The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent

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ABSTRACT **Plasminogen activator inhibitor 1 (PAI-1) is believed to control proteolytic activity and cell migration during angiogenesis. We previously demonstrated in vivo that this inhibitor is necessary for optimal tumor invasion and vascularization. We also showed that PAI-1 angiogenic activity is associated with its control of plasminogen activation but not with the regulation of cell–matrix interaction. To dissect the role of the various components of the plasminogen activation system during angiogenesis, we have adapted the aortic ring assay to use vessels from gene-inactivated mice. The single deficiency of tPA, uPA, or uPAR, as well as combined deficiencies of uPA and tPA, did not dramatically affect microvessel formation. Deficiency of plasminogen delayed microvessel outgrowth. Lack of PAI-1 completely abolished angiogenesis, demonstrating its importance in the control of plasmin-mediated proteolysis. Microvessel outgrowth from PAI-1$^{-/-}$ aortic rings could be restored by adding exogenous PAI-1 (wild-type serum or purified recombinant PAI-1). Addition of recombinant PAI-1 led to a bell-shaped angiogenic response clearly showing that PAI-1 is proangiogenic at physiological concentrations and antiangiogenic at higher levels. Using specific PAI-1 mutants, we could demonstrate that PAI-1 promotes angiogenesis at physiological (nanomolar) concentrations through its antiproteolytic activity rather than by interacting with vitronectin.—Devy, L., Blacher, S., Grignet-Debrus, C., Bajou, K., Masson, V., Gerard, R. D., Gils, A., Carmeliet, G., Carmeliet, P., Declerck, P. J., Noël, A., Foidart, J. M. The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *FASEB J.* 16, 147–154 (2002)**

Key Words: angiogenesis · cell migration · proteolysis · serine protease · PAI-1

Plasminogen activator inhibitor 1 (PAI-1) is a key regulator of the plasminogen activation system, a proteolytic cascade implicated in various physiological and pathological processes including vascular thrombolysis, inflammation, wound healing, tumor invasion, and neovascularization (1). PAI-1 is the primary inhibitor of urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), which both activate plasminogen (Plg) into its active form, plasmin (2). uPA is secreted as an inactive precursor that binds with high affinity to a specific cell surface-anchored receptor (the uPA receptor or uPAR) (3). It is generally believed that uPA at the cell surface initiates a protease cascade, which in turn leads to the breakdown of the extracellular matrix and thereby promotes cellular migration. This is supported by the fact that uPA and uPAR are highly expressed by tumor cells or surrounding stromal cell and both are independent prognostic indicators in human cancer (4).

We recently demonstrated the key role played by PAI-1 in tumor invasion and angiogenesis in PAI-1-deficient mice in which implanted malignant keratinocytes were unable to induce vascularized tumors (5). Our observation partially explains the paradoxical clinical data that high levels of PAI-1 in the primary tumor tissue of patients with various types of solid cancer correlate with disease recurrence and reduced survival (for review, see ref 6). However, experimental data have yielded contradictory results. Tumor cells transduced with PAI-1 cDNA showed a reduction in primary tumor growth, tumor-associated angiogenesis, and metastasis (7). In another study, the deficiency of host PAI-1 or overexpression of murine PAI-1 in transgenic mice did not affect primary tumor growth or pulmonary metastasis after inoculation of melanoma cells (8). Conversely, PAI-1 was found to accelerate lung metastasis formation of human fibrosarcoma cells (9). These conflicting results might be a consequence of differences in tumor models or PAI-1 concentrations used. Alternatively, they may be due to the multiple functions

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of PAI-1. Indeed, there is a growing body of evidence that PAI-1 not only functions as a classical serine protease inhibitor, but also plays a crucial role through a mechanism independent of its uPA/tPA inhibitory capacity (10). PAI-1 may govern the balance between adhesion/detachment and migration of normal and tumor cells (1, 2, 11). By blocking the interaction between vitronectin, uPAR, and integrins, PAI-1 may induce cell detachment from the extracellular matrix and promote cellular migration and perhaps tumor invasion (11). The relevance of the latter mechanism to overall tumor growth and angiogenesis has not been conclusively confirmed in vivo. By using adenoviral gene transfer of PAI-1 mutants in PAI-$1^{-/-}$ mice transplanted with malignant keratinocytes (5), we demonstrated that PAI-1 promotes tumor angiogenesis not by interacting with vitronectin, but rather by inhibiting proteolytic activity (12). The importance of controlled plasmin-mediated proteolysis was further confirmed by a delay of tumor angiogenesis in plasminogen-deficient mice (12).

