

Plasminogen Activator Inhibitor-1 Protects Endothelial Cells from FasL-Mediated Apoptosis

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SUMMARY

Plasminogen activator inhibitor-1 (PAI-1) paradoxically enhances tumor progression and angiogenesis; however, the mechanism supporting this role is not known. Here we provide evidence that PAI-1 is essential to protect endothelial cells (ECs) from FasL-mediated apoptosis. In the absence of host-derived PAI-1, human neuroblastoma cells implanted in *PAI-1*-deficient mice form smaller and poorly vascularized tumors containing an increased number of apoptotic ECs. We observed that knockdown of *PAI-1* in ECs enhances cell-associated plasmin activity and increases spontaneous apoptosis in vitro. We further demonstrate that plasmin cleaves FasL at Arg144-Lys145, releasing a soluble proapoptotic FasL fragment from the surface of ECs. The data provide a mechanism explaining the proangiogenic activity of PAI-1.

SIGNIFICANCE

Plasminogen activator inhibitor-1 (PAI-1), the central regulator of plasmin generation, is a predictor of poor clinical outcome in cancer patients. We previously reported that PAI-1 has a proangiogenic function, but the mechanism has remained poorly understood. Here we describe a mechanism for the proangiogenic function of PAI-1 by providing evidence that PAI-1 protects endothelial cells (ECs) against Fas/FasL-mediated apoptosis. We demonstrate that in the absence of PAI-1 in ECs, there is an increase in plasmin generation and the release by plasmin of a soluble FasL fragment. This plasmin-generated soluble FasL activates Fas and is a potent inducer of apoptosis in ECs. Our results suggest that PAI-1 could be a target for antiangiogenic and antivascular therapies.

INTRODUCTION

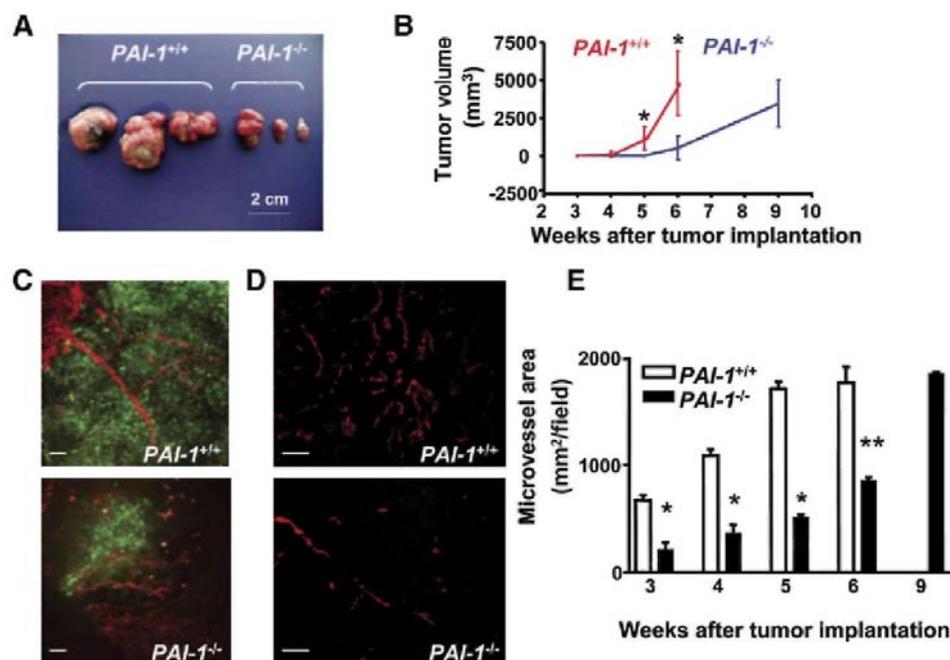
Angiogenesis, the process leading to the formation of new blood vessels, plays a central role in solid tumor growth and metastatic dissemination (Carmeliet, 2003). This process requires a coordinated and temporal regulation of adhesive, proteolytic, and migratory events. During angiogenesis, endothelial cells (ECs) stimulated by hypoxia and angiogenic factors leave a state of quiescence to proliferate and migrate through the extracellular matrix (ECM). During this process, ECs become more sensitive to apoptosis (Folkman, 2003). The Fas/FasL pathway is a key mediator of EC apoptosis and controls angiogenesis. The expression of *Fas* and *FasL* is upregulated in activated ECs and upon EC detachment, hypoxia, or exposure to vascular endothelial growth factor (VEGF) (Bouchet et al., 2002; Cardier et al., 1999; Chavakis and Dimmeler, 2002). Natural inhibitors of angiogenesis like thrombospondin 1 (TSP1) and pigment epithelium-derived factor (PEDF) derive their specific antiangiogenic function from upregulating *FasL* and enhancing apoptosis in ECs (Volpert et al., 2002). The ability of ECs to become resistant to Fas-mediated apoptosis is therefore critical for angiogenesis to succeed. Among the known mechanisms of resistance is EC attachment to the ECM, which modulates Fas-mediated apoptosis by downregulating *Fas* and upregulating *c-Flip*, an endogenous antagonist of caspase-8 (Aoudjit and Vuori, 2000, 2001). However, beyond this mechanism, little is known of other potential ways for ECs to escape.

During angiogenesis, ECs express and activate enzymes that degrade the ECM as they invade surrounding tissues. Stimulated ECs produce urokinase-type plasminogen activator (uPA), which converts plasminogen into its active form, plasmin (Pepper, 2001). uPA itself is secreted as an inactive precursor form (pro-uPA) that binds with high affinity to a specific cell surface glyco-sylphosphatidylinositol-anchored receptor designated uPAR

(Blasi and Verde, 1990). When activated, uPAR-bound uPA enhances the generation of plasmin at the cell surface, promoting ECM degradation, the activation of matrix metalloproteinases (MMPs), and the release and activation of ECM- and cell surface-anchored growth factors (Dano et al., 2005). Plasminogen activator inhibitor-1 (PAI-1) is the physiological inhibitor of uPA and therefore controls the activation of plasminogen into plasmin. In addition to interacting with uPA, PAI-1 also binds to vitronectin. The binding of PAI-1 to vitronectin results in its stabilization but also interferes with the binding of integrins of the α_v family to the RGD domain of vitronectin and therefore with cell migration (Deng et al., 1996). The two inhibitory functions of PAI-1 in proteolysis and in cell attachment initially led to the prediction that PAI-1 would have an antiangiogenic function. It was therefore surprising to discover that high rather than low levels of PAI-1 were predictive of poor outcome in patients suffering from several types of cancers like breast, colon, and neuroblastoma (Foekens et al., 1995; Ganesh et al., 1994; Sugiura et al., 1999). Consistent with a positive effect of PAI-1 on cancer progression, we and others have published data providing some explanation for the paradoxical effect of PAI-1 in cancer. We demonstrated that in the absence of host-derived PAI-1, there is a defect of tumor vascularization in mice implanted subcutaneously with transformed keratinocytes (Bajou et al., 1998, 2001) and that this is related to the antiproteolytic activity of PAI-1. The effect of PAI-1 on angiogenesis is dose dependent, with a stimulatory role reported at low physiological concentrations and an inhibitory role at pharmacological concentrations (Bajou et al., 2004). The exact mechanism by which inhibition of proteolysis by PAI-1 promotes angiogenesis, however, has not been determined. Here we provide evidence for such a mechanism by demonstrating that PAI-1 expression by ECs is critical to protect them against FasL-mediated apoptosis.

Figure 1. Absence of Host-Derived PAI-1 Reduces Human Neuroblastoma Tumor Growth and Angiogenesis

(A) Representative neuroblastoma tumors collected 6 weeks after orthotopic implantation in *PAI-1^{+/+}Rag-1^{-/-}* or *PAI-1^{-/-}Rag-1^{-/-}* mice.
 (B) Tumor volume in *PAI-1^{+/+}* (n = 6-8) and *PAI-1^{-/-}* (n = 6-8) mice over time. The data represent means \pm standard deviation (SD).
 (C) Representative images of fluorescence angiographies obtained 3 weeks after tumor implantation. Tumor cells are EGFP positive (green), and blood vessels (red) are recognized by the presence of the biotinylated tomato lectin-Texas red avidin D complex.
 (D) Representative microphotographs of tumor sections stained for PECAM-1 /CD31 4 weeks after tumor implantation. (E) Microvessel area values were determined in tumor sections stained for PECAM-1 /CD31 at the indicated time points as described in Chantrain et al. (2004). The data represent means \pm SD of 6 sections examined in each of 6-8 tumors per time point.
 * $p < 0.05$, ** $p < 0.01$ in (B) and (E). Scale bars = 100 μ m in (C) and (D).



