Modulation of Plasminogen Activator Inhibitor-1 and Urokinase in Retinal Pigmented Epithelial Cells

Sean F. Hackett and Peter A. Campochiaro

**Purpose.** To examine the effect of several agents that either stimulate or inhibit neovascularization on plasminogen activator inhibitor-1 and urokinase in retinal pigmented epithelial cells and vascular endothelial cells.

**Methods.** Steady-state levels of messenger RNA were assessed by Northern blots and dot blots and protein levels were assessed by immunoprecipitation.

**Results.** Data indicate that messenger RNA levels for plasminogen activator inhibitor-1 are modulated in similar fashion in both cell types, being increased by incubation with transforming growth factor-beta, dexamethasone, tumor necrosis factor, phorbol myristate acetate, and thrombin. Levels of urokinase messenger RNA in retinal pigmented epithelial cells are significantly increased only by phorbol myristate acetate and are decreased by dexamethasone and transforming growth factor-beta, whereas in endothelial cells plasminogen activator urokinase messenger RNA is increased by each of the stimuli except dexamethasone, which causes a decrease. Immunoprecipitation experiments demonstrate similar modulation of inhibitor-1 proteins secreted by retinal pigmented epithelial cells, whereas urokinase is difficult to detect.

**Conclusions.** These data suggest that retinal pigmented epithelial cells may help to alter proteolytic activity in the subretinal space and thereby participate, along with endothelial cells, in the regulation of choroidal neovascularization.
this study, we examined the effect of several agents known to modulate neovascularization in vivo, for their ability to modulate uPA and PAI-1 in RPE cells.

MATERIALS AND METHODS

Human transforming growth factor-beta (TGF-beta) was obtained from R&D Systems (Minneapolis, MN), human recombinant tumor necrosis factor-alpha was obtained from Amgen (Thousand Oaks, CA), and thrombin, phorbol myristate acetate, and dexamethasone were obtained from Sigma (St Louis, MO). Human RPE cells were cultured from postmortem eyes obtained from the Old Dominion Eye Bank (Richmond, VA) using a procedure that has been previously described. In the authors' opinion, methods for securing human tissue were humane, included proper consent and approval, and complied with the tenets of the Declaration of Helsinki. Cultures from donors aged 6 months, 48 years, and 73 years were used. Human umbilical vein endothelial cells (HUVEC) were harvested as previously described. RPE cells at passages 2–5 and HUVEC at passages 10–15 were grown in 150 mm plates until confluent. RPE cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan UT) and HUVEC were grown in medium 199 (Gibco) supplemented with 100 μg/ml heparin (Sigma, St Louis, MO), 20% fetal bovine serum and 50 μg/ml of Endothelial Cell Growth Supplement (Collaborative Research, Bedford, MA). After reaching confluence, the RPE cells were placed in fresh serum-free media with various test substances. Endothelial cells were placed in serum-containing media along with the test substances. After 4 or 24 hours, the cells were washed and total RNA was isolated by the method of Chomczynski and Sacchi.

Northern blots and dot blots were performed using a 3.0 kb PAI-1 human complementary DNA probe (cDNA) (provided by Dr. David Loskutoff) and 1.5 kb urokinase type plasminogen activator human cDNA (American Type Culture Collection). A 4.8 kb 18S ribosomal RNA mouse cDNA (generously provided by Dr. Ramared Gunkata) was used to rehybridize dot blots after stripping for standardization of RNA loading. The cDNA were labeled with 32P using the Schleicher and Schuell random priming kit following the manufacturer’s instructions.

Fractionation of denatured RNA on formaldehyde-containing 1.4% agarose gels and blot transfer to Nytran paper (Cuno, Inc., Meriden, CT) was performed as described by Thomas. Dot blots were also prepared on Nytran paper by adding serial dilutions of 5 μg of each RNA sample to each row of a dot blot apparatus (Schleicher and Schuell) connected to low suction. Northern blots and dot blots were prehybridized in a shaker bath at 42°C in 5X Denhardt’s reagent, 50% formamide, 5X SSPE (1 X SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM NaPO4, pH 7.7), 0.1% SDS, and 150 μg/ml denatured salmon sperm DNA for 2–18 h. Hybridization was performed for 20 hr at 42°C in 2X Denhardt’s reagent, 50% formamide, 5X SSPE, 0.1% SDS, 10% dextran sulfate and 150 μg/ml salmon sperm DNA containing 1.5 X 106 cpm/ml of labeled probe. After hybridization, the blots were washed twice in 2X SSPE and 0.1% SDS for 15 min at 40°C, once in 0.1X SSPE and 0.1% SDS for 15 min at 40°C, and once in 0.1X SSPE and 0.1% SDS for 30 min at 60°C. The washed blots were exposed to XRP film (Kodak, Rochester, NY) with an intensifier screen (lightning plus; Dupont Co, Wilmington, DE) at −80°C. Exposure times were varied for optimal visualization of results. The density of autoradiographic signals on dot blots was quantitated with a scanning densitometer and specially designed software. Dot blots were stripped and reprobed with the cDNA for 18S ribosomal RNA to standardize for the amount of RNA added to each row.