To provide insight into the mechanism of PAI-1 action and further determine the respective role of the different members of the Plg/plasminogen activator (PA) system, we used an aortic ring assay developed initially for rat vessels (13). By using intact vascular explants embedded in collagen gels, this model bridges the gap between in vivo and in vitro models by more accurately reproducing the environment in which angiogenesis takes place than isolated endothelial cell models. In the present study, we have adapted the aortic ring assay to use vessels from knockout mice. The single deficiency of Plg delayed the angiogenic response, but the lack of tPA, uPA, tPA/uPA, or uPAR did not block microvessel outgrowth. PAI-1 appears proangiogenic at low concentrations and antiangiogenic at higher concentrations, thereby providing an explanation for the apparently contradictory results previously reported. By using wild-type (WT) and mutated forms of PAI-1, we have demonstrated that at low concentrations the inhibitory activity is responsible for the restoration of angiogenesis, whereas at high concentrations the interaction of PAI-1 with vitronectin might take over the effect.

MATERIALS AND METHODS

Genetically modified mice

Brother-sister mating generated all knockout mice in a C57B16/129 background and their corresponding WT littermates. Homozygous deficient mice and their corresponding WT with a mixed genetic background of 75% C57B16 and 25% 129 SV/SL strain (tPA$^{-/-}$, uPA$^{-/-}$, tPA$^{-/-}$/uPA$^{-/-}$, Plg$^{-/-}$, or uPAR$^{-/-}$ mice) or 87.5% C57B16 and 12.5% 129 SV/SL strain (for PAI-$1^{-/-}$ mice) were generated as described previously (14–16). Mice between 8 and 12 wk old of both sexes were used.

Preparation of the 3-dimensional aortic ring cultures

Angiogenesis was studied by culturing rings of mouse aorta in 3-dimensional collagen gels with some modifications of the method originally developed for the rat aorta (17). Thoracic aortas were removed from mice killed by cervical dislocation and immediately transferred to a culture dish containing ice-cold serum-free minimum essential medium (MEM, Life Technologies Ltd., Paisley, Scotland). The periaortic fibroadipose tissue was carefully removed with fine microdissecting forceps and iridectomy scissors, paying special attention not to damage the aortic wall. One mm-long aortic rings (~15 per aorta) were sectioned and rinsed extensively in five consecutive washes of MEM. Ring-shaped explants of mouse aorta were then embedded in a rat tail interstitial collagen gel (1.5 mg/ml) (18) prepared by mixing 7.5 volumes of 2 mg/ml collagen (Collagen R; Serva, Heidelberg, Germany), 1 volume of 10× MEM, 1.5 volumes of $NaHCO_3$ (15.6 mg/ml), and ~0.1 volume 1 M NaOH to adjust the pH to 7.4. The collagen gels containing the aortic rings were polymerized in cylindrical agarose wells (17) and kept in triplicate at 37°C in 60 mm diameter Petri dishes (bacteriological polystyrene; Falcon, Becton Dickinson, Lincoln Park, NJ). Each dish contained 6 ml of MCDB131 (Life Technologies Ltd.) supplemented with 25 mM $NaHCO_3$, 2.5% mouse serum, 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were kept at 37°C in a humidified environment for a week and examined every second day with an Olympus microscope at appropriate magnification.

Quantification of angiogenesis

Image analysis was performed on a Sun SPARC30 work station with the software ′ Visilog 5.0 ≈ from Noesis. We used an improved computer-assisted image analysis (19) that allows automatic measurements of the geometrical and morphological parameters. After generation of binary image, the following automatic measurements were performed: *1)* the number of microvessels (N_v), maximal microvessel length (L_{max}), and the total number of branches in microvessels (N_b); *2)* the size and form of the aortic explant.

Fluorescent staining of endothelial cells

To stain the endothelial cells before sectioning, rinsing, and collagen embedding, aortas were incubated for 4 h at 37°C in a solution of 10 μg/ml fluorescent acetylated low density lipoprotein (Dil-Ac-LDL, acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-3,3, 3′,3′-tetramethylindocarbocyanine perchlorate; Biomedical Technologies, Stoughton, MA), which is selectively taken up by endothelial cells (20). Cultures were then examined by fluorescent microscopy using rhodamine excitation and emission filters. Dil-Ac-LDL had no effect on endothelial cell growth rate at the concentration used for labeling cells.

Construction, production, and characterization of active recombinant PAI-1 and mutants

A stable PAI-1 variant referred to as PAI-1-stab, harboring the mutations Asn150His, Lys154Thr, Gln301Pro, Gln319Leu, and Met354Ile and demonstrating a functional half-life of ~150 h, was produced as described (21) and used in all experiments where active recombinant PAI-1 is noted.

The mutants PAI-1-stab-Q123K (with impaired vitronectin binding properties), PAI-1-stab-R346M/M347S, and PAI-1-stab-R346A (with impaired inhibitory activity) were created by a method based on the Quickchange™ Site-Directed Mu-

tagenesis Kit of Stratagene (LaJolla, CA) using pIGE20-PAI-1-stab as template and the appropriate synthetic oligonucleotides (i.e., 5′ CGGTCAAGAAAGTGGACTTTTCAGAGG 3′, 5′ GTCATAGTCTCAGCCATGAGTGCCC-CCGAGG 3′, 5′ GTCATAGTCTCAGCCGCCATGGCCCCCGAGG 3′, and their complementary oligonucleotides for introduction of Q123K, R346M/M347S, and R346A mutations, respectively).

