Stimulation of PAI-1 Expression in Endothelial Cells by Cultured Vascular Smooth Muscle Cells

Marisa Gallicchio, Stella Argyriou, Gina Ianches, Enrico L. Filonzi, Hans Zoellner, John A. Hamilton, Katherine McGrath, Johann Wojta

Abstract  Regulation of endothelial cell (EC) plasminogen activator inhibitor type-1 (PAI-1), the primary physiological inhibitor of tissue-type plasminogen activator (TPA) and urokinase-type plasminogen activator (UPA), by various stimuli has been well characterized. We report the upregulation of secreted and intracellular PAI-1 in human umbilical ECs when cocultured with human smooth muscle cells (SMCs) on amniotic membranes or incubated with SMC conditioned medium (CM) under serum-free conditions as determined by enzyme-linked immunosorbent assay. Cocultured human umbilical vein ECs and SMCs, or human umbilical artery ECs and SMCs, displayed a 73% and 68% increase, respectively, in released PAI-1. SMC-derived stimulatory factor release showed tissue specificity, since only human aortic, umbilical vein, and umbilical artery SMCs upregulated PAI-1 synthesis, whereas SMCs from human mammary artery, pulmonary artery, and saphenous vein did not. Stimulation of EC PAI-1 by SMC CM was both time and concentration dependent, with as much as five- and fourfold increases in supernatants and lysates, respectively. PAI-1 synthesis and activity in ECs from other vascular beds were also upregulated by SMC CM.

Northern blot analysis paralleled the protein results, showing as much as a 2.7-fold increase in specific EC PAI-1 mRNA expression after incubation with SMC CM for 8 hours. PAI-1 stimulatory activity in SMC CM was completely abolished by boiling or incubation with protamine sulfate and was reduced by transient acidification or heparin-Sepharose pretreatment by 33% or 48%, respectively. The stimulatory factor(s) appeared to have a molecular mass of 23 kD as determined by gel filtration. Heat and acid lability precluded transforming growth factor-β involvement. SMC CM also proved negative for interleukin-1α activity, tumor necrosis factor-α activity, and basic fibroblast growth factor antigen. These results suggest that a soluble factor(s) secreted constitutively by SMCs is probably distinct from transforming growth factor-β, interleukin-1α, tumor necrosis factor-α, and basic fibroblast growth factor and may influence intravascular fibrinolysis through regulation of PAI-1 gene expression. (Arterioscler Thromb. 1994;14:815-823.)

Key Words  •  plasminogen activator inhibitor type-1  •  endothelial cells  •  smooth muscle cells  •  fibrinolysis

Initiation of fibrinolysis occurs with the generation of the active serine protease plasmin by tissue-type (TPA) and urokinase-type (UPA) plasminogen activators. In blood, plasminogen activator inhibitor type-1 (PAI-1) is the major physiological inhibitor of plasminogen activation; PAI-1 is tightly complexed with TPA and is thought to prevent fibrin clot dissolution. PAI-1 also inhibits UPA and thus plays a pivotal role in cell migration, neoplasia, angiogenesis, and tissue remodeling in addition to intravascular clot formation and dissolution.

Because net fibrinolytic activity reflects the balance between plasminogen activators and plasminogen activator inhibitors, identification of the factors involved in the regulation of PAI-1 is important. In cultured endothelial cells (ECs), PAI-1 is upregulated by various stimuli, including dexamethasone, thrombin, lipopolysaccharide, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and lipoprotein(a).6-8

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Smooth muscle cells (SMCs), which are separated from ECs by the basal lamina and are the major cellular component of the blood vessel wall, are a potential source of at least some of these factors. In addition SMCs synthesize TPA, UPA, PAI-2, and PAI-1,9-12 the last of which is modulated by platelet-derived growth factor, TGF-β, and thrombin.13-15 In coculture with ECs, SMCs contribute to the proteolytic activation of latent TGF-β.16-18 Via these mechanisms, SMCs may influence net fibrinolysis of the blood vessel wall. However, there is a paucity of information on how SMC and EC interactions affect the fibrinolytic potential of the vascular wall.17-20 It was the aim of the present studies, therefore, to look at the effects on PAI-1 biosynthesis of EC-SMC cocultures.

