**Type 1 Plasminogen Activator Inhibitor Synthesis of Endothelial Cells Is Downregulated by Smooth Muscle Cells**

By Günter Christ, Dietmar Seiffert, Peter Hufnagl, Alois Gessl, Johann Wojta, and Bernd R. Binder

Plasminogen activator inhibitor type 1 (PAI-1), the physiological inhibitor of both tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), is a major biosynthetic product of endothelial cells in vitro; endothelial cells in vivo, in contrast, do not appear to produce significant amounts of PAI-1 as made evident by in situ-hybridization studies in normal mice. This suggests that the high rate of PAI-1 synthesis of endothelial cells in vitro might be a result of the culture conditions. When human umbilical vein endothelial cells (HUVEC) were grown on human amniotic membranes, resembling the natural growth support instead of coated plastic, their morphology was changed from the cobblestone-like appearance on plastic to an in vivo like flagstone pattern. However, this morphological change had no significant effect on the synthesis and secretion of PAI-1. When smooth muscle cell (SMC) conditioned media (CM) were added to HUVEC cultures, PAI-1 antigen secretion of HUVEC was reduced by 40% to 60% as measured by enzyme-linked immunosorbent assay (ELISA). Immunoprecipitation experiments using 35S-methionine metabolically labeled HUVEC and Northern blot analysis of HUVEC PAI-1 mRNA indicate that this reduction was attributable to decreased PAI-1 synthesis and reduced steady-state levels of both the 3.2 kb and 2.2 kb form of PAI-1 mRNA. This effect was dose-dependent and observed under serum-containing as well as serum-free conditions, in the absence or presence of endothelial cell growth supplement (EGCS, 0 to 100 μg/mL) and attributable to a nondialyzable factor. Our data suggest that the high level of PAI-1 biosynthesis of endothelial cells in vitro may be attributable to the lack of a soluble factor produced by SMC, which controls and suppresses PAI-1 biosynthesis of endothelial cells in vivo.

© 1993 by The American Society of Hematology.

**MATERIALS AND METHODS**

**Cell culture.** HUVEC were isolated by mild collagenase treatment as described. Cells were seeded into Petri dishes (Costar, Cambridge, MA) coated with gelatine (BioRad, Richmond, CA) and grown to confluency in Medium 199 (Sigma, St Louis, MO) containing 20% supplemented calf serum (SCS; HyClone, Logan, UT), 50 IU/mL penicillin, 50 μg/mL streptomycin, 250 ng/mL amphotericin B (all JRH Biosciences, Lenexa, KS), 50 μg/mL endothelial cell growth supplement (ECGS) (unless otherwise stated) prepared as described and 5 U/mL heparin (Liquemin Roche IV, Hoffmann-LaRoche, Basel, Switzerland) in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Cells were confirmed to be endothelial cells by their cobblestone morphology, by positive immunofluorescence using anti-von Willebrand factor antibodies (Cappel, Cochranville, PA).

From the Laboratory for Clinical Experimental Pathology, and the Department of General and Experimental Pathology, University of Vienna, Vienna, Austria.

Submitted April 2, 1992; accepted October 29, 1992.

Supported by Grant Nos. P8011 and 7617 from the Austrian Fund for the Promotion of Scientific Research.

Address reprint requests to Bernd R. Binder, MD, Laboratory for Clinical Experimental Pathology, University of Vienna, Schwarzspännerstraße 17, A-1090 Vienna, Austria.

The publication costs of this article were defrayed in part by charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.

0006-4971/93/$00.00/0
and by uptake of acetylated low-density lipoprotein (LDL). All HUVEC used in this study were between passage 3 and 4.

