The Anti-angiogenic Activity of rPAI-1<sub>23</sub> Inhibits Fibroblast Growth Factor-2 Functions*<sup>§¶</sup>

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Many angiogenesis inhibitors are breakdown products of endogenous extracellular matrix proteins. Plasmin and matrix metalloproteinase-3 generate breakdown products of matrix-bound plasminogen activator inhibitor-1 (PAI-1). We produced a truncated form of PAI-1, rPAI-1<sub>23</sub>, that possesses significant anti-angiogenic activity and stimulates high levels of apoptosis in quiescent arterial endothelial cells. Quiescent endothelial cells are less susceptible to apoptosis than angiogenic endothelial cells. The present study was designed to determine the mechanism of the rPAI-1<sub>23</sub> effects in bovine aortic endothelial cells. Apoptosis was measured in annexin V and caspase 3 assays. Expression of death and survival signaling molecules were examined by Western blot and kinase activity. Fibroblast growth factor 2 (FGF2) functions were analyzed in angiogenesis assays. The early response to rPAI-1<sub>23</sub> was an increase in annexin V-positive cells and phosphorylated (p) JNK isoform expression followed by an increase in p-Akt and p-c-Jun expression. Caspase 3 was activated at 4 h, whereas p-Akt was reduced to control levels. By 6 h of rPAI-1<sub>23</sub> treatment cell number was reduced by 35%, and p-c-Jun and p-JNK were degraded by proteasomes. Confocal microscopic images showed increased amounts of FGF2 in the extracellular matrix. However, rPAI-1<sub>23</sub> blocked FGF2 signaling through FGF receptor 1 and syndecan-4, inhibiting cell migration, tubulogenesis, and proliferation. Exogenous FGF2 stimulation could not reverse these effects. We conclude that rPAI-1<sub>23</sub>, stimulation of apoptosis in BAEC triggers a cascade of death versus survival events that includes release of FGF2. The rPAI-1<sub>23</sub> anti-angiogenic activity inhibits FGF2 pro-angiogenic functions by blocking FGF2 signaling through FGF receptor 1 and syndecan-4 and downstream effectors p-Akt, p-JNK, and p-c-Jun.

The vascular endothelium is normally maintained in a differentiated, quiescent state. Pro-angiogenic factors destabilize the quiescent endothelium into migratory, proliferative endothelial cells that are attenuated by anti-angiogenic factors. The pro- and anti-angiogenic molecules have cell survival and death functions that are tightly controlled to maintain a balance (1–4).

Many negative regulators of angiogenesis are cleavage products of an existing cellular protein that is not inhibitory in its normal intact conformation (5–7). Induction of endothelial cell apoptosis is one characteristic common to these inhibitors (8). Endothelial cells are more susceptible to apoptosis when they are activated (angiogenic) as compared with quiescent (9, 10). Survival of the latter is dependent upon angiogenic growth factors in the local environment, which can be blocked by anti-angiogenic factors. If growth factor functions are blocked, endothelial cells are removed by apoptosis (4, 9, 11).

Apoptosis can be induced by diverse stimuli that initiate specific signal transduction pathways. The apoptosis pathways are regulated by pro- and anti-apoptotic molecules that are controlled in part by kinases (12) and proteosomal degradation (13). The c-Jun NH<sub>2</sub>-terminal kinase (JNK)<sup>§</sup> signaling pathway is activated by cellular stress, and its role in apoptosis versus survival remains unclear (14). The role of JNK in cellular proliferation, apoptosis, differentiation, and motility is dependent upon the activity and stability of JNK isoforms and their associated substrates (15–18). The cellular function associated with JNK-substrate complexes is tightly regulated by proteosomal activity (19, 20).

