Abstract. The plasminogen (Plg)/plasminogen activator (PA) system plays a key role in cancer progression, presumably via mediating extracellular matrix degradation and tumor cell migration. Consequently, urokinase-type PA (uPA)/plasmin antagonists are currently being developed for suppression of tumor growth and angiogenesis. Paradoxically, however, high levels of PA inhibitor 1 (PAI-1) are predictive of a poor prognosis for survival of patients with cancer. We demonstrated previously that PAI-1 promoted tumor angiogenesis, but by an unresolved mechanism. We anticipated that PAI-1 facilitated endothelial cell migration via its known interaction with vitronectin (VN) and integrins. However, using adenoviral gene transfer of PAI-1 mutants, we observed that PAI-1 promoted tumor angiogenesis, not by interacting with VN, but rather by inhibiting proteolytic activity, suggesting that excessive plasmin proteolysis prevents assembly of tumor vessels. Single deficiency of uPA, tissue-type PA (tPA), uPA receptor, or VN, as well as combined deficiencies of uPA and tPA did not impair tumor angiogenesis, whereas lack of Plg reduced it. Overall, these data indicate that plasmin proteolysis, even though essential, must be tightly controlled during tumor angiogenesis, probably to allow vessel stabilization and maturation. These data provide insights into the clinical paradox whereby PAI-1 promotes tumor progression and warrant against the uncontrolled use of uPA/plasmin antagonists as tumor angiogenesis inhibitors.

Key words: angiogenesis • tumor invasion • proteolysis • migration • serine protease

Introduction

Tumor progression involves the disruption of anatomical barriers and penetration of tumor cells into normal adjacent host tissues, as well as the infiltration of normal host cells into the tumor. Such migratory and tissue remodeling events are, among others, regulated by different proteolytic systems. Among the proteases that play an active role in these processes are the serine proteases (the uPA receptor [uPAR]). Concomitant binding of pro-uPA to uPAR and Plg to nonspecific binding sites at the cell surface strongly enhances plasmin generation.

Urokinase-type (uPAs) and tissue-type plasminogen activators (tPAs) both activate the zymogen Plg into its active form, plasmin. uPA is secreted as an inactive precursor (pro-uPA) that binds with high affinity to a specific cell surface glycosylphosphatidylinositol-anchored receptor (the uPA receptor [uPAR]). Concomitant binding of pro-uPA to uPAR and Plg to nonspecific binding sites at the cell surface strongly enhances plasmin generation.

It is generally believed that uPA at the cell surface initiates a proteinase cascade, which in turn leads to breakdown of the extracellular matrix and thereby promotes cellular migration. This conclusion is supported by the fact that uPA and uPAR are highly expressed by tumor cells or by surrounding stromal cells, and that they are both independent prognostic indicators in human cancer (Dano et al., 1994; Reuning et al., 1998; Stephens et al., 1999). In
addition, the use of antisense mRNA for uPA and uPAR, of natural or synthetic serine protease inhibitors, or of uPAR antagonists, all reduced tumor invasion (Min et al., 1996; Carmeliet and Collen, 1998). Consequently, uPA/uPAR/plasmin antagonists are currently being developed as therapeutic strategies to inhibit tumor angiogenesis and progression.

PA inhibitor 1 (PAI-1) is the primary physiological inhibitor of uPA and tPA. It not only regulates the proteolytic activity of uPA, but also determines the level of uPA bound to uPAR by promoting the rapid endocytosis of the trimolecular uPA-PAI-1-uPAR complex (Conese and Blasi, 1995; Blasi, 1997). However, it has also been implicated in modulating cell migration via alternative mechanisms (Deng et al., 1996; Stefansson and Lawrence, 1996; Blasi, 1997; Loskutoff et al., 1999), even though conclusive in vivo evidence is lacking. By blocking the interaction between vitronectin (VN), uPAR, and integrins, PAI-1 may induce cell detachment from the extracellular matrix and thereby promote cellular migration and tumor invasion. However, the relevance of the latter mechanism in overall tumor growth and angiogenesis has not been confirmed in vivo.