Methods

Cell Culture

Human vascular SMCs were isolated by explant techniques from pieces of human aorta, mammary artery, pulmonary artery, and saphenous vein that were discarded after surgery.21 SMCs were also isolated by explant techniques from the umbilical artery and vein of cords obtained after vaginal delivery. Briefly, tissue specimens were cut into 10 to 20 pieces 1 to 2 mm in diameter, placed in a gelatin-coated (Bio-Rad) Petri dish (100-mm diameter, Costar), and covered with a drop of Medium 199 (M199, Sigma Chemical Co) containing 20% supplemented calf serum (SCS, Hyclone), 50 IU/mL penicillin, 50 µg/mL streptomycin (both from Flow), 250 ng/mL amphi-
tericin (GIBCO), and 50 μg/mL EC growth supplement (EGCS) prepared as described. After 3 to 5 days the explants became adherent, and the Petri dish was then filled with M199 containing SCS and ECGS as described above. SMCs that grew out from explants were grown to confluency and subcultured using a split ratio of 1:3. Cells were confirmed as SMCs by their typical "hill-and-valley" morphology and positive immunofluorescence staining with a monoclonal antibody against α-actin (Boehringer Mannheim). All SMCs used in this study were between passages 3 and 5.

ECs from human umbilical vein (HUVECs), human umbilical artery (HUAECs), and human saphenous vein (HSVECs) were isolated and characterized as described. Briefly, cells isolated by mild collagenase treatment were seeded onto gelatin-coated Petri dishes, grown to confluency as described above in M199 containing 5 U/mL heparin (Fisons), and subcultured using a 1:3 split ratio. Human foreskin microvascular ECs (HFMECs) were isolated from material discarded after circumcision as described. Cells were confirmed as ECs by their "cobblestone" morphology, uptake of acetylated low-density lipoprotein (Biomedical Technologies), and positive immunofluorescence with anti-α-von Willebrand factor antibodies (Sigma). ECs used were between passages 2 and 4. Tissues were collected with the patients' informed consent and approval by the Royal Melbourne Hospital Board of Medical Research Ethics Committee.

Preparation of Amniotic Membranes

Amniotic membranes were prepared as described. Briefly, human amniotic membranes from placertas were fastened to polystyrenefluorocarbon rings, and the amniotic epithelium was lysed by overnight incubation in 0.25 mol/L NH4OH at 4°C followed by scraping with a rubber policeman. Membrane-covered rings were then placed into six-well culture plates (Costar), thus creating an upper (0.7 mL medium) and a lower (2.3 mL medium) compartment. ECs were grown on the upper basa lamina side of the amnion and SMCs on the stromal surface facing the lower compartment. After coculture in M199 without heparin (as above) for 5 hours, cells were rinsed twice with Hanks' balanced salt solution (HBBS, Sigma) containing 10 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid. Serum-free M199 containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL Na2SeO3 (M199-ITS, Sigma) was added for 16 hours before harvesting the supernatants. ECs and SMCs were grown separately on membranes. Upper- and lower-compartment supernatants were pooled before assaying for PAI-1 antigen.

Preparation of SMC Conditioned Medium (CM)

SMCs were seeded into Petri dishes. Confluent cultures were washed twice with HBSS and given M199-ITS. After 24 hours the SMC CM was collected, centrifuged at 1000g for 5 minutes to remove cellular debris, and frozen at −70°C.

Treatment of ECs With SMC CM

HUVECs, HSVECs, and HFMECs were grown to confluency in 24-well dishes (104 cells per well, Costar) and rinsed twice with HBSS. SMC CM, undiluted or diluted in M199-ITS, was added to the cells, and the CM of treated cells was collected after 24 hours or at the times indicated, centrifuged at 1000g, and stored at −70°C. After they were rinsed twice with HBSS, ECs were lysed with phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Triton X-100. Cell lysates were centrifuged and stored at −70°C.

PAI-1 Antigen Assay

PAI-1 antigen in CM and cell lysates was determined by a sandwich enzyme-linked immunosorbant assay (ELISA) using monoclonal antibodies that recognize active, latent, and complexed PAI-1.

TPA, UPA, and PAI-2 Antigen Assays

PAI-1, UPA, or PAI-2 antigen in CM and cell lysates was measured by sandwich ELISAs as described.

Assay for PAI-1 Activity

PAI-1 activity was measured by a modified ELISA. Excess TPA was bound to ELISA plates precoated with a monoclonal anti-TPA antibody (MP3W3VPA). Samples were added and any active PAI-1 became complexed to immobilized TPA. PAI-1 bound to TPA was quantified by a peroxidase-labeled monoclonal anti-PAI-1 antibody.