Plasminogen activator inhibitor-1 (PAI-1) has been shown to have both pro- and anti-angiogenic activity (21–24). It has been suggested that PAI-1 pro-angiogenic versus anti-angiogenic activity is based on the relative amounts of the inhibitor that are in active versus inactive conformations (22, 25). Proteolytic molecules plasmin (26) and matrix metalloproteinase 3 (27) cleave and inactivate PAI-1. Potential functions of cleaved PAI-1 have not been studied. We made truncated PAI-1 cDNAs and produced truncated PAI-1 proteins (rPAI-1) to investigate potential PAI-1 functions in the absence of the reactive center loop (“inactive PAI-1”). One rPAI-1 protein, rPAI-1<sub>23</sub>, has significant anti-angiogenic activity (28, 29). The reactive center loop at the carboxyl terminus and part of the heparan sulfate binding domain at the amino terminus were removed from PAI-1 cDNA to produce rPAI-1<sub>23</sub> (28). The striking anti-angiogenic feature of rPAI-1<sub>23</sub> is its ability to induce an unusually high level of apoptosis in endothelial cells. The goal of this study

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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The abbreviations used are: JNK, c-Jun NH<sub>2</sub>-terminal kinase; PAI-1, plasminogen activator inhibitor-1; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FGF, fibroblast growth factor; FGFR, FGF receptor; BrdUrd, bromodeoxyuridine; BAEC, bovine aortic endothelial cells; p-, phosphorylated; SAPK, stressed-activated protein kinase.
was to determine the mechanisms responsible for high levels of rPAI-123-stimulated apoptosis.

**EXPERIMENTAL PROCEDURES**

Recombinant PAI-1 Protein Purification—Recombinant, truncated PAI-1 protein, rPAI-123, was produced by truncating poPAI-1 cDNA and subsequent expression of the gene products in pGAPZ-α, a *Pichia pastoris* expression vector (Invitrogen) (28, 29). The secreted protein is purified on a heparin-Sepharose column containing 0.02% sodium azide. The rPAI-123 protein is eluted in 400 mM NaCl and then dialyzed in a Sepharose column containing 0.02% sodium azide. The secreted protein is purified on a hep- trated cells were harvested and labeled with annexin V followed by staining with propidium iodide (Sigma). Cell number was measured by flow cytometry as described (28, 29).

Endothelial Cell Culture Conditions—Primary BAEC were isolated from a *Bos taurus* aorta. All experiments were performed on confluent bovine aortic endothelial cells (BAEC), passage 3, to mimic quiescence and obtain maximum receptor expression. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, l-glutamine (0.29 mg/ml) and penicillin/streptomycin (100 IU/ml). Untreated cells served as a control. Concentrations of exogenous proteins in all experiments were as follows: 0.6 nM rPAI-123, 150 nM angiotatin (Calbiochem), 2 nM PAI-1 (American Diagnostica, Stamford, CT), and 1.47 nM fibroblast growth factor 2 (FGF; BD Biosciences).

Annexin V Detection of Externalized Phosphatidylserine—Confluent BAECs were treated with a single dose of rPAI-123 or angiotatin, and growth was continued for 1, 2, or 4 h. The treated cells were harvested and labeled with annexin V following the manufacturer’s protocol (Roche Applied Science). Cells were analyzed for annexin V by fluorescent microscopy (510 nm) and flow cytometry as described (28, 29).

**Measurement of Active Caspase 3 in rPAI-123-treated Cells**—BAEC were incubated with rPAI-123 or angiotatin for 2, 4, and 6 h at 37 °C then analyzed for caspase 3 activity using an EnzChek caspase-3 assay kit (Invitrogen). Adherent cells (1 × 10⁴) from each treatment group were lysed before incubating with 17 μM caspase 3 substrate (Z-DEVD-R110) according to the manufacturer’s directions. Fluorescence was measured at 490 nm on a Labsystems Multiskan MCC/340 microplate reader.

**Measurements of Cell Viability**—Confluent BAECs were treated with a single dose of rPAI-123, angiotatin, or PAI-1, and growth was continued for 1, 2, 4, or 6 h. Detached cells in the culture medium and adherent cells were collected at each time point, pelleted, and resuspended in PBS containing 5 μg/ml propidium iodide (Sigma). Cell number was measured by hemacytometer in a Nikon inverted microscope, and the number of propidium iodide-positive cells was detected with a 580-nm filter.

