A Truncated Plasminogen Activator Inhibitor-1 Protein Blocks the Availability of Heparin-binding Vascular Endothelial Growth Factor A Isoforms* 

We have made deletions of the porcine plasminogen activator inhibitor-1 (PAI-1) gene to obtain recombinant truncated PAI-1 proteins to examine functions of the PAI-1 isoforms. We previously reported that one recombinant truncated protein, rPAI-123, induces the formation of angiostatin by cleaving plasmin. The rPAI-123 protein is also able to bind urokinase plasminogen activator and plasminogen and then reduce the amount of plasmin that is formed. We have now prepared three different truncated rPAI-1 proteins and demonstrate that PAI-1 conformations control the release of heparin-binding vascular endothelial growth factor (VEGF) isoforms. The rPAI-123 isoform can regulate the functional activity of heparan sulfate-binding VEGF-A isoforms by blocking the activation of VEGF from heparan sulfate. The rPAI-123 conformation induced extensive apoptosis in cultured endothelial cells and thus reduced the number of proliferating cells. The rPAI-123 isoform inhibited migration of VEGF-stimulated sprouting from chick aortic rings by 65%, thus displaying a role in anti-angiogenic mechanisms. This insight into anti-angiogenic functions related to PAI-1 conformational changes could provide potential intervention points in angiogenesis associated with atherosclerotic plaques and cancer.

Angiogenesis is the formation of new capillary blood vessels as outgrowths of pre-existing vessels. This tightly regulated process normally occurs during development (1, 2), tissue repair and remodeling (1, 2), and abnormally in pathologic diseases (3). At the onset of angiogenesis, the quiescent endothelium is destabilized into migratory, proliferative endothelial cells. The angiogenic (activated) endothelium is maintained primarily by positive regulatory molecules. In the absence of such molecules, the endothelium remains in a differentiated, quiescent state that is maintained by negative regulatory molecules, which act as angiogenesis inhibitors (4, 5). Normally, the negative and positive activities are balanced to maintain the vascular endothelium in quiescence (5, 6). A shift in the balance of the positive and negative regulatory molecules can alter the differentiated state of the endothelium from the non-angiogenic, quiescent to the angiogenic state (5). In the switch to pro-angiogenesis, the quiescent endothelial cells are stimulated to migrate toward a chemotactic stimulus, lining up in a tube (sprout) formation (6). They also secrete proteolytic enzymes that degrade the endothelial basement membrane, thus allowing the migrating endothelial cells to extend into the perivascular stroma to begin a new capillary sprout. The angiogenic process is characterized by increased proliferation of endothelial cells to form the extending capillary (6–9).

Vascular endothelial growth factor (VEGF) is a mitogenic factor that stimulates pro-angiogenic properties, including endothelial cell migration and proliferation. VEGF induces the expression of plasminogen activator proteolytic pathway proteins that participate in cellular invasive and remodeling processes (10–12). VEGF-A RNA can undergo alternative splicing to produce four isoforms (13–15). Three of those isoforms, VEGF-A165, VEGF-A189, and VEGF-A206, bind to heparan. VEGF-A165 and VEGF-A206 isoforms have a greater affinity for heparin (16, 17) than VEGF-A165, which can also be active in the soluble form (16). Pro-VEGF affinity for heparin/heparan sulfate appears to be important in the regulation of the availability of VEGF at the cell surface (18), where it can interact with its tyrosine kinase receptors to exert its activity (19). VEGF-A165 can be released from heparan sulfate in an inactive or active form (20, 21). Plasmin and uPA cleave pro-VEGF into an active form of varied sizes depending upon the isoform and the activator molecule (21). Soluble pro-VEGF-A165 can be cleaved by plasmin to produce a smaller active molecule that can directly bind VEGFR-1 and VEGFR-2 independent of heparan sulfate binding (17).

There are naturally occurring molecules that serve as negative regulators of angiogenesis. Angiostatin, one of those negative regulators, is a 38- to 45-kDa cleavage product of plasminogen, containing kringle domains 1–4 (K1–4) (22, 23). Plasminogen, the precursor of plasmin, is activated when it is cleaved at the carboxyl terminus by plasminogen activators. The amino terminus contains five consecutive kringle domains, each of ~9 kDa. The greatest inhibitory activity of angiostatin

* This work is supported by Research Grant R01-HL59590 from the NHLBI, National Institutes of Health, and by a Pacific Vascular Research Foundation Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dartmouth Medical School, Borwell 530 E., 1 Medical Center Dr., Lebanon, NH 03756. Tel.: 603-650-8597; Fax: 603-650-4928; E-mail: Mary.J.Mulligan-Kehoe@dartmouth.edu.

The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; uPA, urokinase plasminogen activator; K1–4, kringle domains 1–4; PAI-1, plasminogen activator inhibitor-1; tPA, tissue plasminogen activator; RCL, reactive center loop; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HBSS, Hank’s balanced salt solution; FITC, fluorescein isothiocyanate; PI, propidium iodide; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; BBE, bovine brain extract; DTT, dithiothreitol; PARC, porcine aortic endothelial cells; BAEC, bovine aortic endothelial cells; MWM, molecular weight marker.
Truncated PAI-1 Blocks Availability of VEGF-A

Anti-angiogenic Effects of rPAI-1 Proteins

Characterization of the rPAI Protein Interactions with Heparin, uPA, and Plasminogen—The functionality of the rPAI-1 proteins was first tested by incubating each truncated protein with uPA and plasminogen to assess the proteolytic activity of the products of the reaction, as described (55). The functionality of each rPAI-1 isoform was then tested in a reaction with heparin bound to Sepharose beads. First, the rPAI-103, rPAI-123, rPAI-1Hep23, and rPAI-124 proteins (20 µg) were each incubated with 50 µl of heparin-bound Sepharose beads (Amersham Biosciences, Piscataway, NJ) for 2 h at 37 °C. The unbound protein was separated from the heparin-Sepharose-bound protein complex by microcentrifugation at 4 °C for 15 min. The proteins bound to the Sepharose beads were washed in TBS/Tween 20 followed by TBS washes.

is contained within kringle 1–3 (24) and kringle 1–5 (25). The mechanism for angiotatin inhibition of endothelial cell growth in vitro and angiogenesis in vitro is unclear.

PAI-1, a serpin family, serine protease inhibitor, is a multifunctional regulatory protein in the plasminogen activator proteolytic (26, 27) and fibrinolytic pathways (28, 29). Active PAI-1 (vitronectin-bound) inhibits proteolytic degradation of the extracellular matrix by inhibiting uPA/PAI, which in turn inhibits cell migration and invasion (30).

PAI-1 can exist in an active, inactive, or substrate cleaved conformation (31, 32). The PAI-1-strained reactive center loop (RCL), located at amino acids 320–351 (33, 34), interacts with uPA at Arg-346 (35, 36) to form a stable PAI-1–uPA complex to inactivate uPA (37). In the inactive/latent configuration of PAI-1 (not bound to vitronectin) the RCL spontaneously inserts into the β-sheet of strand 4a to stabilize the PAI-1 structure (38, 39).

