

# Human Fibroblasts Downregulate Plasminogen Activator Inhibitor Type-1 in Cultured Human Macrovascular and Microvascular Endothelial Cells

By Jing-Chuan Zhang, Astrid Fabry, Ljubomir Paucz, Johann Wojta, and Bernd R. Binder

We have previously reported that plasminogen activator inhibitor type-1 (PAI-1) expression in endothelial cells (ECs) can be modulated differently by smooth muscle cells depending on their origin. Human pulmonary artery smooth muscle cells (HPASMCs) strongly downregulated PAI-1 expression in ECs. Fibroblasts (FBs) are another cell type that could come in close contact with ECs. Therefore, it was the aim of this study to investigate whether FBs could also influence the fibrinolytic potential of ECs. As in the case of HPASMCs, PAI-1 antigen produced by human umbilical vein ECs (HUVECs) cocultured with human skin FBs (HSFBs) was significantly lower as compared with the sum of PAI-1 secreted by the respective cell types cultured separately. Not only HUVECs but also human skin microvascular ECs (HSMECs) responded in a dose-dependent way to serum-free conditioned media (CM) from HSFBs from one individual donor. Similar results were obtained when CM from HSFBs from four other individual donors were used. PAI-1 mRNA

decreased in HUVECs incubated for 6 hours with HSFB-CM to 24% to 55% of control, depending on the preparation of HSFBs used. A significant PAI-1 downregulatory effect was only observed when CM from low-passage HSFBs (up to passage no. 5) was used, whereas no reduction in EC PAI-1 production was observed with CM obtained from HSFBs in passage no. 8. This PAI-1 downregulatory activity present in HSFB-CM was heat-labile and had a molecular mass of approximately 5 kD. When CM from HPASMCs was analyzed in the same way, an almost identical elution profile was found. In conclusion, our data showed that FBs can decrease the expression of PAI-1 in ECs. Such an effect could be operative during wound-healing and at other capillary sites where FBs could render ECs profibrinolytic, thereby facilitating processes requiring an increase in proteolytic activity such as EC migration and proliferation.

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**P**LASMINOGEN ACTIVATOR INHIBITOR type-1 (PAI-1) is a member of the serine protease inhibitor (serpin) superfamily. It is the major physiological inhibitor of tissue-type PA (t-PA) and urokinase-type PA (u-PA) and, therefore, appears to be the major inhibitor of plasminogen activation in blood.<sup>1</sup> However, the cellular source of plasma PAI-1 is not clear. The results of recent *in vivo* studies indicate that PAI-1 is expressed in a variety of different tissues.<sup>2</sup> This finding has led to the speculation that a common cell type, eg, the endothelial cell (EC) or the smooth muscle cell (SMC), within these tissues might be the source of origin for circulating PAI-1. The results of *in situ* hybridization studies in mice have shown PAI-1-specific mRNA in SMCs but not in ECs.<sup>3</sup> These findings correlate with data showing that freshly isolated ECs in culture do not contain detectable amounts of PAI-1-specific mRNA.<sup>4</sup> Nevertheless, PAI-1 is still a major biosynthetic product in passaged EC cultures *in vitro*, accounting for up to 12% of total protein secreted.<sup>5</sup> In view of this contrasting evidence from *in vivo* and *in vitro* studies and the fact that PAI-1 is present only in trace amounts in plasma, one must ask the question of whether the secretion pattern of PAI-1 in cultured ECs reflects the situation *in vivo*.<sup>6-8</sup>

We have recently shown that PAI-1 expression in ECs in culture is upregulated by SMCs isolated from aorta, umbilical artery, and vein but is downregulated by conditioned media (CM) obtained from human pulmonary artery SMCs (HPASMCs).<sup>9,10</sup> This was, to our knowledge, the first report showing that PAI-1 expression in ECs can be attenuated by a cell type that is *in vivo* in close contact with the endothelium. Therefore, it was the aim of this study to investigate whether fibroblasts (FBs), which are also in close contact with ECs *in vivo* (especially in the capillary vasculature that accounts for the vast majority of the endothelium *in vivo*), would affect the expression of PAI-1 in ECs in a manner similar to HPASMCs.