Northern Blots

Northern blot analysis was performed using HUVECs grown in six-well dishes, with each well having a surface area of 9.6 cm2. HUVECs in triplicate wells were treated with undiluted human umbilical vein SMC CM. Total cellular RNA was collected and pooled by the guanidine isothiocyanate/cesium chloride method. Agarose gel electrophoresis was used to size-fractionate RNA on a formaldehyde-containing 1.4% (wt/vol) agarose gel. RNA was transferred from the gel to a BioTrace HP membrane (Gelman Sciences) using a transfer buffer of 1.5 mol/L NaCl and 0.5 mol/L NaOH. Hybridizations were performed overnight by using cDNA fragment probes that encode PAI-1 (kindly provided by Dr D. J. Loskutoff, La Jolla, Calif) and the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (kindly provided by Dr O. Bernard, Melbourne, Victoria, Australia). Probes were labeled by random priming with [32P]dCTP (Amersham) and added to a 50% (vol/vol) formamide hybridization buffer to achieve a minimum specific activity of 2×106 cpm/mL. After hybridization the membranes were washed in reducing concentrations of saline–sodium citrate (SSC) to a final concentration of 0.2× SSC/0.1% (wt/vol) sodium dodecyl sulfate (SDS) at 65°C. Autoradiography was performed with Kodak XAR-5 film at −70°C. Films were scanned by densitometry (LKB 220 2 Ultrascan).

Characterization of SMC-Derived PAI-1 Stimulating Factor

SMC CM was subjected to heat treatment for 5 minutes at 100°C, acidified to pH 2 with 10 mol/L HCl for 1 hour at 4°C and the pH adjusted back to 7.4 with 10 mol/L NaHCO3; and incubated with heparin–Sepharose CL-4B (Pharmacia) overnight at 4°C or treated with 100 μg/mL protamine sulfate (Boots) before addition to HUVEC monolayers.

Gel Filtration of SMC-Derived CM

Human umbilical vein SMC CM (450 mL) was freeze-dried, resuspended in 20 mL distilled water, and dialyzed against PBS, pH 7.4, at 4°C overnight. Gel filtration of this material was performed by using a Fractogel TSK HW-55 column (Toso Soda) equilibrated in PBS, pH 7.4, in a 2.6×90-cm column (Pharmacia) at 4°C and a flow rate of 13 mL/h. The column was calibrated with low-molecular-weight standards (Pharmacia) and the void volume determined with blue dextran (Pharmacia). Fractions of 2.6 mL were collected and screened for protein at an absorbance of 280 nm. Aliquots of all fractions were diluted 1:5 in M199-ITS and added to confluent monolayers of HUVECs for 12 hours. As a control, PBS at pH 7.4 was diluted 1:5 in M199-ITS and added to cells. After incubation, PAI-1 antigen in HUVEC CM was determined by ELISA as described above.

IL-1α and TNF-α Assays

SMC CM was tested for IL-1α activity in a murine thymocyte comitogenesis assay and for TNF-α activity in an actinomycin D–treated L929 cell assay as described.
TABLE 1. Effect on PAI-1 Synthesis by ECs and SMCs in Coculture

<table>
<thead>
<tr>
<th></th>
<th>PAI-1, ng/16 h</th>
<th>Percent Increase</th>
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<tbody>
<tr>
<td>HUVECs</td>
<td>306 ± 116</td>
<td></td>
</tr>
<tr>
<td>HUVSMCs</td>
<td>690 ± 149</td>
<td></td>
</tr>
<tr>
<td>Cocultured HUVECs/HUVSMCs</td>
<td>1722 ± 328*</td>
<td>73</td>
</tr>
<tr>
<td>HUAECs</td>
<td>1001 ± 252</td>
<td></td>
</tr>
<tr>
<td>HUASMCs</td>
<td>336 ± 104</td>
<td></td>
</tr>
<tr>
<td>Cocultured HUAECs/HUASMCs</td>
<td>2241 ± 103†</td>
<td>68</td>
</tr>
</tbody>
</table>

Confluent monolayers of human umbilical vein and artery endothelial cells (HUVECs and HUAECs) and human umbilical vein and artery smooth muscle cells (HUVSMCs and HUASMCs) were co-cultured separately or together on amniotic membranes for 16 hours, in Medium 199-5 μg/mL insulin/5 μg/mL transferrin/5 ng/mL Na₂SeO₃. Conditioned medium was harvested and plasminogen activator inhibitor type-1 (PAI-1) determined as described in "Methods." PAI-1 antigen levels are expressed in nanograms per 16 hours and represent the mean±SD of triplicate cultures.

