Tumor Development Is Retarded in Mice Lacking the Gene for Urokinase-Type Plasminogen Activator or Its Inhibitor, Plasminogen Activator Inhibitor-1

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ABSTRACT

In vivo tumor progression in mice with targeted deficiencies in urokinase-type plasminogen activator (UPA\(^-/-\)) and its inhibitor, plasminogen activator inhibitor-1 (PAI-1\(^-/-\)), was studied using a fibrosarcoma tumor model. Murine T241 fibrosarcoma cells were s.c. implanted into three groups of mice with the following genotypes, wild-type (WT), UPA\(^-/-\), and PAI-1\(^-/-\). A significantly diminished primary tumor growth in UPA\(^-/-\) and PAI-1\(^-/-\) mice occurred, relative to WT mice. Tumors in UPA\(^-/-\) and PAI-1\(^-/-\) mice displayed lower proliferative and higher apoptotic indices and displayed a different neovascular morphology, as compared with WT mice. These results are consistent with the decreased growth rates of this tumor in these gene-deleted mice. Immunohistochemical analyses of the tumors revealed a decrease in vascularity and vascular endothelial growth factor expression only in tumors in PAI-1\(^-/-\)/mice. Analyses of the relative extents of corneal angiogenesis in these same animals, induced by basic fibroblast growth factor, corroborated the resistance of PAI-1\(^-/-\) mice to neovascularization. The results obtained suggest that the host fibrinolytic system plays an important role in tumor growth in this model. Alterations in host expression of components of this system may alter tumor growth and dissemination by affecting the balance between tumor cell death and proliferation, as well as extracellular matrix changes needed for invasiveness and angiogenesis.

INTRODUCTION

Activation of the fibrinolytic system results from the proteolytic conversion of plasma Pg\(^+\) to the serine protease, Pm. uPA, itself a serine protease, is one of the enzymes that catalyzes this process via cleavage of the Arg\(^{550}\)Val\(^{562}\) peptide bond in Pg. PAI-1 is one natural serpin-type inhibitor of this process that functions through its ability to directly inhibit uPA (1). The localization of Pm activity at the site of a developing thrombus, as well as the short-term protection of Pm and Pg activators from circulating inhibitors, are critical events that regulate the rate of clot dissolution (2).

In addition to their roles in clot dissolution, components of the fibrinolytic system, including Pg, uPA, uPAR, PAI-1, and tPA have been shown to be important for other processes, such as ECM degradation, in some cases via activation of MMPs (3), wound healing (4), atherosclerosis (5), angiogenesis (6), and cancer (7). In this latter case, observations that neoplastic cells express elevated levels of uPA, tPA, PAI-1, and uPAR suggest that these proteins may be involved in tumor growth, metastasis, and angiogenesis (8–11). Angiogenesis-dependent tumor progression (12) has also been associated with interaction of integrins with uPA and uPAR, thus linking cellular proteolysis and ECM interactions (13). Confirming the importance of fibrinolytic components and the cancer phenotype, numerous clinical studies have demonstrated that high levels of uPA (14–17) and PAI-1 (18, 19) are associated with a poorer prognostic outcome in certain human cancers, and recent data show a similar correlate with the receptor for uPA, uPAR (20–22).

On a molecular level, uPA/uPAR complex formation on the surface of tumor cells can focus Pm-catalyzed proteolysis, which in turn can facilitate tumor cell invasion and metastasis by either directly degrading ECM proteins or indirectly performing this same function via activation of MMPs. Immunohistochemical studies of primary tumors and metastasis of Lewis lung carcinoma cells using uPA antibodies have demonstrated intense immunoreactivity at the sites of invasive growth of the tumor and degradation of normal tissue (23). Thus, although there appears to be a correlation between enhanced expression of components of the fibrinolytic system and tumor growth and metastasis, the specific mechanisms that underlie the contributions of this system to neoplasia are unclear. In an attempt to illuminate some of these issues, the current study used a fibrosarcoma model in WT, UPA\(^-/-\), and PAI-1\(^-/-\)/C57BL/6J mice to directly assess the role of host expression of these proteins in tumorigenesis. The choice of this tumor model was governed by its syngenicity with, and rapid growth characteristics in, the host C57BL/6J mice; its high degree of vascularization; its expression of high levels of the angiogenic factor, VEGF; and its predictable route of metastasis. A summary of the results of this investigation is presented herein.