## MATERIALS AND METHODS

**Cell culture.** Human umbilical vein ECs (HUVECs) were isolated by mild collagenase treatment as described.<sup>11</sup> Human skin microvascular ECs (HSMECs) were isolated from normal skin biopsy specimens obtained after surgical operations according to the method of Jackson et al.<sup>12</sup> The ECs were seeded into Petri dishes (Costar, Cambridge, MA) coated with 1% gelatine (Biorad, Richmond, CA) and grown to confluence in Medium 199 (M199; 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 from JRH Biosciences, Lenexa, KS), 50 µg/mL EC growth supplement (ECGS; Technoclone, Vienna, Austria) and 5 U/mL heparin (Liquemin Roche, Hoffmann-LaRoche, Basel, Switzerland) in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C. After 5 to 8 days, ECs reached confluence and were subcultured using a split ratio of 1:3. Cells were confirmed to be ECs by their typical cobblestone morphology, by positive immunofluorescence using anti-von Willebrand Factor antibodies (Cappel, Cochranville, PA) and by uptake of acetylated low-density lipoprotein.<sup>13-15</sup> All ECs obtained from human umbilical cords and human skin biopsy specimens used in this study were between passages no. 2 and 3.

Human skin FBs (HSFBs) and HPASMCs were isolated, using the explant technique, from normal skin biopsy specimens or from pulmonary artery after surgery, respectively.<sup>9</sup> Briefly, the respective tissue specimen was cut into 5 to 10 pieces of 1 to 2 mm in diameter. These pieces were placed in a Petri dish (100 mm) coated with gelatine and covered with a drop of M199 containing 20% SCS.

From The Department of Vascular Biology and Thrombosis Research, University of Vienna, Austria.

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Address reprint requests to Johann Wojta, PhD, Department of Vascular Biology and Thrombosis Research, University of Vienna, Schwarzschanerstraße 17, A-1090 Vienna, Austria.

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After 3 to 5 days, the explants became adherent, and the Petri dish was filled with M199 containing SCS and ECGS as described above. FBs growing out from the explants were grown to confluence and subcultured in Petri dishes coated with 1% gelatine using a split ratio of 1:3. Cells were grown to confluence in M199 containing 20% SCS and antibiotics at the above concentrations. HSFBs did not stain with either anti-von Willebrand Factor antibodies or anti-SMC actin antibodies and did not take up acetylated low-density lipoprotein. Furthermore, they showed a typical spindle-shaped FB-like morphology, forming multiple layers. From these data, the purity of the respective preparation of HSFBs was judged to be greater than 98%. HPASMCs showed positive immunofluorescence (>98%) with anti-SMC actin and showed a typical "hill and valley" morphology. HSFBs and HPASMCs used in this study were between passages no. 3 and 5, except when indicated otherwise.

**Coculture system.** HUVECs and HSFBs were cocultured using the Transwell system (Costar). Briefly HSFBs were seeded into 6-well plates (Costar) and grown to confluence under conditions described above. HUVECs were seeded onto collagen Transwell inserts (pore size, 0.4  $\mu\text{m}$ ) precoated with 1% gelatine and also were grown to confluence as described above. Such inserts with confluent monolayers of HUVECs were rinsed with Hank's balanced salt solution (HBSS; Sigma) and transferred to 6-well plates with and without confluent monolayers of HSFBs, which had also been rinsed with HBSS, yielding cocultures of HUVECs and HSFBs or cultures of HUVECs alone, respectively. Furthermore, inserts without HUVECs were added to 6-well plates containing monolayers of HSFBs to yield cultures of HSFBs alone. The cocultures and the respective cells in separated cultures were incubated with M199 containing 4% SCS for the indicated time periods, and cell culture supernatants were collected, processed, and tested as described below.

**Preparation of HSFb-CM.** Confluent monolayers of HSFBs were rinsed twice with HBSS and cultured with serum-free M199 in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  at 37°C. After 24 hours, HSFb-CM was harvested, centrifuged at 1,000g for 5 minutes to remove cell debris, and frozen at -70°C until used. Unless otherwise indicated, SCS was added to serum-free HSFb-CM to give a final concentration of 4% SCS.

**Treatment of ECs with HSFb-CM.** EC monolayers were rinsed with HBSS and were incubated with different concentrations of HSFb-CM or with M199 containing 4% SCS as a control. At the indicated time points, cell supernatants were harvested, centrifuged at 1,000g for 5 minutes to remove cell debris, and stored at -70°C until tested.

**Assays for PAI-1 antigen, u-PA antigen and t-PA antigen.** PAI-1 antigen and t-PA antigen in the samples were determined by specific enzyme-linked immunosorbent assays (ELISAs) using specific monoclonal antibodies for PAI-1, u-PA, and t-PA (Technoclone). The test ranges for these assays are 1.0 to 30.0 ng/mL and 0.3 to 2.5 ng/mL for PAI-1 and for u-PA and t-PA, respectively. The PAI-1 ELISA measures active, complexed, and latent PAI-1, the u-PA ELISA detects single-chain and two-chain u-PA and two-chain u-PA in complex with PAI-1, and the t-PA ELISA detects free t-PA and t-PA in complex with PAI-1.