The expression and purification of PAI-1-stab, PAI-1-stab-Q123K, PAI-1-stab-R346M, M347S, and PAI-1-stab-R346A were performed as described (22). Biochemical characterization of the PAI-1 variants revealed a 10^4–10^5 slower reaction rate with uPA for both the PAI-1-stab-R346M, M347S ($k=7.5\pm2.5\ 10^2\ M^{-1}s^{-1}$) and the PAI-1-stab-R346A ($k<10^2\ M^{-1}s^{-1}$) vs. PAI-1-stab ($k=1.3\pm0.16\ 10^7\ M^{-1}s^{-1}$). Binding of PAI-1-stab-Q123K to murine vitronectin was 40-fold lower than observed with PAI-1-stab.

Adenovirus-mediated PAI-1 cDNA transfer

E1-deleted recombinant adenovirus vectors expressing wild-type human PAI-1 (AdCMVPAI-1), *Escherichia coli* β-galactosidase (AdCMVβgal) or no transgene (AdRR5) were propagated as described previously (23–24). Recombinant viruses expressing mutant PAI-1 proteins AdCMVPAI-1^{Q123K} and AdCMVPAI-1$^{R346M, M347S}$ were generated after substitution of a restriction fragment containing the desired mutation into the pACCMVPAI-1 (WT) shuttle plasmid (23). PAI-1 with the Q123K mutation had a specific 40-fold decrease in affinity for vitronectin but retained full inhibitory activity (10). The double point mutant R346M, M347S (25) bound to vitronectin with the same affinity as WT PAI-1 but did not inhibit PA activity (12).

Immediately after resection, intact aortas were exposed to recombinant adenoviruses encoding either *E. coli* β-galactosidase (AdCMVβgal) or human PAI-1 (WT or mutants; see above) at 5×10^9 plaque-forming units in 0.5 ml of DMEM at 37°C in 5% CO_2. After 1 day of incubation, aortas were cut into 1 mm-long fragments that were extensively rinsed with DMEM, embedded in collagen gel, and cultured with WT or PAI-1$^{-/-}$ serum. After 6 days of culture, explants were examined by microscopy and image analysis was performed. To evaluate the efficiency of transduction, PAI-1$^{-/-}$ aortic explants were exposed to AdCMVβgal, cultured in WT serum for 6 days, fixed for 15 min with cold paraformaldehyde 1% (w/v), and stained with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal; Sigma, St. Louis, MO) as described (26).

Statistical analysis

Experiments including four explants per condition were repeated at least twice. Student's *t* test was used to evaluate whether differences among groups were significant. Statistical significance was set at $P < 0.05$.

RESULTS

Mice aortic explants lead to neovessel formation

We successfully adapted the aortic ring assay initially developed using rat aorta (13). Segments of mouse thoracic aortas embedded in a collagen gel were cultured in MCDB131 medium containing autologous serum (2.5% final concentration). During the first 4 days of culture, isolated fibroblast-like cells migrated into the gel (data not shown). Subsequently, microvessel outgrowths arose from the edges of the parental vessel where the basement membrane had been ruptured (**Fig. 1*a***). The initially linear sprouts of endothelial cells progressively branched and anastomosed and gave rise to a complex microvascular network (Fig. 1*b, c*). The number of vessels (N_v) and degree of branching (N_b) reached a maximal value at day 6 (Fig. 1*d*). After this, progressive remodeling of the microvascular network and subsequent loss in the number of microvessels was observed. From day 4 to day 8, the length of microvessels increased (0.5 mm$<L_{max}<$1.00 mm). The endothelial nature of the microvessels was demonstrated by staining the aortic rings with Dil-Ac-LDL, which is selectively taken up by endothelial cells. In accord with their endothelial origin, the tubular outgrowths were fluorescent whereas the individual cells were unstained (Fig. 1*e, f*).