RESULTS

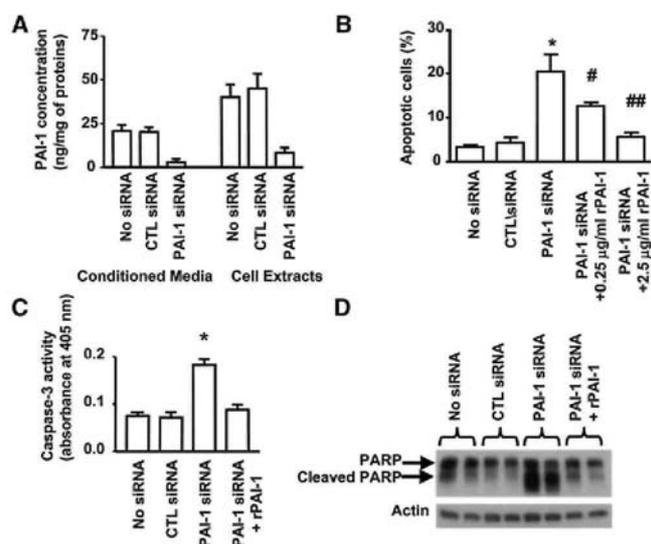
Absence of Host-Derived PAI-1 Reduces Human Neuroblastoma Tumor Growth and Angiogenesis

To investigate the importance of PAI-1 in tumor progression, we took advantage of the fact that neuroblastoma cells do not produce PAI-1 (Sugiura et al., 1999) by orthotopically implanting fragments from human SK-N-

BE(2) neuroblastoma tumors grown subcutaneously in the adrenal gland of *PAI-1^{+/+} Rag-1^{-/-}* mice and *PAI-1^{-/-} Rag-1^{-/-}* mice. The growth of these tumors was monitored by sacrificing mice 3, 4, 5, 6, and 9 weeks after implantation; measuring the tumor volume; and examining the tumors for vascularization, malignant cell proliferation, and apoptosis. We observed a significant difference in the tumor volume between the two groups of mice, with the presence of much smaller tumors in *PAI-1^{-/-}* mice compared with *PAI-1^{+/+}* mice at weeks 5 and 6 after implantation (Figures 1A and 1B). By week 6, all tumor-bearing *PAI-1^{+/+}* mice had to be sacrificed due to the presence of large tumors (average volume 5 cm³). However, by week 9 postimplantation, *PAI-1^{-/-}* mice had developed tumors of size similar to those of *PAI-1^{+/+}* mice at week 6. Because PAI-1 has been shown to play a regulatory role in angiogenesis, the vasculature of these xenotransplanted tumors was specifically examined. This analysis revealed a delay in the formation of the vasculature between weeks 3 and 6 in tumors derived from *PAI-1^{-/-}* mice compared with tumors derived from *PAI-1^{+/+}* mice as indicated by angiographies (Figure 1C) and measurements of the microvessel area in tumor sections immunostained for the platelet/endothelial cell adhesion molecule-1 (PECAM-1)/CD31 antigen (Figures 1D and 1E). At week 9, the mean microvessel area of tumors from *PAI-1^{-/-}* mice was similar to values observed in tumors from *PAI-1^{+/+}* mice at week 6. The inhibition of angiogenesis in tumors derived from *PAI-1^{-/-}* mice was associated with an inhibition of tumor cell proliferation as indicated by a lower percentage of BrdU-positive tumor cells between weeks 3 and 5 (see Figures S1A and S1B available online). In contrast, the presence or absence of *PAI-1* in host cells did not affect tumor cell apoptosis (Figures S1C and S1D). However, we observed a higher percentage of apoptotic ECs in tumors obtained from *PAI-1^{-/-}* mice at weeks 3 and 4 after tumor implantation when compared to tumors implanted in *PAI-1^{+/+}* mice (Figures S1E and S1F). This increase in apoptotic ECs was not observed in tumors obtained at weeks 5 and 6 after implantation. The data thus indicate that in the absence of host-derived PAI-1, there is a delay in tumor growth associated with an inhibition of angiogenesis and an increase in EC apoptosis. These point to a possible protective effect of PAI-1 against EC apoptosis.

Figure 2. *PAI-1 Protects Endothelial Cells from Apoptosis*

- (A) The levels of PAI-1 in conditioned medium and in cell extracts of endothelial cells (ECs) transfected with different siRNAs were measured by ELISA. The data represent the means \pm SD of triplicate samples.
 (B) The percentage of apoptotic ECs was determined 72 hr after siRNA transfection by TUNEL assay. rPAI-1 (0.25 and 2.5 μ g/ml) was added 24 hr after transfection. The data represent the means \pm SD of triplicate samples.
 (C) Caspase-3 activity in the experimental conditions described in (B) was determined by colorimetric assay as described in Experimental Procedures. The data represent the means \pm SD of triplicate samples.
 (D) PARP cleavage was examined by western blot analysis in the same experimental conditions as in (B). rPAI-1 was added at 2.5 μ g/ml 24 hr after siRNA transfection.
 * $p < 0.01$ versus control (CTL) siRNA; # $p < 0.05$, ## $p < 0.01$ compared to *PAI-1* siRNA in (B) and (C).



PAI-1 Protects Endothelial Cells against Apoptosis

To test this possibility, we examined the effect of downregulation of *PAI-1* expression on apoptosis in human brain microvascular ECs (HBMECs) using small interfering RNA (siRNA). Transfection of HBMECs with a *PAI-1* siRNA inhibited the expression of PAI-1 protein in cell extracts and conditioned media by 80% 72 hr after transfection compared with untransfected cells or cells transfected with a control scrambled siRNA (Figure 2A). Inhibition of *PAI-1* expression in HBMECs transfected with *PAI-1* siRNA was associated with a 5-fold increase

in spontaneous apoptosis (Figure 2B). Depending on the conditions, an 8- to 9-fold increase in apoptosis was observed in other experiments. A 9-fold increase in apoptosis was also documented when another *PAI-1* siRNA sequence was tested (data not shown). Consistent with this effect being the result of an inhibition of PAI-1 production and secretion in the culture medium, the percentage of apoptotic HBMECs decreased by 40% with the addition of 0.25 µg/ml of stable recombinant PAI-1 (rPAI-1) to the culture medium and decreased to baseline levels at a concentration of 2.5 µg/ml. The induction of HBMEC apoptosis upon *PAI-1* downregulation was associated with a 2-fold increase in caspase-3 activity compared with HBMECs transfected with scrambled siRNA (Figure 2C) and was also associated with the detection of poly-(ADP-ribose) polymerase (PARP) cleavage products by western blot analysis (Figure 2D). These two effects were suppressed by the addition of exogenous rPAI-1. Altogether, the data suggest that PAI-1 has a protective effect against spontaneous apoptosis in ECs that is associated with an inhibition of caspase-3 activation.