SMC were either isolated by collagenase treatment of normal pulmonary artery obtained by surgery, or from human umbilical vein by using the explant technique. SMC from pulmonary artery were separated from endothelial cells by fluorescence-activated cell sorter (FACS) analysis (FACS III; Becton Dickinson, Mountain View, CA) using acetylated LDL as an endothelial cell-specific marker, and characterized by staining with monoclonal anti-SMC actin antibodies (FACS 111; Becton Dickinson, Mountain View, CA) separated from endothelial cells by fluorescence-activated cell sorter by using the explant technique. SMC from pulmonary artery were previously. Briefly, pieces of human amniotic membrane were fastened to teflon rings and the amnion epithelium was lysed by incubation in 0.25 mol/L NH₄OH for 2 hours followed by scraping with a rubber policeman. Such teflon rings were placed into 6-well culture plates (Costar), creating an upper compartment (0.5 mL medium) and a lower compartment (2.0 mL medium) communicating only through the membrane. HUVEC were placed onto the stromal surface, facing the upper compartment of the amnion. Confluent HUVEC monolayers were stained with silver nitrate as described. Cultures were flooded sequentially with the following reagents at room temperature: 5% glucose for 30 seconds, 0.25% AgNO₃ for 30 seconds, 5% glucose to rinse, 1% NH₄Br for 30 seconds, 5% glucose to rinse, 3% CoBr₂ for 30 seconds, 5% glucose to rinse, 5% formalin to fix for 15 minutes. Cells were counterstained with Wrights stain (Sigma) for 15 minutes.

Preparation of conditioned media (CM). SMC were seeded into 24-well culture plates (Costar) and reached confluency within 3 to 5 days at a density of about 1.2×10⁵ cells per well. Confluent cultures were washed twice with Hanks' balanced salt solution (HBSS; Sigma) containing 10 mmol/L HEPES and given either 1 mL Medium 199 (plus 5 U/mL heparin and 50 μg/mL ECGS, unless otherwise stated) containing 20% SCS or 1% bovine serum albumin (BSA; Behring, Marburg, Germany), or 1 mL RPMI 1640 (Sigma) (plus 5 U/mL heparin without ECGS and serum).

After 24 hours the SMC-conditioned Medium 199 was collected, centrifuged (1,000g, 5 minutes) to remove cell debris, and immediately transferred to HUVEC, grown to confluence in 24-well plates and washed twice with HBSS before experiment. For each experiment, separate 24 hour SMC CM was collected from cultures (between passages 3 and 5), either cultured in 24-well plates (n = 16) or in 100 mm Petri dishes (n = 3). SMC CM was transferred to corresponding HUVEC cultures also grown either in 24-well plates (n = 16) or 100 mm Petri dishes (n = 3). Cultures in 24-well plates were used for antigen determination (n = 13) as well as immunoprecipitation analysis (n = 3). Cultures in 100 mm Petri dishes were used for RNA extraction (n = 3). For control experiments, CM from confluent HUVEC cultures was processed in the same way.

For immunoprecipitation analysis, SMC CM (RPMI 1640) was processed as above and sequentially dialyzed against phosphate-buffered saline (PBS), pH 7.4 (4 hours, 4°C) and methionine deficient RPMI 1640 (GIBCO/BRL, Grand Island, NV) plus 5 U/mL heparin (16 hours, 4°C), using a dialyzing membrane with a cutoff of 1,000 daltons (Spectra/Por; Spectrum, Los Angeles, CA). For control experiments fresh RPMI 1640 was processed in the same way. Viability of cells was determined by standard Trypan Blue exclusion.