**Adhesion Assay**—BAEC were harvested, washed twice in PBS, then seeded into 6-well plates at a density of 4 × 10⁴ cells/ml. The wells contained DMEM supplemented with 1% FBS (to enable attachment) and either rPAI-123, PAI-1, or no treatment. The cells were incubated at 37 °C for 1, 2, 4, and 6 h, washed in PBS, fixed in 40% methanol, 10% acetic acid for 1 h, stained with Coomassie Blue, and destained in fixative.

**Immunoblots Probed for Akt**—Equivalent lysates of protein from untreated or rPAI-123- or angiotatin-treated cells were gel-resolved and transferred to nitrocellulose membranes. The immunoblots were probed for total and phosphorylated Akt (Ser⁴⁷³ and Thr⁴⁰⁸) in an overnight, 4 °C binding reaction (1 μg/ml anti-total Akt antibody, 0.5 μg/ml anti-p-Akt antibody, Cell Signaling, Beverly, MA). A horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (Amersham Biosciences) was used to amplify the binding reaction, which was detected with SuperSignal West Pico chemiluminescent substrate (Pierce). Lane loading equivalence was assessed with a goat anti-human actin polyclonal antibody specific for the carboxyl terminus of α, β, and γ isoforms (Santa Cruz, Santa Cruz, CA). The binding reaction was amplified in a 1-h room-temperature incubation with a rabbit anti-goat secondary antibody (Pierce), and a horseradish peroxidase-conjugated donkey anti-rabbit tertiary antibody provided detection with an ECL substrate.

**Akt Kinase Activity Assay**—Akt was immunoprecipitated from 200 μg of lysate protein with 4 μg of an anti-Akt antibody (Cell Signaling) and protein G-Sepharose beads (Pierce). Each immobilized, washed sample was incubated at 30 °C for 15 min in a 40-μl reaction mixture under conditions previously described (30).

**Detection of Active Caspase 3 in Lysate Proteins**—Immunoblots containing equivalent amounts of lysate proteins were probed for active caspase 3 with a rabbit polyclonal antibody specific for the cleaved p17 fragment (Chemicon, Temecula, CA). The binding reaction was amplified and detected as described for total Akt.

**Immunoblots Probed for Stress-activated Protein Kinase/JNK**—Lysate proteins were probed for total SAPK/JNK with a rabbit polyclonal antibody (Cell Signaling). Amplification and detection of the binding reaction were as described for Akt. A monoclonal antibody specific for phosphorylated SAPK/JNK (residues Thr¹⁸³/Tyr¹⁸⁵, Cell Signaling) was incubated overnight at 4 °C with membranes containing lysate proteins. The binding reaction was amplified at room temperature for 1 h with a rabbit anti-mouse secondary antibody (Pierce) followed by a 1-h room temperature incubation with horseradish peroxidase-conjugated tertiary donkey anti-rabbit antibody. SuperSignal West PICO substrate was used to detect the binding reaction.

**Immunoblots Probed for c-Jun**—Immunoblots containing equivalent amounts of lysate proteins were probed for c-Jun and phospho-c-Jun overnight at 37 °C using rabbit polyclonal antibodies specific for residue Ser⁷³ (Cell Signaling). Amplification and detection were as described for Akt.

**JNK Kinase Activity Assay**—To measure JNK kinase activity 200 μg of lysate proteins were incubated with 4 μg of anti-JNK antibody (Cell Signaling) and protein G-Sepharose beads. A glutathione S-transferase-c-Jun (residues 1–89) fusion protein (Cell Signaling) served as the JNK substrate. Immobilized JNK complexes were mixed with 40 μl of reaction buffer (20 mM Hepes, pH 7.6, 20 mM MgCl₂, 25 mM β-mercaptoethanol, 100 μM sodium orthovanadate, 2 mM dithiothreitol). The reaction was started by adding 10 μg of substrate, 20 μM ATP, 0.25 μM Ca²⁺.
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[γ-32P]ATP then incubation at 25 °C for 0.5 h. The remainder of the reaction was as described for Akt kinase activity (30). Radioactivity was determined in a scintillation mixture.