uPA-Mediated Plasminogen Activation Induces Apoptosis in HBMECs

To determine whether this protective effect against apoptosis involves the antiproteolytic activity of PAI-1, we first examined whether downregulation of *PAI-1* affects the expression of uPA and its receptor uPAR by western blot analysis. The data (Figure 3A) revealed higher levels of cell-associated uPA and uPAR in HBMECs transfected with *PAI-1* siRNA. The addition of stable rPAI-1 to HBMECs transfected with *PAI-1* siRNA decreased the amount of cell-associated uPA, although not uPAR. Changes in uPAR levels were not associated with changes in uPAR transcription since we did not observe changes in *uPAR* mRNA upon *PAI-1* downregulation by RT-PCR (Figure 3A, lower panel). When binding to uPA, PAI-1 promotes the internalization of a uPA/uPAR/PAI-1 complex, which results in an initial decrease in the expression of uPAR at the cell surface (Nykjaer et al., 1997). Consistently, analysis by flow cytometry revealed an increase in cell surface expression of uPA and uPAR in HBMECs upon downregulation of *PAI-1* that reflects a decrease in internalization of the uPA/uPAR complex in the absence of PAI-1 (Figure 3B). Downregulation of *PAI-1* was also associated with higher levels of uPA-associated proteolytic activity in cell extracts and conditioned media that were not observed upon addition of rPAI-1 or a function-blocking antibody against the proteolytic activity of uPA (Figure 3C). As anticipated and documented by casein zymography, higher levels of cell-associated uPA activity in HBMECs transfected with *PAI-1* siRNA resulted in an increase in plasminogen activation, which was suppressed in the presence of stable rPAI-1 (Figure 3D) or an anti-uPA antibody (data not shown). Thus, the data indicate that PAI-1 controls the proteolytic activity of uPA at the surface of ECs not only through inhibition of uPA activity but also through inhibition of uPA and uPAR expression. This raises the question of whether the protective effect of PAI-1 against HBMEC apoptosis can be a direct consequence of the antiproteolytic effect of PAI-1 on uPA and the subsequent decrease in plasmin generation, or, in other words, whether there is a causal link between EC apoptosis and cell-associated plasmin activity. To test this possibility, we examined the effect of blocking uPA and plasmin activity on apoptosis in HBMECs transfected with *PAI-1* siRNA. This experiment indicated a suppression of spontaneous apoptosis in *PAI-1*-deficient HBMECs in the presence of an antibody against the proteolytic function of uPA, but not in the presence of a control normal IgG. There was also a significant decrease in apoptosis in the presence of aprotinin or when plasminogen was depleted from the serum in the culture medium. The data thus confirm that the increase in uPA-generated plasmin upon *PAI-1* downregulation is responsible for inducing apoptosis in HBMECs (Figure 3E). Because plasmin has been shown to activate other proteases, in particular MMP-3/stromelysin-1 (Nagase et al., 1990) and indirectly MMP-9/gelatinase B (Ramos-DeSimone et al., 1999), it was conceivable that the effect of plasmin on apoptosis might be an indirect effect of MMP activation by plasmin. To eliminate this possibility, we performed experiments in the presence of an MMP inhibitor (AG3340). The data indicate that treatment with AG3340 does not prevent ECs from undergoing apoptosis upon *PAI-1* downregulation (Figure 3F), indicating that the effect of plasmin on apoptosis is therefore MMP independent.

Figure 3. uPA-Mediated Plasminogen Activation Induces EC Apoptosis

(A) Top: western blot analysis of cell-associated (CA) and conditioned medium (CM)-secreted uPA and CA uPAR in ECs transfected with siRNA as indicated at top. rPAI-1 was added at 2.5 $\mu\text{g}/\text{ml}$. Bottom: RT-PCR analysis of uPAR and β -actin expression in cell extracts obtained in the same conditions.

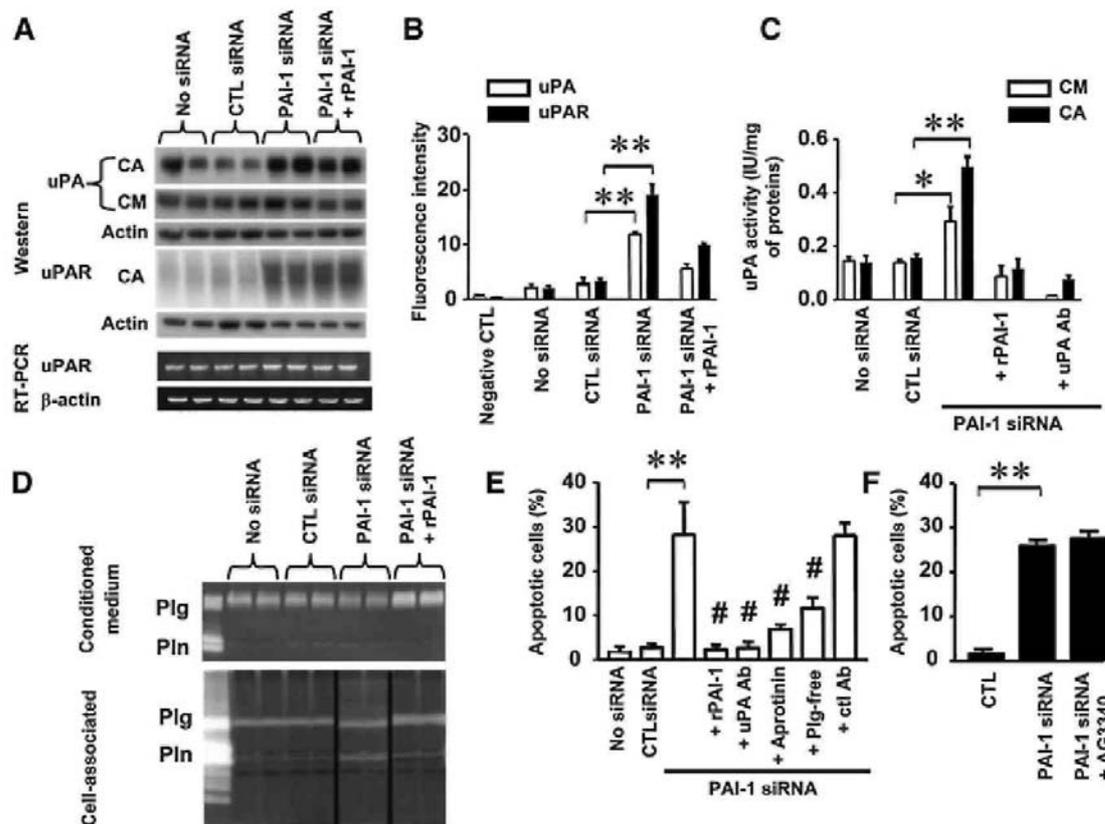
(B) The presence of uPA and uPAR at the surface of cells treated as in (A) was examined by flow cytometry. The data represent the means \pm SD of triplicate samples and are representative of three experiments showing similar results.

(C) The proteolytic activity of uPA in CA and uPA secreted in CM was determined by colorimetric assay as described in Experimental Procedures. The data represent the means \pm SD of triplicate samples.

(D) The presence of plasminogen (Plg) and plasmin (Pin) secreted in CM and in cell extracts (CA) was examined by casein zymography in the same experimental conditions as in (A).

(E and F) The percentage of apoptotic cells in ECs transfected with *PAI-1* siRNA and control siRNA and treated as indicated was determined by flow cytometry. The data represent the means \pm SD of triplicate dishes and are representative of two separate experiments showing similar results. rPAI-1 was added at 2.5 $\mu\text{g}/\text{ml}$, aprotinin at 200 $\mu\text{g}/\text{ml}$, and anti-uPA antibody or mouse IgG control at 7 $\mu\text{g}/\text{ml}$. AG3340 was added at 10 $\mu\text{g}/\text{ml}$.

* $p < 0.05$, ** $p < 0.01$ for *PAI-1* siRNA versus control siRNA in (B), (C), (E), and (F); # $p < 0.01$ for values versus *PAI-1* siRNA in (E).