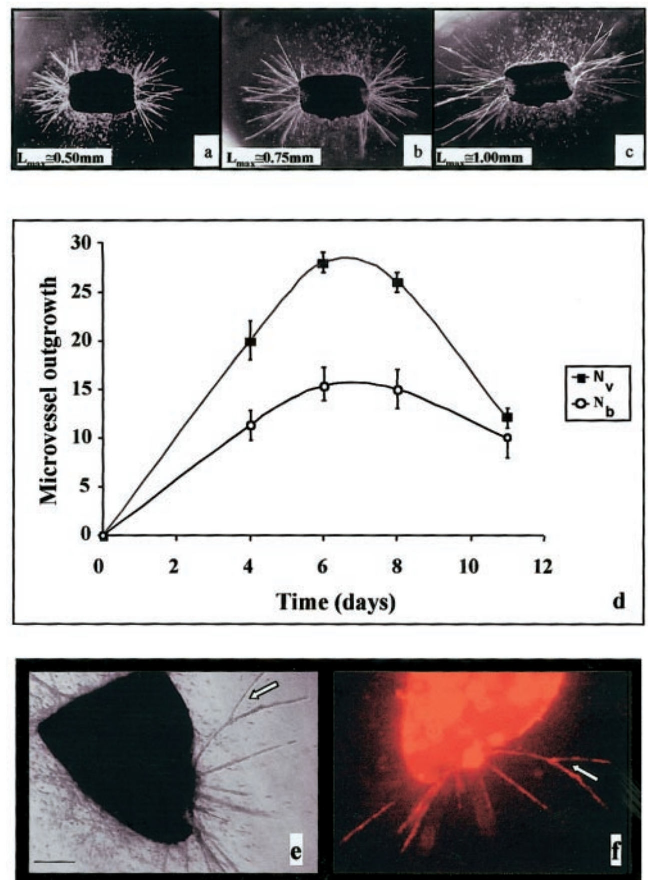

Figure 1. Capillary outgrowth from mouse aortic rings. Photomicrographs showing the angiogenic response of a collagen-embedded explant isolated from WT mice and cultured for 4 (*a*), 6 (*b*), or 8 (*c*) days in autologous serum (2.5% final concentration). Bar, 500 μm. Microvessels were quantified by computer-assisted image analysis and plotted as a function of the number of days in culture (*d*). N_v, number of microvessels; N_b, number of branchings, L_{max}, maximal microvessel length. $n = 4$; error bars = SE. Photomicrograph of a typical WT aortic explant (*e*) cultured for 6 days in autologous serum showing endothelial cells organized as branched microvessels (white arrow) and isolated fibroblast-like cells. Endothelial cells were identified by their capacity to take up Dil-Ac-LDL (*f*). The white arrow indicates a branch point. Bar, 250 μm.

Microvessel outgrowth in $Plg^{-/-}$ mice, $tPA^{-/-}$, $uPA^{-/-}$, $tPA^{-/-}/uPA^{-/-}$, and $uPAR^{-/-}$ mice

To determine whether microvessel outgrowth was dependent on other components of the Plg/PA system, aortic explants resected from single Plg, tPA, uPA, uPAR, or combined tPA/uPA-deficient mice were embedded in collagen gels in the presence of autologous serum. As early as day 4, stimulation of microvessel outgrowth was observed in $uPAR^{-/-}$ explants compared with WT (**Fig. 2*a***) ($P<0.05$). At this time, no differences were observed between $uPA^{-/-}$, $tPA^{-/-}$, double $tPA^{-/-}/uPA^{-/-}$, and WT explants whereas a significant reduction in microvessel outgrowth was observed from $Plg^{-/-}$ vessels. At day 6, when the angiogenic response of the WT, $uPA^{-/-}$, $tPA^{-/-}$, and $tPA^{-/-}/uPA^{-/-}$ explants was maximal, a marked delay in outgrowth from the $Plg^{-/-}$ explants was still observed [65% ($P<0.001$) of WT values] (Fig. 2*b*). On day 8, except for the $Plg^{-/-}$ explants in which angiogenesis had progressed, a regression in both the number of microvessels and the number of branches was observed in all vessels tested (Fig. 2*c*). Despite a time-dependent response, the single deficiency of Plg, tPA, uPA, or uPAR as well as the combined tPA and uPA deficiency did not dramatically impair the angiogenic response in the mice aorta ring assay.

