A Novel Function of Plasminogen Activator Inhibitor-1 in Modulation of the AKT Pathway in Wild-type and Plasminogen Activator Inhibitor-1-deficient Endothelial Cells*

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Cell proliferation, an event associated with angiogenesis, involves coordinated activities of a number of proteins. The role of plasminogen activator inhibitor-1 (PAI-1) in angiogenesis remains controversial. Utilizing proliferating PAI-1−/− endothelial cells (EC), the impact of a host PAI-1 deficiency on Akt activation was evaluated. Hyperactivation of Akt(Ser(P)473) was observed in PAI-1−/− EC, and this was probably due to enhanced inactivation of tumor suppressor PTEN, thus rendering the cells resistant to apoptotic signals. Higher levels of activated caspase-9 in PAI-1−/− EC led to lower levels of procaspase-3 and cleaved caspase-3, thereby promoting survival. These effects were reversed when recombinant PAI-1 was added to PAI-1−/− EC. Additional studies demonstrated that regulation of proliferation is dependent on its interaction with low density lipoprotein receptor-related protein. Thus, PAI-1 is a negative regulator of cell growth, exerting its effect on the phosphatidylinositol 3-kinase/Akt pathway and allowing controlled cell proliferation.

Angiogenesis is an important biological process involving the formation of new capillaries from preexisting blood vessels, and it occurs in normal as well as pathophysiological settings. It involves a highly orchestrated series of events, including the stimulation of endothelial cells (EC) by growth factors, degradation of the extracellular matrix, migration and proliferation of EC, and formation of new capillary tubes (1, 2). Components of the fibrinolytic pathway have been implicated in the formation of angiogenic vessels, notably plasminogen activators, viz. urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator. The activation product, plasmin, a trypsin-like serine protease, facilitates degradation of the extracellular matrix, either directly or indirectly through activation of matrix metalloproteases. This process allows migration of EC and subsequent events leading to neovessel formation. However, specific inhibitors control proteolysis of the extracellular matrix, and PAI-1 is an important inhibitor of the plasminogen activation system (3, 4). Considering the important role of the plasminogen activator-PAI-1 system in angiogenesis and the availability of genetically engineered mice lacking various components of this system, several investigators have utilized transgenic approaches to establish the functional potential of components of the plasminogen activator-PAI-1 system in angiogenesis.

The role of PAI-1 in angiogenesis and tumor formation has been extensively studied, but results were determined to be dependent on the experimental setting (5–8), the stage of cancer progression, and the origin of the cells (9–13). It has been demonstrated that implanted malignant keratinocytes were unable to induce vascularization of tumors in PAI-1-deficient mice (PAI-1−/−) (5). Similarly, T241 fibrosarcoma tumor growth was suppressed in PAI-1−/− mice relative to WT mice (6). In vitro studies demonstrated a lack of microvessel generation in an aortic ring assay derived from aortas of PAI-1−/− mice (7). Laser-induced choroidal neovascularization was also reduced in PAI-1−/− mice (14). However, both of these observations were reversed when the experimental system was exposed to exogenous PAI-1. This is in concordance with clinical studies that correlate high levels of PAI-1 with disease recurrence and reduced survival for patients from a variety of tumors (15). Results from these studies suggest that PAI-1 is proangiogenic. However, contradictory data have also been documented, whereby a PAI-1 deficiency had no impact on metastasis of melanoma cells (9) or on murine mammary tumor virus-PymT-induced breast cancer progression (10). In yet another related investigation, an antiangiogenic effect of PAI-1 was observed through the inhibition of fibroblast growth factor-2-induced angiogenesis in the chicken chorioallantoic membrane assay (11). Other studies have shown that the presence of active PAI-1 inhibited M21 human melanoma tumor growth and angiogenesis in Matrigel implants in mice in a dose-dependent manner (12). Additionally, a dose-dependent effect of PAI-1 has been reported using in vitro (7) and in vivo (14) models of angiogenesis. Hence, the influence of PAI-1 in angiogenesis is still controversial.

Since vascular EC are the principal initiators of angiogenesis, they are highly relevant cells in which to gain insight into the
role of PAI-1 in cell functions associated with the angiogenic process. This laboratory has previously reported that a complete deficiency of PAI-1 is associated with increased proliferation of aortic EC in vitro (16), an effect associated with higher levels of Akt(Ser(P)473). Akt-dependent signaling is known to be activated by the interaction of growth factors with their cognate receptors, thereby promoting EC survival (17, 18). In particular, it has been demonstrated that vascular EC growth factor (VEGF), via the phosphotyrosine kinase KDR/Flk-1 receptor, activates Akt and induces an antiapoptotic response in different cell types (19). More recently, the role of VEGF receptor-3 has been elucidated in proliferation, migration, and survival of primary EC via activation of the Erk, Akt, and Jnk pathways (20). In PAI-1−/− EC, interaction of VEGF with VEGF receptor-1 stimulated the phosphorylation of Akt (16). It has been documented that activated Akt phosphorylates a number of downstream substrates involved in cell survival, cell cycle progression, glucose metabolism, and protein synthesis, which are biological functions of the EC that contribute to angiogenesis (21, 22). The crucial role of the PI3K/Akt pathway in cell survival is supported by the observation that the tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten), which is inactivated in a number of human cancers, possesses a 3′-phosphoinositol-phosphatase activity, thereby inactivating the PI3K/Akt pathway (23). It is also known that Akt promotes survival through activation of caspase-9 (24), activation of the transcription factor NF-κB (25, 26), and different components of the apoptotic machinery, such as BAD and forhead transcription factor (27).