MATERIALS AND METHODS

Mice. UPA\(^-/-\), PAI-1\(^-/-\), and WT mice, 5–6 weeks of age, in a C57BL/6J genetic background (at least eight times back-crossed into this strain), were used for this study. All animals were maintained in accordance with the NIH guide for the care and use of laboratory animals, and experimental protocols were approved by the Institutional Animal Care and Use Committee.

Cell Culture. The C57BL/6J-derived murine fibrosarcoma tumor cell line, T241, was grown in McCoy’s modified with l-glutamine (RPMI 16290) containing essential vitamins, 10% fetal bovine serum (Life Technologies, Rockville, MD), and antibiotics. These cells were maintained in a humidified incubator at 37°C, 5% CO\(_2\).

Implantation of Tumor Cells in Mice. At confluency, adherent cells were detached by incubation with 0.25% trypsin (Hyclone, Rockville, MD). The cells were centrifuged and then resuspended in a sterile solution of PBS at a final concentration of about 1.0 × 10\(^6\) cells/ml. A 100-μl aliquot of resuspended cells (about 1.0 × 10\(^6\) cells) was injected s.c. between the shoulder blades ~3 cm from the tail. Visible and small palpable tumors were measured using a digital caliper (MyCal; Thomas Scientific, Swedesboro, NJ). Tumor volumes were calculated using the formula: (width\(^2\) × length) / 2.5, as described (24).

RT-PCR. Total RNA was isolated from tumors implanted in WT, UPA\(^-/-\), and PAI-1\(^-/-\) mice and from control WT livers, kidneys, intestines, spleen, and testes. Additionally, total RNA was obtained from T241 fibrosarcoma cells grown in culture. The UltraSpec RNA isolation system (Biotech, Houston, TX) was used in each case for the first strand cDNA synthesis, which was carried...
Histology and Immunohistochemistry. Mice were sacrificed 3 weeks after tumor implantation. Tumors were excised and fixed overnight in 4% formalin in PBS. Mouse tissues were processed, embedded in paraffin, and for the mouse cornea studies, whole eyes were bisected longitudinally posterior to the lens after a 10-min fixation in 10% neutral buffered formalin. The frontal aspect was fixed for an additional 2 h and then routinely processed and embedded in paraffin. The sections were then preincubated with 10% normal horse serum (20% normal rabbit serum, 10% normal goat serum, or 3% bovine serum albumin), deparaffinized, and rehydrated. Following this, the slides were coated slides, deparaffinized, and rehydrated. After washing, the slides and allowed to incubate for 1 h. Detection of the antibody was accomplished with the chromogen, 3-amino-9-ethylcarbazole (Vector, Burlingame, CA), conjugated to horseradish peroxidase, was applied to the sections. Staining with H&E.

TUNEL. The TUNEL assay was performed according to a published method (25). Tissue sections were incubated with 5 μg/ml of proteinase K for 15 min at room temperature to remove proteins. Sections were then covered with a buffer containing 30 μM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, and 1 mM CoCl2. An aliquot of 0.2 μl of terminal deoxynucleotidyl transferase (Boehringer Mannheim, Gaithersburg, MD) and 10 μM biotinylated dUTP (Boehringer Mannheim) were added to the sections. The slides were incubated in a humidified chamber at 37°C for 60 min, washed with 50 mM Tris-HCl (pH 7), and finally with PBS (0.06 M sodium phosphate/0.15 M NaCl, pH 7.3). The sections were then incubated for 30 min with ABC system, and DAB was used as the chromogen.