Increase in FasL-Mediated EC Apoptosis in the Absence of PAI-1

We next determined whether the protective effect of PAI-1 against apoptosis involves interference with the intrinsic or the extrinsic apoptotic pathway. In a first experiment, we examined the effect of *PAI-1* downregulation on the expression of phospho-Bcl-2, Bcl-X_L, Bak, and Bax and the activation of Akt and ERK1/2. This analysis revealed no differences in the expression and/or activation of these proteins upon downregulation of *PAI-1* by siRNA (Figure S2). In contrast, we observed an increase in cleaved active caspase-8 and in caspase-8 activity in the absence of activation of caspase-9. This effect was suppressed by the addition of stable human rPAI-1. Altogether, these data suggest that in the absence of PAI-1, there is an activation of the extrinsic apoptotic pathway in ECs. Considering the central role played by the Fas/ FasL pathway in EC apoptosis, we asked whether plasmin could affect the activity of Fas/ FasL. We first documented that HBMECs expressed Fas and FasL under unstimulated conditions (Figure 4A). The Fas receptor was functional because the cells underwent apoptosis when exposed to a recombinant soluble FasL (sFasL) (Figures 4B and 4C). More direct evidence supporting the involvement of FasL in HBMEC apoptosis upon downregulation of *PAI-1* was then obtained by demonstrating that blocking Fas/ FasL interaction with an anti-Fas antibody (ZB4) inhibited apoptosis in HBMECs transfected with *PAI-1* siRNA (Figure 4B) whereas a nonspecific mouse IgG had no effect. In contrast, treatment of SK-N-BE(2) neuroblastoma cells with sFasL did not induce apoptosis

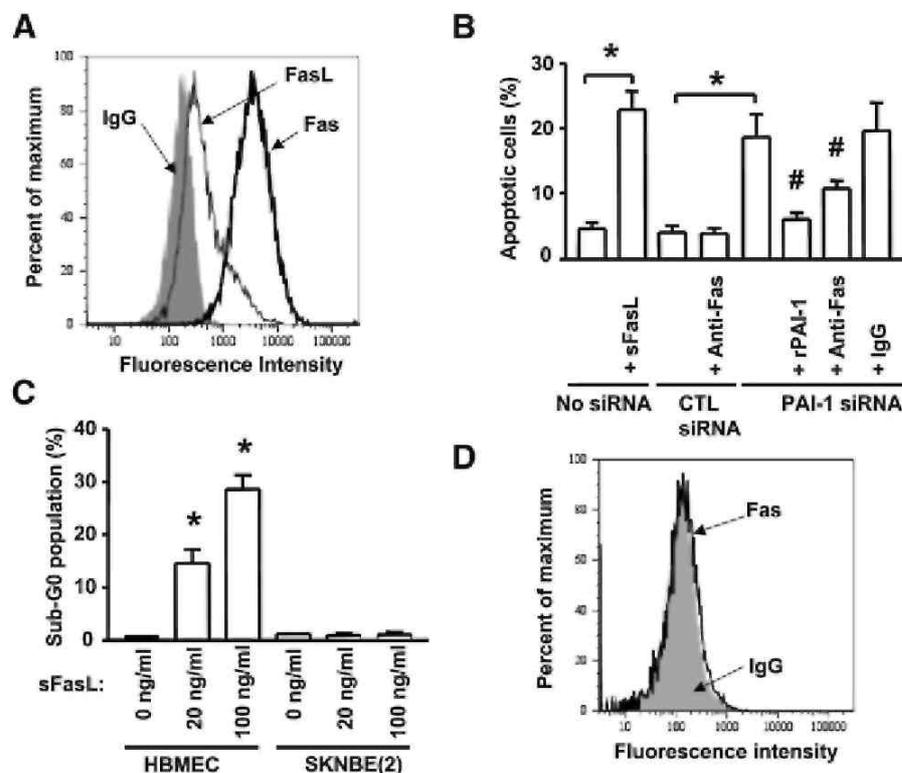
(Figure 4C) because these cells did not constitutively express Fas as determined by fluorescence-activated cell sorting (FACS) analysis (Figure 4D). In a second experiment, we tested whether *PAI-1* downregulation would induce apoptosis in HBMECs in the absence of FasL. To accomplish this, we generated a *FasL* siRNA and first demonstrated that it inhibited the expression of FasL in HBMECs by 65% (Figure 5A). We then examined the effect of simultaneously downregulating *PAI-1* and *FasL* in HBMECs by dual siRNA transfection. This experiment (Figure 5B) indicated a significant reduction in the percentage of apoptotic HBMECs when *FasL* was simultaneously downregulated with *PAI-1*, but not when a control scrambled *FasL* siRNA sequence was used. Downregulation of *FasL* alone had no effect, consistent with Fas/FasL having no effect on basal apoptosis but having an effect on apoptosis in the absence of PAI-1. Consistently, the cleavage of PARP observed upon transfection of ECs with *PAI-1* siRNA was significantly reduced upon simultaneous transfection with a *FasL* siRNA, but not with a control scrambled siRNA (Figure 5C). These data thus provide evidence that FasL is a downstream target of PAI-1 and a necessary intermediate in the induction of apoptosis in ECs upon *PAI-1* downregulation. On the basis of these observations, we postulated that the increase in plasmin activity in the absence of PAI-1 might solubilize sFasL and enhance Fas-dependent apoptosis.

Plasmin Cleaves FasL at Arg144-Lys145

This possibility was first tested by examining the proteolytic activity of plasmin on a soluble recombinant glycosylated FLAG-FasL protein extending from residue 103 to 281. The data revealed a time-dependent cleavage of the 36 kDa FLAG-FasL in the presence of plasmin and the generation of a cleavage product with a molecular mass of approximately 21.5 kDa (Figure 6A). This cleavage was inhibited by leupeptin. Neither uPA nor plasminogen cleaved FasL, but cleavage occurred in the presence of both plasminogen and uPA and was blocked in the presence of aprotinin, leupeptin, or rPAI-1 (Figure 6B). To identify the plasmin cleavage site of FasL, the N-terminal amino acid sequence of the ~21.5 kDa FasL plasmin-generated fragment was determined by Edman degradation (Figure 6C; Figure S3A). This analysis revealed the N-terminal sequence Lys-Val-Ala-His-Leu-Thr, indicating that there is a plasmin cleavage site in FasL at Arg144-Lys145 in the trimerization domain, located in the extracellular segment of the protein. This analysis predicted the release of a soluble FasL fragment of 137 amino acids with a molecular mass of 15.7 kDa (nonglycosylated) and higher pending the degree of glycosylation. We next examined whether plasmin could also cleave native membrane-associated FasL. For this experiment, human HT1080 cells (which do not express *FasL*) were transfected with a pcDNA plasmid containing either the full-length sequence of wild-type (WT) *FasL* or the same sequence in which the plasmin cleavage site Arg144-Lys145 was mutated to Val144-Ala145 (μ *FasL*). To demonstrate that this mutation did not affect the apoptotic activity of FasL, we cocultured WT *FasL*- and μ *FasL*-expressing HT1080 cells with Jurkat T cells and examined the effect on apoptosis in Jurkat T cells (Cappellesso et al., 2000). This experiment (Figure S4) revealed similar increases in apoptosis of Jurkat T cells in the presence of either WT *FasL*- or μ *FasL*-expressing HT1080 cells. Thus, the mutation at Arg144-Lys145 does not affect FasL apoptotic activity. To demonstrate that the mutation affects the cleavage of FasL, we subjected membrane extracts from HT1080 cells expressing WT or μ *FasL* to plasmin digestion and examined the extracts for the presence of FasL by western blot (Figure 6D). The data indicated a loss of the 40 kDa membrane-associated FasL protein and the presence of an ~21.5 kDa fragment in HT1080 cells expressing WT *FasL*, but not in cells overexpressing μ *FasL*. The data thus confirm that plasmin cleaves cell-associated FasL at the specific Arg144-Lys145 site. To further demonstrate that plasmin promotes the cleavage and release of FasL, we performed a series of experiments in cultured HBMECs measuring the amount of FasL released in the culture medium upon exposure to plasmin. The data (Figure 6E) indicated an increase in sFasL in the medium in the presence of plasmin at a concentration of 0.01 to 10 μ g/ml that was inhibited by the addition of α 2 anti-plasmin but not by the addition of an MMP inhibitor. This is consistent with plasmin having a direct effect on releasing sFasL from the surface of HBMECs that does not involve MMP activation.

Figure 4. Increase in FasL-Mediated Apoptosis in the Absence of PAI-1

(A) The expression of Fas and FasL in human brain microvascular ECs (HBMECs) was determined by flow cytometry.
 (B) The percentage of apoptotic cells in HBMECs transfected with siRNAs in the absence or presence of an anti-Fas blocking antibody or rPAI-1 (2.5 µg/ml) was determined by flow cytometry after staining with propidium iodide. The data represent the means ± SD of triplicate samples.
 (C) Apoptosis in HBMECs and SK-N-BE(2) tumor cells was determined by flow cytometry as in (B) after the cells were treated for 24 hr with sFasL at the indicated concentrations. The data represent the means ± SD of triplicate samples.
 (D) The expression of Fas in SK-N-BE(2) tumor cells was determined by flow cytometry.
 #p < 0.01, *p < 0.001 in (B) and (C).