Although a number of earlier studies have focused on the effect of PAI-1 or its deficiency on tumor development and vascularization, wound healing, and inflammation, our study is the first to mechanistically underline the effect of PAI-1 on EC survival via the PI3K/Akt signal transduction pathway (16) (this study). Given that a proangiogenic signal (VEGF) in proliferating PAI-1−/− EC stimulates activation of Akt, the current study focused on an investigation of the EC-based consequences of this enhanced activation. The results of these investigations are presented herein.

**EXPERIMENTAL PROCEDURES**

**Reagents**

The α-tubulin antibody was obtained from Sigma and from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit antibodies against mouse Akt, mouse Akt(Ser(P)473), human PTEN, human PTEN(Ser(P)409), mouse caspase-9, human caspase-9(Thr(P)125), and human caspase-3 were purchased from Cell Signaling Technology (Beverly, MA). Where required, the secondary antibody used was horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG (Bio-Rad) and horseradish peroxidase-conjugated goat anti-mouse IgM antibody (Santa Cruz Biotechnology Inc.). The rabbit 2629 anti-human LRP polyclonal antibody was a generous gift from Dr. D. K. Strickland. Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences) was used for the Western blots.

To prepare complete medium (CM), RPMI 1640 (Mediatech, Herndon, VA) was supplemented with 20% fetal bovine serum (BioWhittaker, Walkersville, MD), 1% antibiotic/antimycotic mixture (100 units of penicillin, 0.1 mg of streptomycin, 0.25 μg of amphotericin B), 50 μg/ml endothelial growth factor supplement (BD Biosciences), 2 mM glutamine (Mediatech), 0.1 mM amino acids (Invitrogen), and 1 μl/ml β-mercaptoethanol (Invitrogen). EC serum-free defined medium was purchased from Cell Applications (San Diego, CA). Recombinant WT murine PAI-1 and the variants, recombinant PAI-1 (R76E) and recombinant PAI-1 (R346A) were expressed and purified as described (28). Endotoxin levels were evaluated using the Limulus Amebocyte Lysate kit QCL-1000 (Cambrex, Walkersville, MD). Final endotoxin levels of the PAI-1 protein preparations used were less than 0.01 enzyme units/ml.

**Mice**

The characteristics of PAI-1−/− mice have been previously described (29, 30). PAI-1−/− mice were back-crossed into the C57BL/6J strain (>F3). WT C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were used as controls. Male mice between 8 and 12 weeks of age were utilized for this study. The mice were anesthetized, intraperitoneally, with a rodent mixture (0.015 mg of xylazine, 0.075 mg of ketamine, and 0.0025 mg of aceprozamine/g of body weight). Experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Notre Dame.

**Harvesting of Aortic Trees and Isolation of EC from Aortic Tissues**

The methods for harvesting of aortas and the isolation, purification, maintenance, and confirmation of homogeneity of primary EC have been described previously (16). All cell culture experiments were performed at 37 °C in a humidified 5% CO2 incubator. At least six mice/genotype/preparation have been used for each n value.

**Cell Proliferation Assays**

Cell proliferation assays were performed as previously described (16). EC resuspended in CM at a density of 2 × 10⁴ cells/ml were added to collagen-coated 24-well plates and allowed to adhere overnight. The medium was then aspirated, and fresh CM without EC growth supplement was added (t = 0). The cells were then allowed to proliferate for 24, 48, 72, and 96 h after the change in medium. For proliferation assays, where the addition of PAI-1 protein was required, the cells were plated as above, but at 72 h, 10 ng/ml rPAI-1 was added to the cells. The cells were then counted after 24 h. Manual cell counts were performed after the cells were fixed in 4% paraformaldehyde and then stained with hematoxylin (Vector Laboratories, Burlingame, CA). The cells were viewed with a Nikon Eclipse TE200 microscope, and images were recorded using the SPOT camera and the SPOT advanced version 4.0.9 software (Diagnostic Instruments, Inc., Sterling Heights, MI). Cell counts were determined in triplicate/time point/genotype (three fields/well).