It has been shown that, when PAI-1 is cleaved between residues P and P' in the RCL, PAI-1 is converted to a substrate cleaved conformation (31, 32). The PAI-1-strained reactive center loop (RCL), located at amino acids 320–351 (33, 34), interacts with uPA at Arg-346 (35, 36) to form a stable PAI-1–uPA complex to inactivate uPA (37). In the inactive/latent configuration of PAI-1 (not bound to vitronectin) the RCL spontaneously inserts into the β-sheet of strand 4a to stabilize the PAI-1 structure (38, 39).

Biochemical and in vitro studies demonstrate that PAI-1 can be cleaved into smaller fragments by thrombin (41, 42), plasmin (43), and MMP-3 (44). The PAI-1 cleavage products, ranging from 22 to 44 kDa, render PAI-1 inactive.

The PAI-1 region distant from the RCL contains many binding domains for regulatory molecules involved in the proteolytic and fibrinolytic pathways. This region of PAI-1 has active sites for vitronectin (45–49), heparin (49, 51), tPA and uPA (52, 53), and thrombin and fibrin (49). We and others (53–57) have demonstrated that, through its interactions with some of the same regulatory molecules in the proteolytic and fibrinolytic pathways, PAI-1 (active and inactive) is also able to play a role in anti-angiogenic mechanisms.

We have recently reported that, when a truncated porcine PAI-1 protein, rPAI-1123, is incubated with plasminogen and urokinase plasminogen activator (uPA), it induces formation of an angiostatin-like protein with proteolytic activity (55). In this second mechanism, rPAI-1123 fragments from plasmin. This truncated PAI-1 appears to expose sites that partially interact with uPA at Arg-346 (35, 36) to form a stable PAI-1–uPA complex that inactivates uPA (37). In the inactive/latent configuration of PAI-1 (not bound to vitronectin) the RCL spontaneously inserts into the β-sheet of strand 4a to stabilize the PAI-1 structure (38, 39).

We have previously shown by zymography the importance of PAI-1 binding domains for regenerating amounts of rPAI-123 that are available for binding uPA and/or plasminogen. In this second mechanism, rPAI-1123, rPAI-1Hep23, and rPAI-1124, are encoded by DNA sequences identical to the functional regulatory protein in the plasminogen activator procoagulant activity of endothelial cells in vitro and sprouting in an organ culture.

MATERIALS AND METHODS
PAI-1 Gene Deletions

The DNA encoding each truncated PAI-1 protein was obtained by deleting the porcine PAI-1 gene (poPAI-1) (55, 61). The selection of gene fragments was based on the poPAI-1 sequences that correspond to the human PAI-1 gene (huPAI-1) (62) sequences reported to code for functional domains in human PAI-1 (Fig. 1) (55). Each DNA fragment was isolated from porcine aortic endothelial cells by reverse-transcribing RNA into cDNA. The cDNA was made double-stranded in a PCR reaction containing porcine PAI-1-specific primers. The primers for rPAI-1123, rPAI-1Hep23, and rPAI-1124 (underlining denotes restriction enzyme sequences): rPAI-1123, 5′ primer, 5′-GGAATTCTAGTATCATACGCAGGCGTGTGAGGATGAGGAGAAGGGC-3′ (amplifies poPAI-1 nucleotides 471–999 corresponding to huPAI-1 nucleotides 625–1346); rPAI-1Hep23, 5′ primer, 5′-GAGACTTACGAGATCACTGCATTGCTGATGAGGATGAGGAGAAGGGC-3′ (amplifies poPAI-1 nucleotides 390–999 corresponding to huPAI-1 nucleotides 184–783); rPAI-1124, 5′ primer, 5′-GGAGTTAAGGAGACTGCTGATGAGGATGAGGAGAAGGGC-3′ (amplifies poPAI-1 nucleotides 444–1346 corresponding to huPAI-1 nucleotides 238–1182). The PCR conditions for all three genes were as described previously (55). The PCR-amplified rPAI-1 DNA fragments were double-digested with EcoRI and Xbal (Roche Molecular Biochemicals, Indianapolis, IN) to activate the incorporated restriction enzyme sites. The restricted DNA was ligated into a Pichia pastoris yeast shuttle vector, pGAPZaA (Invitrogen, Carlsbad, CA). The TOP 10 strain of Escherichia coli was transformed by electroporation as described (55). Following an overnight incubation at 37 °C, colonies were selected and grown in low salt LB broth for 5–7 h at 37 °C. The DNA from each colony growth was isolated (Qiagen, Inc., Valencia, CA) to identify a clone containing each gene insert. Positive isolates from restriction enzyme digests were verified by sequencing. Each recombinant protein was expressed in P. pastoris as described (55) and purified by affinity chromatography. The deletions of poPAI-1 are shown diagrammatically in Fig. 1. The sequences of the rPAI-1123, rPAI-1Hep23, and rPAI-1124 DNA matched the known sequence of the corresponding segment of porcine PAI-1 DNA. Each rPAI-1 protein corresponded to its expected molecular weight (data not shown).

The functionality of each rPAI-1 isoform was then tested in a reaction with heparin bound to Sepharose beads. First, the rPAI-103, rPAI-123, rPAI-1Hep23, and rPAI-1124 proteins (20 µg) were each incubated with 50 µl of heparin-bound Sepharose beads (Amersham Biosciences, Piscataway, NJ) for 2 h at 37 °C. The unbound protein was separated from the heparin-Sepharose-bound protein complex by microcentrifugation at 4 °C for 15 min. The proteins bound to the Sepharose beads were washed in TBS/Tween 20 followed by TBS washes.

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Bound samples were reacted with uPA (0.5 IU/μg of rPAI-1 protein) at 37 °C for 1 h followed by a second 1-h, 37 °C incubation with plasminogen (1 IU/μg of rPAI-1 protein). The reaction samples were analyzed on a 15% SDS-polyacrylamide gel containing 1.3% casein (zymogram) (63, 64). The electrophoresed samples were incubated overnight at 37 °C in a 50 mM Tris-HCl, pH 7.9, buffer containing 5 mM CaCl2 to enable plasmin degradation of casein.

Identification of Plasminogen Kringle Domains—Protein products from the reaction of rPAI-1, uPA, and plasminogen were electrophoresed on a 4–20%, SDS, non-reducing polyacrylamide gel, and transferred to nitrocellulose. The blots were probed with an antibody (1 μg/ml) specific for plasminogen kringle 1–3 (R&D Systems, Inc., Minneapolis, MN). Following a 1-h, room temperature incubation with the primary antibody, a secondary rabbit anti-goat IgG HC (Pierce, Rockford, IL) polyclonal antibody at a concentration of 1 μg/ml was incubated with the anti-kringle probe membrane for 1 h at room temperature. A horse radish peroxidase-conjugated antibody (donkey anti-rabbit IgG, Amersham Biosciences, Arlington Heights, IL) diluted 1:5000 amplified the binding reaction, which was ultimately detected by addition of a chemiluminescent substrate (Amersham Biosciences). Protein products, the rPAI-1 proteins, were plated into six-well culture plates at a density of 10^4/ml to assess their proliferative properties in the presence of rPAI-1 proteins. The cells were trypsinized and counted on a hemacytometer plate at 48 and 96 h after adding exogenous rPAI-1. To further ascertain the proliferative properties of the rPAI-1-treated cells, a BrdUrd labeling assay was performed using a FITC-labeled BrdUrd-specific antibody. The addition of propidium iodide (PI) enabled a microscopic count of the proliferating cells relative to total number of cells. BrdUrd was added to the culture medium to obtain a final concentration of 10 μM. The cells were incubated for 30 min at 37 °C in a CO2 incubator. The cells were washed twice in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. FITC-conjugated anti-BrdUrd was diluted 2.5-fold in 0.5% Tween 20/PBS and added directly to the cell culture medium for 30 min at room temperature. The cells were washed in PBS and incubated with PI for 1 min. Incorporation of BrdUrd was calculated by counting the number of cells containing FITC stain (green) or PI stain (red) in five fields per sample in triplicate experiments.

