Perturbations in the Fibrinolytic Pathway Abolish Cyst Formation But Not Capillary-Like Organization of Cultured Murine Endothelial Cells

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Fibrinolytic activity and its relation to morphogenesis was investigated in several transformed murine endothelial cell lines and primary cultures of endothelial cells. Two in vitro systems, fibrin gels and Matrigel (Collaborative Research, Bedford, MA), were used. Fibrin gels model a fibrin-rich extracellular matrix that frequently supports neovascularization in vivo, and Matrigel models the basement membrane surrounding quiescent endothelial cells in vivo. The transformed endothelial cell lines have higher levels of plasminogen activator (PA) mRNA than primary cultures of endothelial cells, and an increased PA-mediated proteolytic activity was correlated with formation of cysts in fibrin gels. Addition of neutralizing anti-u-urokinase antibodies, plasminogen depletion, or addition of a plasmin inhibitor prevented cyst formation. Addition of plasminogen restored the ability to form cysts in the plasminogen-depleted system. Normal endothelial cells organized into capillary-like structures in fibrin gels regardless of manipulations affecting the fibrinolytic pathway. In Matrigel, both transformed and primary cultures of endothelial cells rapidly formed a capillary-like network that was not affected by plasminogen depletion or addition of plasmin inhibitors. Thus, elements of the fibrinolytic pathway necessary for cyst formation are not critical in capillary-like structure formation on a reconstituted basement membrane. These results suggest that plasmin is essential for hemangioma formation but is not critical to the organizational behavior of normal endothelial cells. © 1994 by The American Society of Hematology.

The invasion of cells of one tissue into neighboring tissues occurs during several normal and pathologic processes such as embryonic development, angiogenesis, and the spread of tumors. Invading cells must breach barriers such as the basement membrane and the stalk matrix. In many instances, cells release proteolytic enzymes during invasion. Therefore, the regulation of the proteolytic balance of a cell is associated with invasion. Numerous transformed cell lines have a higher proteolytic activity than their normal counterparts.

Proteolytic balance is modulated in part by the relative production of plasminogen activators (PAs) and their inhibitors. PAs are serine proteases, and their main function is to catalyze the conversion of inactive plasminogen to plasmin. Plasmin degrades fibrin and has specific effects on the integrity of the extracellular matrix. For example, plasmin is able to degrade fibronectin and laminin and to activate latent collagenase. Two molecularly different PAs have been described, the urokinase-type (uPA) and tissue-type (tPA) plasminogen activators. Plasminogen activator activity is implicated in invasion by both tumor and normal cells.

Neovascularization also requires degradation of basement membrane, and endothelial cells produce a number of proteolytic enzymes that degrade basement membranes and extracellular matrices. PA inhibitors are synthesized by all cultured endothelial cells. Rifkin et al. have proposed that PA activators are a subset of serine proteases in this process. The serine proteases are a large class of molecules that includes trypsin, kallikrein, and plasmin.

An endothelial cell line, Py-4-1, was isolated from transgenic mouse hemangiomas induced by expression of the polyoma early region gene. Py-4-1 cells have retained endothelial cell properties such as active uptake of acetylated low-density lipoprotein and expression of von Willebrand factor. Although the cells are contact inhibited and anchorage dependent, they also form hemangiomas when reinjected into host-compatible mice. Because of these properties, this cell line provides a good model to investigate the role of PA-mediated proteolysis in normal angiogenesis and vascular tumor formation.

In this report, we compared the PA-mediated proteolytic activity of Py-4-1 cells with that of other murine transformed endothelial cells and primary cultures of endothelial cells. Py-4-1 cells, as well as three other transformed endothelial cell lines, displayed an increased fibrinolytic activity compared with primary cultures of endothelial cells. Increased PA-mediated proteolytic activity was correlated with formation of cysts by transformed endothelial cells in fibrin gels. Urokinase (uPA) and plasminogen were required in hemangioma-like cyst formation, showing that the fibrinolytic pathway was responsible for cyst formation. In contrast, organization of primary cultures of endothelial cells into capillary-like tubes occurred regardless of manipulations affecting the fibrinolytic pathway. Taken together, these results suggest that the fibrinolytic pathway is important in the organizational behavior of endothelial cells during hemangioma formation but not critical to normal capillary-like organization.

EXPERIMENTAL PROCEDURES

Materials and animals. Heparin, fibrinogen from bovine plasma, thrombin from human plasma, human urokinase, α-2-antiplasmin,
and L-Lysine Sepharose 4B were obtained from Sigma Chemical Co (St Louis, MO). Plasminogen from human serum and aprotinin were purchased from Boehringer Mannheim (Indianapolis, IN). Plasminogen was also obtained from Sal Pizzo (Duke University, Durham, NC). Rabbit antimonuse Urokinase IgG was from American Diagnostica Inc (Greenwich, CT). Endothelial cell growth supplement and basement membrane Matrigel were from Collaborative Research (Bedford, MA). Agar was obtained from DIFCO Laboratories (Detroit, MI). B62F1 mice were purchased from Jackson Laboratories (Bar Harbor, ME).