Analysis and Scoring. MVCs were determined by operational modifications of a published method (26). MVCs were calculated by analyzing vWF-stained sections. Sections were scanned at low magnification (×100); three areas per section were considered to have the highest microvessel densities within the tumor, and adjacent peripheral tissues were defined as hot spots. Each hot spot was examined at high magnification (×400), and a computer-digitized image was taken and used for counting the vessels. The mean of the MVCs of three hot spots was designated as the total MVCs/hot spot. Two investigators blinded to the genotype of the animal analyzed the same samples. Three microscopic fields/slide were counted.

Cell proliferation was determined by counting the number of BrdUrd-positive cells. The results were expressed as the percentage of the total number of tumor cells within a field. The apoptotic index was obtained by dividing the number of apoptotic cells by the total number of cells counted within a field and then multiplying the results by 100.

Electron Microscopy. Ultrastructural analyses were performed on tumors from WT, as well as UPA+/− and PAI-1−/− mice. The tumors were fixed with Karnovsky solution (27), rinsed twice with a solution of 0.1 M sodium cacodylate (pH 7.3), postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol solutions, and then embedded in epoxy resins (Polysciences, Warrington, PA). Thick sections (0.5 μm), stained with toluidine blue, were used to choose the area of interest, after which corresponding ultrathin sections (90 nm) were cut and stained in 2% uranyl acetate and Reynold’s lead stain (28). Sections were viewed and photographed using a transmission electron microscope (Hitachi H600, Tokyo, Japan) at 75 KV accelerating voltage.

Table 1. Primers used for RT-PCR

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<th>K1-3 fragment of murine Pg</th>
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Fig. 1. Primary tumor growth of T241 fibrosarcoma is altered in UPA+/− and PAI-1−/− mice. The tumors were fixed with Karnovsky solution (27), rinsed twice with a solution of 0.1 M sodium cacodylate (pH 7.3), postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol solutions, and then embedded in epoxy resins (Polysciences, Warrington, PA). Thick sections (0.5 μm), stained with toluidine blue, were used to choose the area of interest, after which corresponding ultrathin sections (90 nm) were cut and stained in 2% uranyl acetate and Reynold’s lead stain (28). Sections were viewed and photographed using a transmission electron microscope (Hitachi H600, Tokyo, Japan) at 75 KV accelerating voltage.
of xylazine, 0.0675 mg of ketamine, and 0.00225 mg of ACE prozamine/g weight of animal. A corneal pocket was made in one eye by a transverse incision, centrally, halfway through the cornea. The pocket was ~1 mm from the limbic vessel toward the corneal center (29). Hydron-coated pellets of sucrose aluminum sulfate containing 100 ng of bFGF were placed in the corneal pocket. On days 3 and 7, bFGF-induced corneal angiogenesis was evaluated using a slit-lamp ophthalmology scope (Nikon) and photographed with a digital camera (Olympus DP-10). Through the use of a 180° reticule in the slit-lamp, two measurements were made: maximal vessel length (mm) and neovascular area (mm²) was calculated as described (29, 30).

Statistical Analysis. Statistical analyses of the tumor growth measurements and immunohistochemistry scores were performed with nonparametric ANOVA and Fisher’s test. The calculations were performed using the StatView system for Macintosh (Abacus Concepts, Berkeley, CA). P < 0.05 was considered significant. Where appropriate, values were expressed as the mean ± SE.