For proliferation assays involving inhibition of PI3K, EC were seeded at a density of 1 × 10⁵ cells/well in a 6-well collagen-
Akt Phosphorylation Assay

Data were expressed as percentage of WT activity. For measurement of the levels of total Akt and Akt-
 phosphorylated) dye for 1 h at 37 °C and 5% CO2. The cells were
 incubated with LEHD-
 caspase-9 activity using the colorimetric caspase-9
 assay kit (Calbiochem) as per the manufacturer’s instructions.
 An aliquot of 2 × 106 cells were lysed in lysis buffer, and the
 protein concentration of the supernatant was assayed. An ali-
quots of 400 μg of protein was diluted in cell lysis buffer and
 incubated with LEHD-p-nitroanilide substrate at 37 °C for 2 h,
 and samples were read at 405 nm in a microplate reader (SPEC-
 TRAmax Plus; Molecular Devices, Sunnyvale, CA). This assay
 was also performed using EC treated with exogenous PAI-1. Although this kit assays for both early (caspase-7) and
 late apoptosis (caspase-3), data presented herein are for late
 apoptosis.

Caspase-9 Assay

A proliferation assay was set up as described under “Analysis
 of EC Apoptosis.” At the 96 h time point, WT and PAI-1−/−
cells (in the presence or absence of 10 ng/ml PAI-1) were ana-
lyzed for caspase-9 activity using the colorimetric caspase-9
 assay kit (Calbiochem) as per the manufacturer’s instructions.
 An aliquot of 2 × 106 cells were lysed in lysis buffer, and the
 protein concentration of the supernatant was assayed. An ali-
quots of 400 μg of protein was diluted in cell lysis buffer and
 incubated with LEHD-p-nitroanilide substrate at 37 °C for 2 h,
 and samples were read at 405 nm in a microplate reader (SPEC-
 TRAmax Plus; Molecular Devices, Sunnyvale, CA). This assay
 was also performed using EC treated with exogenous PAI-1.
 Data were expressed as percentage of WT activity.

Akt Phosphorylation Assay

Cells were plated at a density of 1 × 105 cells/ml in a 6-well
 collagen-coated plate and allowed to adhere overnight. After
 this time, the medium was aspirated, and fresh CM without EC
 growth factor supplement was added. Cells were then allowed
to grow for 72 h and then washed three times with
 phosphate-buffered saline. After this, they were incubated in
 EC serum-free defined medium (Cell Applications, Inc.) over-
night and then treated with 150 or 200 nM wortmannin for 1 h.
The cells were directly lysed with 100 μl of SDS gel loading
 buffer. For measurement of the levels of total Akt and Akt-
 phosphorylated) primary antibodies (Cell Signaling Technology).

Western Blot Analysis

For detection of total Akt, Akt(Ser(P)473), α-tubulin, caspase-3, PTEN, PTEN(Ser(P)180), and caspase-9, cells were
 added to lysis buffer (1% Igepal CA-630, 50 mM Tris-HCl (pH
 7.5), 100 mM NaCl, 5 mM EDTA, 250 mM NaF, 200 mM β-glyc-
erophosphate, 2 μg/ml aprotinin and leupeptin, and 1 mM
 phenylmethylsulfonyl fluoride). The cell lysates were cen-
 trifuged, and supernatants were fractionated on gels, blotted on
 polyvinylidene difluoride membranes (Osmonics Inc.), and
 immunoassayed according to the manufacturer’s protocol. For
 some experiments, WT rPAI-1 was added to proliferating cells
 at 72 h, and the cells were lysed 24 h later. Cleaved caspase-3
 and caspase-9 analyses were performed by fractionating the cell
 lysates on 10–20% linear gradient Tris-HCl gels (Bio-Rad). To
 enable distinct separation of the cleaved caspase-9 bands, the
gel was run at 205 V until the dye front reached the bottom of
 the gel, and then the gel was run for an additional 10 min.
 Densitometric analyses of Western autoradiograms were per-
 formed using the Scion program downloaded from NIH (avail-
able on the World Wide Web at www.scioncorp.com).

RT-PCR and Western Blot Analysis for LRP Detection

RT-PCR Analysis—Total RNA from WT and PAI-1−/− EC
 was isolated using the RNeasy mini method (Qiagen, Valencia,
 CA). LRP mRNA levels were detected by TaqMan analysis
 (Sequence Detection System 7700, Applied Biosystems, Foster
 City, CA) using primer sequences 5’-TGCCGGGCCCTCAT-
 TGAC-3’ (forward) and 3’-GCCGTTATACCGAACA-
 CCA-3’ (reverse) to yield a predicted 144-bp product. The
 reverse transcription-PCR was performed using the SYBR®
 Green Master mix (Applied Biosystems). The reaction was
 loaded on a 1.5% agarose gel stained with ethidium bromide to
 observe the PCR band.

Western Blot Analysis—Actively growing WT and PAI-1−/−
cells at a confluence of 65–70% were lysed, and total protein was
 loaded on a 6% SDS-polyacrylamide gel under nondenaturing
 conditions. Electrobloottng was performed overnight, at first
 using 70 V for 1 h and then at 30 V overnight, followed by 70 V
 for 45 min. The membrane was blocked in blocking buffer (1×
 TBS, 0.1% Tween 20, 5% milk powder, 1 mM CaCl2). The blot
 was exposed to the primary antibody, 1 mg/ml rabbit 2629 anti-
 human LRP polyclonal antibody, at room temperature for 2 h.