Aortic arch ring assay—Aortic arches were removed (R. Auerbach, University of Wisconsin, personal communication) from fertilized chicken eggs (Oliver Merrill & Sons, Londonderry, NH) at day 14 of embryonic development. The eggs were cracked into a sterile 100-mm culture dish. The embryo was removed from its surroundings by cutting away the associated membranes and yolk sac. The chick embryo was placed ventral side up to surgically expose the heart and aortic arches. The heart and aortic arch were removed and placed into a sterile culture dish containing PBS to which 1% penicillin-streptomycin (Invitrogen, Gaithersburg, MD) was added. Arches, from which the surrounding adventitia had been removed.
been removed, were cut into 0.8-mm sections. Each arch was placed on a 1–5-μl Matrigel (66) that was deposited on the bottom of a six-well culture plate just prior to adding the ring. An additional 300 μl of ice-cold Matrigel was spread in a circle surrounding each aortic arch. The Matrigel was allowed to solidify before adding 2 ml of human endothelial-SFM basal growth medium (Invitrogen). An rPAI-1 protein and bovine brain extract (BBE) (Clonetics, San Diego, CA), at 30 nm and 10%, respectively, were added to each well and incubated at 37 °C, 5% CO2. To assess the characteristics of the new sprouts that could be ascribed to VEGF and the characteristics that were inhibited by rPAI-123, VEGF-A (100 ng/ml) and rPAI-13 (30 nm) were added to the culture medium containing the aortic rings. At 48 h, additional medium containing BBE or VEGF and rPAI-1 protein was added to the aortic rings. Cultures with VEGF-A at 37 °C was continued for an additional 48 h. Quantitative evaluation of tubule formation was performed by a blinded observer on a scale of 1–5 (least to maximum sprouting).

Biochemical Interactions of a VEGF-heparin Complex with rPAI-1 Proteins, uPA, and Plasmin—VEGF was isolated from bovine aortic endothelial cells by first incubating the cells overnight in serum-free DMEM. The medium was changed before adding 100 μg/ml heparin (Sigma, St. Louis, MO) for 4 h at 37 °C. The serum-free DMEM containing the heparin-VEGF complex was precipitated in 80% ethanol. The serum-free medium from which the VEGF-heparin complex was isolated, was probed for VEGF-A123 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to ensure that this non-heparin binding VEGF isoform did not participate. Following the incubation with the VEGF-heparin complex, VEGF-A123 was not detected. The heparin-VEGF complex was incubated with rPAI-123, rPAI-124, or rPAI-1Hep23 protein (3, 15, and 30 nm) for 2 h at 37 °C. Either uPA (0.25 IU) or plasmin (made from 1.0 IU plasminogen and 0.25 IU uPA) was added to the VEGF-heparin-rPAI-1 reaction. After an additional 2-h incubation at 37 °C with uPA or plasmin, DTT at a final concentration of 0.1 M was added to one half of the reaction mixture for 3.5 h at 37 °C. An equal volume of each reaction mixture was denatured at 95 °C, then electrophoresed on a 4–20% SDS-polyacrylamide gel. The VEGF in each sample was visualized on a nitrocellulose membrane probed with a monoclonal antibody to human VEGF-A165, 165, 206 (BD Pharmingen, San Diego, CA). A secondary rabbit anti-mouse IgG H+L (Jackson Immunoresearch, Rockford, IL) polyclonal antibody was diluted to 1 μg/ml in TBS, pH 8.0, containing 5% skim milk, and incubated with the anti-VEGF probed membranes for 1 h at room temperature. A hors eradish peroxidase-conjugated antibody (donkey anti-rabbit IgG, Ams her sham Biosciences, Arlington Heights, IL) diluted 1:500 amplified the binding reaction, which was ultimately detected by addition of a chemic lentiminesubstrate (Ams her sham Biosciences).

VEGF-A in the Culture Medium of rPAI-1-treated Endothelial Cells—Bovine aortic endothelial cells were seeded into six-well culture plates and grown to confluence in DMEM, as described. The confluent (quiescent) cells were treated with a single dose of rPAI-1 protein at a final concentration of 1.2 nm. At 6, 12, 15, 24, 30, 45, and 72 h after the onset of treatment, the culture medium was removed from cells treated with each rPAI-1 protein. Equivalent amounts of protein were incubated with 0.1 M DTT for 2.5 h at 37 °C. The protein samples were electrophoresed on a 4–20% gradient, SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and probed for VEGF-A165, 165, 206 (BD Pharmingen, San Diego, CA). A secondary rabbit anti-mouse IgG H+L (Jackson Immunoresearch, Rockford, IL) polyclonal antibody was diluted to 1 μg/ml in TBS, pH 8.0, containing 5% skim milk, and incubated with the anti-VEGF probed membranes for 1 h at room temperature. A horseradish peroxidase-conjugated antibody (donkey anti-rabbit IgG, Am sh er sham Biosciences, Arlington Heights, IL) diluted 1:500 amplified the binding reaction, which was ultimately detected by addition of a chemiluminescence substrate (Am sh er sham Biosciences).