The 21.5 kDa Plasmin-Generated FasL Fragment Is Released upon PAI-1 Downregulation and Is Proapoptotic

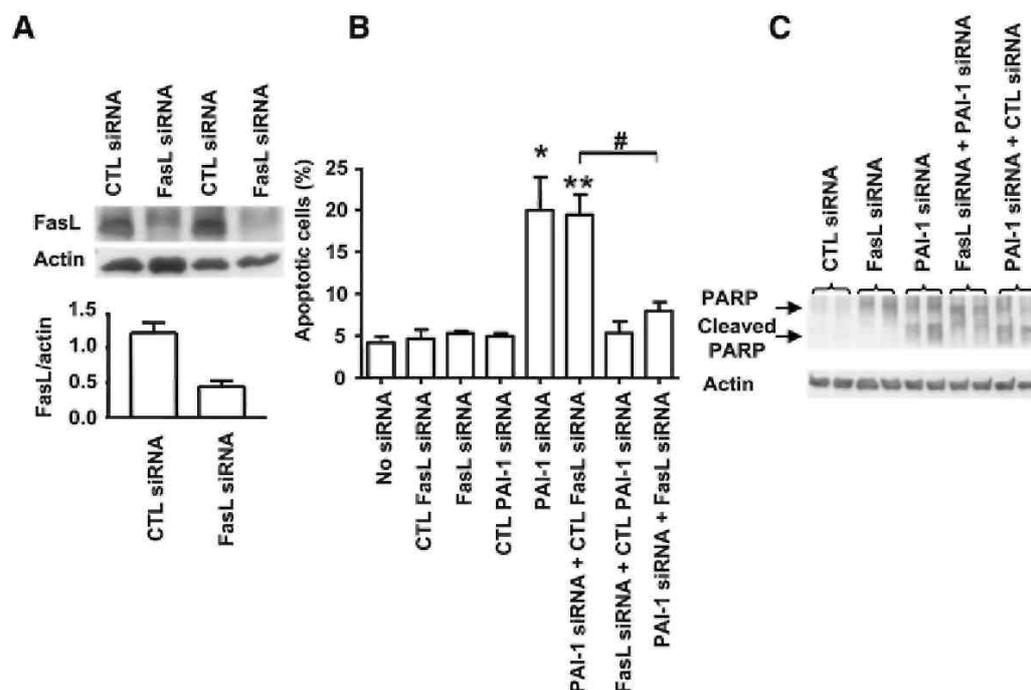
Using immunoprecipitation, we next documented the presence of the ~21.5 kDa plasmin-generated FasL peptide in the conditioned medium of HBMECs upon downregulation of *PAI-1*. Consistent with our previous data, this fragment was detected in the supernatant of HBMECs transfected with a *PAI-1* siRNA but not with the control siRNA or upon addition of rPAI-1 (Figure 7A). Further evidence supporting a key role for the cleavage of FasL by plasmin in increasing apoptosis in *PAI-1*-deficient ECs was obtained by comparing the effect of *PAI-1* downregulation in HBMECs expressing either WT *FasL* or mu *FasL*. This experiment (Figures 7B and 7C) indicated a significantly higher level of apoptosis associated with an increased presence of cleaved PARP in cells that overexpressed WT *FasL* and in which *PAI-1* was downregulated. Such an increase, above the level of apoptosis typically observed in ECs expressing endogenous *FasL* and transfected with *PAI-1* siRNA, was not observed in ECs that overexpressed mu *FasL*. Under these conditions, the level of apoptosis was similar to the level observed in cells expressing endogenous *FasL*. Altogether, these data provide direct evidence that plasmin cleaves membrane-associated FasL at Arg144-Lys145 to release an ~21.5 kDa soluble fragment in the supernatant of HBMECs upon *PAI-1* knockdown and that apoptosis upon *PAI-1* downregulation is dependent on the release of sFasL.

To obtain confirmation that the soluble FasL fragment generated by plasmin is proapoptotic in HBMECs, we generated a recombinant FasL fragment starting at Lys145 and containing a 6x His tag on its N terminus using the pRSET bacterial expression vector. This plasmid was transfected into competent BL21 *E. coli*, and the 6xHis-tagged protein (6xHisLys145FasL) was extracted from the bacterial lysate and purified in a single step on a nickel-chelating column by elution with 250 mM imidazole (Figures S3B and S3C). A preparation containing

70 µg/ml of the protein was tested for its effect on EC apoptosis. The data indicated that 6 x HisLysl 45FasL protein is a potent inducer of apoptosis in HBMECs at concentrations ranging from 10 to 500 ng/ml and that its proapoptotic activity is inhibited in the presence of an anti-Fas blocking antibody (Figure 7D). This apoptotic effect was confirmed by the detection of PARP cleavage products by western blot analysis (Figure S3D). Altogether, these data are consistent with PAI-1 having a protective effect against EC apoptosis by inhibiting the release by plasmin of a sFasL fragment that is an activator of caspase-8 and -3 in ECs. Because it has been suggested that FasL needs to form a trimer to be active and plasmin cleaves FasL in the trimerization domain, we examined whether 6 x HisLysl 45FasL forms a trimer. FLAG-sFasL and 6 x HisLysl 45FasL were incubated with the crosslinking agent disuccinimidyl suberate (DSS) and examined for trimer formation by western blot (Figure S5A). The data showed that FLAG-sFasL forms a trimer in the presence of DSS but that 6x HisLysl 45FasL does not, indicating that cleavage in the trimerization domain by plasmin prevents trimer formation. However, we showed that 6x HisLysl 45FasL is proapoptotic (Figure 7D). To confirm the proapoptotic activity, we tested whether 6xHisLysl45FasL activates Fas by examining the presence of Fas/FADD complexes in lysates of HBMECs exposed to FLAG-sFasL or 6x HisLysl 45-FasL. This experiment (Figure S5B) indicated the presence of FADD associated with Fas in HBMECs treated with FLAG-sFasL and 6x HisLysl 45FasL and thus confirms that 6x HisLysl 45-FasL is active in the absence of trimer formation.

Figure 5. FasL Is Required for EC Apoptosis upon PAI-1 Downregulation

(A) HBMECs were transfected with a scrambled (CTL) siRNA sequence or a *FasL* siRNA and examined after 72 hr for the presence of FasL in the cell lysate by western blot. A quantitative analysis of the data obtained by scanning three separate blots is shown in the lower panel. The data are the mean ratios ± SD of FasL/actin.
 (B) HBMECs were transfected with *FasL* siRNA, *PAI-1* siRNA, or a combination of both siRNAs and their respective scrambled controls and examined after 72 hr for apoptosis by flow cytometry. The data represent the mean numbers ± SD of apoptotic cells from triplicate samples, * $p < 0.025$, ** $p < 0.01$, # $p < 0.005$.
 (C) Cell lysates of HBMECs treated as indicated were examined for PARP cleavage by western blot analysis.



PAI-1 Control of Angiogenesis In Vitro Is FasL Dependent

It was important to determine whether PAI-1's control of angiogenesis in vitro is FasL dependent. We therefore plated HBMECs on Matrigel and examined the effect of *PAI-1* downregulation on the formation of a network of cords of ECs that mimic a vascular network. The data (Figures 8A and 8B) revealed a significant inhibition in the number of branches in the network formed by HBMECs in the presence of sFasL or when cells were transfected with *PAI-1* siRNA. The reduction in branching in HBMECs transfected with *PAI-1* siRNA was markedly prevented in the presence of rPAI-1, a blocking antibody against uPA, aprotinin, or plasminogen-

depleted serum. Consistent with the effect of PAI-1 on angiogenesis being dependent on Fas/FasL, the addition of a blocking antibody against Fas also prevented the reduction in branching. These data are consistent with our data on apoptosis and indicate that PAI-1 affects angiogenesis in vitro by a mechanism that is Fas dependent.