Proteins synthesized by RPE and secreted into their media were radiolabeled by the following procedure. Confluent cultures were washed with PBS and then placed in serum-free media containing 4 μM methionine, 100 μg/ml BSA, 100 uCi per ml [35S]methionine (1097 Ci per mmol; New England Nuclear, Boston, MA), and various test substances. After 24 hours, the media were harvested, clarified by centrifugation, and after addition of 0.01% Tween-80, were frozen for use in immunoprecipitation experiments.

Goat antibodies directed against human PAI-1 and uPA were obtained from American Diagnostica (Greenwich, CT). One milliliter of media containing radiolabeled proteins was incubated with a 1:250 dilution of antibody overnight at 4°C. Rabbit anti-goat Immunobeads (Bio-Rad) were used to precipitate the immune complexes. After washing, the beads were boiled in Laemmli sample buffer for 3 min, and proteins were resolved by SDS-polyacrylamide gel electrophoresis in slab gels according to the procedure of Laemmli. The upper stacking gel contained 4% acrylamide and the lower separating gel contained 10% acrylamide. After electrophoresis, the gels were analyzed for labeled proteins by autoradiography and for cross-reactivity to PAI-1 and uPA by immunoblotting. For immunoblotting, proteins were transferred to nitrocellulose using a buffer containing 50 mM Tris base, 95 mM glycine, 20% methanol, and 0.01% SDS. The nitrocellulose sheets were soaked in Tris-buffered saline containing 1% nonfat dry milk (Carnation) for 1 hr at room temperature to block additional protein binding sites and then were incubated overnight at
Urokinase and PAI-1 In RPE

4°C in Tris-buffered saline/1% milk containing the indicated antiserum (1:500 dilution). The nitrocellulose sheets were washed three times (10 min each wash) with Tris-buffered saline/1% milk and then incubated for 1 hr at room temperature in Tris-buffered saline/1% milk containing a 1:200 dilution of an affinity purified rabbit antigoat IgG conjugated to alkaline phosphatase (Kirkegaard and Perry, Cabin John, MD). After washing, the sheets were incubated for 30-40 min at room temperature in phosphate substrate solution containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Kirkegaard and Perry).

RESULTS

Total RNA prepared from human RPE and HUVEC was analyzed by Northern blotting as described in Materials and Methods using a 3.0 kb PAI-1 human cDNA corresponding to the PAI-1 coding region or a 1.5 kb uPA human cDNA. Two PAI-1 messenger RNA (mRNA) species, approximately 3.2 and 2.3 kb, were detected in both RPE and HUVECs, whereas a single uPA mRNA species of 2.5 kb was detected in both cell types.

Several agents known to modulate cellular processes implicated in neovascularization were assessed for their effect on steady-state levels of PAI-1 and uPA mRNA in RPE and HUVEC. In preliminary experiments, several concentrations of each agent were used and the most effective concentrations were selected for comparative studies. Transforming growth factor-beta (TGF-beta), dexamethasone, tumor necrosis factor-alpha (TNF), phorbol myristate acetate (PMA), and thrombin were found to increase PAI-1 mRNA levels in very similar fashion in RPE and endothelial cells (Figure 1 and Table 1). Tumor necrosis factor caused a very large increase after 4 hr of incubation that was sustained after 24 hr of incubation. Dexamethasone and TGF-beta both caused a large increase after 4 hr of incubation that was somewhat less after 24 hr of incubation. Incubation for 4 hr with phorbol myristate acetate caused a large increase in PAI-1 mRNA in both cell types and it was sustained after 24 hr of incubation in RPE, but less so in HUVEC. Thrombin caused a moderate increase that was fairly well sustained in both cell types. Thus the modulation of PAI-1 mRNA levels seems to be under very similar control in RPE and HUVEC.