rPAI-1 Protein Interactions and Anti-angiogenic Activity in Vitro

rPAI Proteins Interactions with Heparin, uPA, and Plasminogen—We first analyzed the effects of the recombinant truncated rPAI-1 molecules on proteinase activity in the presence of 1) uPA and plasminogen or 2) heparin, uPA, and plasminogen. The zymogram, Fig. 2, lanes 4–7, shows that the interaction of three of the four truncated rPAI-1 proteins with uPA and plasminogen resulted in production of one or more proteolytic fragments that migrate at 34–38 kDa. The rPAI-123 protein (lane 6) induces formation of two 34- to 38-kDa proteolytic angiotensin fragments from plasmin. However, the rPAI-123 protein (lane 5) and rPAI-124 (lane 7) proteins each have a single band that corresponds to one of the 34–38-kDa fragments visualized in the rPAI-123 products. In the case of rPAI-123 (lane 5), a proteolytic band appears at or near the size of the lower proteolytic fragment induced by rPAI-123 cleavage of plasmin. The rPAI-124 (lane 7) protein induces a proteolytic fragment at or near the molecular mass corresponding to the larger of the two plasmin cleavage products induced by rPAI-123. The rPAI-124 (lane 4) does not produce a proteolytic fragment at 34–38 kDa (lane 4). The rPAI-123 protein (partial heparin binding domain) maintains its activity when bound to heparin (lane 3). Similarly, the proteolytic activity associated with a reaction mix containing uPA, plasminogen, and rPAI-123 (complete heparin domain) is not altered in the presence of heparin (lane 2). The rPAI-123 protein (lacks a heparin binding domain) and the rPAI-124 protein (contains the RCL and a partial heparin binding domain) did not demonstrate proteolytic plasmin cleavage products when incubated with heparin. The proteolytic proteins near 80-kDa correspond to plasmin. In lanes 3 and 6 there are proteolytic proteins near 50 kDa that may represent a different plasmin cleavage product containing a greater number of plasminogen kringle domains. The function of the rPAI-
Fig. 3. Verification of plasminogen kringles 1–3 domains in rPAI-1, uPA and plasminogen reactions. Each rPAI-1 protein (3, 15, and 30 nM) was incubated with uPA for 1h at 37°C before adding plasminogen for a 2nd 1h incubation at 37°C. The final products of the reaction mixture were transferred to nitrocellulose and probed for plasminogen kringle domains 1–3. In the legend, the rPAI-1 proteins are identified as their subscript: rPAI-123 = 23; rPAI-123 – 24; rPAI-1Hep23 = Hep23. Lane 1, MWM; lane 2, plasminogen; lane 3, uPA + plasminogen; lane 4, 23 (3 nM) + uPA + plasminogen; lane 5, 23 (15 nM) + uPA + plasminogen; lane 6, 23 (30 nM) + uPA + plasminogen; lane 7, 23 (3 nM) + uPA + plasminogen; lane 8, 23 (15 nM) + uPA + plasminogen; lane 9, 23 (30 nM) + uPA + plasminogen; lane 10, 24 (3 nM) + uPA + plasminogen; lane 11, 24 (15 nM) + uPA + plasminogen; lane 12, 24 (30 nM) + uPA + plasminogen; lane 13, Hep 23 (3 nM) + uPA + plasminogen; lane 14, Hep 23 (15 nM) + uPA + plasminogen; and lane 15, Hep 23 (30 nM) + uPA + plasminogen.

1Hep23 isoform (complete heparin domain) in a reaction with uPA and plasminogen as compared with the rPAI-123 isoform (partial heparin binding domain) shows that binding to heparin does not alter the inability of rPAI-1Hep23 to mediate the formation of proteolytic fragments at a molecular mass near 34 kDa. These experiments show that a full heparin-binding domain can block the ability of PAI-1 to induce proteolytic proteins corresponding to or near the molecular mass of angiostatin containing kringle 1–3.

Plasminogen Kringle Domains 1–3 Contained in rPAI-1 Reactions—A nitrocellulose membrane containing the plasminogen (plasmin) cleavage products formed in reactions containing rPAI-1 proteins, uPA, and plasminogen and probed for kringle 1–3 (angiostatin) is shown in Fig. 3. The samples containing all three concentrations of rPAI-124 (lanes 4–6) have fragments containing kringle 1–3 at a molecular mass corresponding to the size that was visualized byzymography in Fig. 2, lane 6. Additionally, there are fragments containing kringle 1–3 at a molecular mass near: (a) 45 kDa, which corresponds to the reported size of kringle 1–4, and (b) 70–80 kDa, which corresponds to the size of the plasmin control in lane 3. The reaction product of 15 nM rPAI-123, uPA, and plasminogen contained angiostatin kringle 1–3 at 34–36 kDa (lane 8). However, that fragment was absent when 3 or 30 nM rPAI-123 were part of the reaction with uPA and plasminogen (lanes 7 and 9). In the reactions containing either rPAI-124 (lanes 10–12) or rPAI-11Hep23 (lanes 13–15), plasminogen kringle 1–3 are not present at 34–36 kDa. There are detectable fragments containing kringle 1–3 near 45 kDa in the reactions containing 3 nM rPAI-124 (lane 10). These data show that the reaction of uPA and plasminogen with all concentrations of rPAI-123 resulted in the formation of plasminogen kringle 1–3 (angiostatin) at 34–36 kDa, which is consistent with the molecular mass of the proteolytic fragments observed on thezymogram (Fig. 2). Additionally, there is a greater amount of kringle 1–3 contained within the 45-kDa fragments, which is representative of a less potent angiostatin.

Apoptosis—Apoptosis of bovine aortic endothelial cells treated with exogenous rPAI-1 proteins was evaluated in an Annexin V binding assay. The adherent cells, 39% of the rPAI-123 and 19% of rPAI-11Hep23-treated cells, were undergoing apoptosis (Fig. 4). Whereas, only 4–7% of the rPAI-11Hep23, rPAI-124, and yeast-treated cells were apoptotic, a value comparable to the endothelial cell control. This suggests that heparin bind-
that unlike rPAI-123 and rPAI-1Δ23, the rPAI-1Hep23 does not:
1) induce the formation of proteolytic fragments at a 34- to 38-kDa molecular mass corresponding to plasminogen kringles 1–3 and 2) induce apoptosis in BAEC with a concomitant reduction in cell number.

Migration appeared to be VEGF-dependent. By day 4, The...
rPAI-123-VEGF-treated rings (Fig. 7B) display a reduction in the rate of migration similar to that measured in the rPAI-123/BBE-treated samples (Fig. 6A). The significant differences in the structure of the VEGF-treated control tubules (Fig. 7B) and the rPAI-123-VEGF-treated samples (Fig. 7A) should be noted. These data show that rPAI-123 and rPAI-1Hep23 are able to inhibit the migratory function of new sprouts from chick aortic rings stimulated with BBE. The rPAI-123 protein inhibits the migration of new sprouts stimulated with VEGF. In the rPAI-123- and rPAI-1Hep23-treated aortic rings we observed apoptosis of sprouting endothelial cells. The apoptosis appears to result in breakage of the tubule.

Analyses of Biochemical Interactions of a VEGF-Heparin Complex with rPAI-1 Proteins, uPA, and Plasmin

We next determined if the rPAI-1 proteins could regulate the release or activation of VEGF-A from a complex with heparin. Such information could provide insight into the mechanisms by which rPAI-123 inhibits the migration of VEGF-stimulated sprouts from chick aortic rings. Therefore, variable concentrations of each rPAI-1 protein were incubated with a VEGF-heparin complex to examine the release and/or activation of VEGF-A. VEGF activation and release from the complexes were then tested in the presence of activator molecules uPA or plasmin. Western blots of the complexes were probed with an antibody to heparin-binding VEGF-A isoforms (Fig. 8, A–D, and Fig. 9, A–C).