Surprisingly high, rather than low, levels of PAI-1 are predictive of poor survival prognosis for patients suffering from a variety of different cancers (Pedersen et al., 1994a,b). To date, the molecular mechanisms of this apparent paradox remain largely unexplained, raising concerns about whether therapeutic strategies to suppress tumor growth and angiogenesis should be aimed at inhibiting or increasing uPA/plasmin proteolysis. These questions have been difficult to address, largely because uPA promotes, and PAI-1 suppresses, tumor growth in most available experimental tumor models. However, recently PAI-1 was found to promote tumor growth and angiogenesis (Bajou et al., 1998) in a highly reproducible mouse tumor model. The availability of this model now allows us to examine the molecular mechanism by which PAI-1 promotes tumor angiogenesis (i.e., either by inhibiting the proteolytic activity or by interrupting the interaction between VN and uPAR or integrins). To address this question and to further determine the respective role of the different members of the Plg/plasmin system, we have transplanted malignant keratinocytes into wild-type (WT) mice and mice lacking uPA, tPA, uPA and tPA, uPAR, Plg, PAI-1, or VN. We also performed adenoviral gene transfer experiments using PAI-1 mutants that selectively inhibit PA activity or no longer bind to VN. We provide direct evidence that plasmin is involved in the formation of new vessels by host endothelial cells, but that PAI-1 control of proteolytic breakdown is required. These data not only help to explain the apparent paradox that high levels of a proteinase inhibitor promote tumor angiogenesis and are positively correlated with tumor progression in patients, but also warrant against the uncontrolled use of uPA/plasmin inhibitors for suppression of tumor angiogenesis. Furthermore, they indicate that neutralization of PAI-1 may be an attractive new target for antiangiogenic therapy.

### Materials and Methods

#### Mice

Homozygous mice with single (uPA−/−, tPA−/−, uPAR−/−, and VN−/−) or combined deficiencies (tPA−/−uPAR−/− mice) and their corresponding WT with a mixed genetic background of 75% C57BL/6 and 25% 129 SV/SL strain were generated as described previously (Carmeliet et al., 1994; Zheng et al., 1995; Carmeliet and Collen, 1998). Tumor angiogenesis and invasion in PAI-1−/− (Carmeliet et al., 1993a,b) and WT mice were evaluated in three different sets of experiments using mice with different genetic background obtained by two, three, or four backcrosses with C57BL/6 strain. In each set of experiments, similar results were obtained and therefore the data presented in Table I were pooled. The Plg−/− mice were derived from those generated previously (Bugge et al., 1995; Ploplis et al., 1998). They have been backcrossed 1 or 11 times with C57BL/6 strain, yielding similar results in two different sets of experiments presented in Table I. Mice of either sex used for experiments were littermate mice, produced by mating of heterozygous brothers and sisters between 8 and 12 wk old. Genotypes of mice were established using tail biopsy DNA preparation by either Southern blot analysis or PCR assays (Carmeliet et al., 1993b, 1994; Bugge et al., 1995).

#### Cell Culture

Malignant murine keratinocytes (PDVA cells; Fusenig et al., 1978) were routinely grown in DME containing a fourfold concentration of amino acids and vitamins (GIBCO BRL), 10% FCS (GIBCO BRL), and antibiotics in a humidified incubator at 37°C. Cells (2 × 10⁵) were plated on collagen gel (4 mg/ml of type I collagen isolated from rat tail tendons) inserted in teflon rings (Renner GmbH) and maintained in culture for 1 d before transplantation into mice as described previously (Fusenig et al., 1983; Bajou et al., 1998). For transplantation assays in VN−/− mice, keratinocytes were precultured on the collagen gels in the presence of 10% serum derived from VN−/− mice.

#### Transplantation Assay in Mice

The cell-coated collagen gels were covered with a silicone transplantation chamber (Renner GmbH) and implanted in toto onto the dorsal muscle fascia of mice as described previously (Skobe et al., 1997; Bajou et al., 1998). 2 wk later, transplants were resected, embedded in Tissue Tek (Miles Laboratories, Inc.), and either frozen in liquid nitrogen for cryostat sectioning or embedded in paraffin after zinc formalin fixation.