**Cells.** Py-4-1 cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% newborn calf serum. The End cell lines (bEnd.3 and eEnd.2) were grown in DMEM supplemented with 10% fetal calf serum (FCS). Murine heart endothelial cells (sHMVEC) (for isolation technique, see ref 21), murine endothelioma cells (EOMA) (both gifts from Robert Auerbach, University of Wisconsin, Madison), and Swiss 3T3 mouse cells (3T3) from the American Type Culture Collection (ATCC; Rockville, MD) were grown in DMEM supplemented with 10% FCS. Murine brain endothelial cells (MBE) (gift from Robert Auerbach) were grown in DMEM supplemented with 20% FCS, 20% tumor-conditioned medium (S180 from the ATCC were used to generate the tumor-conditioned medium), nonessential vitamins, L-glutamine at 2 mmoi/L, endothelial cell growth supplement at 50 μg/mL, and heparin at 25 μg/mL.

**RNA isolation and RNA protection assay.** Cytoplasmic RNAs were isolated from confluent cells using urea/sodium dodecyl sulfate (SDS) as described.24 RNAs from mouse tissues were isolated using a modification of the hot phenol method described previously.25 tPA RNA probe was synthesized from a T3 RNA promoter in the pDB4501 plasmid (gift from Erwin Wagner, Institute for Molecular Pathology, Vienna, Austria) were cells (3T3) from the American Type Culture Collection (ATCC; Rockville, MD) were grown in DMEM supplemented with 10% FCS. The End cell lines (bEnd.3 and eEnd.2) were grown in DMEM supplemented with 20% FCS, 20% tumor-conditioned medium (S180 from the ATCC were used to generate the tumor-conditioned medium), nonessential vitamins, L-glutamine at 2 mmoi/L, endothelial cell growth supplement at 50 μg/mL, and heparin at 25 μg/mL.

**Transcription assays.** Cytoplasmic RNA was isolated from confluent cells using urea/sodium dodecyl sulfate (SDS) as described.24 RNAs from mouse tissues were isolated using a modification of the hot phenol method described previously.25 tPA RNA probe was synthesized from a T3 RNA promoter in the pTAM66 plasmid (gift from Sidney Suickland, SUNY at Stony Brook, NY) containing a 680-bp EcoRI-EcoRI fragment of tPA cDNA cloned into the EcoRI site of pT3/T7-18 (Bethesda Research Laboratories [BRL], Bethesda, MD). uPA RNA probe was synthesized from an sp6 RNA promoter in the pDB4501 plasmid (gift from D. Belin and J.-D. Vassalli, CMU, University of Geneva, Switzerland) containing the 660-bp Pst I-HindIII fragment of uPA cDNA clone pDB29 cloned in the cognate sites of pSP64 (Promega, Madison, WI). For in vitro transcription with RNA polymerase, pTAM66 and pDB4501 were linearized with AvaiI and EcoRI, respectively.

**Reverse zymographic assays for PA activity.** Single or composite tissue extracts were subjected to polyacrylamide gel electrophoresis (SDS-PAGE). For reverse zymographic assays, 0.001 U/mL urokinase was also added to substrate gels. Zymograms and reverse zymograms were photographed under dark-field illumination after incubation at 37°C at the times indicated.

**PA plaque assay for PAs.** For detection of PA activity by single cells, a method adapted from that of Goldberg26 was used. Cells were plated at 1 × 10⁴ cells/35-mm dish. Twenty-four hours later, the cells were washed three times with PBS and the overlay mixture (250 μL) was pipetted on the plates and spread rapidly by tilting. The plates were incubated at 37°C in 5% CO₂ for the times indicated, fixed, stained using a solution of 0.2% Coomassie brilliant B in methanol:acetic acid:water (7:1:2), and rinsed 2 hours later with water. The overlay mixture consisted of 1.2% agar, 1.33% commercial instant nonfat dry milk, DMEM, and plasminogen at 0.28 U/mL. Plaques resulting from caseinolysis were counted using phase-contrast microscopy.

**PA measurement.** Heat-inactivated FCS was depleted of plasminogen by passage of 50 mL serum through a 30-mL bed volume L-Lysine-Sepharose 4B column.27 Fractions were determined to be free of plasminogen by the absence of caseinolysis in a substrate gel containing 1% agar, 2.5% commercial instant nonfat dry milk, and 0.001 U/mL urokinase and compared with plasminogen-containing serum.

**Culture of endothelial cells in fibrin gels and on Matrigel.** For incorporation into fibrin gels, cells were harvested from confluent cultures using 3 mmoi/L EDTA-0.5% trypsin, counted, adjusted to the desired concentration, and centrifuged in a plastic tube. Cells (1 × 10⁴) for Py-4-1, MBE, sHMVEC, or 0.8 × 10⁴ cells for bEnd.3, eEnd.2, and EOMA were then suspended in a polymerizing fibrinogen solution prepared as described.18 The mixture was immediately transferred to 24-well culture dishes and allowed to gel for 2 minutes before adding medium. For culture of cells on Matrigel, 250 μL of the reconstituted basement membrane Matrigel28 was applied to a 24-well culture dish and incubated at 37°C for 30 minutes. Cells were washed twice with PBS and resuspended in DMEM. Cells (1 × 10⁴) for Py-4-1, MBE, sHMVEC, and 5 × 10⁴ cells for bEnd.3, eEnd.2, and EOMA) were pipetted onto the gel. For both fibrin gel and Matrigel cultures, the culture medium consisted of DMEM supplemented with 10% FCS under normal conditions, and DMEM supplemented with 10% plasminogen-depleted FCS under depleted conditions. Where appropriate, the medium was supplemented with aprotonin at 1,000 KIU/mL or α-2-antiplasmin at 1 μg/mL, 5 μg/mL, and 20 μg/mL. Two sources of plasminogen were used in experiments involving addition of plasminogen. Commercial plasminogen (Boehringer Mannheim) was used, and a pure preparation of plasminogen (a generous gift from Sal Pizzo) that was purified according to Deutsch and Mertz29 and analyzed for purity on an 11% polyacrylamide gel was also used. Where appropriate, plasminogen from both sources was added at 1.8 μg/mL, 3.6 μg/mL, and 7.2 μg/mL. Cultures were subsequently incubated at 37°C for the times indicated. The culture medium was changed every 3 days with addition of fresh aprotonin, α-2-antiplasmin, or plasminogen where appropriate. The cultures were observed and photographed with phase-contrast optics using an Olympus CK2 inverted microscope (Lake Success, NY). Activity of α-2-antiplasmin was determined using a chromogenic substrate assay (Instrumentation Lab Co, Lexington, MA). In this assay, α-2-antiplasmin is incubated with an excess of plasmin, and the residual plasmin activity is measured on a synthetic chromogenic substrate.