Release or Activation of VEGF-A165, 189, 206—In this set of experiments, blot membranes were probed for released and/or activated VEGF-A165, 189, 206 from an rPAI-1-VEGF-heparin complex containing activator molecules uPA or plasmin. When rPAI-123 is part of the complex, there are only traces of the 46-kDa activated VEGF-A165, 189, 206 (Fig. 8A, lanes 5–13). Fig. 8B shows the effect of rPAI-1Hep23 in a reaction mixture containing VEGF-heparin and uPA or plasmin. VEGF is released from heparin in the absence (lanes 6–8) or presence of either uPA (lanes 9–11) or plasmin (lanes 12–14). Similarly, the presence of rPAI-124 in the mixture with VEGF-heparin results in the release of active VEGF at all concentrations of rPAI-124 as shown in Fig. 8C, lanes 6–14. In the rPAI-124-containing reactions, the VEGF release occurs in the presence of uPA (lanes 9–11) or plasmin (lanes 12–14). Reaction mixtures containing rPAI-1Hep23 (Fig. 8D, lanes 6–14) show activated VEGF in reactions containing low concentration of rPAI-1Hep23 in the absence (lane 6) or presence of either uPA (lane 9) or plasmin (lane 12). At higher concentrations of rPAI-1Hep23 there is no VEGF release and an increase in VEGF-containing fragments between 60 and 80 kDa (Fig. 8D, lanes 7, 8, 10, 11, 13, and 14). The high molecular mass fragments containing VEGF have cross-reactivity with VEGF-B186 (data not shown) and are indicative of the 60- to 62-kDa active VEGF-B186 homodimer (23). The greater than 80-kDa molecular mass containing VEGF also exists in samples containing rPAI-123, rPAI-1Hep23, and rPAI-124. However, the samples containing rPAI-1Hep23 clearly have a greater amount of VEGF-A complexed at a high molecular mass. These experiments show that rPAI-123 and rPAI-124 do not block the activation or release of VEGF from a complex with heparin. Although rPAI-123 and rPAI-124 both have a partial heparin-binding domain, only rPAI-124 has a RCL. The RCL may alter the conformation such that the partial heparin domain in rPAI-124 is obscured and unable to block the release of activated VEGF. On the other hand, both rPAI-123 and rPAI-1Hep23 are able to block the release of VEGF from a complex with heparin. These data suggest that the heparin-binding domain in each of these two isoforms participates in blocking the release of active VEGF-A and that partial...
Fig. 8. Western blot analysis of VEGF-A<sub>165,189,206</sub> activation in a VEGF-heparin complex with rPAI-1 proteins, uPA, or plasmin. Each rPAI-1 protein (3, 15, and 30 nm) was incubated with a VEGF-heparin complex for 2 h at 37 °C before adding either uPA (0.25 IU) or plasmin (0.25 IU of uPA). The incubation continued at 37 °C for 2 additional h. The samples were electrophoresed on a 4–20% polyacrylamide gel. Membranes containing the transferred protein were probed in 23°C for 2 h. The samples were electrophoresed on a 4–20% polyacrylamide gel. Membranes containing the transferred protein were probed.

### A rPAI-1<sub>23</sub>

- Lane 1: VEGF-heparin + uPA
- Lane 3: Hep 23 (3 nM)
- Lane 5: Hep 23 (15 nM)
- Lane 8: Hep 23 (30 nM)
- Lane 10: Hep 23 (50 nM)
- Lane 12: VEGF-heparin + plasmin
- Lane 13: VEGF-heparin + plasmin
- Lane 14: rPAI-1<sub>23</sub>

### B rPAI-1<sub>14</sub>

-Lane 1: VEGF-heparin + uPA
- Lane 3: Hep 23 (3 nM)
- Lane 5: Hep 23 (15 nM)
- Lane 8: Hep 23 (30 nM)
- Lane 10: Hep 23 (50 nM)
- Lane 12: VEGF-heparin + plasmin
- Lane 13: VEGF-heparin + plasmin
- Lane 14: rPAI-1<sub>14</sub>

### C rPAI-1<sub>Hep23</sub>

- Lane 1: VEGF-heparin + uPA
- Lane 3: Hep 23 (3 nM)
- Lane 5: Hep 23 (15 nM)
- Lane 8: Hep 23 (30 nM)
- Lane 10: Hep 23 (50 nM)
- Lane 12: VEGF-heparin + plasmin
- Lane 13: VEGF-heparin + plasmin
- Lane 14: rPAI-1<sub>Hep23</sub>

### D rPAI-1<sub>1Hep23</sub>

- Lane 1: VEGF-heparin + uPA
- Lane 3: Hep 23 (3 nM)
- Lane 5: Hep 23 (15 nM)
- Lane 8: Hep 23 (30 nM)
- Lane 10: Hep 23 (50 nM)
- Lane 12: VEGF-heparin + plasmin
- Lane 13: VEGF-heparin + plasmin
- Lane 14: rPAI-1<sub>1Hep23</sub>

VEGF-A in the Culture Medium of rPAI-1-treated Endothelial Cells

The results of the analysis of VEGF-A contained within the culture medium of rPAI-1-treated endothelial cells is shown in Fig. 10 (A–C). The rPAI-1<sub>23</sub>-treated cells (Fig. 10A, lanes 3–8) primarily contain VEGF-A fragments at a molecular mass (30 nm) + VEGF-heparin + plasmin, C, rPAI-1<sub>23</sub>: lane 1, VEGF-heparin; lane 2, VEGF-heparin + uPA; lane 3, MWM; lane 4, VEGF-heparin + plasmin (0.25 IU uPA); lane 5, VEGF-heparin + plasmin (0.25 IU of uPA); lane 6, 24 (3 nm) + VEGF-heparin; lane 7, 24 (15 nm) + VEGF-heparin; lane 8, 24 (30 nm) + VEGF-heparin + uPA; lane 9, 24 (15 nm) + VEGF-heparin + uPA; lane 10, 24 (30 nm) + VEGF-heparin + uPA; lane 11, 24 (30 nm) + VEGF-heparin + plasmin; lane 12, 24 (15 nm) + VEGF-heparin + plasmin; lane 13, 24 (15 nm) + VEGF-heparin + plasmin; lane 14, 50 kDa.

Reducing Release or Activation of VEGF-A<sub>165,189,206</sub>—We wanted to determine which VEGF isoforms were complexed with heparin-rPAI-1<sub>23</sub> at the higher molecular mass as seen in the previous Fig. 8A. To address this question, DTT was added to the final reaction mixtures containing VEGF-heparin-rPAI-1<sub>23</sub> + uPA (or plasmin) for 2.5 h at 37 °C (Fig. 9, A–C). The rPAI-1<sub>23</sub>-VEGF-heparin complexes in Fig. 9A (lanes 3–5) contained multiple-sized VEGF fragments representative of mature or active VEGF-A<sub>165,189,206</sub> 1) fragment of less than 34 kDa corresponds to activated VEGF-A<sub>165</sub> (18, 21); 2) the 34- to 38-kDa fragments correspond to plasmin or uPA-activated VEGF-A<sub>165,189</sub> (18, 21); 3) the 40- to 45-kDa fragments correspond to uPA-matured VEGF-A<sub>189</sub> or VEGF-A<sub>165</sub> (18, 21); and 4) the 50- to 52-kDa band is the molecular mass of mature but not active VEGF (18).