**FGF2 Imaged by Confocal Microscopy**—Confluent BAEC were incubated with rPAI-1_{23} at 37 °C for 2, 4, and 6 h. After the incubation period, cells were washed in PBS and then incubated at −20 °C for 10 min with a 1 methanol:1 acetone fixative. The fixed cells were air-dried before blocking for 1 h at 4 °C in 3% bovine serum albumin. The blocked cells were probed for FGF2 at room temperature for 1 h using a rabbit anti-bovine FGF2 polyclonal antibody (5 μg/ml Tris-buffered saline, 1% bovine serum albumin, 0.03% Tween 20) (Sigma). A goat anti-rabbit secondary antibody conjugated to Alexa 568 (Invitrogen) amplified and detected the binding reaction. Z-stack images were acquired on a Zeiss LSM-510 META point scanning confocal microscope (Thornwood, NY).

**Quantification of FGF2 by Enzyme-linked Immunosorbent Assay**—Endothelial cells were treated as described for confocal microscopy. The cells were washed extensively in ice-cold PBS after the incubation period. Cells and extracellular matrix were isolated in cell dissociation buffer (Sigma). Equivalent amounts of protein from each test group were analyzed for FGF2 expression in an FGF2 enzyme-linked immunosorbent assay kit following the manufacturer’s protocol (Calbiochem).

**Co-immunoprecipitation of p-FGFR1 in rPAI-1_{23} Lysates**—Lysate proteins (250 μg) from cells stimulated with rPAI-1_{23} or FGF2 were incubated overnight at 4 °C with 6 μg of anti-phosphotyrosine antibody (Upstate, Charlottesville, VA). The anti-body-ligand complexes were immobilized with 50 μl of protein G-coupled magnetic beads (Invitrogen). The protein complexes were resolved on 10% polyacrylamide gels. Membranes containing the transferred proteins were probed with a monoclonal anti-p-FGFR1 antibody (Tyr_653/654, Cell Signaling). The reaction was amplified and detected as described for phosphorylated SAPK/JNK. Lane loading was assessed by cutting the membrane above p-FGFR1 and probing with the anti-phosphotyrosine antibody.

**Detection of Syndecan-4 in rPAI-1_{23}, Lysate**—Lysate proteins from cells treated with rPAI-1_{23}, FGF2, or no treatment were probed with a 1:200 dilution of a polyclonal antibody raised against the cytoplasmic domain of syndecan-4 (a kind gift from Dr. Nicholas Shworak). The binding reaction was amplified and detected as described for c-Jun.

**Proteasomal Inhibition with Lactacystin**—Proteasomal degradation of signaling molecules in BAEC treated with rPAI-1_{23} was evaluated by preincubating the cells with 25 μM lactacystin (Sigma) for 1.5 h at 37 °C, then adding rPAI-1_{23}. The 37 °C incubation continued until the designated time points before harvesting the cells in lysis buffer. BAEC treated with rPAI-1_{23} in the absence of lactacystin served as the control.

**FGF2 Rescue of rPAI-1_{23} Inhibition**—BAEC were incubated with rPAI-1_{23} and FGF2 in three different conditions. In condition 1 cells were exposed to rPAI-1_{23} for 30 min at 37 °C before adding FGF2 and continuing the incubation for an additional 5.5 h. For condition 2 FGF2 and rPAI-1_{23} were added simultaneously to culture medium and incubated for 6 h at 37 °C. In condition 3, FGF2 was added to the medium and incubated for 30 min before adding rPAI-1_{23} and incubating for 5.5 additional hours.

**Endothelial Cell Migration into a Scratch Wound**—Cell migration was assessed using a standard wounding assay. After the wounding procedure, 1 ml of DMEM containing either rPAI-1_{23} or FGF2 was added to duplicate wells, and the extent of migration was measured after 6 h of incubation at 37 °C. Cell migration in serum-free medium was used as a control. For FGF2 rescue experiments, FGF2 and rPAI-1_{23} were added to culture medium in three conditions as described.