*P<.03 compared with the sum of PAI-1 from cells cultured separately.
†P<.04 compared with the sum of PAI-1 from cells cultured separately.

bFGF Western Blot

SMC CM was probed for bFGF by Western blot. Briefly, concentrated SMC CM diluted in buffer (62 mmol/L Tris-HCl, pH 6.8, 0.2% SDS, 10% glycerol) was separated on a 10% SDS gel by electrophoresis proteins were transferred to nitrocellulose sheets (Bio-Rad) by using transfer buffer (25 mmol/L Tris, 192 mmol/L glycine, 20% methanol). The nitrocellulose was then blocked in Blotto (5% skim milk powder in PBS, pH 7.4) and probed with rabbit anti-bFGF antibody (Sigma) diluted 1:100 in Blotto. bFGF (Sigma) was used as a positive control. Bound antibody was detected with a chemiluminescence system (Amersham). Molecular-weight markers (Bio-Rad) in the range of 18 to 106 kD were used.

Statistical Analysis

Data were compared statistically by Student's t test for paired observations. Values of P<.05 were considered significant. Results are expressed as the mean±SD. For the results in Fig 2A and 2B, a repeated-measures ANOVA was performed.

Results

Production of PAI-1 by Cocultured ECs and SMCs

Corresponding ECs and SMCs derived from the same vascular bed were cocultured on amniotic membranes. In a representative experiment shown in Table 1, cocultured cells derived from umbilical vein or artery displayed significantly increased PAI-1 antigen release into the medium relative to the sum of PAI-1 released by ECs and SMCs cultured separately (73%, n=3 [number of wells], P<.03; 68%, n=3, P<.04, respectively). Cocultures were repeated at least 10 times in triplicate, with increases in PAI-1 antigen expression of approximately 80% compared with ECs and SMCs cultured separately (results not shown). Corresponding cocultured ECs and SMCs from saphenous vein and mammary artery did not display augmented PAI-1 levels (results not shown).

Effect of CM From Amniotic Membrane–Cultured ECs and/or SMCs on PAI-1 Synthesis by HUVECs

To determine whether increases in PAI-1 were derived from ECs and/or SMCs, monolayers of HUVECs grown in wells were incubated with CM collected from cells grown on amniotic membranes. As shown in Fig 1A, incubation of confluent HUVEC monolayers with SMC CM alone or cocultured SMCs and ECs resulted in a similar significant increase of PAI-1 antigen secretion into the supernatant of 64% (n=3, P<.01) and 56% (n=3, P<.01), respectively, relative to untreated controls. There was no statistically significant difference between PAI-1 stimulation by CM from SMCs cultured alone or SMCs cocultured with ECs (NS). EC CM had no significant effect on PAI-1 synthesis by human umbilical vein SMC monolayers grown in wells relative to untreated SMC controls (Fig 1B). Thus, SMCs released a soluble factor(s) that upregulated PAI-1 antigen levels in ECs.

Concentration Response and Time Course of PAI-1 Synthesis in HUVECs Exposed to SMC CM

The effect of SMC CM serially diluted in M199-ITS on PAI-1 antigen levels in supernatants and lysates of HUVECs was determined (Fig 2A and 2B, respective-
FIG 2. Line plots of concentration-dependent upregulation of plasminogen activator inhibitor type-1 (PAI-1) antigen synthesis by human umbilical vein endothelial cells incubated in smooth muscle cell (SMC) conditioned medium (CM). Confluent monolayers of human umbilical vein endothelial cells were incubated for 24 hours with various dilutions of human umbilical vein SMC CM (A), human umbilical artery SMC CM (o), and human aortic SMC CM (•) under serum-free conditions. PAI-1 antigen in supernatants (A) and cell lysates (B) was determined as outlined in "Methods." SMC-derived PAI-1 was determined simultaneously and subtracted from each value. Data are given as nanograms per 10⁵ cells per 24 hours and represent mean±SD of triplicate cultures. Experiments were performed twice, with a representative experiment shown. *P<.01, tP<.02, compared with untreated cells.

As determined by repeated-measures ANOVA, increasing concentrations of three different SMC CM resulted in highly significant increases of PAI-1 antigen in CM (Fig 2A, P<.001) and cell lysates (Fig 2B, P<.001) of HUVECs. Furthermore, ANOVA indicated that human umbilical artery SMC CM was the most potent and human umbilical vein SMC CM the weakest stimulus of PAI-1 antigen in EC CM (Fig 2A, P<.001). Finally, ANOVA revealed that the slope of the curve for the PAI-1 increase observed in HUVEC CM resulting from human umbilical artery and aortic SMC CM was significantly steeper than that caused by human umbilical vein SMC CM (Fig 2A, P<.001).