There were much more heterogeneous effects on uPA mRNA levels and in general, stimulatory effects were more prominent in endothelial cells than in RPE. The response to dexamethasone and thrombin were similar, with dexamethasone causing a decrease and thrombin causing little change in uPA mRNA levels in both cell types (Fig 2, table). Phorbol myristate acetate caused an increase in both cell types, but the effect was

<table>
<thead>
<tr>
<th>Agent</th>
<th>RPE (4 hr)</th>
<th>RPE (24 hr)</th>
<th>HUVEC (4 hr)</th>
<th>HUVEC (24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-beta (200 pg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone (2 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF (10 ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin (1 U/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA (100 ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 1. Modulation of plasminogen activator inhibitor-1 (PAI-1) mRNA accumulation in human retinal pigmented epithelial (RPE) cells and human umbilical vein endothelial cells (HUVEC). After reaching confluence, cells were placed in serum-free medium or serum-free medium containing (A) transforming growth factor-beta (TGF-beta; 200 pg/ml); (B) dexamethasone (2 μM); (C) tumor necrosis factor-alpha (TNF; 10 ng/ml); (D) thrombin (1 U/ml); or (E) phorbol myristate acetate (PMA; 100 ng/ml). Total RNA was extracted after 4 or 24 hr and analyzed by Northern blotting with probes for PAI-1 (upper portion of each panel). The bottom portion of each panel shows the ethidium-bromide-stained agarose gel corresponding to each blot before transfer to demonstrate the amount of RNA loaded in each lane. Controls incubated in medium alone for 4 hr or 24 hr showed no differences and therefore only 4 hr controls are shown. For each panel, the lanes contain 10 μg of RNA from: (1) lane 1—RPE control; (2) lane 2—RPE incubated for 4 hr with listed agent; (3) lane 3—RPE incubated for 24 hr with listed agent; (4) lane 4—HUVEC control (5) lane 5—HUVEC incubated for 4 hr with listed agent (6) lane 6—HUVEC incubated for 24 hr with listed agent.
somewhat greater in RPE, particularly after 4 hr of incubation. The response to TGF-beta and TNF were very different in RPE and HUVEC. In RPE, TGF-beta caused a decrease in uPA mRNA levels that was comparable to that seen with dexamethasone, but in HUVEC there was a substantial increase in uPA mRNA after 24 hr of incubation with TGF-beta. Tumor necrosis factor caused a prominent, sustained increase in uPA mRNA in RPE, but had little effect in RPE.

Immunoprecipitation experiments were performed to determine if protein levels of PAI-1 and uPA were modulated in RPE by the various agents listed above as would be expected based on the alterations in mRNA. After a 24-hr incubation and labeling period, there was a prominent increase in newly synthesized PAI-1 secreted by RPE cultures in the presence of each of the agents except phorbol myristate acetate (Fig. 3). This correlates well with the mRNA levels. Using two different antibodies directed against PAI-1 and uPA, that based on its molecular weight, was felt to be uPA.20 In this study, we measured steady-state levels of PAI-1 and uPA mRNA in RPE. Human umbilical vein endothelial cells express two species of PAI-1 mRNA, a 2.3 kb transcript and a polyadenylated 3.2 kb transcript.29-30 They also express a single 2.5 kb transcript of uPA mRNA.25 In this study, we have demonstrated that RPE cells also express 2.3 kb and 3.2 kb transcripts of PAI-1 mRNA and a 2.5 kb transcript of uPA.

There is evidence that uPA is involved in the degradation of extracellular matrix that accompanies and is required for neovascularization.9 The activity of uPA is controlled largely by the production of PAI that bind and inactivate uPA.10,11 Several peptides that modulate neovascularization in vivo have been demonstrated to modulate mRNA levels of PAI-1 and/or uPA in vascular endothelium.12-19 In this study, we demonstrated that many of these agents also cause modulation in PAI-1 mRNA and to a lesser extent uPA mRNA in RPE. Although differences in culturing conditions suggest that one should exercise caution in comparing results in RPE and HUVECs, results in HUVECs provide a frame of reference to help interpret results in RPE. Human choroidal vascular endothelial cells (HCVEC) could theoretically provide an even better frame of reference when considering the pathophysiology of choroidal neovascularization, but there are several reasons why HUVEC are a reasonable alternative. (1) It would be very difficult to culture

### DISCUSSION

We previously demonstrated that RPE cells secrete PAI-1 and a PA, that based on its molecular weight, was felt to be uPA.20 In this study, we measured steady-state levels of PAI-1 and uPA mRNA in RPE. Human umbilical vein endothelial cells express two species of PAI-1 mRNA, a 2.3 kb transcript and a polyadenylated 3.2 kb transcript.29-30 They also express a single 2.5 kb transcript of uPA mRNA.25 In this study, we have demonstrated that RPE cells also express 2.3 kb and 3.2 kb transcripts of PAI-1 mRNA and a 2.5 kb transcript of uPA.