**Antibody-blocking studies.** Py-4-1 cells (1.6 × 10⁴) and 0.8 × 10⁴ bEnd.3 cells were incorporated into fibrin gels in 24-well culture dishes in the presence of neutralizing rabbit antimonose uPA IgG (American Diagnostica Inc, Greenwich, CT) at the indicated concen-
trations. One milliliter of medium supplemented with an identical concentration of anti-tPA IgG was then added in each well. The cultures were fed every 3 days with addition of anti-tPA IgG. At day 5 for bEnd.3 and at day 9 for Py-4-1 cells, 50 structures from random fields were analyzed in each well and scored as cysts or starlike structures. The fields were chosen with special care to represent all areas of the well. Cysts were scored as large three-dimensional balls with no extensions, and starlike structures were flattened with short extensions from a center. Controls consisted of cultures that received an equivalent concentration of normal rabbit serum, rabbit anti-β-galactosidase IgG (Cappel, Durham, NC), and cultures with no additions.

Quantitative analysis of capillary-like tube formation. MBE cells (1.6 × 10⁴) were incorporated into fibrin gels as described above. Capillary-like tube formation was quantitated 4, 5, and 6 days after plating. Cells were washed twice with PBS, fixed in a solution containing 1% glutaraldehyde, 0.1 mmol/L sodium phosphate buffer (pH 7), and 1 mmol/L MgCl₂ for 15 minutes at room temperature, washed twice with PBS, and stored in 70% glycerol. The extent of capillary-like tube formation was judged by scoring the number and length of the tubes formed. In addition, the number of primary branches per tube, the length of primary branches, and the number of secondary branches per primary branch were calculated where appropriate. Because the cells were plated sparsely, the capillary-like structures were easily distinguished from one another. In addition, the primary branches were easily distinguished from the initial tube because of their shorter length. Capillary-like tube formation was quantitated directly from the wells using an Olympus IMT-2 inverted microscope connected to a Cohu 4810 CCD camera (Cohu Inc, San Diego, CA). The measurement process was performed through use of the Image Pro II software (Media Cybernetics, Silver Spring, MD) and the Image Measure software (Phoenix Technology, Federal Way, WA). Capillary-like tubes were analyzed in random fields chosen from each well (triplicate wells were set up for each group). Areas of the wells where the meniscus gave a distortion of optics were not used in scoring.

Electron microscopy. Ultra-thin sections for electron microscopy were processed according to standard procedure. Briefly, cells were washed twice with prewarmed DMEM and fixed in 3% glutaraldehyde in DMEM for 3 hours at room temperature, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Araldite-502 Electron Microscope Sciences, Fort Washington, PA). Ultra-thin sections were cut at 70 nm, stained in 4% aqueous uranyl acetate and 0.5% lead citrate, and observed with a Zeiss 10A Transmission Electron Microscope (Carl Zeiss, Oberkochen, Germany) at a voltage of 80 kV.

RESULTS

PA-mediated proteolytic activity is increased in transformed endothelial cells. We investigated PA-mediated proteolytic activity in Py-4-1 cells and compared it with that of other murine transformed endothelial cells and primary cultures of endothelial cells. The murine transformed cell lines included bEnd.3 and eEnd.2 that were derived from brain endothelial cells and embryonic hemangiomas, respectively. Both were transformed by the polyoma virus middle T oncogene. The EOMA cell line was derived from a spontaneously occurring murine hemangiona. The normal murine endothelial cell lines included MBE and sMHEC, both endothelial cell cultures that were greater than 95% pure.

To determine if Py-4-1 cells had increased levels of PA activity compared with normal endothelial cells, RNase protection assays were used to identify tPA and uPA RNAs on a semi-quantitative basis. Riboprobe constructs containing a portion of the tPA or uPA cDNA (see Experimental Procedures) were transcribed in vitro to generate 32P-labeled antisense RNA probes. tPA mRNA should protect 600 bases of the 670-base tPA probe, and uPA mRNA should protect 465 bases of the 467-base uPA probe. The results presented in Fig 1 show that tPA (Fig 1A-a) and uPA (Fig 1A-b) transcripts were present in all the murine endothelial cell lines. Comparison of labeled protected fragment intensity between the different endothelial cell lines indicates that both tPA and uPA RNA levels were increased in Py-4-1 cells as well as in bEnd.3, eEnd.2 (as described by Montesano et al for uPA), and EOMA cells compared with normal brain endothelial cells (Fig 1A-a and A-b, compare lanes 2 through 6 with 7). The uPA and tPA protected fragments were hardly visible in brain endothelial cells in Fig 1A, although they were present at low levels on the original autoradiograph. Comparison of labeled protected fragments also indicates that uPA levels were increased in Py-4-1 cells to a lesser extent than in the other tumoral cell lines (Fig 1A-b, compare lanes 2 and 3 with lanes 4 through 6). Increased levels of tPA and uPA RNA in Py-4-1 cells were shown not to be passage dependent (Fig 1A-a and A-b, compare lanes 2 and 3).