RESULTS

T241 Fibrosarcoma Tumor Cells Express Fibrinolytic Proteins in Vitro. This investigation was focused on the role of uPA and PAI-1 in tumor growth and dissemination by use of UPA- and PAI-1-deficient mice in which the murine fibrosarcoma tumor cell line, T241, had been implanted. This is a rapidly growing hypervascular metastatic tumor that offers advantages in studying tumor angiogenesis. Analyses of the expression of these components of the fibrinolytic system were performed by RT-PCR on cultured cells (not shown), mRNA for uPA, PAI-1, u-PAR, and tPA was observed in this cell line. However, Pg was detected only in the liver control and not in the fibrosarcoma cell line.

Tumor Growth Suppression in uPA- and PAI-1-deficient Mice. To compare tumor progression in UPAn−/− and PAI-1−/− mice relative to their WT counterparts, equivalent amounts of T241 fibrosarcoma cells were injected, s.c., in all animals. The sizes of the primary tumors were measured weekly. All of the WT mice grew tumors, which appeared earlier and were larger in mass relative to those in the UPAn−/− and PAI-1−/− mice; some of which never grew tumors (Fig. 1A). These differences proved to be statistically significant by the third week (Fig. 1B). After 3 weeks, the tumor-bearing mice were sacrificed, and the primary tumors were removed. Tumors from WT mice (Fig. 2A) were consistently larger in mass than the tumors of the UPAn−/− and PAI-1−/− mice and were also more poorly delineated and hemorrhagic (Fig. 2A). Tumors that grew in UPAn−/− and PAI-1−/− mice were small, well circumscribed, and easy to dissociate from host tissue (Fig. 2B). Masson’s Trichrome staining of the tumors extracted from UPAn−/− and PAI-1−/− mice (Fig. 2, D and E) showed an increase in collagen deposition, predominantly in the periphery of the tumor, which appeared to form a fibrous and thick pseudocapsule. This pseudocapsule was almost undetectable in the tumors of WT mice (Fig. 2C).

Decrease and Delay of Metastasis in UPAn−/− and PAI-1−/− Mice. A variety of organs from tumor-bearing mice were examined histologically for metastasis. Metastases in WT animals were only detected in lung and brain (Fig. 2, F and G). Although almost all of the WT mice displayed these metastases, only a very small number of UPAn−/− and PAI-1−/− mice, i.e., those that survived for longer periods (2 months) after the injection, had evidence of metastasis to these same locations. Because the numbers of gene-deficient animals that grew tumors were so small and had significantly aged during this period, the only organs that were examined were lung and brain. Metastases in UPAn−/− and PAI-1−/− organs (Fig. 2, D and E) were significantly smaller, well delineated, and demonstrated fewer hemorrhagic areas than tumors from WT mice. Masson’s Trichrome staining demonstrated increased collagen deposition in UPAn−/− (D) and PAI-1−/− (E) mice (×100 and ×400, respectively). A thick pseudocapsule was present around the tumors in the gene-deficient mice that was not as evident in tumors of WT mice (×100). All magnifications are original values. H&E staining of tissues from WT mice indicated that metastasis occurred mainly in the lung (F) and brain (G).
Expression of Fibrinolytic Factors by Implanted Fibrosarcoma Tumors in WT, UPA+/−, and PAI-1−/− Mice. Some of the tumors excised from the mice were immediately frozen in liquid nitrogen, and RT-PCR was performed using the same probes as that described for RT-PCR of cultured cells. All tumors were found to express the same fibrinolytic proteins as observed in the cultured cell line (Fig. 3).

Proliferation and Apoptotic Indices in Tumor Tissue. To investigate the effects of uPA and PAI-1 deficiencies on tumor cell proliferation, mice were injected with BrdUrd. A higher percentage of BrdUrd-positive tumor cells were observed in WT mice (30.05 ± 6.72%, n = 4), as seen in Fig. 4, A and D, whereas only scattered BrdUrd-positive cells were detected in the periphery of the tumors in UPA+/− and PAI-1−/− mice (4.07 ± 1.82, n = 3, and 2.20 ± 0.47, n = 3, respectively), as is evident in Fig. 4, B–D.