### Table I. Tumor Angiogenesis and Invasion in WT, PAI-1−/−, and Plg−/− Mice Injected or Not with WT or Mutated hPAI-1 Adenovirus

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Virus Mice</th>
<th>Plasma level of hPAI-1</th>
<th>Angiogenesis Invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>ng/ml</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>AdRR5 control</td>
<td>11 &lt;0.8</td>
<td>+++</td>
</tr>
<tr>
<td>PAI-1−/−</td>
<td>AdRR5 control</td>
<td>10 &lt;0.8</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td>AdCMVPAI-1</td>
<td>4  300</td>
<td>+ +</td>
</tr>
<tr>
<td>(WT hPAI-1)</td>
<td>102</td>
<td>0 2</td>
<td>1,699</td>
</tr>
<tr>
<td></td>
<td>2,805</td>
<td>+ + + + +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AdPAI-1R123K</td>
<td>6 106</td>
<td>+ + +</td>
</tr>
<tr>
<td>(mutated hPAI-1)</td>
<td>250</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>(defective in VN binding)</td>
<td>1,853</td>
<td>+ +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6,722</td>
<td>+ +</td>
<td>26,840</td>
</tr>
<tr>
<td></td>
<td>20,618</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td>AdPAI-1R346MMS375</td>
<td>5 579</td>
<td>0 0</td>
</tr>
<tr>
<td>(mutated hPAI-1)</td>
<td>1,043</td>
<td>0 0</td>
<td>1,426</td>
</tr>
<tr>
<td>(defective in PA inhibition)</td>
<td>1,816</td>
<td>0 0</td>
<td>4,435</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td>WT</td>
<td>5</td>
<td>ND</td>
<td>+++</td>
</tr>
<tr>
<td>Plg−/−</td>
<td>5</td>
<td>ND</td>
<td>++</td>
</tr>
<tr>
<td>None</td>
<td>3</td>
<td>ND</td>
<td>++</td>
</tr>
</tbody>
</table>

Tumor invasion and angiogenesis were scored as described in Materials and Methods.
In all assays, the take rate and growth of transplants were verified by classical histology and some samples were not taken into account. The exclusion criteria were the loss of transplantation chamber, the absence of cells on top of the collagen gel, and the failure of collagen gel adherence onto the host tissue. Based on these parameters, we excluded ~20 and 50% of samples resected from mice untreated or injected with adenovirus (see above), respectively. There were no differences in exclusion rates related to the genotypes of the mice or the type of virus injected.

**Adenovirus-mediated PAI-1 cDNA Transfer**

A recombinant adenovirus vector bearing WT human PAI-1 (hPAI-1) (AdCMVPAI-1 and control adenovirus [AdRR5]) were propagated as described previously (Gerard and Meidell, 1995; Carmeliet et al., 1997). Recombinant viruses expressing mutant hPAI-1, AdCMVPAI-1R346LM347S, and AdCMVPAI-1R346LM347S were generated after substitution of a restriction fragment containing the desired mutation into the pACCMVPAI-1(WT) shuttle plasmid (Gerard and Meidell, 1995). PAI-1 with mutation of Gln 123 to Lys had a specific 100-fold decrease in affinity for VN, but retained antiplasmin activity (Bajou et al., 1997). The double point mutant, Arg 346 to Met and Met 347 to Ser (Shubeita et al., 1990), bound to VN with the same affinity as WT PAI-1 but did not inhibit PA activity. We verified that hPAI-1 (WT or mutant forms) interacts efficiently with murine PA and murine VN according to the procedure described previously (Vleugels et al., 2000).  

1 d after cell transplantation, mice were intravenously injected with 200 μl of control or recombinant adenovirus (7 × 10⁹ pfu). After 5 d, blood was sampled from the retroorbital sinus and PAI-1 antigen was measured as described (Bajou et al., 1998). On day 14, mice were killed and transplants were excised and processed as described above. The hPAI-1 cDNA was used in this study to allow ELISA measurement of circulating PAI-1 levels in all animals, including WT mice.