To determine if the increase in tPA and uPA RNAs in transformed endothelial cells was correlated with an increase in tPA and uPA proteolytic activity, fibrin-agar indicator gels containing plasminogen were used. These gels were used because tPA requires fibrin as a cofactor to display its full proteolytic activity. In cultured endothelial cells, PA proteolytic activity has been found in both cell extract and culture medium. To determine the total PA proteolytic activity in the transformed cell lines, cell extracts and culture media of confluent cells were assayed. In fibrin-agar plasminogen indicator gels, tPA proteolytic activity was found to be extremely low in cell extracts and culture media of all endothelial cell lines including Py-4-1, bEnd.3, eEnd.2, EOMA, MBE, and sMHEC. This low activity could not be photographed under dark-field illumination (data not shown). This result suggests that the increased levels of tPA RNAs in tumoral endothelial cells are not correlated with an increase in tPA proteolytic activity. However, uPA proteolytic activity was shown to be increased in all transformed cell lines compared with primary cultures of endothelial cells (data not shown).

To allow a better comparison of the uPA proteolytic activity among the different endothelial cell lines, different quantities of cell extract and culture medium were used in a zymographic assay rather than a fibrin gel assay. The murine cell lines produced a 48-kD band with activity that corresponds in molecular size to uPA. In cell extracts and culture media of confluent cells, Py-4-1 cells as well as bEnd.3, eEnd.2, and EOMA cells showed increased uPA proteolytic activity when compared with normal endothelial cells (Fig 1B, compare lanes 2 through 5 with lane 6, and data not shown for sMHEC).

Because net proteolytic activity is also determined by PAI activity, reverse zymography was performed. In confluent
Fig 1. (A) RNA protection analysis of tPA and uPA RNA. Each experiment was repeated at least three times for each cell line or tissue. F1 refers to RNA obtained from B6D2F1 mice. Lane 1, Swiss 3T3 cells as a positive control; lanes 2 and 3, Py-4-1 cells at passage 24 and 82, respectively; lane 4, bEnd.3; lane 5, eEnd.2; lane 6, EOMA; lane 7, MBE; lane 8, F1 liver as a negative control; lane 9, F1 brain (a) and F1 kidney (b) as positive controls; lane 10, reaction with no RNA. (a) Autoradiogram of an RNA protection assay using the tPA probe. Fifteen micrograms, or 30 μg for lane 7, of total RNA was used. The probe and fragment corresponding to protection by tPA transcript are indicated in the left margin. Exposure time was 5 days with an intensifying screen at -80°C. (b) Autoradiogram of an RNA protection assay using the uPA probe. Fifteen micrograms, or 30 μg for lane 7, of total RNA was used. The probe and fragment corresponding to protection by uPA transcript are indicated in the left margin. Exposure time was 17 hours with an intensifying screen at -80°C. The size markers indicated in the right margin are pBR322 digested with HpaII. (B) Zymographic analysis of uPA in cell extracts and culture media of confluent cells. Cell extracts and culture media were fractionated by SDS-PAGE and PA activity was localized in casein-agar gels. The dark areas correspond to caseinolysis zones. To facilitate comparison of Py-4-1 cells with the other cell lines, different quantities of samples were used: Py-4-1 cells (lane 2) were used at 1× (corresponding to cell extracts or culture media of 1,000 cells); 3T3 (lane 1) and MBE (lane 6) at 10×; and bEnd.3 (lane 3), eEnd.2 (lane 4), and EOMA (lane 5) at 0.5×. Cell extracts (a) and culture media (b) of confluent cells. Zymograms were allowed to develop for 20 hours. The left margin indicates migration of murine uPA (m uPA). The size markers indicated in the right margin are bovine serum albumin (69 kD) and ovalbumin (46 kD). (C) Reverse zymographic analysis of PAI in confluent cells. Culture media were fractionated by SDS-PAGE and PAI activity was localized on casein-agar gels supplemented with urokinase. The bright areas correspond to zones where caseinolysis was inhibited. To facilitate comparison of Py-4-1 cells with the other cell lines, different quantities of samples were used: 3T3 (lane 1), Py-4-1 (lane 2), bEnd.3 (lane 3), eEnd.2 (lane 4), and EOMA (lane 5) cells were used at 10× (corresponding to conditioned media of 10,000 cells); MBE (lane 6) and sMHEC (lane 7) were at 5×. Reverse zymograms was allowed to develop for 2.5 hours. The left margin indicates migration of murine PAI-1 (m PAI-1). The size markers indicated in the right margin are bovine serum albumin (69 kD), ovalbumin (46 kD), and carbonic anhydrate (30 kD).
The End cells form cysts in fibrin gels. To determine if MBE and sMHEC cells at 6 to 8 hours (Table 1). bEnd.3, proteolytic activity is correlated with an increase in the level than any other endothelial cell lines because plaques were these structures were apparent as soon as 2 days after plating, of lytic activity around individual cells. Plaques of caseinolysis plaques appeared around Py-4-1 (Table 1). Caseinolysis plaques appeared around bEnd.3 and eEnd.2, and EOMA cells were faster in degrading casein (Fig 2A). bEnd.3 and eEnd.2 cells formed cysts in fibrin and sMHEC cells on the original gel. These results indicate that transformed endothelial cells showed little or no increase in PAI compared with primary cultures of endothelial cells.