In VEGF-heparin complexes containing lower concentrations of rPAI-1<sub>23</sub> protein, there are activated VEGF fragments between 30 and 42 kDa, as shown in lanes 3 and 4, which correspond to the reported sizes for processed VEGF-A<sub>165,189,206</sub>. The fragments in Fig. 9A (lanes 3 and 4) that migrate at 36–38 kDa are also in reactions containing uPA (Fig. 8B, lanes 4 and 5) or plasmin (Fig. 8C, lanes 2 and 5). The most predominant VEGF fragment released from a VEGF-heparin-rPAI-1<sub>23</sub> complex is seen near 50 kDa (mature VEGF) in the absence of uPA and plasmin. When the rPAI-1<sub>23</sub> protein concentration is increased to 30 nm, the active VEGF at 50 kDa is absent and the products are ~30, 38–42 kDa, which corresponds in size to uPA-matured VEGF-A<sub>189</sub> and VEGF-A<sub>165</sub>, or plasmin-activated VEGF-A<sub>189</sub> (Fig. 9, A, lane 5, B, and C, lanes 6). The data obtained from the reducing reactions demonstrate that the rPAI-1<sub>23</sub> conformation blocks the release of multiple forms of matured, activated, and processed (uPA and plasmin-cleaved) VEGF-A fragments reported for heparin-binding VEGF-A<sub>165,189,206</sub> isoforms. In mixtures containing a VEGF-heparin-rPAI-1<sub>1Hep23</sub> complex, the associated VEGF remains at a higher molecular mass (greater than 80 kDa) (data not shown), except when rPAI-1<sub>1Hep23</sub> is at a low concentration. The rPAI-1<sub>1Hep23</sub> protein conformation maintains VEGF-A<sub>165,189,206</sub> in a complex with heparin.

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heparin binding is more effective in blocking VEGF-A activation and/or release. These findings suggest that the ability of rPAI-1<sub>23</sub> to block growth and vessel sprouting may be due to its ability to block VEGF activation.
greater than 50 kDa. A small fraction of VEGF fragments at a molecular mass less than 50 kDa (lanes 4–8) are also observed in the rPAI-123-treated samples. The fragments greater than 50 kDa are representative of mature or pro-VEGF and those less than 50 kDa correspond to active VEGF. In the culture medium samples collected from rPAI-123-treated cells (Fig. 10A, lanes 9–14) there is an abundance of VEGF-A fragments at a molecular mass of ~36–45 kDa at all time points. This molecular mass corresponds to active or uPA/plasmin processed, active VEGF-A. All rPAI-123-treated samples also contain mature or pro-VEGF at a molecular mass greater than 50 kDa. One of the inactive fragments in the 15-h time (lane 10) is absent in lanes 9, 11–14. The culture media from all untreated (Fig. 10B, lanes 4–8) and rPAI-123-treated cells (Fig. 10B, lanes 9 and 10), contain active VEGF and a small amount of processed, active VEGF. The culture medium samples from the rPAI-14-treated cells (Fig. 10C) primarily contain inactive VEGF and lesser amounts of processed, active VEGF. The results of these experiments clearly show that VEGF in rPAI-123-treated culture medium contains a much greater amount of active VEGF, mostly representative of uPA or plasmin processed VEGF. Similar VEGF fragments are absent or present to a lesser degree in the untreated or rPAI-123-, rPAI-14, and rPAI-124-treated cells. The culture media from rPAI-14-treated and untreated cells contain a greater amount of active, unprocessed VEGF than the media from rPAI-123-, rPAI-123-, or rPAI-14-treated cells. These data correspond with the biochemical analysis of VEGF release from a complex with heparin and rPAI-1 proteins (Fig. 8, A–D). The molecular mass of all of the VEGF fragments correspond to dimeric VEGF-A despite the rigorous reducing conditions applied to all samples. Such results have been reported by others (20).

VEGF-A Bound to Heparan Sulfate in rPAI-1-treated Endothelial Cells

In the series of experiments shown in Fig. 11 (A and B), two different antibodies were used to delineate differences in heparan sulfate-bound VEGF-A isoforms in rPAI-1-treated cells. In Fig. 11A, immunoblots were probed for VEGF-A with an antibody specific for epitopes common to VEGF-A165, 189, 205. The results of this set of experiments showed that, at the 12-h time point, the rPAI-14-probed (Fig. 11A, lane 6) and rPAI-124-treated (lane 11) cells contained two fragments of VEGF-A at a molecular mass near 58 kDa. In the rPAI-123-treated (lanes 3 and 4), rPAI-123-treated (lanes 8 and 9), and untreated (lane 7) samples, VEGF-A is absent or barely visible. However, when two competing antibodies specific for VEGF-A were simultaneously incubated with immunoblots containing proteins released from a heparinase digest (Fig. 11B), the rPAI-123-treated (lane 1), rPAI-123-treated (lane 10), and untreated (lane 6) cells show VEGF-A released from heparan sulfate. Among those samples, the rPAI-123-treated cells at the 6-h time point had the greatest amount of detectable VEGF-A released in the enzymatic digest. The VEGF is seen as two distinct fragments with a small difference in molecular mass. Each fragment corresponds to the molecular mass of dimeric VEGF, despite the rigorous reducing conditions. By 12 h, VEGF is not detected in rPAI-14-treated cells (lane 2). However, at the 12-h time point, the rPAI-123-treated (lane 10) and the untreated cells (lane 6) show a variable molecular mass in VEGF released as a result of the digest. The VEGF fragments in the rPAI-123-treated samples are very close to 50 kDa, whereas, the VEGF fragments in the untreated and the rPAI-14-treated cells correspond to a molecular mass that is more representative of VEGF-A165 or VEGF-A206. The rPAI-14-probed and rPAI-123-treated cells do not show the release of heparan sulfate-bound VEGF when the blot is probed with competing antibodies (lanes 5, 6, 13, and 14). The results of these sets of experiments show that different VEGF-A isoforms were released with the heparinase digest, depending upon the rPAI-1 treatment. The use of competing antibodies exposed binding sites in rPAI-1-treatet cells (lane 2). However, at the 12-h time point, the rPAI-123-treated (lane 10) and the untreated cells (lane 6) show a variable molecular mass in VEGF released as a result of the digest. The VEGF fragments in the rPAI-123-treated samples are very close to 50 kDa, whereas, the VEGF fragments in the untreated and the rPAI-14-treated cells correspond to a molecular mass that is more representative of VEGF-A165 or VEGF-A206. The rPAI-14-probed and rPAI-123-treated cells do not show the release of heparan sulfate-bound VEGF when the blot is probed with competing antibodies (lanes 5, 6, 13, and 14).
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Analysis of VEGF Activation in rPAI-123-treated BAEC versus PAEC