The effects of deficiencies of uPA and PAI-1 on tumor apoptosis were determined by TUNEL analysis. A lower apoptotic index was observed in WT mice compared with UPA+/− and PAI-1−/− mice (0.212 ± 0.105, n = 4, for WT versus 2.23 ± 0.35, n = 3, and 3.98 ± 0.38, n = 3, respectively), as illustrated in Fig. 5, A–C. Apoptotic nuclei in the tumors were scattered and located in the surrounding tissue of the tumors in WT mice, whereas in UPA+/− and PAI-1−/− mice, they were located within the tumor and less so in the surrounding tissue and pseudocapsule. Central necrosis was observed in all of the mice, and some distinct apoptotic nuclei were observed in these areas. The apoptotic indices were significantly different between the UPA+/− and PAI-1−/− mice, relative to WT mice (Fig. 5D).

Effects of Deficiencies of UPA and PAI-1 in Vascularity and VEGF Expression in Implanted Fibrosarcoma Tumors. Tumor sections were stained for vWF expression and were analyzed by two investigators blinded to the genotypes. A significant decrease in vascular density (intratumor and peripheral tissue) was observed in PAI-1−/− mice (Fig. 6, A–C). Vessels were scattered or not evident at
all in PAI-1<sup>−/−</sup> mice. Tumors in UPA<sup>−/−</sup> mice demonstrated vascular densities (26.7 ± 1.7, n = 3) approximately the same as for tumors in WT mice (23.5 ± 4.8, n = 4; Fig. 6G). However, tumors in PAI-1<sup>−/−</sup> mice showed significantly lower vascular densities (4.0 ± 2.1, n = 3) when compared with tumors in WT or UPA<sup>−/−</sup> mice (Fig. 6G). Positive vessels were localized both in the periphery of the tumor as well as in the center of the tumors close to necrotic areas (Fig. 6, A–C). Because vascularity is not necessarily related to angiogenesis, immunohistochemical analysis of the VEGF expression in these tumors was performed (Fig. 6, D–F). In general, VEGF expression was higher in the center and periphery of the tumors. A few cells in necrotic areas were also positive for VEGF. VEGF staining was intense in the WT mice and moderate in UPA<sup>−/−</sup> mice (Fig. 6, D and E). A decrease in intensity and number of positive cells was observed in tumors from PAI-1<sup>−/−</sup> mice (Fig. 6F) and no increase in staining intensity was observed in the periphery of these tumors.

Fig. 5. Apoptosis is enhanced in UPA<sup>−/−</sup> and PAI-1<sup>−/−</sup> mice. Immunochemical TUNEL analyses indicated more apoptotic cells (arrowheads) in tumors from UPA<sup>−/−</sup> (×200; B) and PAI-1<sup>−/−</sup> (×200; C) mice, compared with WT mice (×200; A). D, cell counts showed significantly higher apoptotic indices in the tumors of UPA<sup>−/−</sup> (n = 3, red column) and PAI-1<sup>−/−</sup> (n = 3, blue column) mice, relative to WT mice (n = 4, black column). The following Ps were calculated for the different mouse groupings: P = 0.0035 for PAI-1<sup>−/−</sup>/UPA<sup>−/−</sup>; P < 0.0001 for PAI-1<sup>−/−</sup>/WT; P = 0.007 for UPA<sup>−/−</sup>/WT. Bars, SE.