The net proteolytic activity of Py-4-1 cells was assayed by a substrate overlay procedure that allows for the detection of lytic activity around individual cells. Plaques of caseinolysis were observed after 4 hours of incubation around Py-4-1 cells (Table 1). Caseinolysis plaques appeared around MBE and sMHEC cells on the original gel. These results indicate that transformed endothelial cells showed little or no increase in PAI compared with primary cultures of endothelial cells.

To determine if increased uPA activity was involved in cyst formation, neutralizing antibodies to mouse uPA IgG, cyst formation was inhibited in a dose-dependent manner (Table 2). Cyst formation was completely abrogated at a concentration of anti-uPA IgG of 10 μg/mL for 1.6 × 10^4 Py-4-1 cells and 0.8 × 10^4 bEnd.3 cells initially plated, respectively (Table 2). In both cell lines, cystic structures were replaced by starlike structures. Cyst formation was not affected when an equivalent concentration of normal rabbit serum or rabbit anti-β-galactosidase IgG was added to Py-4-1 and bEnd.3 cell cultures (Table 2).

Table 1. Time Course of Caseinolysis Measured by PA Plaque Assay

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>No. of Plaques/35-mm Dish</th>
<th>(Time in hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3</td>
<td>30</td>
<td>5-20</td>
</tr>
<tr>
<td>Py-4-1</td>
<td>4</td>
<td>500-800</td>
</tr>
<tr>
<td>bEnd.3</td>
<td>0.7</td>
<td>20</td>
</tr>
<tr>
<td>eEnd.2</td>
<td>0.7</td>
<td>10</td>
</tr>
<tr>
<td>EOMA</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>MBE</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>sMHEC</td>
<td>8</td>
<td>70</td>
</tr>
</tbody>
</table>

* The PA plaque assay was performed in triplicate and led to an identical time for each category. Plaque numbers were scored every 20 minutes for the first 8 hours, every 2 hours for the next 10 hours, and then three times a day for 7 days. Controls without plasminogen in the overlay were performed in parallel for each cell line and did not show any plaques after a 7-day incubation.

To determine if serine proteases were involved in the cysts formed by Py-4-1 and EOMA cells, as has been shown for the End cell lines, inhibitors of serine proteases were used. Addition of aprotinin, a serine protease inhibitor of plasmin but not PAs, modified the morphogenetic properties of Py-4-1 and EOMA cells in fibrin gels (Fig 2B). Py-4-1 cells and EOMA no longer formed cysts and the cells organized into starlike structures (Fig 2B), similar to the control bEnd.3 and eEnd.2 cells. Fibrinolysis was not observed with aprotinin as long as 1 month after the initial plating, whereas under normal conditions appearance of cysts was concomitant with fibrinolysis.

Aprotinin has been shown to inhibit both circulating plasmin and fibrin-bound plasmin, so the effect of a more specific circulating plasmin inhibitor, α-2-antiplasmin, was assayed. When plasmin interacts with fibrin, the kinetics of inactivation of plasmin by α-2-antiplasmin are considerably slower. All cells formed cysts in the presence of α-2-antiplasmin, and no difference was observed in the morphogenetic properties of the transformed cells (Py-4-1, bEnd.3, eEnd.2, and EOMA) or the normal murine endothelial cells (MBE and sMHEC) when compared with culture in fibrin gels without the inhibitor (data not shown). α-2-Antiplasmin activity was tested using a chromogenic substrate assay, and was found to be 75% of the normal human plasma α-2-antiplasmin activity (data not shown). These results suggest that either plasmin is not involved in cyst formation or that plasmin formed by PA proteolytic activity was coupled with fibrin in our system so that an inhibitor of circulating plasmin, α-2-antiplasmin, was ineffective. The possibility that the concentration of α-2-antiplasmin used might not have been sufficient to block plasmin activity cannot be excluded.