**Immunofluorescence and Morphometry**

Cryostat sections (5 μm thick) were fixed in acetone at −20°C and in 80% methanol at 4°C and then incubated with the primary antibodies (Abs). For double immunofluorescence labeling studies, sections were first incubated for 1 h at room temperature with the two primary Abs: antitype IV collagen Ab (rabbit polyclonal Ab, diluted 1:100 and antikeratin Ab (guinea pig polyclonal Ab, diluted 1:20; Sigma-Aldrich). The sections were washed three times in PBS for 10 min each and subsequently FITC- or Texas red–conjugated appropriate secondary Abs were applied for 30 min: swine anti–rabbit (diluted 1:40; Dakopat) and/or mouse anti–guinea pig (diluted 1:40; Sigma-Aldrich). After three washes in PBS for 10 min each and a final rinse in 10 mM Tris-HCl buffer, pH 8.8, coverslips were mounted and specific labeling was observed using an inverted microscope equipped with epifluorescence optics.

Morphometric measurements of cell invasion (average distance of penetration) were performed as described previously (Bajou et al., 1998): migration <50 μm was scored as 0; migration from 50 to 150 μm, +; migration from 150 to 300 μm, ++; and migration 300 μm, +++. Morphometric assessment of angiogenesis was scored as follows: vessels undetected in the collagen gel, 0; vessels infiltrating the collagen without reaching the malignant epithelial layer, +; blood vessels in close apposition to the epithelial layer, ++; and blood vessels intermingling with invasive epithelial tumor sprouts, +++.  

**In Situ Zymography**

Cryostat sections were coated with a mixture containing 2% skim milk, 0.9% agar, and 600 μg/ml of Plg (Sigma-Aldrich) (Bajou et al., 1998). An 8% milk stock solution was prepared in PBS, heated at 95°C for 30 min, and centrifuged briefly at 3,000 rpm to remove insoluble material. Slides were incubated at 37°C in a humidified chamber for 2 h for assessment of total PA activity and for 24 h in the presence of uPA-specific inhibitor.
amiloride (2 mM; Sigma-Aldrich) for assessment of tPA activity. Caseinolysis was monitored by examination under a dark field microscope.

Results

Plasmin Proteolysis in Tumor Angiogenesis and Invasion

Malignant murine keratinocytes (PDVA cells; Fusenig et al., 1978) cultured on a collagen gel were implanted onto the dorsal muscle fascia of WT and transgenic mice. In response to angiogenic stimuli (produced by tumor cells; Skobe et al., 1997), new blood vessels formed in the underlying stroma, invaded the collagen gel, and reached the malignant epithelial layer. Thereafter, the malignant keratinocytes formed tumor sprouts that invaded downwards into the granulation tissue (Fig. 1 a). Within 2 wk after transplantation, these tumor islets were intermingled with closely apposed new vessels. The degree of tumor cell invasion was scored by calculating the average distance over which the tumor cells infiltrated in the host mesenchyme, whereas tumor angiogenesis was semiquantitatively scored after staining for collagen type IV, a component of the capillary basement membrane (Fig. 2 a). Compared with WT mice, tumor angiogenesis (score +++) developed to a similar degree and invasion of tumor cells occurred over a similar distance (>150 μm, ++ or ++++) in uPA−/−, tPA−/−, uPAR−/− mice, and double tPA−/−/uPA−/− mice (Fig. 1, b–e, and Fig. 2, b–e). Both tumor invasion and stromal angiogenesis were reduced in Plg−/− mice (Table I, Figs. 1 f and 2 f).