### TABLE 1. Quantification of Changes in Steady State Levels of mRNA for Plasminogen Activator Inhibitor-1 and Urokinase-Type Plasminogen Activator in Retinal Pigmented Epithelial and Vascular Endothelial Cells by Dot Blot Analysis

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Retinal Pigmented Epithelium</th>
<th>Vascular Endothelium</th>
<th>Stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[PAI-1 mRNA (% of Control)]/</td>
<td>[uPA mRNA (% of Control)]/</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[Ribosomal RNA (% of Control)]/</td>
<td>[Ribosomal RNA (% of Control)]/</td>
<td></td>
</tr>
<tr>
<td>TGF-β, 4 hr</td>
<td>3.3 ± 0.27*</td>
<td>0.69 ± 0.03†</td>
<td>3.05 ± 0.13*</td>
</tr>
<tr>
<td></td>
<td>0.69 ± 0.03†</td>
<td>1.97 ± 0.10†</td>
<td>2.34 ± 0.32†</td>
</tr>
<tr>
<td>Dexamethasone, 4 hr</td>
<td>3.21 ± 0.31*</td>
<td>0.66 ± 0.05†</td>
<td>2.09 ± 0.15†</td>
</tr>
<tr>
<td></td>
<td>0.78 ± 0.01†</td>
<td>2.73 ± 0.07†</td>
<td>0.83 ± 0.11</td>
</tr>
<tr>
<td>TNF, 4 hr</td>
<td>3.16 ± 0.54*</td>
<td>0.93 ± 0.05</td>
<td>4.50 ± 0.36*</td>
</tr>
<tr>
<td></td>
<td>0.78 ± 0.11</td>
<td>4.70 ± 0.44†</td>
<td>2.98 ± 0.11*</td>
</tr>
<tr>
<td>Thrombin, 4 hr</td>
<td>2.61 ± 0.07</td>
<td>1.08 ± 0.05</td>
<td>1.78 ± 0.12†</td>
</tr>
<tr>
<td></td>
<td>1.13 ± 0.08</td>
<td>1.88 ± 0.11†</td>
<td>1.19 ± 0.02</td>
</tr>
<tr>
<td>PMA, 4 hr</td>
<td>2.91 ± 0.28†</td>
<td>1.38 ± 0.37</td>
<td>3.43 ± 0.15†</td>
</tr>
<tr>
<td></td>
<td>1.99 ± 0.44†</td>
<td>1.06 ± 0.22</td>
<td>0.98 ± 0.12</td>
</tr>
</tbody>
</table>

Cells were incubated with the listed agents at the same concentrations and in the same manner as described in the legends to Figures 1 and 2. Serial dilutions of total RNA were applied to nylon membranes using a dot blot apparatus, and hybridization was performed as described in Materials and Methods using probes for PAI-1 or uPA. After exposure to x-ray film, the blots were stripped and rehybridized using a probe for ribosomal RNA. This was repeated three times using three different cell lines. The integrated optical density of each dot was determined using a scanning densitometer and image analysis. Rows of dots in the linear range of exposure were used to calculate percent of control integrated optical density for each of the stimuli, and the values for PAI-1 and uPA were divided by that for ribosomal RNA to normalize for the amount of RNA loaded. Each value represents the mean ± SEM from three separate experiments.

* P < 0.01 versus unstimulated control (ANOVA).
† P < 0.05 versus unstimulated control (ANOVA).
Modulation of urokinase type plasminogen activator (uPA) mRNA accumulation in human retinal pigmented epithelial (RPE) cells and human umbilical vein endothelial cells (HUVEC). After reaching confluence, cells were placed in serum-free medium or serum-free medium containing (A) transforming growth factor-beta (TGF-beta, 200 pg/ml; (B) dexamethasone (2 μM); (C) or tumor necrosis factor-alpha (TNF, 10 ng/ml); (D) thrombin (1 U/ml) or (E) phorbol myristate acetate (PMA; 100 ng/ml). Total RNA was extracted after 4 hr or 24 hr and analyzed by Northern blotting with probes for uPA (upper portion of each panel). The bottom portion of each panel shows the ethidium-bromide-stained agarose gel corresponding to each blot before transfer to demonstrate the amount of RNA loaded in each lane. Controls incubated in medium alone for 4 or 24 hr showed no differences and therefore only the 4 hr controls are shown. For each panel, the lanes contain 10 μg of RNA from: (1) lane 1—RPE control; (2) lane 2—RPE incubated for 4 hr with listed agent; (3) lane 3—RPE incubated for 24 hr with listed agent; (4) lane 4—HUVEC control; (5) lane 5—HUVEC incubated for 4 hr with listed agent; (6) lane 6—HUVEC incubated for 24 hr with listed agent.