Immunoprecipitation of Cleaved Caspase-3

An aliquot of 75 μg (for detecting cleaved caspase-3) or 100 μg (for detecting caspase-9(Thr(P)163)) of WT and PAI-1−/−
cell lysates were incubated at 4 °C with 1:100 diluted caspase-3
 antibody or 1:50 diluted caspase-9(Thr(P)163) antibody (recog-
nizes Thr(P)163 of mouse caspase-9) in a total volume of 200 μl
 overnight with gentle rocking. To this mixture 20 μl of 50% bead
 slurry of Protein A-agarose beads (Pierce) was added and
 incubated for 3 h under the same conditions. The beads were
 coated plate and allowed to adhere overnight. The medium was
 then aspirated, and fresh complete medium without EC growth
 supplement was added (t = 0). The cells were allowed to grow
 for 72 h and treated with 200 nM wortmannin for 2 h. After this
 time, the medium was aspirated, and cells were washed three
times with 1× phosphate-buffered saline and then incubated in
 EC-defined serum-free medium with 200 nM wortmannin and
 20% serum where indicated. Cells were harvested after 18 h of
 incubation, and total cell counts were performed.
**Modulation of Akt Signaling in PAI-1<sup>−/−</sup> EC**

washed five times under cold conditions with cell lysis buffer and resuspended in 20 μl of gel loading buffer, and equal volumes were loaded onto an SDS-polyacrylamide gel, which was used to perform Western blot analysis.

**Statistical Analysis**

Where applicable, results represent the mean ± S.E. Differences in mean values were analyzed using Student’s t test. p values of ≤0.05 were considered to be statistically significant.

**RESULTS**

**PI3K Mediates Akt Activation and Affects Proliferative Activity in WT and PAI-1<sup>−/−</sup> EC**—In unstimulated cells, Akt is present in the cytoplasm, and the two regulatory phosphorylation sites at Thr<sup>308</sup> and Ser<sup>473</sup> are nonphosphorylated. Upon growth factor stimulation, PI3K recruits Akt to the plasma membrane, where Akt is sequentially phosphorylated at Thr<sup>308</sup> and Ser<sup>473</sup>. Phosphorylation at both sites is required for full activation of proliferative function in WT and PAI-1<sup>−/−</sup> was observed. These results support the conclusion that the presence of serum, which activates Akt, an increase in cell counts (Fig. 1A). The kinase responsible for phosphorylation at both sites at Thr308 and Ser473 are nonphosphorylated. Upon growth factor stimulation, PI3K recruits Akt to the plasma membrane, where Akt is sequentially phosphorylated at Thr308 and Ser473. Phosphorylation at both sites is required for full activation of Akt (21). Whereas the ratio of Akt(Ser<sup>P</sup>473)/Akt was 1.53 in PAI-1<sup>−/−</sup> EC compared with 0.12 for WT cells at 96 h, the levels of Akt(Thr<sup>P</sup>308) in the two genotypes were similar, as well as the levels of PDK1(Ser<sup>P</sup>241) (3-phosphoinositide-dependent protein kinase-1), a protein that facilitates phosphorylation of Akt(Thr<sup>P</sup>308) (data not shown). The kinase responsible for phosphorylation of Akt(Ser<sup>P</sup>473) has not been identified (31).

To determine whether Akt activation is transduced by PI3K, proliferating WT and PAI-1<sup>−/−</sup> EC were treated for 1 h with wortmannin, a selective inhibitor of PI3K. Immunoblot analysis using anti-Akt(Ser<sup>P</sup>473) and anti-Akt antibodies, demonstrated that at doses of 150 nM or above, wortmannin inhibited phosphorylation of Akt at Ser<sup>P</sup>473 in both cell types, whereas total levels of Akt were not altered (Fig. 1A). These results suggest that, as in WT cells, Akt activation in PAI-1<sup>−/−</sup> cells is mediated by PI3K.

It has been reported that wortmannin is known to block growth factor-induced antiapoptotic activity as well as proliferative activity in different types of EC. Additionally, it has been demonstrated that wortmannin inhibits angioptotin-1 (Ang-1)-induced antiapoptotic effects due to inhibition of basal PI3K activity in serum-deprived human umbilical vein EC (18). Similarly, wortmannin is known to block the protective action of nerve growth factor (32) and platelet-derived growth factor in serum-deprived PC12 cells (33), as well as VEGF-mediated antiapoptotic activity in human umbilical vein EC (17). To examine the involvement of PI3K in the proliferative process, proliferating WT and PAI-1<sup>−/−</sup> EC were incubated with 200 nM wortmannin and assayed for total cell counts (Fig. 1B). Wortmannin decreased the total cell counts in both WT and PAI-1<sup>−/−</sup> EC. The decrease in cell counts may be due to inhibition of basal PI3K activity present in serum-deprived cells. In the presence of serum, which activates Akt, an increase in cell counts was observed. These results suggest the conclusion that the proliferative function in WT and PAI-1<sup>−/−</sup> EC is mediated via PI3K.