Fas, FasL, uPA, and uPAR Are Expressed in Neuroblastoma Tumors Orthotopically Implanted in Mice

To obtain evidence that Fas/FasL and the plasminogen system contribute to EC apoptosis in tumors, we examined by immunofluorescence the expression of FasL, Fas, uPA, and uPAR in neuroblastoma tumors orthotopically implanted in mice. The data (Figure S6) revealed a strong expression of uPAR in cells aligned along the basement membrane (type IV collagen) of blood vessels, consistent with uPAR being expressed in vascular ECs. uPA also was coexpressed with PECAM-1/CD31-positive ECs in the tumor vasculature. There was no obvious difference in uPA and uPAR expression between tumors grown in WT mice and *PAI-1*^{-/-} mice. We also demonstrated the coexpression of PECAM-1/CD31 and Fas or PECAM-1/CD31 and FasL in vascular structures in these tumors (Figure S6). The data thus indicate that Fas/FasL and uPA/uPAR are expressed in ECs in vivo. Although the data do not demonstrate that they are responsible for the increased apoptosis observed in the absence of PAI-1, the selective expression of these proteins in vascular ECs in tumors is consistent with their role in controlling EC apoptosis in vivo.

In summary, our data identify a cleavage site for plasmin in FasL that results in the release of a proapoptotic sFasL peptide and provide a mechanism explaining the previously reported proangiogenic activity of PAI-1 (Figure S7).

DISCUSSION

Our data provide a mechanism explaining the protective effect of PAI-1 against apoptosis through its control of the pericellular activity of plasmin and FasL solubilization. Evidence for such a FasL-dependent mechanism is provided by the following data: (1) induction of apoptosis in *PAI-1*-deficient ECs is blocked in the presence of an anti-Fas antibody; (2) *PAI-1*-deficient ECs do not undergo apoptosis in the absence of FasL; (3) there is no additional increase in apoptosis in *PAI-1*-deficient ECs over-expressing FasL in which the plasmin cleavage sequence has been mutated; and (4) FasL is released as a 21.5 kDa soluble protein in the culture medium of ECs upon *PAI-1* downregulation or plasmin treatment. We also report a cleavage site in FasL that is sensitive to plasmin. Membrane-associated FasL (mFasL) is typically solubilized by MMPs, and its cleavage by plasmin has not been reported thus far.

The effect of sFasL on apoptosis is complex, differs among cell types, and is in part governed by cell polarity. In nonpolarized cells like hematopoietic and mesenchymal cells and lymphocytes, the solubilization of FasL results in Fas inactivation (Tanaka et al., 1995). In contrast, in polarized epithelial cells, sFasL is active and induces apoptosis (Powell et al., 1999). The cleavage of mFasL by MMP-7 in prostate epithelial cells induces Fas-mediated apoptosis (Vargo-Gogola et al., 2002), whereas it protects tumor cells from drug cytotoxicity (Mitsiades et al., 2001). In ECs under hypoxia, FasL is solubilized by MMPs and forms a 70 kDa trimeric complex that inhibits Fas and prevents hypoxia-induced apoptosis (Mogi et al., 2001). Our data are different, as we report here the cleavage of FasL not by an MMP but by plasmin and demonstrate that this cleavage enhances rather than prevents apoptosis in ECs. Because plasmin is a known activator of several MMPs, it was important to demonstrate that the effect of plasmin on the solubilization of sFasL and apoptosis in vivo was also a direct effect as shown in a test tube assay. Our data demonstrating an absence of effect of AG3340 on the release of sFasL by plasmin in cultured HBMECs and on HBMEC apoptosis upon *PAI-1* downregulation are consistent with plasmin having a direct effect independent of MMP activation. The cleavage of FasL by plasmin was demonstrated not only for a recombinant protein but also for FasL expressed by HT1080 cells. The absence of cleavage in cells overexpressing mu *FasL* also confirms the specificity of the cleavage. It is interesting to note that in the case of MMP-7, two cleavage sites have been reported. A primary cleavage site at a leucine residue in the sequence ELAELR between the transmembrane domain and the self-assembly (trimerization) domain generates a 25 kDa fragment, and a secondary ELR site in the self-assembly domain generates an approximately 21 kDa fragment. In the case of plasmin, the cleavage site was identified close to the secondary MMP-7 cleavage site at RK in the KELRKV sequence in the self-assembly domain and generated a 21.5 kDa fragment. Consistent with the plasmin cleavage site being within the trimerization domain, we demonstrated an absence of trimer formation in the recombinant 6xHisLys145FasL protein corresponding to the plasmin-generated sFasL. However, we demonstrated that this fragment is proapoptotic in ECs and activates Fas. These data are also consistent with the observation of Vargo-Gogola et al. (2002) that MMP-7, which also cleaves FasL in its trimerization domain, increases apoptosis in prostate epithelial cells and indicate that trimerization of sFasL is not necessary for its activity. This may explain why our results differ from the data of Mogi et al. (2001).

Although these authors did not identify the MMP cleavage site in FasL, they reported the presence of soluble FasL trimers that prevented Fas-mediated apoptosis. It is thus conceivable that the absence of the trimerization domain in the case of cleavage by plasmin (and MMP-7) may be responsible for the proapoptotic activity of sFasL.

Figure 6. Cleavage of Recombinant and Membrane-Associated FasL by Plasmin

(A and B) Western blot analysis of FLAG-FasL (10 µg/ml) treated in the conditions indicated at the top using an anti-FLAG antibody (upper panels) or an anti-FasL antibody (lower panels). Concentrations used were as follows: leupeptin, 500 µg/ml; plasmin (Pin), 1 µg/ml; uPA, 600 IU/ml; plasminogen (Pig), 1 µg/ml; aprotinin, 1 mg/ml; rPAI-1, 50 µg/ml.
 (C) Silver staining of a polyacrylamide gel of recombinant sFasL incubated in the presence or absence of Pin as indicated in (A) and (B).
 (D) Western blot analysis with an anti-FasL antibody of membrane proteins extracted from HT1080 cells expressing wild-type (WT) *FasL* or a mutant (*mu*) *FasL* and incubated for 1 hr in the presence or absence of plasmin (1 µg/500 µg of lysate) at 37° C before SDS-PAGE.
 (E) Levels of sFasL in serum-free culture medium of HBMECs incubated in the presence of plasmin and inhibitors as indicated. α2 anti-plasmin was added at a concentration of 100 µg/ml and AG3340 at a concentration of 10 µg/ml. The data represent the mean concentrations ± SD of sFasL in the culture medium of triplicate samples and are representative of two experiments showing similar results, * $p < 0.025$, ** $p < 0.01$, *** $p < 0.005$ versus untreated cells; # $p < 0.005$ versus plasmin-treated cells; NS, not significant.