Fig. 10. VEGF-A in culture medium of rPAI-1-treated endothelial cells. Bovine aortic endothelial cells were treated with a single dose of exogenous rPAI-1 protein over a period of 72 h. The culture medium was isolated from individual culture plates at selected time points. Equivalent amounts of protein were treated with 0.1 M DTT at 37 °C for 2.5 h before electrophoresis on a 4–20% SDS-polyacrylamide gel. The reduced VEGF samples migrate at a molecular mass corresponding to dimeric VEGF-A. Immunoblots containing the transferred proteins were probed for VEGF-A. Kodak X-Omat Blue film was used in development procedure. Final figures were auto adjusted in Photoshop to enable clear visualization of proteins. A, endothelial cells treated with rPAI-123 or rPAI-123, rPAI-123, is named 23. In this figure rPAI-123 is named 23 and rPAI-123 will be named 23. Lane 1, molecular weight marker; lane 2, empty well; lane 3, 23-treated, 6 h; lane 4, 23-treated, 15 h; lane 5, 23-treated, 24 h; lane 6, 23-treated, 30 h; lane 7, 23-treated, 48 h; lane 8, 23-treated, 72 h; lane 9, 23-treated, 6 h; lane 10, 23-treated, 15 h; lane 11, 23-treated, 24 h; lane 12, 23-treated, 30 h; lane 13, 23-treated, 48 h; lane 14, 23-treated, 72 h. B, endothelial cells untreated or treated with rPAI-123. In this figure rPAI-124 is named 24. Lane 1, molecular weight marker; lane 2, empty well; lane 3, empty well; lane 4, untreated, 6 h; lane 5, untreated, 15 h; lane 6, untreated, 24 h; lane 7, untreated, 30 h; lane 8, untreated, 72 h; lane 9, 24-treated, 6 h; lane 10, 24-treated, 30 h. C, endothelial cells untreated or treated with rPAI-124. In this figure rPAI-124 is named 24. Lane 1, molecular weight marker; lane 2, 24-treated, 6 h; lane 3, 24-treated, 15 h; lane 4, 24-treated, 24 h; lane 5, 24-treated, 30 h; lane 6, 24-treated, 48 h; lane 7, 24-treated, 72 h.

Fig. 11. VEGF-A bound to heparan sulfate in rPAI-1-treated endothelial cells. Bovine aortic endothelial cells were treated with rPAI-1 proteins for 6 or 12 h. At the designated time points, the medium was removed from each cell culture well. The cells were washed with HBSS before adding 0.05 IU of heparinase III/ml HBSS. The treated and untreated cells were incubated with heparinase for 1 h at 37 °C. The HBSS containing heparinase and proteins released from heparan sulfate in the enzymatic digestion were collected. Equivalent amounts of protein from each sample were treated with 0.1 M DTT for 2.5 h at 37 °C. The reduced proteins were separated by electrophoresis on a 4–20% SDS-polyacrylamide gel. The proteins were transferred to an immunoblot where they were probed for VEGF-A. The reduced VEGF samples migrate at a molecular mass corresponding to dimeric VEGF-A. Kodak X-Omat Blue film was used in development procedure. Final figures were auto adjusted in Photoshop to enable clear visualization of proteins. A, immunoblots probed for VEGF-A165, 189, 205. In this figure, rPAI-1 proteins are denoted as follows, rPAI-123 = 23, rPAI-123 = 23, rPAI-123 = 23, rPAI-124 = 24, Lane 1, molecular weight marker; lane 2, molecular weight marker; lane 3, 23-treated, 6 h; lane 4, 23-treated, 12 h; lane 5, 23-treated, 12 h; lane 6, 23-treated, 12 h; lane 7, untreated, 12 h; lane 8, 23-treated, 6 h; lane 9, 23-treated, 12 h; lane 10, 24-treated, 6 h; lane 11, 24-treated, 12 h. B, immunoblot simultaneously probed for VEGF-A165, 189, 205 and VEGF-A205 active site. In this figure, rPAI-1 proteins are denoted as follows, rPAI-123 = 23, rPAI-123 = 23, rPAI-124 = 24, Lane 1, 23-treated, 6 h; lane 2, 23-treated, 12 h; lane 3, 23-treated, 12 h; lane 4, 23-treated, 12 h; lane 5, untreated, 6 h; lane 6, untreated, 12 h; lane 7, empty well; lane 8, molecular weight marker; lane 9, 23-treated, 6 h; lane 10, 23-treated, 12 h; lane 11, 24-treated, 6 h; lane 12, 24-treated, 12 h.

DISCUSSION

Truncated PAI-1 proteins were used as biological tools to study anti-angiogenic functions of PAI-1. We demonstrate that, in the absence of the reactive center loop that was contained within deleted residues 262–379, the rPAI-1 Hep23 isoform, which contains the full heparin (heparan sulfate)-binding domain, does not inhibit angiogenic functions. Cultured endothelial cells treated with rPAI-1 Hep23 did not undergo apoptosis, and they proliferated at a rate comparable to controls. Angiogenic tubules from embryonic chick aortic rings migrated and proliferated at levels equivalent to the control samples.

When varied concentrations of rPAI-1 Hep23 were reacted with VEGF-A and heparin, higher concentrations prevented
the release of VEGF-A from that complex (Fig. 8D). VEGF-A165 appears to be the isoform bound to heparan sulfate in the extracellular matrix of bovine aortic endothelial cells after the initial 12 h of rPAI-1Hep23 treatment. The culture medium primarily contains inactive populations of VEGF-A isoforms, as well as small amounts of activated VEGF-A165 and/or VEGF-A189. Immunoblots probed for VEGF-B show that the rPAI-1Hep23 VEGF-heparin complex also contains VEGF-B (data not shown). This interesting aspect of rPAI-1Hep23 is being investigated.

The rPAI-1Hep23 isoform, which only has deletions at the amino terminus and, therefore, has the RCL, did not: 1) block the release of activated VEGF-A isoforms in vitro or in biochemical reactions, 2) induce apoptosis in BAEC, and 3) inhibit the migration and proliferation of angiogenic tubules in an organ culture. This suggests that the residues at the carboxyl terminus introduce a conformation in rPAI-1Hep23 that alters the accessibility of important binding domains that enable rPAI-1Hep23 to function in anti-angiogenic mechanisms.

The rPAI-1Hep23 isoform, in which the entire heparin-binding domain and residues 262–379 have been deleted, is able to: 1) mediate the production of proteolytic fragments that contain angiostatin kringles 1–3, 2) induce apoptosis in 22% of the treated BAEC, and 3) reduce tubule migration by 50% in an organ culture. However, rPAI-1Hep23 does not block the release of activated heparin-binding VEGF-A in biochemical reactions. That evidence is supported by the in vitro data (Fig. 10A), which shows an abundance of active VEGF-A of various sizes in the culture medium of endothelial cells treated with rPAI-1Hep23. The VEGF-A fragments correspond in size to those reported for active VEGF-A165 and uPA or plasmin-activated VEGF-A165 and/or VEGF-A189. The interesting aspect of uPA and plasmin activated VEGF-A165,189 is their ability to bind directly to VEGFR-1 and VEGFR-2 independently of heparan sulfate. A comparison of the levels of activated VEGF-A in the culture medium of rPAI-1Hep23 and rPAI-1Hep23-treated cells would suggest that plasmin- and uPA-activated VEGF-A are either not important in the pro-angiogenic mechanisms or the active VEGF is bound to VEGF receptors in rPAI-1Hep23-treated cells. In contrast, the culture media from rPAI-1Hep23-treated cells contain substantial amounts of activated VEGF-A that are either: 1) representative of a percentage of the total pool of active VEGF where a portion is bound and unbound or 2) the activated VEGF-A is available to bind the receptors, but apoptosis occurs through a mechanism other than VEGF inhibition, thereby, reducing the number of VEGF receptors. The data would suggest that cleavage of plasmin into angiostatin is the apoptosis mechanism that may limit VEGF receptor numbers. The active VEGF-A may bind the surviving endothelial cells, enabling them to proliferate. Such a mechanism would account for the rPAI-1Hep23 and rPAI-1Hep23 differences in cell number (Fig. 5), percentage of apoptotic cells (Fig. 4), and migration of sprouting tubules in an organ culture (Fig. 6).