Human mammary artery, pulmonary artery, and saphenous vein SMC CM did not affect HUVEC PAI-1 synthesis (results not shown). Because at least two donors of each vessel type were tested and yielded consistent results, this finding appears to be a vessel-specific difference. When HUVECs were incubated with SMC CM for various times, a significant time-dependent increase in PAI-1 antigen was evident in supernatants (24 hours, n=3, P<.05) (Fig 3) and cell lysates (results not shown) in treated but not untreated cells. In the same experiments changes in UPA, TPA, and PAI-2 antigen levels in HUVEC CM and cell lysates were not seen.

Effect of SMC CM on PAI-1 Activity

Part of the increased PAI-1 antigen synthesis induced by SMC CM involved increased synthesis of active PAI-1 into the CM, as shown in Table 2. Human umbilical artery and vein CM increased PAI-1 activity in ECs by 12- and 27-fold, respectively, relative to untreated ECs. At the same time PAI-1 antigen was also stimulated.

Effect of SMC CM on Other ECs

SMC CM was further tested on other ECs in addition to HUVECs. As shown in Table 3, SMC CM from
TABLE 2. Effect of SMC CM on PAI-1 Activity in ECs

<table>
<thead>
<tr>
<th>PAI-1 Antigen, (ng/10^5 Cells)/24 h</th>
<th>PAI-1 Activity, (U/10^5 Cells)/24 h</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>171±57</td>
</tr>
<tr>
<td>HUASMC CM</td>
<td>1761±122*</td>
</tr>
<tr>
<td>HUVSMC CM</td>
<td>1535±35*</td>
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Confluent monolayers of human umbilical vein (HUV) endothelial cells (ECs) were cultured in Medium 199–5 μM insulin/5 μM transferrin/5 ng/mL Na2SeO3 (control) or conditioned medium (CM) from human umbilical artery (HUASMC) or vein (HUVSMC) smooth muscle cells. CM was harvested and plasminogen activator inhibitor type-1 (PAI-1) antigen or activity was determined as described in "Methods." SMC-derived PAI-1 antigen and activity were determined simultaneously and subtracted from each value. PAI-1 activity levels are expressed in tissue-type plasminogen activator inhibiting units per 10^5 cells per 24 hours and, as with PAI-1 antigen levels, represent mean±SD of triplicate cultures.

*P<.001, †P<.02, §P<.05 compared with untreated control cells.

human aortic, umbilical vein, and umbilical artery stimulated PAI-1 synthesis in the supernatants and cell lysates of HFMECs and HSVECs, consistent with the findings for HUVECs. In accordance with the results for HUVECs, human saphenous vein and mammary artery SMC CM did not stimulate PAI-1 synthesis in HFMECs and HSVECs. Pulmonary artery SMC CM exhibited a slight upregulatory effect on HSVECs, whereas saphenous vein SMC CM exhibited a downregulatory effect on HFMECs.

Effect of Culture Conditions on Stimulatory Factor Production

Human umbilical artery and aortic SMCs were grown in the presence of M199 containing 5 μM insulin, 20% SCS, and 50 μg/mL ECGS. CM was harvested and tested for stimulatory activity on monolayers of HUVECs. As shown in Table 4, PAI-1 stimulatory activity was seen under these culture conditions as well as when SMCs were cultured under serum-free conditions (Figs 1 through 3).

Effect of SMC CM on PAI-1 mRNA Levels in HUVECs

Stimulation of PAI-1 expression in HUVECs by SMC CM was also seen at the mRNA level (Fig 4A and 4B). Steady-state PAI-1 mRNA levels were increased 2.4- and 2.7-fold after 4 and 8 hours’ incubation with SMC CM (Fig 4A, lanes 3 and 5, respectively) compared with untreated controls at the same times (lanes 2 and 4), as determined by densitometry. PAI-1 mRNA relative to glyceraldehyde-3-phosphate dehydrogenase mRNA appeared to decrease with time in untreated EC controls (Fig 4B; 0h con, 4h con).