In situ zymography in WT mice revealed that uPA activity (Fig. 3 a) was mainly localized to the cellular front, containing both invading tumor cells (these malignant keratinocytes are in all experiments WT, and hence produce uPA and tPA; Bajou et al., 1998) and newly formed blood vessels, whereas weak tPA activity was largely restricted to the upper part of the transplant, predominantly consisting of tumor cells (Fig. 3 b). In uPA−/− mice, tPA activity was not only restricted to the tumor-rich upper region, but also expressed at increased levels over the invasion zone, containing both infiltrating tumor cells and newly formed vessels (Fig. 3 c and d). These results may suggest a compensatory upregulation of tPA activity in the absence of uPA. In tPA−/− mice, caseinolytic activity appeared similar to that visualized in WT mice and was exclusively mediated by uPA (Fig. 3 e and f). The lack of host uPAR in uPAR−/− mice did not influence the intensity or the localization of uPA-mediated lysis (Fig. 3 g and h). These data indicate that formation of host-derived stromal vessels requires plasmin, normally generated by uPA. However, the two PAs may be somehow redundant.
Normal Tumor Angiogenesis and Invasion in VN−/− Mice

In agreement with our previous findings, host-derived vessels in PAI-1−/− mice were unable to migrate towards the tumor cells and remained confined beneath the collagen gel (Table I, Fig. 2 g). In addition, malignant cells failed to invade the host tissue in PAI-1−/− mice (the average depth of invasion was <50 μm, scored 0) and remained as an irregular stratified epithelium on top of the collagen gel (Fig. 1 f). Since PAI-1 binds strongly to VN and alters the adhesion and migration of cells on this matrix substrate, we anticipated that lack of VN should mimic the impaired tumor angiogenesis and invasion phenotype of PAI-1−/− mice. However, transplantation of malignant keratinocytes into VN−/− mice was associated with normal, and perhaps even accelerated angiogenesis and tumor infiltration (Fig. 4).

Mechanism of the Tumor-promoting Role of PAI-1

We had demonstrated previously that tumor vascularization and invasion in PAI-1−/− mice can be restored by intravenous injection of a recombinant adenovirus expressing human PAI-1 (AdPAI-1; Bajou et al., 1998). To further investigate whether the role of PAI-1 in promoting tumor invasion and angiogenesis depended on its ability to block proteolytic activity, or instead on its ability to bind to VN, two additional adenovirus constructs were produced that expressed a mutant form of hPAI-1 that: (a) exhibited normal binding to VN but was inactive in inhibiting the proteolytic activity of tPA and uPA (AdPAI-1R346M, M347S), or (b) inhibited the PA activity normally but had a dramatically reduced affinity for VN (AdPAI-1Q123K). Intravenous injection of these adenoviruses resulted in 100–1,000-fold increased plasma levels of hPAI-1...
occurs normally in the absence of both PAs and Plg and revealed that angiogenesis during embryonic development (Montesano et al., 1990). However, studies in gene-inactivated mice have re-

ticted with VN but rather by its antiprotease function. This study demonstrates that plasmin proteolysis is in-

Discussion

Proteolytic breakdown of extracellular matrices by uPA/plasmin has been associated with tumor invasion and an-

Figure 4. Invasive behavior of malignant mouse keratinocytes (PDVA cells) 2 wk after implantation into WT mice (a) or Vn−/− mice (b). Histological sections stained with hematoxylin and eosin revealed tumor cells (C) intermingled with host cells (H) in both WT mice (a) and Vn−/− mice (b). Bar, 100 μm.

above normal murine PAI-1 plasma levels of WT mice (2 ng/ml; Table I). Injection of the AdPAI-1Q123K virus into PAI-1−/− hosts restored tumor vascularization and inva-

