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.

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 Dimmel, 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 glycosylphosphatidylinositol-anchored receptor designated uPAR (Blasi and Verde, 1990). When activated, uPAR-bound uPA enhances the generation of plasmin at the cell surface, promoting

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.
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, and therefore with cell migration but also interferes with the binding of integrins of the RGD domain of vitronectin and therefore with cell adhesion. The binding of PAI-1 to vitronectin results in its stabilization but also interferes with the binding of integrins of the αv 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; Sugira 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.

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 (Sugira et al., 1999) by orthotopically implanting fragments from human SK-N-Be2 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 tumor vasculature, 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,
The induction of HBMEC apoptosis upon stable recombinant PAI-1 (rPAI-1) to the culture medium and secretion in the culture medium, the percentage of apoptotic ECs (HBMECs) using small interfering RNA (siRNA). Transfection with a control scrambled siRNA resulted in an increase in cell surface expression of 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.

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-XL, 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 PAI-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 FasL.
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 aprotinin, leupeptin, or rPAI-1 (Figure 6B). To identify the plasmin cleavage site of FasL, the N-terminal amino acid sequence predicted the release of a soluble FasL fragment of 137 amino acids with a molecular mass of approximately 21.5 kDa (Figure 6C). 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 segment of wild-type (WT) FasL or the same sequence in which the plasmin cleavage site Arg144-Lys145 was mutated to Val144-Ala145 (mu FasL). To demonstrate that this mutation did not affect the apoptotic activity of FasL, we cocultured WT FasL- and mu 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 mu 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 mu 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 mu 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 μg/ml that was inhibited by the addition of α2 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 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 6×His tag on its N terminus using the pRSET bacterial expression vector. This plasmid was transfected into competent BL21 E. coli, and the 6×His-tagged protein (6×HisLys145FasL) 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×HisLys145FasL 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 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×HisLys145FasL forms a trimer. FLAG-sFasL and 6×HisLys145FasL 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 6×HisLys145FasL does not, indicating that cleavage in the trimerization domain by plasmin prevents trimer formation. However, we showed that 6×HisLys145FasL is proapoptotic (Figure 7D). To confirm the proapoptotic activity, we tested whether 6×HisLys145FasL activates Fas by examining the presence of Fas/FADD complexes in lysates of HBMECs exposed to FLAG-sFasL or 6×HisLys145-FasL. This experiment (Figure S5B) indicated the presence of FADD associated with Fas in HBMECs treated with FLAG-sFasL and 6×HisLys145FasL, and thus confirms that 6×HisLys145-FasL is active in the absence of trimer formation.

**PAI-1 Control of Angiogenesis In Vitro Is Fas Dependent**

It was important to determine whether PAI-1’s control of angiogenesis in vitro is Fas 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, aprtinin, 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 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 overexpressing 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 apoptosis.

**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 (Pln), 1 μg/ml; uPA, 600 IU/ml; plasminogen (Plg), 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 Pln as indicated in (A) and (B). (D) Western blot analysis with an anti-FasL antibody of membrane proteins extracted from HBMECs 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. s2 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 transfected with a PAI-1 siRNA or a control siRNA and tested for apoptosis by FACS analysis after 72 hr. The data represent the means ± 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 6×HisLys145FasL 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 ± SD of triplicate samples. **p < 0.005.
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 EAELR 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 6×HisLys145FasL 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.

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 that PAI-1, such as α2-anti-plasmin. Although α2-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 TNFα (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-deficient mice (Pig−/−) (Kortlever and Bernard, 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<sup>−/−</sup>) and their corresponding wild-type mice (PAI-1<sup>+/+</sup>) 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<sup>−/−</sup>; B6; 129 s-Rag-1<sup>Tm(mom)</sup>/J) purchased from Charles River Laboratories (L’Arbresle, France) to generate PAI-1<sup>−/−</sup> and PAI-1<sup>+/−</sup> Rag-1<sup>−/−</sup> immunodeficient mice. Mice were anesthetized with avertin, and a 1 mm<sup>2</sup> SK-N-BE(2) c10 subcutaneous tumor fragment was sown on the left adrenal gland as described previously (Chantren 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 (Chantren 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′-AAAGGACCGAG ATCAGGACACA-3′) was used for most experiments. A second nucleotide sequence corresponding to nucleotides 286–306 of human PAI-1 cDNA (5′-AA CTGTGTATTCTCACCTAT-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 TUNEL APO-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.

**Statistical Evaluation**

Results are represented as mean ± standard deviation (SD). For in vivo data, statistical analysis was performed using a nonparametric Mann-Whitney U test.
Plasmin Cleaves FasL and Induces Apoptosis

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. $p \leq 0.05$ 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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