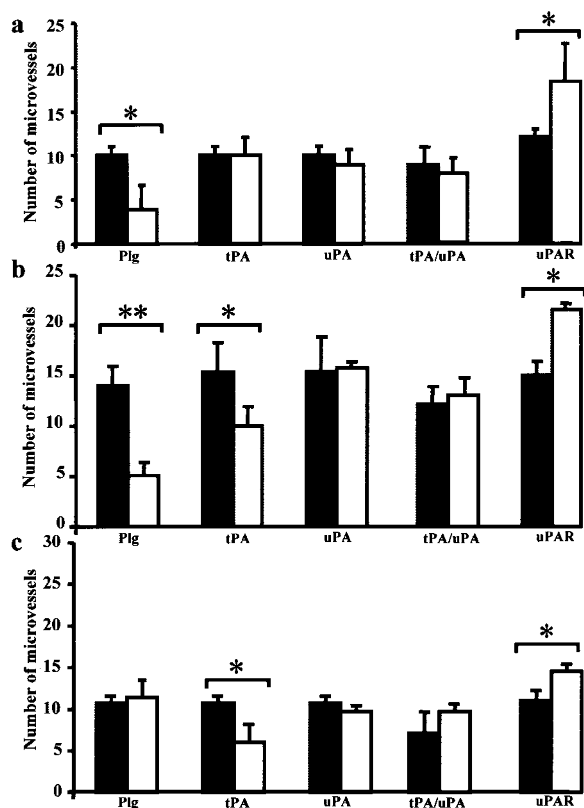

Figure 2. Capillary outgrowth from vessels derived from gene-deficient mice. Aortic explants from $Plg^{-/-}$, $tPA^{-/-}$, $uPA^{-/-}$, $tPA^{-/-}uPA^{-/-}$, or $uPAR^{-/-}$ mice (white bars) and their corresponding WT littermates (black bars) were embedded in collagen gel and cultured in autologous sera. Microvessels were quantified by computer-assisted image analysis after 4 (*a*), 6 (*b*), and 8 (*c*) days of culture *$P<0.05$; **$P<0.001$). $n = 4$; error bars = SE.

Impaired microvessel outgrowth in PAI-1-deficient mice: the dose-dependent effect of exogenous recombinant PAI-1

In contrast to the WT aortic rings (**Fig. 3*a, g***) in which a vigorous angiogenic response occurred ($N_v=27\pm2$ at day 6), microvessels were never generated from PAI-$1^{-/-}$ aortic explants during the 12 day culture period (Fig. 3*b, g*). The migration of fibroblasts from the PAI-$1^{-/-}$ explants was preserved. The importance of PAI-1 to microvessel formation was assessed by culturing aortic rings explanted from PAI-$1^{-/-}$ mice in WT serum (Fig. 3*d, g*). Under these conditions, partial restoration of neovessel formation was observed in the PAI-$1^{-/-}$ mice ($N_v=15\pm1$) (Fig. 3*g*). The physiological concentration of murine PAI-1 quantified by ELISA in the serum of WT mice was 17 ng/ml.

When aortic explants arising from PAI-$1^{-/-}$ mice were cultured in the presence of increasing concentrations of active recombinant PAI-1 (rPAI-1) (0.1–10^5 ng/ml), a bell-shaped dose-response curve was obtained (Fig. 3*h*). Microvessel outgrowth was induced by low concentrations of PAI-1 (<100 ng/ml) and inhibited by higher concentrations (1–100 μg/ml).

Molecular mechanism by which PAI-1 promotes angiogenesis

After infection of the intact PAI-$1^{-/-}$ aorta by a recombinant adenovirus vector expressing nuclear-localized β-galactosidase and culture in WT serum, a large percentage of both branched microvessels (Fig. 3*e*) and individual fibroblasts (Fig. 3*f*) expressed the transgene on day 6. This demonstrates that the recombinant adenovirus was able to transduce the cDNA into both cell types.

To examine the molecular mechanism by which PAI-1 promotes microvascular outgrowth (i.e., either by inhibiting plasminogen activation or interacting with vitronectin), PAI-$1^{-/-}$ aortic explants cultured with their autologous serum were exposed to recombinant adenoviruses expressing mutant forms of human PAI-1. Two adenoviral constructs were produced that *1)* inhibited PA activity normally but had a dramatically reduced affinity for vitronectin (AdCMVPAI-1^{Q123K}) or *2)* exhibited normal binding to vitronectin but was inactive in inhibiting the PA proteolytic activity (AdCMVPAI-$1^{R346M,\ M347S}$). Infection of PAI-$1^{-/-}$ aortic explants by AdCMVPAI-1^{Q123K} partially restored microvessel outgrowth (**Fig. 4*a***). Under these conditions, the number of vessels corresponded to 65% of the value measured after exposure to AdCMVPAI-1. By contrast, incubation of aortic rings with AdCMVPAI-$1^{R346M,\ M347S}$ virus resulted in outgrowth of only few microvessels corresponding to 20% of that observed with AdCMVPAI-1 (Fig. 4*a*).