Below this apparent paradox remained to date un-

The Plg/plasmin system has been implicated in extracel-

latter the typical malignant keratinocyte invasion and tu-

mort vascularization. Second, PAI-1 may affect endothelial

fall that angiogenesis during pathological conditions can occur to a large degree in the absence of either Plg, tPA, or uPA, possibly by redundancy or compensation by other protein-

aggregation factor XII, kininogen, or kalikrein (Colman, 1969; Miles et al., 1983; Carmeliet et al., 1994). The re-

tumor vascularization and invasion in any of the six mice (Table I). Thus, the requirement for PAI-1 in tumor angiogenesis and invasion, in this model, appears not to be due to inhibition of cellular adhesion through its interaction with VN, but rather due to preven-

that angiogenesis during pathological conditions can occur to a large degree in the absence of either Plg, tPA, or uPA, possibly by redundancy or compensation by other protein-

ases (Bugge et al., 1997, 1998; Carmeliet and Collen, 1999; Lund et al., 1999). In accordance with these observations, the present findings show that tumor vascularization was not affected in mice deficient in tPA, uPA, or uPAR. Nota-

bly, stromal/host lack of uPA may in the present tumor model have been compensated by upregulation of tPA ac-

suggests that in vivo neither tPA nor uPA, produced by host cells individually or in combination, is essential for Plg activation; and other enzyme(s) can compensate the lack of uPA and tPA. Alternatively, the presence of active plasmin might result from activation by uPA or tPA pro-

tumor angiogenesis that can be cleaved from Plg by plasmin (O’Reilly, 1997). However, lack of Plg was associated with a decreased, rather than an increased, formation of blood vessels, suggesting that in this model,
angiostatin generation is not a critical event. This is in accordance with findings on other tumor models in Plg-deficient mice (Bugge et al., 1997, 1998). Fourth, PAI-1 may be implicated in inhibiting the proteolytic activity of both PAs, thereby reducing overall plasmin formation. This possibility is supported by the restoration of tumor angiogenesis in PAI-1–deficient mice after adenovirus-mediated transfer of a mutant PAI-1, defective in binding to VN but able to inhibit PA-activity. Presumably, increased plasmin proteolysis in PAI-1–deficient mice may prevent accumulation of fibrin, fibronectin, laminin, and, indirectly via activation of other matrix degrading proteases, of additional extracellular matrix components that are known to stimulate endothelial proliferation and outgrowth. By mediating deposition of such a scaffold, PAI-1 also would allow newly formed vessels to acquire the necessary stability and maturity. Excessive degradation of extracellular matrix is incompatible with efficient cellular migration (Montesano et al., 1990; Pepper and Montesano, 1990). The maintenance of a certain degree of extracellular matrix integrity is indeed an essential requirement for capillary morphogenesis. PAI-1 may thus balance PA-mediated pericellular proteolysis, protecting the stroma from excessive proteolysis during endothelial cell invasion.

In conclusion, our data indicate that in vivo, like previously shown in vitro (Pepper and Montesano, 1990; Liu et al., 1995), a critical balance between proteases and PAI-1 is necessary for optimal invasion. However, in contrast to in vitro data, lack of VN, uPAR, uPA, tPA, or both uPA and tPA did not impair tumor formation. This discrepancy between in vitro and in vivo data probably indicates that compensatory mechanisms are active in vivo but not in vitro. Our observations demonstrate that PAI-1 is essential for tumor angiogenesis, not via its interaction with VN but rather via counterbalancing excessive plasmin generation. Our findings also warrant against the uncontrolled use of inhibitors of proteases (such as of uPA and perhaps also of metalloproteinases) for suppression of tumor angiogenesis. They also indicate that neutralization of PAI-1 activity may be an effective new therapeutic approach for suppression of tumor angiogenesis.

We thank A. Belayew and V. Attenburrow for their collaboration; and P. Gavilleti, V. Laureysens, F. Olivier, and K. Wauthier for their technical assistance.

This work was supported by grants from the Communauté Française de Belgique (Actions de Recherches Concertées), the Commission of European Communities, the Fonds de la Recherche Scientifique Médicale, the Fonds National de la Recherche Scientifique, the DG Biologie, the Fonds de la Recherche Scientifique -Programme National de Recherche et de Formation des Jeunes Chercheurs-, the Fonds de la Recherche Scientifique, the Fédération Belge Concertée, the Commission of European Communities, the Fonds National de la Recherche Scientifique, the Fédération Belge Concertée, and the Fonds National de la Recherche Scientifique, the Fédération Belge Concertée.

References