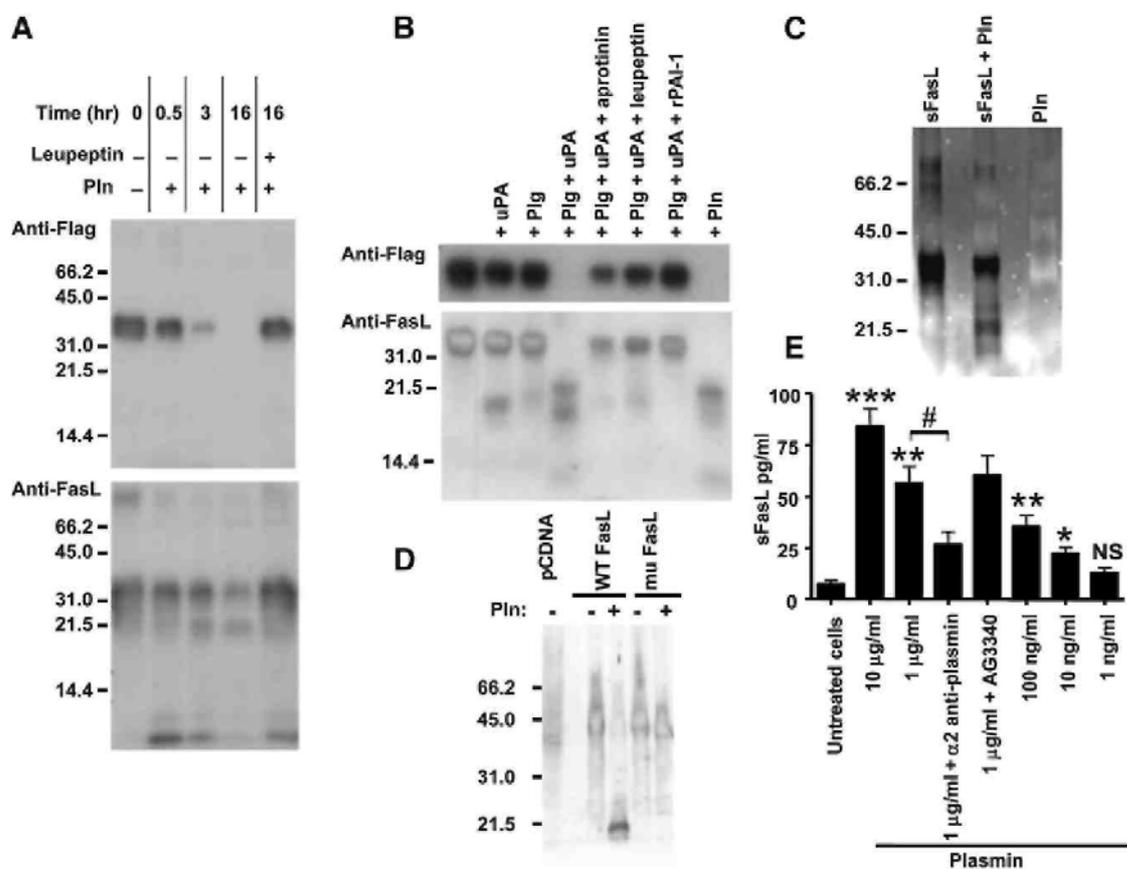


Figure 7. The 21.5 kDa Plasmin-Generated FasL Fragment Is Proapoptotic

(A) The presence of the 21.5 kDa sFasL plasmin-generated fragment was detected by immunoprecipitation and western blot analysis with an anti-FasL antibody in the conditioned medium of HBMECs treated as indicated at top.

(B) HBMECs were transfected with a pCDNA plasmid containing either WT FasL or mu FasL. Stable transfected cells were selected and retransfected with a PAI-1 siRNA or a control siRNA and tested for apoptosis by FACS analysis after 72 hr. The data represent the means \pm SD of triplicate samples. * $p < 0.05$.

(C) Cells treated as described in (B) were examined for the presence of cleaved PARP by western blot analysis.

(D) The recombinant 6xHisLys145FasL protein that corresponds to the 21.5 kDa plasmin-generated FasL fragment obtained as shown in Figure S2C was added at the indicated concentrations to HBMECs for 48 hr, and the percentage of apoptotic cells was measured by flow cytometry. An anti-Fas antibody (ZB4) was added at 500 ng/ml. The data represent the means \pm SD of triplicate samples, ** $p < 0.005$.

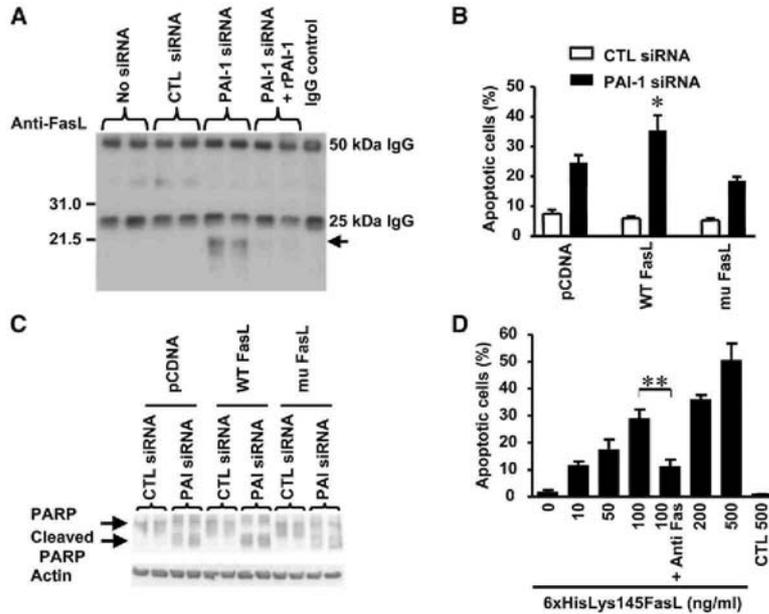
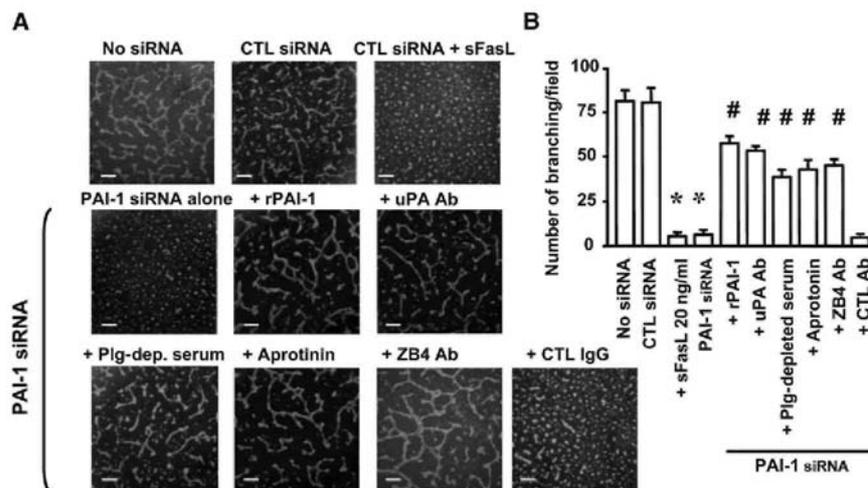


Figure 8. FasL Inhibits EC Branching in Matrigel

(A) Representative photomicrographs of HBMECs plated on Matrigel and treated as indicated. Scale bars = 400 μ m.

(B) Quantitative analysis of the data in (A). The data represent the mean numbers of branches per microscopic field \pm SD from a total of six fields examined in each of three different dishes, * $p < 0.001$, # $p < 0.001$.



The demonstration that plasmin cleaves FasL in vitro does not necessarily mean that plasmin also cleaves FasL in vivo, as plasmin is inhibited in vivo by a series of physiological inhibitors other than PAI-1, such as $\alpha 2$ anti-plasmin. Although $\alpha 2$ anti-plasmin primarily acts on the fibrinolytic system inside the vasculature, it is present in the extravascular space that is invaded by sprouting ECs during angiogenesis and could inhibit the release of sFasL by plasmin as suggested by our in vitro data (Figure 6E). However, PAI-1 may play a more predominant role because it is made by ECs and controls pericellular plasmin activity. The question of the role of PAI-1 as the only regulator of angiogenesis remains open. Nevertheless, our data showing an increase in apoptosis in ECs in PAI-1 null mice indicate that PAI-1 has a contributory function.

The central role of Fas/FasL in controlling EC apoptosis and angiogenesis is well recognized. Although quiescent ECs express low levels of Fas, they become increasingly sensitized to Fas-mediated apoptosis under hypoxic conditions or when stimulated by VEGF or TNF α (Sata and Walsh, 1998), and attachment of ECs to ECM proteins is a known mechanism that protects these cells from Fas-mediated apoptosis. Hypoxia and VEGF have been shown to upregulate *PAI-1* expression in ECs (Dimova and Kietzmann, 2006), which raises the possibility that upregulation of *PAI-1* under hypoxia or by VEGF could be critical for the survival of stimulated ECs. This hypothesis is currently being explored in our laboratory.