Metabolic labeling of cells and immunoprecipitation analysis. This method was performed as described previously. Briefly, HUVEC monolayers were washed twice with HBSS and labeled with 35S-methionine (100 μCi/mL) (ICN Radiochemicals, Irvine, CA) in methionine-free RPMI 1640 prepared as described above. After 6 hours, the CM was collected, cell extracts (CE) were prepared by lysis with 0.5% Triton X-100 (Serva, Heidelberg, Germany) in PBS, and, after washing with distilled water, the extracellular matrix (ECM) was extracted in 0.1% sodium dodecyl sulfate (SDS) (BioRad). Metabolically labeled CM was mixed with an equal volume of immunoprecipitation buffer (PBS, pH 7.4, containing 1% nonfat dry milk, 10 U/L aprotinin [Trasylol, Bayer, Germany], 10 mmol/L benzamidine hydrochloride [Sigma], and 0.1% Triton X-100) and incubated with an excess (20 μg/mL) of polyclonal rabbit anti PAI-1 antibodies (Technoclone, Vienna, Austria) for 2 hours at 37°C followed by overnight incubation at 4°C with protein A Sepharose (Beckman, Fullerton, CA). Unbound proteins were removed from the Sepharose beads by washing twice with 2 mol/L NaCl containing 0.1% Triton X-100 and once with distilled water. Bound proteins were released from the beads by boiling in Laemmli sample buffer for 5 minutes and fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE); subsequently the gels were fixed (30% methanol, 10% acetic acid), soaked in Enlightning (NEN, Boston, MA), dried, and exposed to Kodak XAR x-ray films (Eastman Kodak, Rochester, NY) with intensifying screens. Additionally, the region containing PAI-1 was excised, hydrolyzed in Ready Protein scintillation fluid (Beckman), and subjected to liquid scintillation counting (Beckman LS 7500).

Total protein synthesis was quantified by trichloroacetic acid (TCA) precipitation of CM, CE, and ECM (equal amounts of sample, 0.25 mol/L methionine, 1% BSA, 100% TCA were mixed, diluted 10-fold in PBS, and incubated for 1 hour at 4°C). The precipitate was collected on Whatman GF/A filters (Whatman, Maidstone, England) under light suction and washed twice with ice-cold 10% TCA, once with 70% ethanol, and once with distilled water. The filters were air dried and subjected to scintillation counting in Beckman Ready Protein scintillation fluid (Beckman), and subjected to liquid scintillation counting in Beckman Ready Protein scintillation fluid.

Assay for PAI-1 antigen. PAI-1 antigen in the CM was determined by specific ELISA using monoclonal antibodies recognizing active, latent, and complexed PAI-1 (Technoclone).

Preparation of RNA and Northern blots. Total RNA was prepared from cells cultured in 100 mm Petri dishes (Costar) by acid guanidinium thiocyanate-phenol-chloroform extraction. RNA was electrothrophically separated on 1.2% agarose gel containing 6% formaldehyde, transferred to nitrocellulose (BioRad) and hybridized (10⁶ cpm [Cerenkov/mL]) for 16 hours at 42°C with random-primed α[32P]dCTP-labeled probes (Boehringer Mannheim) for either human PAI-1 (a 1.4 kb EcoR I-Bgl II fragment of polymerase chain reaction [PCR]-amplified cDNA) or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a 1.3 kb PstI cDNA fragment, kindly provided by Dr Busslinger, Vienna). 30 μL hybridization solution, containing 50% vol/vol formamide, 5.0 x SSC, 5.0 x Denhardt's solution, 0.01 mol/L sodium phosphate, pH 7.0, 1 mmol/L EDTA, 0.1% SDS, and 50 μg/mL sonicated and heat-denatured salmon sperm DNA (Boehringer Mannheim), was used per cm² nitrocellulose filter. Blots were washed twice with 1.0 x SSC, 0.1% SDS, 0.1 mol/L EDTA and 0.01 mol/L sodium phosphate, pH 7.0, and once with 0.1 x SSC, 0.1% SDS, 0.1 mmol/L EDTA, and 0.01 mol/L sodium phosphate, pH 7.0, at room temperature, air-dried, and exposed to Kodak XAR x-ray films. Quantitative analysis of films was performed by densitometry (Hirschmann elscript 400, Germany).

Statistical analysis. The results are reported as means ± standard deviation. Student’s unpaired t-test was used for determination of significance levels.