**Endothelial Cell Proliferation Assays**—BAEC were seeded into 6-well cell culture plates and grown to confluence, washed in Hanks’-buffered saline solution, and incubated overnight in serum-free medium at 37 °C. DMEM containing 2% FBS, 25 μM BrdUrd, rPAI-1_{23}, and/or FGF2 was added to triplicate wells and continued in a time course as described for the rescue experiments. BrdUrd-positive cells were detected as previously described (28, 29).

**Endothelial Cell Tube Formation in a Collagen 1 Overlay**—Tissue culture plates were coated with ice-cold collagen 1 (Cohesion, Palo Alto, CA) at a concentration of 1.5 mg/ml and pH 7.0. BAEC (5 × 10^5) were seeded onto the center of each polymerized collagen-coated well and allowed to adhere for 1 h at 37 °C. The cells were then covered with ice-cold collagen 1 and placed in a 37 °C incubator for 1 h. DMEM containing 10% FBS, 2 mM L-glutamine, rPAI-1_{23}, and FGF2 or combinations of rPAI-1_{23} and FGF2 were added to wells and incubated at 37 °C for 72 h. The numbers of endothelial cell enclosures were determined at 24 h using digital imaging.

**Tubule Formation in a Chick Aortic Arch Ring Assay**—Aortic arches were removed from fertilized chicken eggs (Oliver Merrill & Sons, Londonderry, NH) at day 14 of embryonic development and immobilized in Matrigel® (a kind gift from Dr. Hynda Kleinman), as previously described (29). The rings were stimulated with FGF2 (n = 6) or FGF2 and rPAI-1_{23} (n = 6). The sprouting took place over 3 days.

**Statistical Analysis**—Statistical analysis was performed with a two-tailed indirect Student’s t test.

**RESULTS**

To study the signaling mechanisms responsible for rPAI-1_{23}-stimulated apoptosis, we examined early time points (1–6 h) of stimulation in confluent BAEC and compared them with angiostatin, another angiogenesis inhibitor. Annexin V-positive cells at 1 h of rPAI-1_{23} stimulation were 2-fold above the control but 30% less (p < 0.001) than angiostatin-stimulated cells (Fig. 1A). The number of annexin V-positive cells increased another 2-fold at 2 h of rPAI-1_{23} stimulation and then decreased at 4 h. Angiostatin treatment resulted in a decline in annexin V-positive cells at 2 h, which reached control levels by 4 h (27 ± 1% rPAI-1_{23} versus 8 ± 0.5% angiostatin and 10 ± 0.4% control, p < 0.001). We concluded that annexin V detected externalized phosphatidylserine in cells treated with both angiogenesis inhibitors, but the rPAI-1_{23} effects were greater than angiostatin at 2 and 4 h of stimulation.

Caspase 3 activity in adherent cells stimulated with rPAI-1_{23} for 4 h was 12% greater (p = 0.05) than control cells and 25% greater (p < 0.001) than angiostatin-treated cells (Fig. 1B). By
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The mitogen-activated protein kinase stress-induced JNK pathway can activate caspase 3, a protease that cleaves the pro-caspase to its active form. To begin exploring survival signaling cascades that may be modulated by rPAI-123 and angiostatin, we focused on Akt. Cells responded to both inhibitors by activating Akt (Ser473) (Fig. 2A). Kinase activity measurements in adherent cells confirmed that both inhibitors increased Akt activity, but 2 h of rPAI-123 stimulation resulted in 2.5-fold more Akt activity than angiostatin-treated and control cells. Although the activity was reduced at 4 h of rPAI-123 stimulation, it remained significantly greater (p = 0.05) than control cells (Fig. 2B). Thus, both anti-angiogenic molecules stimulate Akt phosphorylation, but the response to rPAI-123 is significantly greater and more prolonged.

We also examined p-Akt levels in cells that detached during rPAI-123 treatment. Degraded p-Akt was detected in detached cell lysates at 4 and 6 h of rPAI-123 treatment but was not found in control or angiostatin samples (Fig. 2C). We then probed the detached cell lysates for active caspase 3, a protease that cleaves and inactivates Akt (31). Active caspase 3 was only detected at 4 and 6 h of rPAI-123 treatment. This set of experiments suggested that rPAI-123 was able to sustain apoptosis by activating caspase 3, which in turn reduced p-Akt levels.