Fig. 6. Angiogenesis is diminished in PAI-1<sup>−/−</sup> mice. vWF immunostaining indicated that there were more vessels (brown) in WT (×200; A) and UPA<sup>−/−</sup> (×200; B) mice than in PAI-1<sup>−/−</sup> (×200; C) mice. VEGF expression was also more intense in tumors from WT mice (×400; D), moderate in UPA<sup>−/−</sup> mice (×400; E), and weaker in PAI-1<sup>−/−</sup> mice (×400; F). G, mean values of total vessel count (tumor and peripheral host tissue) substantiated the significantly lower number of vessels in PAI-1<sup>−/−</sup> mice (n = 3, blue column) compared with WT (n = 4, black column) and UPA<sup>−/−</sup> (n = 3, red column) mice. The number of total vessels in PAI-1<sup>−/−</sup> mice was significantly lower when compared with tumor-bearing WT (P = 0.0225) and UPA<sup>−/−</sup> (P = 0.0009) mice. Bars, SE.
Fig. 7. Tumor neovessels are abnormal in UPA−/− and PAI-1−/− mice. Ultrastructural analysis of fibrosarcoma tumor vessels in mice with deficiencies of PAI-1 and UPA genes. Tumor vessels from WT mice, A, a lumen (+) formed by three endothelial cells and a prominent collagenous interstitium is seen with expanding cellular processes from the endothelial cells (×3,700). Also, pericyte precursors (D, arrow) near the vessels can be found (×3,000). B, vessels in tumors from UPA−/− mice have a large lumen (+), formed by three or more cells, without support from collagenous matrix (×4,600). Open spaces are visible in adjacent cells, and pericyte precursors are not observed. The small cytoplasmic area, with pseudopodia-like projections, is noted with respect to the luminal area. Cellular junctions (arrows) between endothelial cells and tumors are seen (E, ×23,600). C and F, tumor vessels from PAI-1−/− mice contain matrix and pericyte precursors (C, arrow) but are substantially smaller in size than vessels in tumors from WT mice. Short cellular processes project toward the lumen (+) and periphery. Active exocytosis of vessels into the lumen (C, *) is observed. F, tumor cells surround the vessel. C, ×6,200; F, ×3,200.

TEM Analysis of Vessels. Photographs of TEM analysis of the ultrastructures of fibrosarcoma tumor neovessels are shown for WT mice (Fig. 7, A and D) for UPA−/− mice (Fig. 7, B and E), and for PAI-1−/− mice (Fig. 7, C and F).

In the case of tumors derived from WT mice, the main vessel represented (Fig. 7A) consists of three or more endothelial cells with large cytoplasmic processes directed toward the periphery of the vessel wall. A collagenous interstitium is present around the vessels (Fig. 7A), and pericyte precursors with moderate differentiation are seen without foot processes near the endothelial layer or surrounding the vessel (Fig. 7D). These cells have high physiological activity and numerous organelles.

On the other hand, in the case of UPA−/− mice (Fig. 7B), the vessel contains two to three endothelial cells forming the lumen (Fig. 7B), which are larger than those found in tumors from the WT mice. Endothelial cells contained intracytoplasmic vacuoles, suggestive of sprouting and neolumen formation (new lumens in these cells could form by fusing these spaces and generating new branches). Closely packed perivascular tumor cells were seen surrounding thin-walled vessels with no identifiable basement membrane. Cellular junctions, or close cellular contacts, are formed between endothelial cells and these tumor cells (Fig. 7E). Pseudopodia-like structures from the endothelial cells appear along the lumen, and no pericyte precursors were observed. With regard to PAI-1−/− mice, the vessels formed contain small lumens, formed by one to two endothelial cells with numerous invaginations and pseudopodia-like extensions (Fig. 7, C and F). A collagenous interstitium is present, and pericyte precursors next to the vessels are noted.

**Corneal Micropocket Assays.** Neovascularization was further analyzed using a corneal micropocket model. Growth of new capillary vessels from the corneal limbus toward the bFGF pellet was observed in WT animals. Similarly, corneal vascularization was also present in UPA−/− mice. However, as observed in the tumors, PAI-1-deficient mice showed a decrease in vessel growth, and these vessels were considerably shorter than those in UPA−/− mice (Fig. 8, A–C). When areas of neovascularization were calculated in each group, a significant reduction was only observed in PAI-deficient mice (Fig. 9A).