To further explore the basis of enhanced Akt(Ser<sup>P</sup>473) in proliferating PAI-1<sup>−/−</sup> cells, the upstream negative regulator, phosphatase PTEN, was analyzed. PTEN inhibits the PI3K-Akt signaling pathway by dephosphorylating phosphoinositide 3,4,5-triphosphate, which anchors Akt to the plasma membrane, a step that is crucial for allowing activation of Akt (23). Recently, it has been shown that phosphorylation at the C terminus of PTEN inhibits its activity, thus resulting in increased concentrations of phosphoinositide 3,4,5-triphosphate and Akt hyperactivation (34). Therefore, the phosphorylation state of PTEN in proliferating WT and PAI-1<sup>−/−</sup> EC was analyzed. Consistent with enhanced Akt activation, higher levels of the inactivated form of PTEN, PTEN(Ser<sup>P</sup>380), were observed in PAI-1<sup>−/−</sup> cells at 96 h compared with WT cells (Fig. 2, A and B). These results suggest that regulation of PTEN activity plays an important role in controlling proliferation.

**Increased Akt Activation in PAI-1<sup>−/−</sup> EC Is Associated with Decreased Caspase-3 Levels and Increased Caspase-9(Thr<sup>P</sup>163)**—Recent observations suggest that there is an inverse association between expression of PAI-1 and activation of caspase-3. Neuronal cells grown in medium deficient in PAI-1 demonstrated an increase in activation of caspase-3 and induction of apoptosis (35). It has been reported that spontaneous and induced apoptosis were mitigated when exogenous PAI-1 was added to HL-60 promyelocytic leukemia cells (36). Similarly, increased expression of PAI-1 in vascular smooth muscle cells has been observed, concomitant with a decrease in apoptosis and inhibition of caspase-3 activity (37). Immunoblot analyses of pro-caspase-3 levels from WT and PAI-1<sup>−/−</sup> cell lysates at 96 h revealed that levels of procaspase-3 (35 kDa) were lower in PAI-1<sup>−/−</sup> cells at 96 h compared with WT cells (Fig. 3, A and B). Procaspase-3 levels were normalized to α-tubulin.
levels, which were similar in WT and PAI-1−/− EC. Detection of cleaved caspase-3 (17- or 19-kDa) band(s), was facilitated by immunoprecipitation using WT and PAI-1−/− cell lysates at 96 h. In concurrence with lower levels of procaspase-3 observed in PAI-1−/− EC, lower levels of cleaved active caspase-3 bands were observed in these cells (Fig. 3C). The lower levels of procaspase-3 and active caspase-3 in PAI-1−/− EC at 96 h are consistent with our initial findings and support the hyperactivation of Akt at 96 h in these cells.

To explore whether the upstream initiator protein caspase-9 was affected by hyperactivation of Akt, protein levels of procaspase-9 (49 kDa) and its cleaved forms (37 and 39 kDa) were determined by immunoblot analysis using cell lysates from WT and PAI-1−/− EC at 96 h. Caspase-9 is a member of the caspase-signaling pathway, and cleaved caspase-9 activates other caspase molecules, including caspase-3 (38). As observed in Fig. 4A, no differences were observed in the levels of uncleaved procaspase-9 in cells between the two genotypes. However, at 96 h, levels of the cleaved forms, p37 and p39, in PAI-1−/− cells appeared to be lower compared with WT cells (Fig. 4, A and B). Correspondingly, the activity of caspase-9 in PAI-1−/− lysates at 96 h was 60% of that of WT cells (Fig. 4C). Caspase-9 occupies an upstream position in the caspase cascade, and small changes in activated caspase-9 are enough to trigger downstream events. Since activated Akt is known to inactivate mouse caspase-9 by phosphorylation of Thr163 (24), levels of caspase-9(Thr(P)163) were determined at 96 h in the two genotypes. As anticipated, higher levels of caspase-9(Thr(P)163) were seen in PAI-1−/− cells relative to WT cells (Fig. 4D), suggesting that higher levels of Akt(Ser(P)473), in the PAI-1−/− cells, protect against apoptosis.

**Measurement of Cell Counts and Apoptosis in WT and PAI-1−/− EC**—Cell proliferation assays using WT and PAI-1−/− EC, retrieved after collagenase treatment, were employed to analyze total cell counts and apoptosis by flow cytometry. Cell counts were as expected at 72 and 96 h, with higher levels for PAI-1−/− EC relative to WT EC (Fig. 5A). To examine whether lower levels of active caspase-3 in PAI-1−/− EC at 96 h resulted in lower levels of apoptosis compared with WT cells, a specific apoptosis assay was performed. As observed in Fig. 5B, at 72 h, the percentage of apoptosis in both WT and PAI-1−/− EC was similar. However, at 96 h, apoptosis in PAI-1−/− cells was lower compared with WT cells. These findings indicated that at least some percentage of EC grown in culture underwent caspase-dependent programmed cell death and underline the role of Akt in protection against apoptosis in PAI-1−/− cells.