**Preparation of RNA and Northern blot analysis.** Total cellular RNA was isolated from ECs using acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi.<sup>16</sup> The final RNA pellet was resuspended in 50  $\mu\text{L}$  of 0.5% sodium dodecyl sulfate (SDS), and the concentration was determined at 260 nm. For Northern blot analysis, RNA samples were electrophoresed in a 1.2% agarose gel followed by capillary transfer of the fractionated RNA to a Duralon-UV membrane (Stratagene, La Jolla, CA). RNA blots were placed in seal-a-meal bags and prehybridized in 50 mmol/L piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES), 100 mmol/L NaCl, 50 mmol/L sodium phosphate, 1 mmol/L EDTA,

containing 5.0% SDS for at least 3 hours at 57°C. The prehybridization buffer was then discarded and replaced with fresh prehybridization buffer containing  $10^6$  cpm/mL of the  $^{32}\text{P}$ -labeled cDNA probes for human t-PA, human PAI-1, human u-PA, human u-PA receptor (u-PAR), or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Hybridization was performed in a water bath overnight at 57°C. After hybridization, blots were removed from the bag and rinsed for 10 minutes in 100 mL of 5% SDS, 0.2 $\times$  standard saline citrate (SSC) at room temperature. Thereafter, blots were washed for 20 minutes in 400 mL of 5% SDS and 1 $\times$  SSC at the hybridization temperature. After hybridization, the RNA blots were air-dried and exposed to XAR-5 x-ray films (Eastman Kodak, Rochester, NY) at -70°C. To quantify differences in the specific mRNA expression, the developed films were scanned using a densitometer (Hirschmann Elsciprt 400; Hirschmann, Neuried, Germany). The scanning data for each specific mRNA message were compared with the intensity of the GAPDH message.

**cDNA probes.** The following cDNA fragments were used as probes in the hybridization experiments: a 1.5-kb *Sma*I/*Hind*III fragment of human t-PA cDNA (kindly provided by Dr H. Pannekoek, The University of Amsterdam, Amsterdam, The Netherlands); a 1.4-kb *Eco*RI/*Bgl*II fragment of polymerase chain reaction-amplified human PAI-1 cDNA of the 3.2-kb transcript; a 1.5-kb *Pst*I/*Pst*I fragment of human u-PA cDNA (American Type Culture Collection, Rockville, MD); a 1.1-kb *Xba*I/*Eco*RI fragment of human u-PAR cDNA (kindly provided by Dr E.K.O. Kruihof, University Hospital, Geneva, Switzerland); and a 1.2-kb *Pst*I fragment of a rat GAPDH cDNA (kindly provided by Dr M. Busslinger, University of Vienna, Vienna, Austria) that was used as an internal standard probe. The cDNA fragments were radiolabeled by random-priming using a Random Prime DNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany).

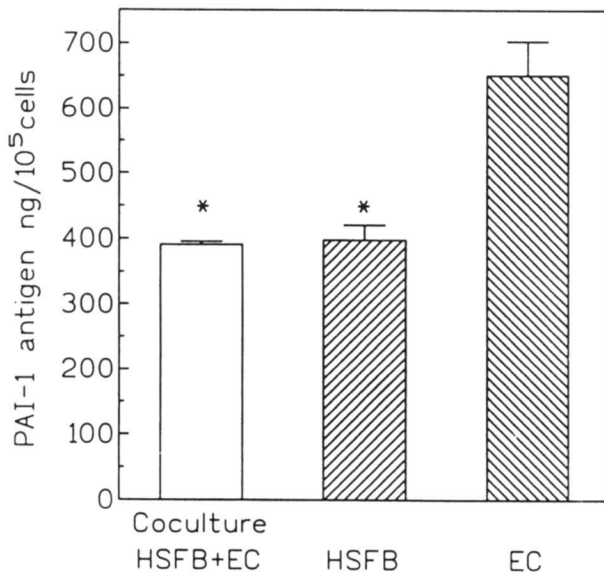
**Characterization of HSFb-derived PAI-1 downregulating activity.** Serum-free CM obtained from HSFBs was subjected to heat treatment for 5 minutes at 100°C, was frozen and thawed twice, was acidified to pH 3.0 with 1.0 mol/L HCl for 1 hour at 4°C and then readjusted back to pH 7.4 with 1.0 mol/L  $\text{NaHCO}_3$ , and was incubated with heparin-Sepharose CL-6B (Pharmacia, Uppsala, Sweden) at 4°C for 24 hours. SCS was added to these media to give a final concentration of 4% before addition to ECs. HUVECs were incubated for 24 hours with these differently treated HSFb-CM, and PAI-1 antigen in the CM of these cells was determined as described above.