The mitogen-activated protein kinase stress-induced JNK pathway can activate caspase 3 (32, 33). Cell lysates probed for total and phospho-JNK detected distinct expression of a phosphorylated 45-kDa JNK isoform (JNK2) at 1–2 h of rPAI-123 stimulation, which could explain the 30% loss in cell number. Low density cells treated with rPAI-123 had adhesion properties similar to untreated and PAI-1 control cells at 4 h (23 cells/μl versus 24 ± 4 untreated and 26 ± 7 PAI-1). We concluded that reduced cell number in response to rPAI-123 was due to apoptosis-induced detachment rather than altered adhesion properties.

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lated by rPAI-123 induced the JNK signaling pathway, but angiostatin did not.

Because proteasomes regulate the expression levels and stability of JNK, we explored the effect of lactacystin, a ubiquitin-mediated proteasomal inhibitor, on JNK expression levels in rPAI-123-treated cells. Immunoblots analyzed for total JNK in cells treated with rPAI-123 for 2 h showed expression of 45-kDa JNK and JNK1 isoforms and a JNK1 cleavage product (immediately below JNK1) (Fig. 4). Proteasomal degradation...
reduced the levels of both isoforms at 6 h of rPAI-123 treatment. Lactacystin treatment depleted JNK1 levels, whereas JNK2 levels were increased in the presence of the inhibitor. The 45-kDa JNK isoform was not detected in control cells unless they were treated with lactacystin, whereas the 54-kDa isoform was detected under all conditions. Immunoblots probed for p-JNK showed similar patterns of proteasomal regulation of JNK isoforms. This series of experiments demonstrated that rPAI-123 treatment modulates differential expression and activation of JNK isoforms at defined treatment times.

Proteasomal activity also controls the stability and expression levels of JNK substrates. Expression levels of phospho-c-Jun (Ser73), a JNK substrate essential for cell proliferation (34), were increased 10-fold at 2 h of rPAI-123 treatment then declined substantially at 6 h (Fig. 4). Proteasomal degradation did not regulate phosphorylated p-c-Jun (Ser73) at 2 h of rPAI-123 treatment but clearly controlled c-Jun levels in control cells. By 6 h, however, p-c-Jun (Ser73) in rPAI-123 lysates was reduced to control levels through proteasomal degradation.

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FIGURE 5. Deposition of FGF2 in extracellular matrix of rPAI-123-treated cells. BAEC treated with rPAI-123 or no treatment were probed for FGF2 (red) and imaged by confocal microscopy (63×). Single images were acquired every 0.5 μm in a Z-stack. Projection images are shown (n = 3). Note the increase in FGF2 in the matrix of rPAI-123-treated cells at 4 and 6 h. Also note the reduced cell number in rPAI-123-treated cells. Data are shown as the mean ± S.D. and p values were determined by Student’s t test (n = 3).
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FIGURE 6. rPAI-123 inhibition of FGF2 signaling and angiogenic functions. BAEC were treated with rPAI-123, FGF2, or no treatment. Lysates were probed for p-FGFR1 and syndecan-4. A, representative immunoblot (IB) and densitometry analysis of experiments that examined lysate proteins for p-FGFR1. Lysate proteins were immunoprecipitated (IP) with a phosphotyrosine (pTyr)-specific antibody. Blots containing phosphotyrosine complexes were gel-resolved under denaturing, non-reducing conditions and probed for p-FGFR1. Anti-phosphotyrosine antibody complex served as lane loading control. Note that p-FGFR1 expression in rPAI-123–treated cells is at control levels (n = 3). B, representative immunoblot analysis and densitometry analysis of syndecan-4 cytoplasmic domain in denatured, reduced lysate proteins obtained from confluent BAEC treated with rPAI-123, FGF2, or no treatment. Note the absence of syndecan-4 at 6 h of rPAI-123 stimulation (n = 3). *, p < 0.05 versus control. **, p < 0.001 versus control. Migration, tubulogenesis, and proliferation of BAEC treated with varied conditions of rPAI-123 and FGF2 were evaluated in a monolayer scratch assay, three-dimensional collagen tube formation assay, and BrdUrd incorporation assay, respectively. rPAI-123 and FGF2 were added either early (immediate), late (30 min later), or simultaneously relative to each other. C, endothelial cell migration. The graphs show the migration distance of confluent BAEC incubated overnight in serum-free medium, then stimulated with FGF2, serum-free medium, rPAI-123, or varied combinations for a total of 6 h (n = 9). Note that FGF2 is not able to rescue cells from the anti-migratory effects of rPAI-123. D, three-dimensional collagen tube formation assay. The extent of closed vascular structures in collagen-1 gel by BAEC was measured at 24 h in the presence of 10% FBS, 10% FBS + rPAI-123, 10% FBS + rPAI-123 + FGF2, or FGF2. Note the reduced number of complete enclosures in cells treated with rPAI-123, or rPAI-123 + FGF2. E, endothelial cell proliferation assay. The amount of BrdUrd uptake was measured in BAEC stimulated with 2% FBS, 2% FBS + FGF2, 2% FBS + rPAI-123, or varied conditions of 2% FBS + rPAI-123 and FGF2 for a total of 6 h (n = 9). Note that FGF2 cannot restore the rate of proliferation in all permutations. *, p < 0.05 versus control. **, p < 0.001 versus control. Data are shown as mean ± S.D., and p values were determined by Student’s t test (n = 3).