TABLE 3. Effect of SMC CM on PAI-1 Synthesis by ECs From Different Vascular Beds

<table>
<thead>
<tr>
<th>PAI-1, (ng/10^5 Cells)/24 h</th>
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<tbody>
<tr>
<td>HFMECs</td>
</tr>
<tr>
<td>CM</td>
</tr>
<tr>
<td>CL</td>
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<tr>
<td>HMASMC CM</td>
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<td>HPASMC CM</td>
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<td>HUVSMC CM</td>
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<td>HASMC CM</td>
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<td>HUASMC CM</td>
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<td>HUVSMC CM</td>
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<table>
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<tr>
<th>HSVECs</th>
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<tr>
<td>CM</td>
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<td>HUVSMC CM</td>
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<td>HASMC CM</td>
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<td>HUASMC CM</td>
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Confluent monolayers of human foreskin microvascular (HFMEC) and saphenous vein (HSVEC) endothelial cells were cultured in Medium 199–5 μM insulin/5 μM transferrin/5 ng/mL Na2SeO3 (control) or conditioned medium (CM) from human mammary artery (HMASMC), pulmonary artery (HPASMC), saphenous vein (HUVSMC), aortic (HASMC), umbilical artery (HUVSMC), and vein (HUASMC) smooth muscle cells. CM and cell lysates (CLs) were harvested and plasminogen activator inhibitor type-1 (PAI-1) was determined as described in "Methods." SMC-derived PAI-1 was determined simultaneously and subtracted from each value. PAI-1 antigen levels are expressed as nanograms per 10^5 cells per 24 hours and represent mean±SD of triplicate cultures.

*P<.01, †P<.02, §P<.03 compared with untreated control cells.

TABLE 4. Effect of Culture Conditions on PAI-1 Stimulatory Factor Synthesis by SMCs

<table>
<thead>
<tr>
<th>PAI-1, (ng/10^5 Cells)/24 h</th>
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<tbody>
<tr>
<td>CM</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>HUASMC CM</td>
</tr>
<tr>
<td>HUVSMC CM</td>
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<th>CL</th>
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<tr>
<td>Control</td>
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<tr>
<td>HUASMC CM</td>
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<td>HUVSMC CM</td>
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Confluent monolayers of human umbilical vein endothelial cells were cultured in M199–5 μg/mL insulin/5 μg/mL transferrin/5 ng/mL Na2SeO3 containing heparin, supplemented cell serum, and endothelial cell growth supplement (control) or human umbilical artery (HUASMC) or vein (HUVSMC) smooth muscle cell conditioned medium (CM) collected in the same medium. CM and cell lysates (CLs) were harvested and plasminogen activator inhibitor type-1 (PAI-1) was determined as described in "Methods." SMC-derived PAI-1 was determined simultaneously and subtracted from each value. PAI-1 antigen levels are expressed as nanograms per 10^5 cells per 24 hours and represent mean±SD of triplicate cultures.

*P<.02, †P<.03 compared with untreated control cells.
Characterization of the SMC-Derived PAI-1 Stimulating Factor

Partial characterization of the SMC-derived stimulating factor was achieved by treating aliquots of SMC CM with heat, acid, protamine sulfate, and heparin-Sepharose before testing for PAI-1 stimulating activity on monolayers of HUVECs. Heating at 100°C for 5 minutes or treatment with 100 μg/mL protamine sulfate completely abrogated SMC CM activity (n=3 for both; P<.001, P<.03, respectively) (Fig 5). Treatment of CM by transient acidification at pH 2 for 1 hour at 4°C or heparin-Sepharose reduced PAI-1 stimulatory activity by 33% (n=3, P<.05) and 48% (n=3, P<.05), respectively.

Gel Filtration of SMC-Derived CM

As shown in Fig 6, a minor protein peak with a molecular weight of 190 kD and a major protein peak with a molecular mass of 39 kD were eluted from the column. The PAI-1 stimulating activity, however, was eluted at a molecular mass of approximately 23 kD. The peak fraction stimulated PAI-1 antigen production in HUVECs by as much as 17-fold.

Fig 4. Upregulation of plasminogen activator inhibitor type-1 (PAI-1) mRNA in human umbilical vein endothelial cells (ECs) by smooth muscle cell (SMC) conditioned medium (CM). Human umbilical vein ECs were incubated for 4 and 8 hours in the presence or absence of undiluted human umbilical vein SMC CM. Northern blots of RNA from these cells were hybridized to cDNA probes for PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as outlined in “Methods” (A). The 3.4- and 2.4-kb mRNA species for PAI-1 are indicated. Lanes 1, 2, 4, untreated ECs at 0, 4, and 8 hours, respectively; lanes 3 and 5, ECs treated with SMC CM for 4 and 8 hours, respectively. The bar graph of Fig 4B was derived from the ratio of PAI-1 and GAPDH densitometric measurements for each lane.