**Plasminogen is required in cyst formation by transformed endothelial cells in fibrin gels.** Cultures of endothelial cells in fibrin gels were incubated in the presence of medium containing plasminogen-depleted serum to determine if the change in morphogenetic behavior of the cells on addition of aprotinin was the result of its inhibitory effect on plasmin. If an increased plasmin level mediated by an increased PA level is involved in cyst formation, then the inactive zymogen plasminogen should be a limiting component. The transformed cell lines no longer formed cysts in plasminogen-depleted serum. Instead they organized into starlike structures identical to the ones observed after addition of aprotinin (Fig 3A). To confirm that plasminogen depletion of the serum did not eliminate some other component necessary for cyst formation, plasminogen was added back to plasminogen-depleted medium. Both commercial and highly purified plasminogens were able to restore cyst formation (Fig 3B, and data not shown). Cyst formation in both cases was optimal after addition of plasminogen at a concentration...
Fig 2. Morphogenetic behavior of transformed and normal murine endothelial cell lines in fibrin gels under normal conditions (A) and on addition of aprotinin at 1,000 KIU/mL (B). Bar represents 200 μm except for Ae and Be where it represents 100 μm. (A) Py-4-1 (a), bEnd.3 (b), End.2 (c), EOMA cells (d), formed round cysts 7, 3, 3, and 7 days after plating in a fibrin gel, respectively. MBE (e) and sMHEC (f) organized in a network resembling capillaries 7 days after plating in a fibrin gel. (B) Py-4-1 (a), bEnd.3 (b), eEnd.2 (c), EOMA cells (d), formed starlike structures on addition of aprotinin to the culture medium; photographs were taken 7, 3, 3, and 7 days after plating in a fibrin gel, respectively. MBE (e) and sMHEC (f) did not show any modification in their morphogenetic properties upon addition of aprotinin; photographs were taken 7 days after initial plating in fibrin gels.
of 3.6 μg/mL, although the cysts took longer to reach the size of controls. This result shows that plasminogen is required for cyst formation by tumoral endothelial cell lines in fibrin gels. Control experiments showed that addition of plasminogen under normal conditions (nondepleted) did not change the morphogenetic properties of the cells.

Capillary-like tube formation by normal endothelial cells is not abolished by fibrinolytic pathway perturbations. In the presence of aprotinin and in plasminogen-depleted medium, MBE and sMHEC formed capillary-like tubes qualitatively similar to those observed under normal conditions (Fig 2, and data not shown). To determine whether capillary-like tube formation in fibrin gels was quantitatively affected by modifications in the fibrinolytic pathway, morphometric analysis was performed on MBE cultured in fibrin gels at 4, 5, and 6 days after plating. Tube formation was not affected by addition of aprotinin (Table 3). In contrast, statistical analysis showed that the tube length of MBE was slightly shorter at days 5 and 6 in plasminogen-depleted medium (Table 3). However, the number of primary branches per tube and the primary branch length were not affected under the same conditions (Table 3). These results suggest that plasminogen/plasmin has an effect on capillary-like tube length but is not critical for capillary-like tube formation of normal endothelial cells.

Transformed endothelial cells form a capillary-like network on reconstituted basement membrane that is not affected by fibrinolytic pathway perturbations. The role of plasmin-mediated proteolysis in cell behavior on the artificial basement membrane, Matrigel, was investigated. After 24 hours, Py-4-1 cells formed a network of branching tubules on Matrigel that resembled capillary blood vessels (Fig 4B). Electron microscopy of Py-4-1 cells cultured on Matrigel showed that the structures were tubes with a lumen (Fig 4C). All murine endothelial cells including transformed and normal cells formed a similar capillary-like network on Matrigel after 24 hours (Fig 5). After 7 days in culture, the endothelial network formed by each endothelial cell line appeared similar to the one observed after 24 hours (data not shown), suggesting that endothelial cells do not proliferate or proliferate slowly when cultured on Matrigel. Furthermore, cells cultured on Matrigel in the presence of aprotinin, α-2-antiplasmin, or in medium depleted of plasminogen still formed capillary-like networks (data not shown). These results indicate that fibrinolytic pathway perturbations do not qualitatively affect the morphogenetic properties of endothelial cells cultured on Matrigel.

**DISCUSSION**

Py-4-1 cells are derived from transgenic mouse hemangiomas induced by the polyoma virus early region gene. Although they retain numerous normal endothelial cell properties, Py-4-1 cells form hemangiomas when injected subcutaneously into histocompatible mice. The hemangiomas consist of growing blood-filled cavities lined with endothelial cells. Thus, the Py-4-1 endothelial cell line provides a good model to investigate the role of the fibrinolytic pathway in both angiogenesis and vascular tumor formation. In this study, proteolytic activity and morphogenetic behavior of Py-4-1 cells as well as three other murine transformed endothelial cell lines were compared with two primary cultures of murine endothelial cells. Three major findings have emerged from this work. First, uPA and plasminogen/plasmin are responsible for hemangioma-like cyst formation by the transformed endothelial cells in fibrin gels. Second, inhibition or depletion of fibrinolytic pathway components did not qualitatively affect the capillary-like organizational behavior of primary cultures of endothelial cells in fibrin gels. Third, primary cultures and transformed endothelial cells organized into a similar capillary-like network on Matrigel regardless of modifications in the fibrinolytic system components. Taken together, these results support the conclusion that the fibrinolytic system is required in cyst formation, but it is not critical for capillary-like endothelial cell organization.

In vitro systems have recently been developed to model the early events of angiogenesis. Normal cultured endothelial cells can organize into structures resembling capillary blood vessels when grown in three-dimensional collagen or fibrin substrata or on reconstituted basement membrane. Three-dimensional fibrin gels are a good in vitro substratum for studying angiogenesis because deposition of fibrin has been shown in pathologic processes involving angiogenesis.