In this study we show that exposure of confluent BAEC to rPAI-123 protein results in a significant induction of apoptosis and inhibition of FGF2-induced cell migration, tube formation, and proliferation, which cannot be rescued by endogenous or exogenous pro-angiogenic FGF2. Blockage of FGF2 functions by rPAI-123 activity is supported by the absence of activated FGF2 signaling pathways. We demonstrate that rPAI-123 is able to sustain its anti-angiogenic activity beyond that of angiostatin by activating caspase 3 and expression levels of specific JNK isoforms that are regulated by proteasomal degradation.

Stimulation of endothelial cell apoptosis is a characteristic of angiogenesis inhibitors. Therefore, measurements of apoptosis markers in BAEC treated with rPAI-123 were compared with angiostatin. Externalized phosphatidylserine is detected by annexin V and as such is used as a marker of early events leading to apoptosis (40). Annexin V–positive cells were detected with rPAI-123 and angiostatin treatment, but the levels remained elevated in rPAI-123–stimulated cells and returned to normal in angiostatin treatment. Active caspase 3 was elevated at 4 h (25%) and 6 h (32%) of rPAI-123 stimulation but was not detected in angiostatin–treated cells, thus indicating that the two inhibitors activate different pathways. Sustained stimulation of pro-apoptotic events enabled rPAI-123 to reduce endothelial cell number by 35% after 6 h of treatment, whereas angiostatin-treated cell numbers were comparable with control cells.

The cellular response to early apoptotic cues stimulated by rPAI-123 was activation of JNK, c-Jun, and Akt. The stress-
induced JNK pathway can activate downstream signaling molecules that are either pro-apoptotic or pro-survival. Immuno- blot analysis demonstrated differential expression of JNK isoforms in rPAI-1_{23}-stimulated cells that were not detected in control and angiotatin-treated cells, thus indicating that these isoforms play an important role in rPAI-1_{23}-stimulated cellular functions. Further evidence was obtained from experiments that demonstrated proteosomal regulation of JNK1 and JNK2 expression in response to rPAI-1_{23}. The levels of c-Jun, a primary JNK substrate that is essential for cell proliferation (15), were also regulated by proteosomal degradation in a pattern similar to the 45-kDa JNK isofrom. These results suggest that a p-JNK2-p-c-Jun complex was degraded to the extent that expression levels of both molecules were reduced to control levels. Others have demonstrated that c-Jun stability and phosphorylation are differentially regulated by JNK1 and JNK2 to control proliferation and apoptosis in fibroblasts (16).