**Effect of Exogenous PAI-1 on PAI-1−/− Cell Proliferation and Components of the Akt Pathway**—LRP is an endocytic receptor, capable of binding to a wide variety of ligands mediating their internalization (39). It is well established that LRP binds to the PAI-1–uPA–uPAR complex (40) through sites localized in PAI-1 and uPA, followed by internalization and degradation of the complex. Some investigators have reported that EC do not express LRP (41, 42),

![Image](image-url)
revealed that the relative ratio of p37/full-length caspase-9 is caspase-9 (49 kDa) and the two cleaved forms of caspase-9 (39 and 37 kDa). There were no detectable differences in the full-length form of caspase-9 between genotypes. B, densitometric analysis of the band intensities revealed that the relative intensity of p37/full-length caspase-9 is ~1.6-fold higher in WT cells at 96 h compared with PAI-1−/− cells. The bars represent ± S.E. of two independent experiments using different lots of cells. C, caspase-9 activity in WT and PAI-1−/− EC at 96 h. Caspase-9 activity using cell lysates of proliferating WT and PAI-1−/− cells. Caspase-9 activity in PAI-1−/− cells was 60% of WT cells. *, p < 0.05. D, levels of caspase-9Thr(P)163 in proliferating WT and PAI-1−/− cells. An aliquot of 100 μg of cell lysates from WT and PAI-1−/− cells at 96 h was used for immunoprecipitation with caspase-9Thr(P)163 antibody, and a Western blot using the same antibody was performed. α-Tubulin was used as a loading control.

To examine whether the molecular mechanism by which PAI-1 affects proliferation is due to its protease inhibitory activity or to its ability to bind to LRP, EC were incubated with rPAI-1 variants. PAI-1 constructs were added to proliferating WT and PAI-1−/− EC. These proteins were either severely diminished in inhibiting the proteolytic activity of uPA-tissue-type plasminogen activator (R346A), or PAI-1-LRP binding ability was compromised (R76E) (28). The addition of the different variants of PAI-1 to WT cells did not affect the cell counts (Fig. 7B). However, in PAI-1−/− cells, only the R346A variant partially reduced the cell counts. Since exogenous WT PAI-1, when added to the cells at the 72 h time point did not decrease the cell counts of PAI-1−/− EC to WT levels, proliferating PAI-1−/− EC were spiked with exogenous PAI-1 twice, once at 48 h and then at 72 h after the medium change. This was done due to observations that enhanced Akt(Ser(P)473) was detected at 72 h in PAI-1−/− EC, and, therefore, exposing cells to PAI-1 24 h earlier could potentially enhance the effects of exogenous PAI-1. Results from these studies indicated that spiking the PAI-1−/− EC twice with WT rPAI-1 and rPAI-1 (R346A) mutant further diminished the cell counts to WT levels (data not shown), whereas the mutant rPAI-1 (R76E) incubated with PAI-1−/− EC did not alter proliferation of PAI-1−/− EC. These results suggest that regulation of cell proliferation by PAI-1 is most likely dependent on its interaction with LRP and does not rely on its inhibitory activity to any significant extent.

To assess if the addition of PAI-1 to WT cells resulted in almost no change in cell counts, the addition of PAI-1 to WT cells resulted in almost no change in cell counts.
PTEN, and procaspase-3 and cleaved caspase-3 were examined using cell lysates from PAI-1-treated cells for immunoblot analyses. As seen in Fig. 8A, the addition of PAI-1 decreased the levels of Akt(Ser(P)473) as well as that of inactivated PTEN(Ser(P)380) in PAI-1−/− cells. An increase of procaspase-3 and cleaved caspase-3 levels was observed in PAI-1−/− cells treated with rPAI-1 relative to WT levels. On the other hand, the addition of exogenous PAI-1 to WT cells did not have an effect on the protein levels of PTEN(Ser(P)380), Akt(Ser(P)473), and procaspase-3. Finally, we also analyzed WT and PAI-1−/− cells exposed to rPAI-1 for caspase-9 activity levels and caspase-3-directed apoptosis. A concomitant increase in caspase-9 activity was observed in PAI-1−/− cells treated with rPAI-1 (Fig. 8B).

The presence of rPAI-1 also increased the percentage of apoptotic activity of PAI-1−/− cells (Fig. 8C), whereas rPAI-1 had no effect on either caspase-9 activity or apoptosis in WT cells.

Collectively, these results suggest that PAI-1 may be acting as a negative growth regulator of murine aortic EC. Thus, PAI-1−/− EC proliferate at a higher rate than WT cells, and the addition of exogenous PAI-1 restores the growth suppression, most likely through the LRP binding domain, resulting in decreased cell proliferation. Results from these studies also suggest that PAI-1 regulates the activation of Akt at Ser473, which may then consequently affect levels of caspase-3, ultimately affecting apoptosis.