Py-4-1 and EOMA cells proliferated and formed cysts resembling hemangiomas in fibrin gels. The cysts were similar to those described by Montesano et al for the End cells. In contrast, under similar conditions, primary cultures of endothelial cells divided and organized to form a capillary-like network. Thus, the morphogenetic organization into
FIBRINOLYSIS AND ENDOTHELIAL MORPHOGENESIS

Fig 3. Morphogenetic behavior of murine transformed endothelial cells in fibrin gels with plasminogen-depleted medium (A) and with addition of plasminogen to plasminogen-depleted medium (B). Bar represents 200 μm except for Ad, Bc, and Bd where it represents 100 μm. (A) In plasminogen-depleted medium, Py-4-1 (a), bEnd.3 (b), eEnd.2 (c), and EOMA cells (d) formed starlike structures identical to those described on addition of aprotinin. Photographs were taken 7, 3, 3, and 7 days after plating in fibrin gels, respectively. (B) Py-4-1 (a), bEnd.3 (b), eEnd.2 (c), and EOMA cells (d) formed round cysts 7, 3, 3, and 7 days after plating in fibrin gels, respectively, when plasminogen was added back to the plasminogen-depleted medium at a concentration of 3.6 μg/mL.

cysts was correlated with tumorigenicity of endothelial cells. Cyst formation was also correlated with elevated PA-mediated proteolytic activity18 (and this report). This increase in proteolytic activity resulted from an increase in uPA proteolytic activity that correlated with an elevated uPA RNA level, and was not compensated for by a concomitant increase in PAI activity. The selective increase in uPA proteolytic activity in all four transformed endothelial cell lines is in agreement with the observation that the induced PA found in malignant cells is usually uPA.53

The role of the fibrinolytic system in cyst formation was investigated by adding inhibitors and depleting components involved in the fibrinolytic pathway. Aprotinin, an inhibitor of circulating and fibrin-bound plasmin and other serine proteases, inhibited cyst formation in transformed endothelial cells. However, addition of α-2-antiplasmin, an effective inhibitor of circulating plasmin but a poor inhibitor of plasmin generated on the cell surface,41 did not abolish cyst formation. These data do not exclude a role for other serine proteases, such as trypsin and kallikrein that are also inhibited by aprotinin, in cyst formation.41 Therefore, the precursor of plasmin, plasminogen, was removed. Plasminogen depletion
abolished cyst formation, and addition of highly purified plasminogen restored cyst formation. These data indicate an essential role for plasminogen in cyst formation.

Because plasminogen has no known biologic function, it is likely that the requirement for plasminogen in cyst formation reflects a requirement for plasmin. Plasmin is active in converting pro-uPA to active uPA and single-chain tPA to active double-chain tPA, so addition of plasmin inhibitors may affect the levels of uPA and/or tPA. Although this possibility has not been directly tested in the PA proteolytic assays, plasmin was not present and a dramatic increase in uPA was demonstrated in all transformed cell lines. Therefore, it is unlikely that the effects of plasmin on cyst formation are mediated through its effects on PA levels. These data fit a model in which elevation of PA-mediated proteolytic activity triggers an increase of plasmin that is responsible for cyst formation via proteolysis of fibrin. The ability of neutralizing uPA antibodies to inhibit cyst formation in a dose-dependent manner further supports the importance of the fibrinolytic pathway in cyst formation by tumoral endothelial cells. The antibody blocks the proteolytic activity of uPA, and the only known substrate of uPA is plasminogen, so it is likely that the conversion of plasminogen to plasmin is prevented in the presence of this reagent.

The manipulations of the fibrinolytic system that prevented cyst formation did not prevent tube formation of primary cultures of endothelial cells in fibrin gels. This result suggests that normal morphogenetic behavior of endothelial cells is independent of the fibrinolytic system. However, it cannot be excluded that a residual fibrinolytic activity is sufficient to support capillary-like tube formation. Other assay systems support the hypothesis that capillary-like endothelial cell organization is independent of the fibrinolytic system. First, inhibitors of plasmin do not inhibit movement of bovine aortic endothelial cells. Second, stimulated bovine capillary endothelial cells can invade a fibrin matrix

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**Table 3. Analysis of Capillary-Like Tube Formation of MBE in Fibrin Gels Under Normal Conditions and Conditions Affecting Fibrinolysis**

<table>
<thead>
<tr>
<th>Day 4</th>
<th></th>
<th>Regular Medium</th>
<th>Regular Medium + Aprotinin</th>
<th>Plasminogen-Depleted Medium</th>
<th>Plasminogen-Depleted Medium + Aprotinin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of structures*</td>
<td>3</td>
<td>30 ± 3 t</td>
<td>31 ± 3</td>
<td>31 ± 4</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>Tube length (µm)</td>
<td>36</td>
<td>231 ± 56</td>
<td>213 ± 62</td>
<td>225 ± 79</td>
<td>234 ± 66</td>
</tr>
<tr>
<td>Day 5</td>
<td></td>
<td>Regular Medium</td>
<td>Regular Medium + Aprotinin</td>
<td>Plasminogen-Depleted Medium</td>
<td>Plasminogen-Depleted Medium + Aprotinin</td>
</tr>
<tr>
<td>No. of structures*</td>
<td>3</td>
<td>31 ± 3</td>
<td>31 ± 2</td>
<td>31 ± 2</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>Tube length (µm)</td>
<td>36</td>
<td>477 ± 147</td>
<td>451 ± 143</td>
<td>395 ± 108 t</td>
<td>415 ± 127 t</td>
</tr>
<tr>
<td>No. of primary branches/tube</td>
<td>36</td>
<td>4.8 ± 1.3</td>
<td>5.0 ± 1.4</td>
<td>4.8 ± 1.3</td>
<td>5.0 ± 1.2</td>
</tr>
<tr>
<td>Primary branch length (µm)</td>
<td>177-182</td>
<td>71 ± 55</td>
<td>70 ± 53</td>
<td>80 ± 65</td>
<td>73 ± 56</td>
</tr>
<tr>
<td>Day 6</td>
<td></td>
<td>Regular Medium</td>
<td>Regular Medium + Aprotinin</td>
<td>Plasminogen-Depleted Medium</td>
<td>Plasminogen-Depleted Medium + Aprotinin</td>
</tr>
<tr>
<td>No. of structures*</td>
<td>3</td>
<td>31 ± 3</td>
<td>32 ± 4</td>
<td>31 ± 3</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Tube length (µm)</td>
<td>30</td>
<td>912 ± 282</td>
<td>813 ± 224</td>
<td>776 ± 156 t</td>
<td>794 ± 198 t</td>
</tr>
<tr>
<td>No. of primary branches/tube</td>
<td>30</td>
<td>7.4 ± 1.6</td>
<td>8.1 ± 1.8</td>
<td>8.1 ± 1.4</td>
<td>8.1 ± 1.7</td>
</tr>
<tr>
<td>Primary branch length (µm)</td>
<td>221-254</td>
<td>164 ± 132</td>
<td>137 ± 96 t</td>
<td>152 ± 100</td>
<td>151 ± 99</td>
</tr>
<tr>
<td>No. of secondary branches'/primary branch</td>
<td>221-254</td>
<td>1.4 ± 1.4</td>
<td>1.4 ± 1.4</td>
<td>1.6 ± 1.6</td>
<td>1.6 ± 1.6</td>
</tr>
</tbody>
</table>