Akt kinase activity was increased in rPAI-1_{23}- and angiotatin-stimulated cells. However, the activity at 2 h of treatment was significantly greater in response to rPAI-1_{23} but then began to decline by 4 h. The decline in p-Akt in adherent cells was inversely proportional to caspase 3 activity. Moreover, degraded p-Akt and active caspase 3 were detected in detached cells treated with rPAI-1_{23}. These data suggest that caspase 3 protease activity contributed in part to p-Akt degradation (31), thus reducing the pool of p-Akt.

FGF2 is a potent angiogenic growth factor that activates proliferation, migration, survival, and differentiation of endothelial cells (41, 42). FGF2 in a complex with FGFR1 activates the Akt and mitogen-activated protein kinase pathways signaling cascades (43). We determined that FGF2 deposited into the extracellular matrix in response to rPAI-1_{23} treatment did not increase FGFR1 phosphorylation above control levels.

Syndecan-4 also acts as a receptor for FGF2 signaling, and its cytoplasmic domain regulates FGF2-stimulated migration and proliferation in the absence of FGF receptors (35–37, 39). We considered that the syndecan-4 signaling pathway could be activated by FGF2 in rPAI-1_{23}-treated cells. Immunoblots showed basal expression of the syndecan-4 cytoplasmic domain at 2 and 4 h of rPAI-1_{23} stimulation and its complete absence at 6 h. The cumulative data demonstrate that rPAI-1_{23} prevents FGF2 signaling through FGFR1 and syndecan-4 in BAEC.

Additional evidence of rPAI-1_{23} inhibition of FGF2 was provided in functional assays that measured endothelial cell migration, tube formation, and proliferation in response to rPAI-1_{23} or combinations of rPAI-1_{23} and exogenous FGF2 stimulation. In all conditions the migration distance, number of complete vascular structures, and BrdUrd incorporation were not significantly different from measurements in cells treated with rPAI-1_{23} alone, thus providing evidence that FGF2 is unable to rescue BAEC from the anti-angiogenic effects of rPAI-1_{23}. These data were supported by the aortic ring ex vivo assays.

The collective data from this study support a mechanistic model (Fig. 8) of the signaling pathways that enable rPAI-1_{23} to reduce cell number by 35% in the first 6 h of stimulation and reduce migration, tube formation, and proliferation of the remaining semi-confluent cell population to levels less than or equivalent to quiescent BAEC. These effects are in part due to rPAI-1_{23}-stimulated blockage of FGF2 signaling and proteosomal regulation of JNK isoforms.

Apoptosis plays an important role in regulating homeostasis, differentiation, and development, which is achieved by complex signaling cascades that balance death and survival (44, 45). Quiescent endothelial cells are less susceptible to apoptosis than angiogenic endothelial cells (9, 10). Survival of angiogenic endothelial cells is dependent upon localized expression of angiogenic growth factors. Anti-angiogenic factors can block pro-angiogenic growth factor functions, which results in removal of endothelial cells by apoptosis (4, 9, 11). This study
**Anti-angiogenic rPAI-1<sub>23</sub> Inhibits FGF2 Functions**

**FIGURE 8. Proposed model of rPAI-1<sub>23</sub> anti-angiogenic mechanisms.** The rPAI-1<sub>23</sub> protein initiates apoptosis in confluent BAEC through a yet unknown molecule (step 1). The early apoptosis activates the JNK signaling cascade (step 2) and overexpression of 54 and 45 kDa p-JNK isoforms (step 3). The cell survival response to rPAI-1<sub>23</sub> is increased levels of p-Akt and phospho-c-Jun (step 4). Proteosomal degradation destabilizes a p-JNK-p-c-Jun (step 5) to reduce their activity. JNK expression activates caspase 3 (step 6), a protease that degrades Akt (step 7) to result in sustained apoptosis (step 8). FGF2 is released into the matrix as a result of cell death (step 9). FGF2 signaling through FGRF1 and syndecan-4 are blocked by the inhibitory effects of rPAI-1<sub>23</sub> stimulation (10). The significant, observable ex vivo difference is inhibition of sprouting tubules in aortic rings stimulated with rPAI-1<sub>23</sub> + FGF2 + 10% FBS.

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