Immunohistochemical support for this conclusion is also seen in Fig. 9, B–D. After a time of 7 days after implantation of the bFGF pellet, vWF immunostaining of corneal sections of WT (Fig. 9B) and UPA−/− (Fig. 9C) mice revealed extensive neovascularization, whereas PAI-1−/− animals (Fig. 9D) showed far less vWF staining, suggesting, in concert with the data of Figs. 8 and 9A, that bFGF-induced neovascularization is greatly attenuated in PAI-1-deficient animals.

**DISCUSSION**

Cell surface expression of specific receptors for Pg and its physiological activators implicate Pm expression in cell-mediated proteolytic processes, such as ECM degradation and directional cell migra-
The present study focused on the contribution of host uPA and PAI-1 in tumor growth and dissemination. To this end, WT, UPA−/−, and PAI-1−/− mice were implanted with the murine fibrosarcoma, T241, and morphological and histological studies were performed to assess alterations in proliferation, cell death, and vascularization of the tumor in these gene-deficient mice, relative to WT mice. The angiogenic capabilities of these same mice, in the absence of tumor burden, were also investigated.

Analyses of the expression of fibrinolytic proteins demonstrated expression of a number of these proteins both in vitro and in vivo. However, ELISA-based analyses indicated a lack of an immune response to these proteins in the relevant gene-deleted mouse. Therefore, the inability of the fibrosarcoma cells to effectively grow in UPA−/− and PAI-1−/− mice relative to WT mice, although these tumors express many of the components of the fibrinolytic system, indicates that host expression of these proteins plays a major role in the growth and dissemination of this tumor.

Other studies have appeared using different model systems that considered the role of components of the fibrinolytic system in tumorigenesis and metastasis. It was concluded that host PAI-1 expression had minimal effects on growth and metastasis of B16 cells (34) in mice, and that elimination of the PG gene slightly influenced tumor size but did not significantly affect lung metastasis in a murine model of Lewis Lung carcinoma (35). On the other hand, there is evidence for a role for uPA in progression of malignancy in a model of cutaneous melanocytic neoplasm (36). Although some of the dilemmas may involve issues such as the appropriateness of i.v. injections (34), in which early metastatic events are bypassed, cell passage numbers, or in the case of Lewis Lung carcinoma, whether the tumor was of the high or low metastatic phenotype, these results also imply that the tumor microenvironment and the effects of host deficiencies of the fibrinolytic system may vary for different tumor models. For example, activation of metalloproteases could directly facilitate tumor growth and indirectly activate other degradative proteins, such as cathepsin, which is secreted by B16 melanoma cells in proportion to their metastatic potential (37). Thus, it is clear, and perhaps even expected, that different tumors could respond variably to any relevant host deficiency. The fibrosarcoma model may be more reflective of general effects of PAI-1 on angiogenesis, because similar defective neovascularization accompanying a PAI-1 deficiency is seen herein with non-tumor-bearing mice in a corneal angiogenesis model and in inflammatory angiogenesis in a completely separate model of remodeling of the vessel wall after oxidative arterial injury (38).