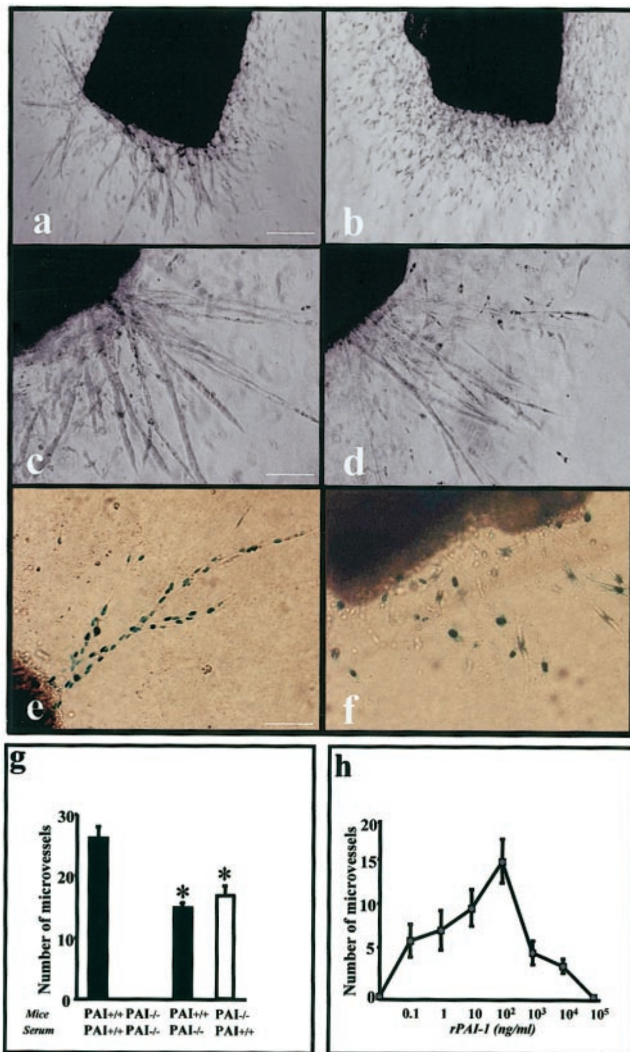

Figure 3. Angiogenesis is impaired in PAI-1$^{-/-}$ mice and modulated by exogenous PAI-1. Neovessel formation was compared in collagen-embedded explants isolated from WT and PAI-1$^{-/-}$ mice and cultured for 6 days in either autologous serum (*a, b*, bar 250 μm) or heterologous serum (*c, d*, bar 100 μm). In contrast to WT aortic rings (*a*), microvessel outgrowth was completely absent in PAI-1$^{-/-}$ aortic explants (*b*) whereas the motility of isolated fibroblast-like cells was preserved. Crossing WT and PAI-1$^{-/-}$ serum between the PAI-1$^{-/-}$ and WT aortic explants leads to partial restoration of capillary outgrowth in the PAI-1$^{-/-}$ mice and a diminution of capillary outgrowth from the WT ones, respectively (*c, d*). Infection by AdCMVβgal demonstrated that branched microvessels (*e*) and individual fibroblasts (*f*) were both infected by virus and continued to express the viral transgene for the duration of the experiment. Bar, 100 μm. The number of microvessels in explants shown in panels *a–d* was determined by computer-assisted image analysis (*g*, *$P<0.05$). Neovessel formation was analyzed in PAI-1$^{-/-}$ aorta rings cultured for 6 days in the presence of increasing concentrations of active recombinant PAI-1 (*h*). $n = 4$; error bars = SE.

PAI-1$^{-/-}$ aortic explants were next cultured in the presence of increasing concentrations of active rPAI-1 and active rPAI-1 variants (from 10 ng/ml to 10 μg/ml). These rPAI-1 variants (i.e., PAI-1-stab-Q123K and PAI-1-stab-R346M, M347S) harbor the same mutations as those present in the adenoviral constructs. In addition, we produced a second rPAI-1 variant (i.e., PAI-1-stab-R346A) impaired in PAI activity. At concentrations ranging from 10 ng/ml to 1 μg/ml, PAI-1-stab-Q123K was able to restore microvessel outgrowth whereas both of the inactive rPAI-1 variants did not. At the highest concentration (10 μg/ml), all rPAI-1 variants induced a partial or complete restoration of microvessel outgrowth (Fig. 4*b*).

DISCUSSION

The plasminogen/plasminogen activator system has been implicated in extracellular proteolysis during