**DISCUSSION**

Regulation of cell proliferation and survival involves a concerted involvement of growth factors, their receptors, and signaling pathways. Among the different pathways activated by the interaction of growth factors and their cognate receptors, the PI3K/Akt transduction signaling pathway has proven to be important for cell survival in many cell types. Altered activation of this pathway correlates with the development and progression of cancers (43). Based on our initial observations that enhanced proliferation of PAI-1−/− EC is probably due to enhanced activation of the Akt signaling pathway (16), further investigations were pursued to determine the effects of this hyperactivation on other cellular functions (e.g. apoptosis). The activation of the PI3K/Akt signaling pathway results in the phosphorylation of a number of Akt substrates that are important in controlling the balance between cell survival and apoptosis (44–46). In both WT and PAI-1−/− EC, the activation of Akt is dependent on PI3K, since levels of Akt(Ser(P)473) were inhibited at relatively low levels of wortmannin (WM). The extent to which WM reduces the cell counts in both WT (29.6%) and PAI-1−/− (28.1%) EC in the presence of serum is almost identical. Multiple intracellular signal transduction pathways, like mitogen-activated protein kinase, extracellular signal-regulated kinase, and signal transducers and activators of transcription, are involved in regulating cell proliferation. It is quite likely that another parallel signaling pathway(s) is hyperactivated in PAI-1−/− EC, which may be reflected by the fact that WM did not reduce the cell counts of PAI-1−/− cells to the same level as WT cells.

Activation of PI3K is a common feature in the signal trans-
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FIGURE 8. Exogenous PAI-1 affects the components of the Akt pathway in PAI-1−/− cells. A, immunoblot of extracts prepared from WT and PAI-1−/− cells treated (with PAI-1) or untreated (C) for 24 h and probed for Akt(Ser(P)473), Akt, PTEN(Ser(P)380), PTEN, pro-caspase-3, and cleaved caspase-3. Lanes 1, WT EC (+PAI-1); lanes 3, PAI-1−/− EC; lanes 4, WT−/− EC (+PAI-1). Intensities of the band revealed that the addition of PAI-1 to PAI-1−/− cells had decreased the phosphorylation levels of the upstream regulator PTEN(Ser(P)380) and thereby affected the phosphorylated levels of downstream Akt(Ser(P)473) as well as downstream caspase-3 target molecules in these cells while having no effect on WT cells. α-Tubulin served as control. Densitometric ratios of Akt(Ser(P)473)/Akt were as follows: WT(C), 1.2; WT + PAI-1, 0.96; PAI-1−/− (C), 2.9; PAI-1−/− + PAI-1, 1.1. Densitometric ratios of PTEN(Ser(P)380)/PTEN were as follows: WT(C), 1.4; WT + PAI-1, 1.1; PAI-1−/− (C), 2.98; PAI-1−/− + PAI-1, 1.9. Densitometric ratios of pro-caspase-3/α-tubulin were as follows: WT(C), 1.33; WT + PAI-1, 1.41; PAI-1−/− (C), 0.78; PAI-1−/− + PAI-1, 1.2. B, effect of PAI-1 on caspase-9 activity on nontreated cells (C) compared with PAI-1-treated cells. *, p < 0.05 for PAI-1−/− EC not exposed to PAI-1 versus PAI-1−/− EC exposed to rPAI-1. C, effect of PAI-1 on caspase-3-mediated apoptosis on nontreated cells (C) compared with PAI-1-treated cells. *, p < 0.05 for PAI-1−/− EC not exposed to PAI-1 versus PAI-1−/− EC exposed to rPAI-1. IP, immunoprecipitation.

duction of the antiapoptotic effects of growth factors (47). It has been reported that VEGF and Ang-1 are capable of inducing Akt phosphorylation at Ser473 in a PI3K-dependent manner and exert antiapoptotic effects (17, 18). Although phosphorylation of both Thr308 and Ser473 is required for full activation of Akt, only Ser473 was hyperphosphorylated in PAI-1−/− cell lysates, whereas levels of Akt(Thr(P)308) were similar in both genotypes. One reason for the aberrant activation of Akt in PAI-1−/− cells could be the higher levels of inactivated PTEN(Ser(P)380) resulting in unopposed Akt activation. This implies that regulation of PTEN phosphorylation in WT cells may aid in protecting the cells by maintaining the balance between proliferative and antiproliferative functions. It has been reported that there is a positive correlation between hyperactivated Akt(Ser(P)473) and pathologic progression of cancer in colorectal carcinoma (48). PTEN, originally identified as a tumor suppressor protein, was found to be inactivated in several types of tumors (e.g. prostate, endometrial, breast, lung, and melanoma), resulting in constitutive activation of the PI3K/Akt pathway (49).