Fig 5. Bar graph of effect of heat, acid, protamine sulfate (prot), and heparin-Sepharose (hep) treatment on the plasminogen activator inhibitor type-1 (PAI-1) stimulating activity of smooth muscle cell (SMC) conditioned medium (CM). Confluent human umbilical vein endothelial cells were treated with Medium 199–5 μg/mL insulin/5 μg/mL transferrin/5 ng/mL Na2SeO3 (control), human umbilical vein SMC CM (CM), or human umbilical vein SMC CM subjected to various treatments: 100°C for 5 minutes (heat); pH 2 for 1 hour (acid); 100 μg/mL protamine sulfate (prot); or heparin-Sepharose (hep). After 24 hours the medium was harvested and PAI-1 antigen determined as described in “Methods.” Results are expressed as percent of control. SMC-derived PAI-1 was determined simultaneously and subtracted from each value, which is given as nanograms per 10^5 cells per 24 hours and represents mean±SD of triplicate cultures. Individual experiments were performed at least twice, with a representative experiment shown. *P<.001 compared with cells incubated with untreated SMC CM; $P<.02 compared with untreated, control cells; $P<.03, §P<.05 compared with cells incubated with untreated SMC CM.

Fig 6. Gel filtration profile of smooth muscle cell (SMC)-derived conditioned medium (CM) on Fractogel TSK HW-55. Human umbilical vein SMC-derived CM was lyophilized, resuspended in water, and dialyzed against phosphate-buffered saline, pH 7.4. The sample was then separated by gel filtration on a Fractogel TSK HW-55 column (2.6x90 cm) equilibrated in phosphate-buffered saline at 4°C. Fractions of 2.6 mL were collected at a flow rate of 13 mL/h. Protein content of respective fractions is shown as absorbance at 280 nm (○). The plasminogen activator inhibitor type-1 stimulating activity was determined as outlined in “Methods” and expressed as fold stimulation over control (△). Molecular masses of respective peaks are indicated by arrows.
IL-1α, TNF-α, and bFGF Assays

PAI-1–stimulating SMC CM proved negative when tested for IL-1α activity, TNF-α activity, and bFGF antigen by bioassay and Western blot analyses, respectively (results not shown).

Discussion

Reports of SMC-mediated modulation of the fibrinolytic potential of ECs are few.17–20 Our studies demonstrate the upregulation of PAI-1 in CM of cocultured human ECs and SMCs grown on amniotic membranes. Subsequent incubation of ECs with cell-free SMC CM induced a marked increase in PAI-1 expression in both cell lysates and supernatants. Conversely, EC CM did not affect PAI-1 synthesis in SMC monolayers. These two findings indicate that upregulated PAI-1 levels of cocultures were EC derived and SMC induced.

Induction of EC PAI-1 synthesis and secretion was not common to all SMCs of various origins, reflecting either tissue specificity of factor expression or differences in culture requirements of SMCs from various vascular beds. Among the SMC CM that stimulated PAI-1 synthesis by ECs, CM obtained from human umbilical artery SMC had the strongest stimulating effect. In addition, a recent study has shown that CM from cultured human umbilical vein and pulmonary arterial SMCs grown in the presence of heparin, serum, and with or without ECGS downregulated PAI-1 expression in HUVECs.19 In contrast, in our studies human pulmonary arterial SMC CM was found not to affect HUVECs or HFMECs and to have a slight upregulatory effect on HSVECs. Additionally, we found that human umbilical vein SMC CM stimulated EC PAI-1 synthesis, and that saphenous vein SMC CM downregulated PAI-1 in HFMECs. In our studies we screened SMCs from six different vascular beds and found that SMCs from three of them were consistent and potent stimulators of PAI-1 in ECs, also derived from various vascular beds. The results were consistent for SMCs isolated from at least two donors. Thus, stimulatory activity appears to rely on the SMC but not the EC source.