The rPAI-1Hep23 isoform, which also has residues 262–379 deleted, but contains a partial heparin domain, is able to: 1) produce, in a reaction with uPA and plasminogen, proteolytic fragments corresponding to angiostatin kringles 1–3; 2) induce apoptosis in 39% of the adherent-treated BAEC; 3) inhibit tubule migration by 65% in an organ culture; 4) block the release of heparin-binding VEGF-A isoforms in biochemical reactions (Figs. 8 and 9); 5) block the release of VEGF-A165 and/or VEGF-A189 from heparan sulfate following 6 h of rPAI-1Hep23 treatment; and 6) reduce the activation of VEGF-A165 and/or VEGF-A189.

The culture medium of rPAI-1Hep23-treated cells contains small amounts of activated VEGF when compared with that found in rPAI-1Hep23-treated cells. Because the rPAI-1Hep23-treated cells are undergoing a higher level of apoptosis after 24 h of treatment, a larger pool of activated, receptor-bound VEGF is not likely. The collective data suggest that rPAI-1Hep23 partial binding to heparan sulfate introduces a conformational change in rPAI-1Hep23 that enables it to control uPA and/or plasmin activation of pro-VEGF bound to heparan sulfate. We demonstrated in Fig. 2 that rPAI-1Hep23 bound to heparin is able to maintain its ability to cleave plasmin. However, plasmin-activated VEGF has reduced mitogenic activity and by itself would not account for the high level of anti-angiogenic activity associated with rPAI-1Hep23 (17). The heparan sulfate-bound VEGF-A in the rPAI-1Hep23-treated cells shows that the active site of VEGF-A165 is exposed, which demonstrates that rPAI-1Hep23 bound to heparan sulfate alters the conformation of VEGF-A. The conformational change could result in one of the following mechanisms: 1) rPAI-1Hep23 binds VEGF-A or 2) rPAI-1Hep23 binds uPA at PAI-1 residues 110–145 to prevent uPA from activating heparan sulfate-bound or soluble VEGF-A. Both mechanisms are currently under investigation.

The rPAI-1Hep23 and rPAI-1Hep23 isoforms demonstrate anti-angiogenic properties. However, there are observed and measured differences between the two isoforms. Based on the differences presented, we propose two mechanisms that contribute to the additional apoptosis in rPAI-1Hep23-treated BAEC. One mechanism is early blockage of VEGF activation, which blocks VEGF-regulated survival signaling pathways to result in apoptosis of endothelial cells. PAEC, which do not express VEGFR-1 or VEGFR-2, were used as a tool to make a distinction between the apoptosis resulting from rPAI-1Hep23 blockage of VEGF functional activity in BAEC and a second apoptosis mechanism. The results of those experiments (Fig. 12) support a mechanism whereby rPAI-1Hep23 blocks activation of VEGF-A to prevent (or limit) VEGF binding to its receptors. The 65% reduction in migration of VEGF-stimulated tubules from rPAI-1Hep23-treated embryonic chick aortic rings supports a mechanism that inhibits VEGF-A function.

The combined data suggest a second apoptosis mechanism induced by rPAI-1Hep23 that is less significant than the apoptosis related to blockage of VEGF functional activity. Angiostatin and other angiogenesis inhibitors are associated with the induction of apoptosis (68–72). The role of rPAI-1Hep23 and rPAI-1Hep23 in the production of angiostatin kringles 1–3 in reactions with uPA and plasminogen suggests that production of angiostatin may be one mechanism that induces apoptosis in

**Fig. 12.** Apoptosis of rPAI-1Hep23-treated BAEC and PAEC. Bovine aortic endothelial cells (BAEC) and porcine aortic endothelial cells (PAEC) were grown in DMEM containing 10% FBS at 37 °C, 5% CO2 until the cells reached confluence, at which time fresh culture medium containing 1.2 nM rPAI-123 was added to the cells. The treated cells were incubated at 37 °C for 36 h, harvested, and analyzed for apoptosis in an Annexin V assay.
rPAI-1<sub>g3</sub> and rPAI-1<sub>Hsp23</sub>-treated endothelial cells. Thus, rPAI-1<sub>g3</sub> has two potential anti-angiogenic mechanisms that result in enhanced apoptosis: blockage of VEGF function and induction of angiostatin production through cleavage of plasmin. We have not reported the specific binding interactions that occur in a reaction containing rPAI-1<sub>g3</sub>, uPA, and plasminogen, but we do show that rPAI-1<sub>g3</sub> is required in the reaction in order for plasmin cleavage to occur. The cleavage of plasmin in this reaction results in production of angiostatin kringle 1–3.

Others have shown that PAI-1 can be cleaved at the P1 and P1' residues of the RCL, turning the inhibitor into a substrate with a molecular mass of 39kDa (42, 73, 74). The amount of substrate PAI-1 (RCL cleaved, inactive) is increased during the interaction of PAI-1 with thrombin in the presence of heparin and vitronectin (42). A smaller cleaved PAI-1 fragment (less than 31 kDa) has been shown to be produced as a result of adding heparin and thrombin to a pre-existing PAI-1-thrombin complex (42) or in reaction of PAI-1 with plasmin (43). The deletions at the carboxyl terminus exclude the RCL in three of the rPAI-1 isoforms that we have utilized in these studies. Each of these proteins functions differently with respect to pro- and anti-angiogenic mechanisms. Therefore, we can conclude that the functional activity of these proteins is not solely dependent upon the absence of the reactive center loop. The structural difference in rPAI-1<sub>g3</sub> and rPAI-1<sub>Hsp23</sub> is the heparin (heparan sulfate)-binding domain, which seemingly accounts for the differences in functional activity of the three proteins. The large amounts of activated VEGF in the culture medium of rPAI-1<sub>g3</sub>-treated cells strongly support the importance of partial PAI-1 binding to heparan sulfate with respect to controlling activation of VEGF.

Data from biochemical and <i>in vitro</i> studies demonstrate that PAI-1 is cleaved by thrombin, matrix metalloproteinase-3, and plasmin. However, there have not been studies to examine the presence of cleaved PAI-1 products in vivo. Identification of cleaved PAI-1 in correlation with tumor growth and atherosclerotic plaque development would provide valuable information with respect to the controversial pro- or anti-angiogenic role of PAI-1 in these two diseases.

Our studies provide evidence that PAI-1 conformational changes in the binding domains that participate in anti-angiogenic mechanisms. Such evidence supports the importance in evaluating the anti-angiogenic role of PAI-1 in tumor growth (76, 77) and atherosclerotic plaques (50, 75) and as a potential therapeutic molecule.

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