Our data indicate that lower amounts of procaspase-3 and cleaved caspase-3 in the PAI-1−/− cells correlated with diminished cellular apoptosis. There appeared to be a direct effect of increased levels of Akt(Ser(P)473) and the inactivated form of caspase-9. Although levels of procaspase-9 protein are present in equivalent amounts in both genotypes at 96 h, its proteolytically processed form p37 is lower in PAI-1−/− EC at 96 h. The phosphorylation levels at Thr1463 in caspase-9 were higher in PAI-1−/− EC at 96 h, which could result in diminished levels of cleaved caspase-9. The data presented here suggest that phosphorylation and inactivation of caspase-9 may be one of several mechanisms employed by Akt to promote cell survival. The activation of Akt plays an important role in EC survival and proliferation. Here we demonstrate that the presence of host or exogenous PAI-1 causes decreased inactivation of PTEN, resulting in decreased activation of Akt at serine 473. As a key regulator of cell survival events, Akt targets a number of different cytoplasmic proteins, including caspase-9. As is well known, phosphorylation of caspase-9 by Akt results in inactivation and inability to activate the proapoptotic pathway.

PAI-1−/− EC were capable of responding to exogenous PAI-1, which suppressed growth. Additionally, exogenous PAI-1 diminished the levels of Akt(Ser(P)473) as well as increased the levels of procaspase-3 in PAI-1−/− cells. It has been reported that some members of the serpin family (e.g. CrmA (50) and PL-9 (51)) are capable of inhibiting caspase activity with varying effectiveness. Studies have documented that PAI-1 is an effective inhibitor of caspase-3 and that the inhibitory site of PAI-1 is required for the interaction between PAI-1 and caspase-3 (37). Our data show that the absence of host PAI-1 is associated with lower levels of procaspase-3 and cleaved caspase-3, allowing for the EC to be more resistant to apoptosis. This effect was reversed by the addition of exogenous PAI-1. Similarly, other studies have demonstrated that PAI-1 induced apoptosis via the caspase-3 pathway in human umbilical vein EC as well as in vascular smooth muscle cells through inhibition of vitronectin-dependent cell adhesion by PAI-1 (52). By contrast, other studies have reported that PAI-1 inhibits spontaneous and induced apoptosis of cancerous and noncancerous cells (36). Inhibition of apoptosis by PAI-1 was also observed in primary cell cultures utilizing vascular smooth muscle cells (37) and neurons (35), and this observation was reversed by withdrawal of PAI-1. In tumor cells, high levels of PAI-1 can potentially increase the aggressiveness of the tumor. Collectively, these observations demonstrate that PAI-1 may affect apoptosis in diverse ways.

Using a biliary duct ligation model, it has been demonstrated that a significant increase in proliferation of cholangiocytes and hepatocytes occurs in bile ducts of PAI-1-deficient mice com-
pared with WT mice. Furthermore, this enhanced proliferative repair response in PAI-1-deficient mice was attributed to elevated levels of hepatic Akt(Ser(P)473) (53). Thus, underscoring the fact that PAI-1 plays an important role in the repair process and inhibiting PAI-1 may be beneficial in attenuating liver injury.

Transforming growth factor-β1, a prototypic cytokine, is a potent regulator of growth. Although it is generally considered an inhibitor of cell growth, studies have shown that it can also stimulate growth. In vitro proliferation studies showed that mouse embryonic fibroblasts isolated from transforming growth factor-β1-deficient mice proliferate at a higher rate compared with cells obtained from WT mice. Transforming growth factor-β1 regulates cell proliferation by an autocrine mechanism and also has the ability to modulate gene expression of select components of the extracellular matrix (e.g. pro-α1(I) collagen, fibronectin, and PAI-1) (54).

PAI-1 is a multifunctional protein. Apart from its role as a serine protease inhibitor, it also binds to vitronectin, which can alter cell/matrix interactions (55). Additionally, it has an LRP-binding site that facilitates the internalization of uPA and its receptor uPAR by PAI-1 (56). To further dissect the functional properties of PAI-1 and their contribution toward proliferation, specific rPAI-1 variants were employed. A reduction in cell counts was observed when PAI-1−/− cells were incubated with WT PAI-1 or a PAI-1 mutant protein with reduced serine protease-inhibitory activity (R346A). On the other hand, variant PAI-1 (R76E), with defective LRP binding properties, was not able to reduce the proliferation of PAI-1−/− cells. This suggests that PAI-1 exerts its regulation of proliferation via its LRP binding domain.

In conclusion, results from this study suggest that PAI-1 may act as a negative regulator of cell proliferation and that it exerts its effect on the prosurvival PI3K/Akt pathway, which plays a crucial role in EC survival. Studies have indicated that PAI-1, in a LRP-dependent manner, activates the Jak/Stat pathway in migrating rat smooth muscle cells (57) and is thought to be relevant in promoting migration and proliferation of these cells during restenosis. LRP is known to bind to a variety of ligands, mediating their internalization and thereby regulating cell survival and migration (57, 58). In addition to its role as an endocytosis receptor protein, LRP is phosphorylated by members of the c-Src family of kinases and is reported to associate with other signaling molecules, such as the Shc adaptor protein, thus playing a role in signal transduction (59). These observations further elucidate a potential mechanism by which PAI-1 regulates the activation of the Akt pathway, resulting in altered cell proliferation and apoptosis.

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