* A structure is defined as a tube on day 4, and a tube and its branches on days 5 and 6.
† Results are expressed as the mean ± SD. Every data point is the result of the analysis of three different wells set up under identical conditions.
‡ Different from control at the 95% confidence level using the Student’s t-test.
§ Different from control at the 90% confidence level using the Student’s t-test.
∥ A secondary branch initiates from a tube primary branch.

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Fig 4. Organization of Py-4-1 cells cultured on gelatin coated plate (A) or on Matrigel (B). The cells formed a confluent monolayer with a cobblestone appearance on gelatin (A), whereas on Matrigel, Py-4-1 cells organized into a capillary-like network (B) that is characterized by branching tubules with a lumen (C). (C) Electron microscopy of Py-4-1 cells plated on Matrigel after 5 days.
Fig 5. Morphogenetic behavior of murine-transformed and normal endothelial cell lines in Matrigel under normal conditions. Py-4-1 (a, b), bEnd.3 (c, d), eEnd.2 (e, f), EOMA (g, h), BEC (i, j), and sMHEC (k, l) organized into a similar network resembling capillaries 20 hours after plating on Matrigel. Bar represents 200 μm except for b, d, f, h, j, and l, where it represents 100 μm.

In vivo endothelial cells are surrounded by basement membranes composed of several extracellular matrix components. Matrigel, a reconstituted extract of the Engelbreth-Holm-Swarm sarcoma basement membrane, was used to study the organizational behavior of endothelial cells in vitro. The transformed endothelial cells formed a network of capillary-like structures similar to that of primary cultures of endothelial cells, suggesting that the increased PA proteolytic activity of the transformed cells does not affect capillary network formation on Matrigel. This hypothesis is supported by the finding that perturbations of the fibrinolytic pathway...
that affected cyst formation did not affect the organizational behavior of the endothelial cells on Matrigel. These data do not rule out the possibility that increased PA proteolytic activity is incompatible with normal organization of endothelial cells, and that PA levels of transformed cells are down-regulated on Matrigel to allow formation of a capillary-like network. Analysis of the PA proteolytic activity of the different cells on Matrigel will clarify the effect of this pathway on normal organizational behavior.

This study suggests that increased plasmin formation mediated by abnormal PA activity is essential for the abnormal organizational behavior of endothelial cells participating in hemangioma formation. Because cyst formation occurs only in fibrin gels and not in Matrigel, it may be that fibrin is a necessary component for cyst formation. The elevated fibrinolysis of transformed endothelial cells may be responsible for the non-directional organization of the cells represented by cyst formation. When fibrinolysis is inhibited, the same endothelial cells grow in a more organized and directional manner. Alternatively, it may be that fibrin does not play a role in cyst formation, but that some other substrate of plasmin that is produced by the endothelial cells is important. However, preliminary experiments indicate that the Py-4-1 endothelial cells do not form cysts in a collagen base matrix (T. Dubose, N. Dubois-Stringfellow, and V.L. Bautch, unpublished results, June 1993). This result suggests that the activation of latent collagenase by plasmin is not responsible for cyst formation and that fibrin is an important component in cyst formation.

The role of the fibrinolytic pathway in cyst formation is relevant to an understanding of tumorigenesis because tumor progression may be facilitated by fibrin deposition and/or fibrinolysis in the local environment. For example, vascular injury that leads to fibrin deposition in the polyoma transgenic mice may initiate tumor formation by endothelial cells that are expressing the polyoma oncogene. A prediction of this model is that perturbations of the fibrinolytic system may affect tumorigenesis, and breeding polyoma transgenic mice with transgenic mice overexpressing uPA may affect hemangioma formation. Fibrin deposition around tumors may also act as a progression factor. If this is the case, blocking fibrin formation and/or fibrinolysis around tumors may affect tumorigenesis. The Py-4-1 cell-induced hemangiomas provide an in vivo system to investigate the effects of fibrinolytic perturbations on tumor progression.

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Perturbations in the fibrinolytic pathway abolish cyst formation but not capillary-like organization of cultured murine endothelial cells

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