The protective role of PAI-1 against EC apoptosis could also be critical for conditions other than tumor angiogenesis. For example, it has been demonstrated that PAI-1 inhibits apoptosis in vascular smooth muscle cells during the formation of atherosclerotic plaques. When mice that are deficient in apolipoprotein E (*ApoE*^{-/-}) and show increased formation of atherosclerotic plaques are crossed with *PAI-1*-deficient mice, there is a decrease in plaque formation that corresponds to an increase in apoptosis in vascular smooth muscle cells (Luttun et al., 2002). Our data suggest that in the absence of plasminogen/plasmin, there would be a stimulation of angiogenesis and wound healing. This is not necessarily the case, however, as there is an impairment in wound healing in plasminogen (*Plg*)-deficient mice (Kortlever and Bernards, 2006) and an acceleration in *PAI-1*-deficient mice (Chan et al., 2001). However, a critical feature in these models is the presence of a provisional fibrin matrix, as suggested by the observation that in mice deficient in plasminogen and fibrinogen there is a stimulation rather than inhibition of wound healing (Bugge et al., 1996). Our studies have not addressed the effect of *PAI-1* deficiency on fibrin and have focused on pericellular plasmin activity and its local control over the release of cell surface-associated proteins like FasL. Although the formation of a provisional fibrin matrix is critical in wound healing, its role in tumor angiogenesis is less well understood. It is conceivable that the selective presence of PAI-1 stabilized by vitronectin in the pericellular space is a mechanism allowing plasmin to act remotely to degrade the provisional fibrin matrix without solubilizing FasL at the surface of ECs. These possibilities clearly deserve further investigation. Our data demonstrating a significant delay in angiogenesis in the absence of host-derived PAI-1 provide important insight into vascular diseases other than tumor angiogenesis. Whether PAI-1 could be a valuable target in controlling angiogenesis is an interesting but presently unanswered question that is currently being examined in our laboratory.

EXPERIMENTAL PROCEDURES

Orthotopic Tumor Model

PAI-1-deficient mice (*PAI-1*^{-/-}) and their corresponding wild-type mice (*PAI-1*^{+/+}) on a mixed genetic background of 87% C57BL/6 and 13% 129 strain (Carmeliet et al., 1993) were mated with *Rag-1*-deficient mice (*Rag-1*^{-/-}: B6; 129 s-*Rag-1*^{tm/Mom/J}) purchased from Charles River Laboratories (L'Arbresle, France) to generate *PAI-1*^{-/-} and *PAI-1*^{+/+} *Rag-1*^{-/-} immunodeficient mice. Mice were anesthetized with avertin, and a 1 mm³ SK-N-BE(2) cl10 subcutaneous tumor fragment was sown on the left adrenal gland as described previously (Chantrain et al., 2004). Mice were sacrificed between 3 and 9 weeks after tumor implantation. Ex vivo angiographies and analysis of tumors were performed as described previously (Chantrain et al., 2004) (see Supplemental Experimental Procedures). All animal experiments were performed in accordance with procedures established by the Institutional Animal Care and Usage Committee of Childrens Hospital Los Angeles under approved protocol #41-05.

RNA Interference

A first siRNA duplex with sequences corresponding to nucleotides 457-477 of human *PAI-1* cDNA (GenBank accession number X12701) (5'-AAGGACGAG ATCAGCACCACA-3') was used for most experiments. A second nucleotide sequence corresponding to nucleotides 286-306 of human *PAI-1* cDNA (5'-AA CGTGGTTTTCTCACCTAT-3') was used to confirm the effect of *PAI-1* knockdown on apoptosis in HBMECs. The siRNA sequence for *FasL* was 5'-AACT GGGCTGTACTTTGTATA-3' (Ji et al., 2005). All siRNA duplex oligonucleotides were synthesized by QIAGEN Sciences. HBMECs were transfected over 5 hr with 40 nM *PAI-1* siRNA, *FasL* siRNA, or a corresponding scrambled siRNA using either Lipofectamine 2000 (Invitrogen) or a TransMessenger Transfection Kit (QIAGEN Sciences). FITC-labeled siRNA was used to assess transfection efficiency.

Measurement of PAI-1 and sFasL Levels

PAI-1 and sFasL levels were determined using commercially available ELISA kits for human PAI-1 (American Diagnostica Inc.) and human sFasL (R&D Systems). The amount of protein in each sample was determined

using a BCA protein assay kit (Pierce), and PAI-1 values were corrected for the amount of protein present in each sample.

Apoptosis

To evaluate apoptosis in HBMECs in culture, we used a TUNELAPO-Direct kit (BD Biosciences PharMingen) or flow cytometry analysis of cells labeled with propidium iodide (5 µg/ml). After nonadherent cells were washed with PBS, adherent cells were harvested using trypsin-EDTA, resuspended in medium containing serum, collected by centrifugation, and resuspended with nonadherent cells in 1 ml of 1% paraformaldehyde in PBS. After several washes with PBS, the cells were resuspended in 70% ethanol and stored at -20°C prior to staining and analysis by flow cytometry.

Flow Cytometry

Analysis of HBMECs by flow cytometry was performed using the following primary antibodies: mouse monoclonal antibody against human uPA (American Diagnostica Inc.), mouse monoclonal antibody against human uPAR (American Diagnostica Inc.), mouse monoclonal antibody against Fas (Alexis), and mouse monoclonal antibody against FasL (Alexis). FITC-conjugated horse anti-mouse IgG (Vector Laboratories) was used as a secondary antibody. Cells were analyzed on a Beckman Coulter Epics ESP cell sorter equipped with a 488 nm argon laser. Data were acquired and analyzed with EXPO32 software (Applied Cytometry Systems).

Caspase-3, -8, and -9 Activities

Caspase-3 and -8 activities were measured using the ApoTarget Caspase-3/ CPP32 and Caspase-8/FLICE Colorimetric Protease Assay (BioSource). The activity of each caspase was determined in 200 µg of cell lysate proteins suspended in 50 µl of extraction buffer. Absorbance at 405 nm using a spectrophotometer was determined after 24 hr of incubation with the substrate for each caspase. The presence of cleaved caspase-8 and -9 was examined by western blot analysis using antibodies from Cell Signaling Technology.

uPA Proteolytic Activity

Ten microliter aliquots of conditioned media or cell extracts were incubated with bovine plasminogen (Sigma, 0.1 mg/ml) and a chromogenic substrate for plasmin (Spectrozyme PL; 0.5 mM; American Diagnostica Inc.) in PBS. Reactions (100 µl) were carried out in the presence or absence of amiloride (2 mM) in 96-well microtitration plates at room temperature for 20 min, and the release of free p-nitroaniline from the chromogenic substrate was measured spectrophotometrically. The activity was determined in reference to a standard curve generated by the activity of a two-chain uPA standard, corrected for the amount of protein in each sample and expressed in international units (IU) per mg of proteins.

Zymographic Analysis of Plasmin Generation

α-casein (1 mg/ml; ICN Biomedicals) was incorporated in 0.1 % SDS/8% poly-acrylamide gels. Aliquots of conditioned media and cell extracts containing equal amounts of proteins were mixed with loading buffer (10% SDS, 4% sucrose, 0.25 M Tris-HCl [pH 6.8], 0.1 % bromophenol blue). After electrophoresis, SDS was eluted from the gels by two washes (30 min each) of 100 mM glycine, 2.5% Triton X-100 buffer (pH 8.0). Gels were incubated overnight at 37°C in 100 mM glycine buffer (pH 8.0), stained with 0.1 % Coomassie brilliant blue, and destained in 20% methanol/10% acetic acid.

Mutagenesis of FasL

The amino acid substitutions Arg144→Val144 and Lys145→Ala145 were introduced in the plasmin cleavage site of *FasL* using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions and a human *FasL* cDNA subcloned into BamHI pcDNA 3.1-Neo (Invitrogen) expression vector (Vargo-Gogola et al., 2002) kindly provided by L. Matrisian (Vanderbilt University). For the substitution, we used the primer pair forward 5'-CCTGAAAAAAGGAGCTGGTGGCAGTGGCCATTAAACAG GC-3'; reverse 5'-GCCTGTAAATGGGCCACTGCCACCAGCTCCTTTTTTTC AGG-3'.

Matrigel Angiogenic Assay

Matrigel (Becton Dickinson) was added at 300 μ l to each well of a 24-well plate and allowed to polymerize for 1 hr at 37°C. HBMECs (4×10^4) in culture medium were then added to the coated wells. Cells were incubated for 24 hr at 37°C, viewed under an Olympus CKX41 microscope, and photographed using PictureFrame 2.1 software (Optronics). The numbers of branches were counted in five different fields in triplicate wells.

Statistical Evaluation

Results are represented as mean \pm standard deviation (SD). For in vivo data, statistical analysis was performed using a nonparametric Mann-Whitney U test. In vitro experiments were performed in triplicate and repeated at least two times, and the analysis was performed using a two-tailed Student's t test, $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and seven figures and can be found with this article online at <http://www.cancerCell.Org/cgi/content/full/14/4/324/DC1/>.

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