RESULTS

Effect of growth substratum on PAI-1 secretion of HUVEC. Endothelial cells grown on amniotic membranes are
Fig 1. (A) Silver nitrate staining of confluent monolayers of HUVEC grown on amniotic membranes, showing the typical in vivo-like "flagstone pattern," compared with (B) HUVEC grown on gelatine coated plastic lacking this staining pattern. (C) PAI-1 antigen levels in the 24-hour CM of HUVEC monolayers grown either on gelatine coated 24-well plastic dishes (□), or on human amniotic membranes (■). Experiments were performed in complete culture media. PAI-1 antigen was determined by specific ELISA (Technoclone), $P = \text{NS.}$
known to display an in vivo–like morphology under silver nitrate staining,3 the so-called “flagstone pattern” (Fig 1A), in contrast to the “cobblestone pattern” of endothelial cells grown on coated plastic (Fig 1B). However, this morphologic change, resembling the endothelium in vivo, was not accompanied by a significant change of PAI-1 secretion ($P = NS$) into the CM (Fig 1C). HUVEC cultures grown on amnion secreted 809 ± 124 ng PAI-1/10⁵ cells into the supernatant within 24 hours compared with 883 ± 101 ng PAI-1/10⁵ cells/24 hours detected in the CM of HUVEC grown on gelatine-coated plastic.

Effect of SMC CM on PAI-1 synthesis and secretion of HUVEC. Incubation of confluent monolayers of HUVEC with the 24-hour SMC CM for 24 hours resulted in a mean decrease of PAI-1 antigen secretion into the supernatant of 56.98 ± 9.1% ($P < .0009$) relative to that of control cells, under serum-containing conditions (20% SCS, 5 U/mL heparin) in the absence as well as in the presence of up to 100 µg/mL ECGS (Fig 2). Under serum-free conditions (1% BSA, 5 U/mL heparin, 0 to 50 µg/mL ECGS) a mean decrease of 35.7 ± 8.9% ($P = .0009$) of PAI-1 antigen secretion compared with control cells can be observed (Fig 2, inset). SMC-derived PAI-1 was determined simultaneously (legend to Fig 2) and subtracted from each measured value. When CM of SMC derived from human umbilical vein were used, a decrease in HUVEC PAI-1 antigen secretion of 12.8 ± 0.3% ($P = .09$)
can be observed. PAI-1 synthesis levels of such SMC were higher (1,745 ± 63 ng/mL) compared with those observed in pulmonary artery SMC (564 ± 190 ng/mL).

Incubation of HUVEC with SMC CM had neither visible influence on the endothelial morphology, as seen under phase-contrast microscopy, nor did any change in viability (91% ± 2.6%) occur as determined by Trypan-Blue exclusion. Freezing (−70°C, 60 minutes) and thawing of SMC CM did not abolish the PAI-1 downregulatory effect on HUVEC.

In control experiments, in which HUVEC monolayers were incubated with 24 hour HUVEC CM, no reduction of PAI-1 antigen secretion was observed. (1,316 ± 240 ng/mL PAI-1 versus 1,380 ± 28 ng/mL; P = NS).

To assure that the influence of SMC CM on synthesis and secretion of PAI-1 is attributable to effects on de novo-synthesized PAI-1, HUVEC were metabolically labeled with 35S-methionine. Incubation of HUVEC monolayers with 24 hour SMC CM (processed as described in Materials and Methods) for 6 hours resulted in a dose-dependent decrease of PAI-1 antigen secreted into the supernatant with a maximal inhibition of 51.9% (P = .0005) by undiluted SMC CM, a 30.9% reduction (P = .03) by 1:2 diluted SMC CM, and no effect by 1:10 dilution of SMC CM compared with control cells (Fig 3). Total protein synthesis remained unaffected as seen by TCA precipitation of CM, CE, and ECM (Fig 3).

Effect of SMC CM on PAI-1 mRNA levels in HUVEC. To determine whether this decrease of de novo synthesis and secretion of PAI-1 protein is also reflected on the steady-state level of PAI-1 mRNA, total RNA of HUVEC under serum-containing (20% SCS, 5 U/mL heparin) and serum-free (1% BSA, 5 U/mL heparin) experimental conditions were prepared. The results show that already after 4 hours, under serum-containing conditions, a 74.4% downregulation and, under serum-free conditions, a 76.5% downregulation (Fig 4) of the steady-state levels of both the 3.2 kb and 2.2 kb form of PAI-1 mRNA occurs.

