear difference in the effect of RPE aggregates and fibroblast aggregates on the measurable level of PA activity released by endothelial cell aggregates into their conditioned media.

Light microscopic analysis showed that the two cell populations in the mixed aggregates were approximately equal, and that the cell counts of the various mixed aggregates were quite similar. This evidence suggests that the PA activity measurements do not reflect large differences in endothelial cell numbers in these mixed aggregates. We observed neither evidence of cell sorting in either kind of mixed aggregate, nor sequestration of one cell population within the center of the aggregate. Although both types of aggregates appeared equally mixed, there is a clear difference in the PA and PAI activity levels of mixed RPE–endothelial cell aggregates and mixed fibroblast–endothelial cell aggregates. The conditioned media of mixed RPE–endothelial aggregates have low PA levels and high PAI levels, whereas fibroblast–endothelial cell aggregates have high PA levels and low PAI levels. The simultaneous presence of PA and PAI in the conditioned media of mixed aggregates probably indicates that there was more than one type of PA and one type of PAI present in the media. It is possible that the PAIs present in the conditioned media did not completely neutralize the available PAs, and therefore that a proportion of both types of substances could be detected simultaneously in the media. These results suggest that RPE cells may have had an effect on the endothelial cells when in direct contact in the mixed aggregate, in addition to any subsequent interaction of molecules released by them, thereby reducing the net measurable PA activity. The fibroblasts, on the other hand, did not have any of these effects on the production and release of PA by the endothelial cells in the mixed aggregates.

Our results indicate that the RPE cells decreased the PA production of endothelial cells in both mixed aggregate and combined aggregate cultures, probably through both direct contact between the cells and diffusible factors. Quantitatively, the direct contact seems to have had a greater effect. Fibroblasts, on the other hand, did not have these effects on endothelial cells. The spatial relationships between endothelial
cells and RPE cells in the mixed aggregates were similar to those occurring in vivo between the endothelial cells of a growing subretinal neovascular membrane and the RPE cells surrounding the membrane. It is possible, therefore, that the inhibitory effect of RPE cells on intraocular neovascularization is mediated at least in part through their ability to reduce PA production by endothelial cells and to neutralize the released PA.

**Key words:** plasminogen activator, aggregate culture, endothelial cells, retinal pigment epithelium, plasminogen activator inhibitor

**References**