Modulation of plasminogen activator inhibitor-1 (PAI-1) in human retinal pigmented epithelial (RPE) cells. After reaching confluence, cells were incubated for 24 hr in serum-free medium containing 100 μCi/ml of [35S]methionine and (1) lane 1—no additions; (2) lane 2—transforming growth factor-beta, 200 pg/ml; (3) lane 3—dexamethasone, 2 μM; (4) lane 4—tumor necrosis factor-alpha, 100 ng/ml; (5) lane 5—thrombin, 1 U/ml; (6) lane 6—phorbol myristate acetate, 100 ng/ml. Immunoprecipitation was performed with an antibody directed against PAI-1 as described in Materials and Methods. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Lane 7 contains an immunoblot of 1 μg of purified PAI-1.

HCVEC in the numbers required for these experiments and since they require special culturing conditions, this difference from RPE would not be eliminated. (2) A substantial amount of in vitro work relevant to neovascularization as been performed using HUVEC. (3) There is currently no information indicating that HUVEC and HCVEC differ in their response to agents that modulate neovascularization in vivo. Therefore, despite possible reservations in using HUVEC as a frame of reference, it is interesting to note that in general, the modulation of PAI mRNA is quite similar in human RPE and a human vascular endothelial cell line and this supports the concept that RPE (like vascular endothelial cells) might participate in the regulation of neovascularization in the outer retina. It is also notable that increases in uPA mRNA are less frequent and less prominent in RPE compared to HUVEC. Unfractionated media conditioned on vascular endothelial cells contains fibrinolytic activity, whereas media conditioned on RPE inhibits fibrinolytic activity. Zymography and our immunoprecipitation results suggest that RPE produce substantial PAI-1 and little uPA. Thus, it appears that RPE may be geared more toward inhibition than stimulation of proteolysis.
Phorbol esters have been demonstrated to enhance proteolytic activity of vascular endothelial cells as well as increase their migration and invasiveness.\textsuperscript{1,2} They have been postulated to play a role in the development of neovascularization.\textsuperscript{1-3,9} Phorbol myristate acetate was the only agent that caused a substantial increase in uPA mRNA in RPE. Because phorbol myristate acetate had very similar effects in RPE and endothelial cells with respect to both PAI-1 and uPA mRNA levels, it is unlikely that RPE would alter the relative balance of proteolysis versus inhibition of proteolysis in the face of this particular stimulus. Tumor necrosis factor and TGF-beta have been demonstrated to stimulate neovascularization in some situations in vivo. In HUVEC, both of these agents caused enhancement of both PAI-1 and uPA mRNA levels.\textsuperscript{14-19} In RPE, TNF and TGF-beta increased PAI-1 mRNA, but not uPA mRNA levels. Thus, for these stimuli RPE might be expected to alter the relative balance of proteolysis versus inhibition of proteolysis in favor of the latter. Dexamethasone has been demonstrated to inhibit choroidal neovascularization in a primate model.\textsuperscript{31} Interestingly, dexamethasone increased PAI-1 mRNA levels and decreased uPA mRNA levels in both vascular endothelial cells and RPE.

Recent clinical observations suggest that the RPE in young patients may function to impede the growth of subfoveal neovascular membranes and promote their involution, and that this function may be impaired in aged RPE.\textsuperscript{7} This possibility is also supported by experimental observations in that laser-induced choroidal neovascular membranes in young primates become engulfed by RPE cells.\textsuperscript{8} The current study supports the concept that the RPE may play a role in the development and regression of choroidal neovascularization by demonstrating that several agents that are known to modulate neovascularization in vivo, alter mRNA levels for PAI-1 and uPA in RPE in vitro. It does not address the issue of the effect on aging on RPE functions, but future investigations in this area are warranted. One potential mechanism by which the RPE could help to limit vascular invasion of the outer retina is to help shift the relative balance of proteolysis vs inhibition of proteolysis in favor of the latter.

**Key Words**

Choroidal neovascularization, retinal pigmented epithelium, vascular endothelium, plasminogen activator inhibitors, urokinase.

**References**