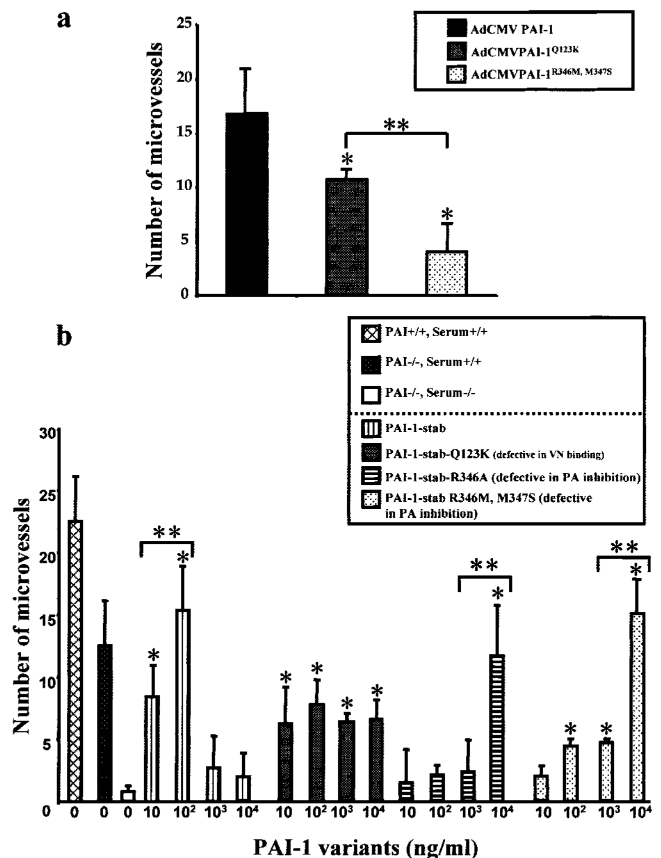

Figure 4. Molecular mechanism of action of PAI-1. Before cutting and embedding into collagen gels, entire aortas resected from PAI-1$^{-/-}$ mice were exposed to recombinant adenoviruses expressing WT or mutated forms of human PAI-1 (AdCMVPAI-1, AdCMVPAI-1^{Q123K}, AdCMVPAI-1$^{R346M, M347S}$). After 6 days of culture in PAI-1$^{-/-}$ serum, explants were examined by microscopy and microvessel quantification based on image analysis was performed (*a*). Aortic rings from WT or PAI-1$^{-/-}$ mice were cultured for 6 days in the presence of serum harvested from WT (serum+/+) or PAI$^{-/-}$ (serum−/−) mice. In the same assay, aortic rings from PAI-1$^{-/-}$ mice maintained in the presence of PAI$^{-/-}$ serum were treated with increasing concentrations of active rPAI-1 or active rPAI-1 variants. Microvessel quantification was based on computer-assisted image analysis as described in Material and Methods (*b*). *$P < 0.01$ compared with the no PAI-1 control, **$P < 0.002$ compared to the other treatment). $n = 4$; error bars = SE.

both physiological and pathological angiogenesis (2, 15). Mice with targeted disruption of genes for members of this proteolytic system develop to term, grow to adulthood, and are fertile. This suggests that either the products of these genes are not essential for physiological angiogenesis or their functions can be substituted by other proteins (27–28). The lack of PAI-1 is associated with impaired tumor angiogenesis (5, 29) whereas deficiency of uPA, tPA, uPAR, or vitronectin does not affect tumor vascularization (12).

To more precisely dissect the contribution of the various components of the Plg/PA system during angiogenesis, we developed a quantitative in vitro model of angiogenesis in which aortic explants derived from WT and gene-deficient mice ($Plg^{-/-}$, $tPA^{-/-}$, $uPA^{-/-}$, double $tPA^{-/-}/uPA^{-/-}$, $uPAR^{-/-}$, and $PAI^{-/-}$) were embedded in a 3-dimensional matrix of collagen. In accordance with our previous in vivo observations (5, 12), the present study shows that angiogenesis was completely inhibited in aortic rings from $PAI\text{-}1^{-/-}$ mice but not dramatically affected in tPA, uPA, uPAR, or double tPA/uPA-deficient mice. It is possible that the deficiency of one plasminogen activator can be compensated for by up-regulation of the other (12). The Plg activation pathway(s) occurring in the double-deficient mice remain(s) to be determined but might involve blood coagulation factor XII, kininogen, or kallikrein (14, 30–31). The delay of microvessel outgrowth observed with aortic rings isolated from $Plg^{-/-}$ mice indicates that plasmin-mediated proteolysis is a key event in angiogenesis and confirms our in vivo observations (12). Nevertheless, microvessel outgrowth was only delayed by Plg deficiency, suggesting that other molecular mechanisms can eventually accomplish the same tasks. uPAR deficiency was associated with a small acceleration in vessel outgrowth. This effect could be independent of the regulation of cell-surface uPA activity, but rather related to a promotion of cell adhesion through its interaction with vitronectin (11). Our data suggest that uPAR is a proadhesive molecule leading to a stationary phenotype.

By contrast, PAI-1 has been proposed as a molecular switch that governs cell detachment by dissociating vitronectin from uPAR, thereby promoting a migratory phenotype (11). Both tumoral and choroidal vascularization are impaired in $PAI\text{-}1^{-/-}$ mice (5, 29, 32) and can be restored in vivo by intravenous injection of a recombinant adenovirus-expressing PAI-1 (5, 32). This is consistent with our current observations that although the migration of fibroblasts was preserved, microvessel outgrowth from aortic rings was impaired in $PAI\text{-}1^{-/-}$ mice. Microvessel sprouting was restored by addition of exogenous PAI-1 as WT serum, purified active rPAI-1, or adenovirus-mediated PAI-1 gene transfer.

By testing different concentrations of active rPAI-1, we provide clear evidence for a subtle role of PAI-1 during angiogenesis. PAI-1 appears proangiogenic at nanomolar concentrations (0.1–100 ng/ml) corresponding to the normal range of concentrations present in murine plasma, but has an antiangiogenic effect at supranormal micromolar concentrations. This is consistent with the recent demonstration that PAI-1 is a potent inhibitor of FGF-2 induced angiogenesis in the chicken chorioallantoid membrane at pharmacological concentrations (33). This dose-dependent effect of PAI-1 may elucidate previous unexplained and paradoxical observations regarding this inhibitor (5, 7–9).