The protease, uPA, is known to regulate the degradation of ECM proteins. Thus, it is not surprising that tumor progression is impeded in UPA−/− mice, wherein a higher apoptotic tumor index and diminished cellular proliferation occur in the fibrosarcoma model, relative to WT mice. Although the tumor itself produces uPA, studies have shown that uPA expression by stromal cells may play an equally important role in cancer cell invasion (39). However, a uPA deficiency did not appear to have a substantial effect on angiogenesis and neovascularization of the tumor. Other studies have demonstrated a temporal relationship between endothelial cell migration and the expression of uPA activity (40). Additionally, fetal bovine aortic endothelial GM 7373 cells overexpressing human uPA demonstrated an enhanced invasive capacity in a Matrigel chemoinvasion assay (41). Thus, it is expected that a lack of host expression of uPA might have a negative effect on endothelial cell migration and resultant neovascularization, to the extent that Pm is involved in this process, unless iPA activation of PG provides an amount of Pm suitable for migratory events that lead to neovascularization. On the other hand, other proteolytic pathways for supporting neovascularization may compensate for a loss of uPA activity. For example, MMP activities
are tightly regulated during angiogenesis, and their activation, and resulting function in this regard, may not necessarily be dependent on Pm activity (42). Clinically, it has been shown in patients with colorectal adenocarcinoma that MMP-2 and MMP-9 expression positively correlated with angiogenesis (43). Additionally, the transcription factor, ETS-1, has been implicated in regulation of endothelial cell conversion to an angiogenic phenotype. Cells that overexpress ETS-1 are highly invasive and demonstrate increased expression of MMP-1, MMP-3, and MMP-9 (44).

VEGF is a potent neovascularization agent in some cells (45–48), and its expression is up-regulated by bFGF and other growth factors (49, 50). VEGF, in turn, also up-regulates the expression of Pg activators and PAI-1 (51), thus providing a possible link between an array of growth factors, angiogenesis, and extracellular protease activity. Furthermore, expression of VEGF, itself, is up-regulated by bFGF. Importantly, PAI-1/mice displayed greatly reduced expression of VEGF and, probably linked to this effect, diminished angiogenesis and neovascularization of the tumor. As a result, the apoptotic index was higher in the tumors of these mice than in WT animals, and cellular proliferation was diminished, thus offering an explanation as to the lack of growth of tumors cells in these gene-deficient animals. This is coupled with ultrastructural analysis, suggesting that neovessel formations in UPA−/− and PAI−/− mice have abnormal features, such as larger and less mature vessels in the UPA−/− mice and small short neovessels in PAI−/−/mice. Without appropriate angiogenesis in the tumor cells, tumors cannot grow and spread effectively.

Previous studies have also shown that PAI-1 blocks cellular binding of uPAR to vitronectin and thus promotes detachment of uPAR-bearing cells from vitronectin, with a resultant increase in cell motility (52). A loss of host PAI-1 expression could result in enhanced adhesion of uPAR-bearing endothelial cells to the matrix protein, vitronectin, and thus adversely affect cell motility and resultant neo-vascularization. Additionally, balanced cell adhesion and proteolytic-mediated detachment through regulation of uPA activity and inhibition could be necessary for protecting the neovascularizing tissue (53). All of these mechanisms may be functioning to enhance angiogenesis in the presence of host PAI-1, and the absence of this protein would then attenuate neovascularization in tumors and other types of cells.

In summary, the studies presented in this report underscore the importance of host expression of components of the fibrinolytic system in supporting growth and dissemination of the murine fibrosarcoma tumor, T241. Lack of host expression of either uPA or its physiological inhibitor, PAI-1, attenuates growth and survival of this tumor. uPA can provide the necessary targeted proteolysis, amplified through Pg activation, to allow the cellular migration and proliferation needed for angiogenesis to occur. However, PAI-1 can provide a control to allow this process to occur in a highly regulated manner. In the absence of PAI-1, unregulated proteolysis may be detrimental to angiogenesis. Furthermore, the absence of PAI-1 may inhibit endothelial cell motility via effects on the uPAR-vitronectin interaction. Additionally, we show that the PAI-1-deficient state may also inhibit neovascularization by affecting VEGF levels, which are needed for this process to occur. Thus, PAI-1 is a complex regulator of neovascularization, and the influence of these different mechanisms likely depends on the nature of the particular angiogenic process that is occurring in different cell types.

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Tumor Development Is Retarded in Mice Lacking the Gene for Urokinase-Type Plasminogen Activator or Its Inhibitor, Plasminogen Activator Inhibitor-1

Linda S. Gutierrez, Alexis Schulman, Teresa Brito-Robinson, et al.


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