DISCUSSION

We could show that the in vivo-like morphology of endothelial cells grown on amniotic membranes, serving as a natural growth support, does not significantly influence the high PAI-1 secretion into the conditioned media observed in endothelial cell cultures in vitro. However we cannot exclude that possible alterations of PAI-1 biosynthesis, although not affecting PAI-1 secretion into the media, might be concealed by changes of PAI-1 deposition into the matrix. The amount of PAI-1 deposited into a natural growth support like the amniotic membrane, however, is likely to be much higher than that deposited into an artificial growth support like plastic.43-44 Therefore, a possible change in PAI-1 synthesis of HUVEC grown on amniotic membranes should rather be an increase than a downregulation.

Because there is growing evidence for complex regulatory cell-cell interactions between the two main cell types of the vascular wall, EC and SMC, concerning cell replication, growth factor activation, and proliferation,45-48 we were interested in possible effects of SMC CM on PAI-1 synthesis and secretion of HUVEC. We found that PAI-1 antigen secretion into the CM by HUVEC decreases when the regular HUVEC media is replaced by media conditioned for 24 hours by SMC. This effect is seen regardless of whether SMC media is conditioned with or without serum and with or without ECGS. Immunoprecipitation analysis of the conditioned media of 35S-methionine–labeled HUVEC, incubated with various dilutions of 24 hours SMC CM, shows a dose-dependent decrease of PAI-1 synthesis and secretion. This downregulation of the synthesized and secreted protein is preceded by an ~70% decrease of the steady-state level of...
PAI-1 mRNA after 4 hours of incubation. These data clearly indicate that media conditioned by SMC contain factor(s) capable of downregulating PAI-1 biosynthesis by EC in vitro. This effect seems to be SMC-specific, because it was seen with CM from pulmonary-artery SMC and umbilical-vein SMC but not with CM from endothelial cells or tumor cells. Furthermore, it does not seem to have an unspecific down-regulatory effect, because total protein synthesis as well as GAPDH expression remains unaffected.

PAI-1 concentrations found in cell culture systems are frequently considerably higher than in plasma. Although the relatively short half-life of PAI-1 in blood (10 minutes) suggests a high biosynthetic rate, the high PAI-1 production of EC in culture might not reflect the behavior of their normal counterparts in vivo. The observation of van den Berg et al., that freshly isolated endothelial cells from umbilical arteries do not contain detectable amounts of PAI-1 mRNA, supports the hypothesis that the high PAI-1 synthesis by EC reflects changes of the biosynthetic properties of EC as they have adapted to the culture conditions. Furthermore, in situ hybridization studies in mice suggest that the primary site of PAI-1 synthesis in the vessel wall, under normal conditions, is the SMC layer. In contrast, no significant amounts of PAI-1 mRNA were detected in EC. However, under pathologic conditions (endotoxin treatment), PAI-1 production is switched from SMC to EC.

The data presented in our study suggest that the high PAI-1 levels observed in endothelial cell cultures in vitro are, at least partially, attributable to the lack of inhibitory influences of vascular SMC. Therefore, SMC not only participate in PAI-1 upregulation in a coculture system via activation of latent TGF-β but also produce soluble factors capable of downregulating PAI-1 biosynthesis in endothelial cells. These data also indicate that the regulation of PAI-1 synthesis and its tissue distribution in vivo may be part of a complex regulatory system involving several paracrine control mechanisms.

REFERENCES
13. van Mourik JA, Lawrence DA, Loskutoff DJ: Purification of an inhibitor of plasminogen activator (antiaactivator) synthesized by endothelial cells. J Biol Chem 259:14914, 1984
15. Francis RB Jr, Neely S: Inhibition of endothelial secretion of tissue-type plasminogen activator and its rapid inhibitor by agents which increase intracellular cyclic AMP. Biochim Biophys Acta 1012:207, 1989
Type 1 plasminogen activator inhibitor synthesis of endothelial cells is downregulated by smooth muscle cells

G Christ, D Seiffert, P Hufnagl, A Gessl, J Wojta and BR Binder