This contrasting ability of SMC CM to influence EC PAI-1 may reflect differences in cell culture conditions, donors, growth state of cells (whether quiescent or actively growing), and extent of passage in culture; however, when our SMCs were grown in the presence of heparin, serum, and ECGS, synthesis of stimulatory activity was unaffected. In our studies SMC CM was collected in the presence of serum-free medium containing insulin and transferrin, known SMC growth promoters.33 The possibility remains that these may induce stimulatory factor synthesis, although they do not appear necessary for factor production, as the stimulatory activity was found in CM under both serum-free and serum-containing culture conditions. SMC heterogeneity in vitro has been noted by other laboratories, even with isolates from the same source and within the same laboratory.7,24,25 and may account for the discrepancy between these results and those of previous studies. In this regard it is interesting to note that SMCs that had previously downregulated EC PAI-1 actually upregulated EC PAI-1 on further passaging.26

The stimulation of EC PAI-1 by SMC CM was both time and concentration dependent, with as much as five- and fourfold increases observed in released and intracellular PAI-1, respectively. As with PAI-1 antigen, stimulation of PAI-1 activity also occurred when ECs were incubated with SMC CM. Northern blot analysis indicated similar increases in EC PAI-1 mRNA, with a greater than twofold increase after 4 hours’ incubation with SMC CM compared with untreated EC controls. Underloading of the zero control sample (Fig 4A, lane 1) as judged by its glyceraldehyde-3-phosphate dehydrogenase transcript highlighted the apparent greater amount of PAI-1 transcript at this time compared with later control times. Additionally, serum has been shown to decrease the fibrinolytic activity produced by ECs. It has been demonstrated that this decrease is associated with a serum-dependent increase in PAI-1.37 This could explain the decreased PAI-1 expression caused by serum withdrawal in our experiments.

A variety of cytokines and growth factors, including IL-1α, TNF-α, bFGF, and TGF-β, have been identified in SMCs and may upregulate EC PAI-1. The possibility that PAI-1 stimulation was caused by any one of these was investigated and found to be unlikely. Latent TGF-β is activated by plasmin when cocultured ECs and SMCs or pericytes are in contact or close proximity,16–18 by heat, and by transient acidification.38 Heat lability of our stimulatory factor precluded TGF-β involvement. The absence of a significant difference in PAI-1 stimulation by SMC CM or coculture CM, in which TGF-β activation may occur, and the apparent acid sensitivity of our substance further precluded TGF-β involvement.

The stimulatory activity bound heparin, as does the growth hormone bFGF; however, the roles of bFGF and the inflammatory cytokines IL-1α and TNF-α in SMC-mediated PAI-1 stimulation are doubtful for several reasons. IL-1α and TNF-α upregulated UPA and PAI-2 synthesis,29 and IL-1 downregulated TPA production in HUVECs.39 bFGF also stimulated UPA production. Yet, we found no changes in UPA, TPA, and PAI-2 levels. Additionally, TGF-β upregulated UPA in ECs.39 These findings cannot exclude the possibility of antagonists that may abrogate and mask possible modulatory effects on plasminogen activators. Nevertheless, SMC CM proved negative when tested for IL-1α activity in a mouse thymus bioassay, for TNF-α activity in an L929 cell bioassay, and for bFGF antigen in a Western blot analysis, making the likelihood of these cytokines’ involvement in stimulatory activity unlikely.

Because only PAI-1 but not TPA was increased by SMC CM, thrombin was also an unlikely candidate, as thrombin has been shown to increase TPA in ECs.6 Furthermore, to our knowledge, SMCs do not secrete thrombin. Upregulation was also inhibited by protamine sulfate, a basic protein, which nonspecifically prevents anachronism of various growth factors, such as vascular endothelial cell growth factor, platelet-derived growth factor, and bFGF, with their respective receptors, suggesting a possible receptor-mediated mechanism of PAI-1 stimulation.40,41

Fractionation of the SMC CM yielded an apparent molecular mass of 23 kD for the PAI-1 stimulating factor. However, this information does not allow us to
distinguish it from other known inducers of EC PAI-1 expression, since many have molecular masses in this range. For instance, IL-1α and IL-1β each have a molecular mass between 15 and 30 kD. TGF-β is 17 kD. Because of their close proximity to vascular ECs, SMCs are a potential source of paracrine activity that can act on the vascular endothelium. Under physiological conditions this suggests a mechanism whereby SMCs may maintain matrix and vessel wall integrity in the immediate vicinity by limiting plasminogen activator activity and protecting against excessive cellular migration and proliferation. These results suggest yet another mechanism by which SMCs may exert an antifibrinolytic influence on ECs in addition to TGF-β activation or PAI-1 expression. The SMC-derived PAI-1 stimulatory factor(s) is probably distinct from TGF-β, IL-1α, TNF-α, bFGF, or thrombin and requires further characterization.

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