Based on in vitro studies, it has been suggested that protease inhibitors play an important permissive role during angiogenesis by limiting extracellular proteolysis to the immediate pericellular environment and thereby preventing excessive or inappropriate matrix degradation (34). Indeed, the stabilization of cell–matrix contacts is essential for cell migration. The colocalization of uPA and PAI-1 on migrating cells emphasizes the importance of the concomitant production of both proteases and inhibitors for cell migration (35) and tumor cell invasion (36–37). These studies have led to the concept of a 'proteolytic balance' in which critical protease-inhibitor equilibrium is necessary for cell migration and differentiation. This concept explains *1)* the impaired angiogenesis observed in vivo (12) and in vitro with both $Plg^{-/-}$ and $PAI\text{-}1^{-/-}$ mice, and *2)* the bell-shaped dose-response curve obtained in vitro with purified active rPAI-1. As PAI-1 levels increased from the minimal amount required for microvessel outgrowth to the maximum level, the angiogenic response increased. However, once the maximal permissive amount of PAI-1 was surpassed, decreased microvessel formation occurred, perhaps because of a lack of matrix degradation or a vitronectin binding effect dominant at high concentrations, or both. This effect may also be related to the fact that PAI-1 is an effective plasmin inhibitor at higher concentrations.

Active PAI-1 inhibits uPAR- or integrin-mediated cell adhesion and migration through interaction with vitronectin and inhibits uPA-mediated pericellular plasminogen activation (10–11, 38). To separate the contribution of PAI-1 protease inhibitory activity from its vitronectin binding properties on microvessel outgrowth in $PAI\text{-}1^{-/-}$ mice, recombinant adenoviruses expressing specific PAI-1 mutants were used. A partial restoration of angiogenesis was observed after aortic incubation with the adenovirus $AdCMVPAI\text{-}1^{Q123K}$ leading to the expression of PAI-1 defective in vitronectin binding. Over a large range of concentrations (10 ng/ml to 10 μg/ml), the corresponding recombinant mutant PAI-1-stab-Q123K was able to induce microvessel outgrowth from $PAI\text{-}1^{-/-}$ aortic rings. On the contrary, few microvessels were formed after addition of $AdCMVPAI\text{-}1^{R346M,\ M347S}$ or corresponding recombinant PAI-1 forms (PAI-1-stab-R346A and PAI-1-stab-R346M, M347S) exhibiting normal vitronectin binding but unable to control PA activity. These two latter PAI-1 mutants restored angiogenesis when used at a high, nonphysiological concentration (10 μg/ml), suggesting that at such concentrations the interaction with

vitronectin may take over the effect and regulate angiogenesis. Alternatively, since these mutated forms show a drastic reduction (10^4-10^5 fold) but not complete abolition of PA inhibition, it is possible that high concentrations of these mutated PAI-1 forms are able to significantly block uPA proteolysis. Nevertheless, our data show that the antiprotease activity is crucial for the proangiogenic effect of PAI-1 displayed at physiological concentrations. Based on the present data and those previously obtained in vivo (12), we cannot exclude the possibility that PAI-1/vitronectin interaction does not participate in the control of angiogenesis. Rather, it can only be concluded that vitronectin binding activity of PAI-1 is less important than PA inhibition for angiogenesis promotion in the absence of other functional deficits or challenging factors. Our results emphasize the dual effects of PAI-1 depending on its concentration and may explain the apparent discrepancy reported in the literature concerning the pro- or antiangiogenic effects of PAI-1 on angiogenesis. Our data argue for a more important role of PAI-1 at physiological concentrations through its capacity to control proteolysis rather than cell adhesion. FJ

The authors wish to thank Guy Roland, Fabrice Olivier, and Els Brouwers for their technical assistance. This work was supported by grants from the Communauté Française de Belgique (Actions de Recherches Concertées), the DGTRE of the 'Region Wallonne' from Belgium, the Commission of European Communities, the Fonds de la Recherche Scientifique Médicale, the Fonds National de la Recherche Scientifique (FNRS, Belgium), the Fédération Belge Contre le Cancer, the Fonds Spéciaux de la Recherche (University of Liège), the Center Anticancéreux près l'Université de Liège, the CGER-Assurances, the Fondation Léon Frédéricq (University of Liège), the Fonds d'Investissements de la Recherche Scientifique (CHU, Liège, Belgium), General RE-Luxembourg, and Roche Diagnostics GmbH, Penzberg, Germany. L.D., V.M., and K.B. are recipients of a grant from FNRS-Télévie. A.N. is a Senior Research Associate from the FNRS.

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Received for publication July 5, 2001.
Revised for publication September 27, 2001.