**Gel filtration of HSFb-CM and HPASMC-CM.** Serum-free CM (450 mL) obtained from HSFBs or serum-free CM (250 mL) obtained from HPASMCs, respectively, was freeze-dried, resuspended in 20 mL distilled water, and dialyzed against phosphate-buffered saline (PBS), pH 7.4, at 4°C overnight. Gel filtration of these materials was performed using a Sephacryl S-200 HP column (Pharmacia) equilibrated in PBS, pH 7.4, in a 2.6- $\times$  90-cm column (Pharmacia) at 4°C and at a flow rate of 13 mL/h. The column was calibrated with molecular weight standards, and the void volume was determined with blue dextran (Pharmacia). Fractions of 2.6 mL were collected and screened for protein (absorbance, 280 nm) and PAI-1 downregulatory activity on HUVECs (fractions were diluted 1:4 with M199 containing 4% SCS).

**Statistical analysis.** The results are reported as means  $\pm$  SDs. A Student's unpaired *t*-test was used to determine significance levels.

## RESULTS

As can be seen from Fig 1, the amount of PAI-1 antigen in cocultures of HUVECs and HSFBs decreased significantly as compared with the sum of PAI-1 antigen secreted by the respective cell type in separated culture (PAI-1 antigen:



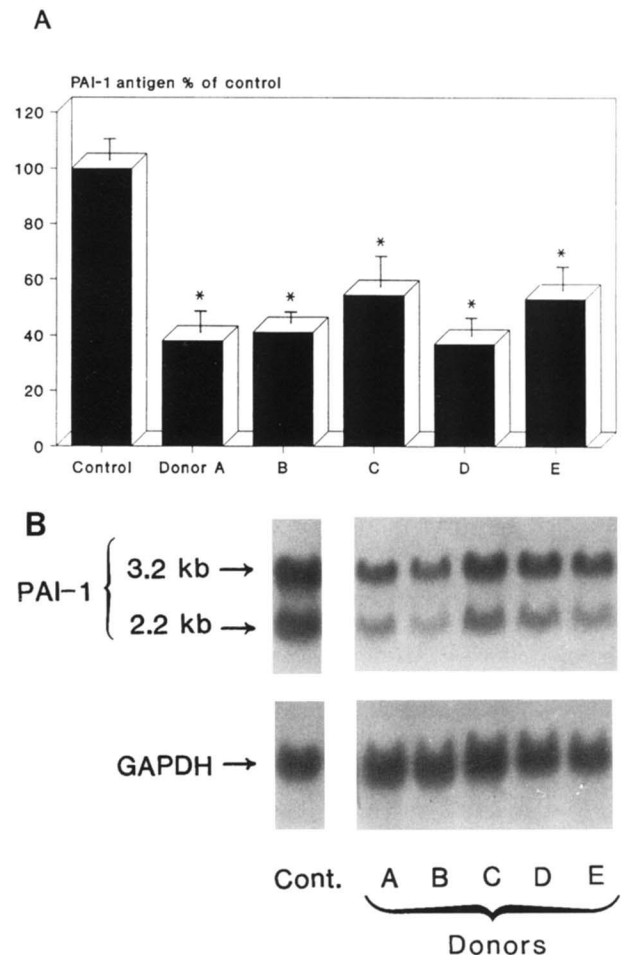
**Fig 1.** PAI-1 antigen in cocultures of HUVECs and HSFBs. HUVECs and HSFBs were cocultured using the Transwell system as described in Materials and Methods. HUVECs were seeded onto the gelatin-coated collagen insert, whereas HSFBs were seeded into gelatin-coated 6-well plates. To obtain a coculture model, inserts with HUVEC monolayers were transferred to 6-well plates with monolayers of HSFBs. Alternatively, HUVECs grown on inserts and HSFBs grown in 6-well plates were cultured alone. CM from these cells were collected after 24 hours and processed and assayed as described in Materials and Methods. PAI-1 antigen is expressed in nanograms per 10<sup>5</sup> cells per 24 hours. The results are the mean values of three experiments, each performed in triplicate. Values are given as mean values ± SD. \*,  $P < .01$  as compared with HUVECs alone.

coculture,  $391 \pm 5$  ng/10<sup>5</sup> cells/24 h; HSFBs,  $398 \pm 22$  ng/10<sup>5</sup> cells/24 h; HUVECs,  $651 \pm 72$  ng/10<sup>5</sup> cells/24 h;  $P < .01$ ). t-PA antigen did not change in HUVECs cocultured with HSFBs as compared with that of ECs alone ( $P > .05$ , data not shown).

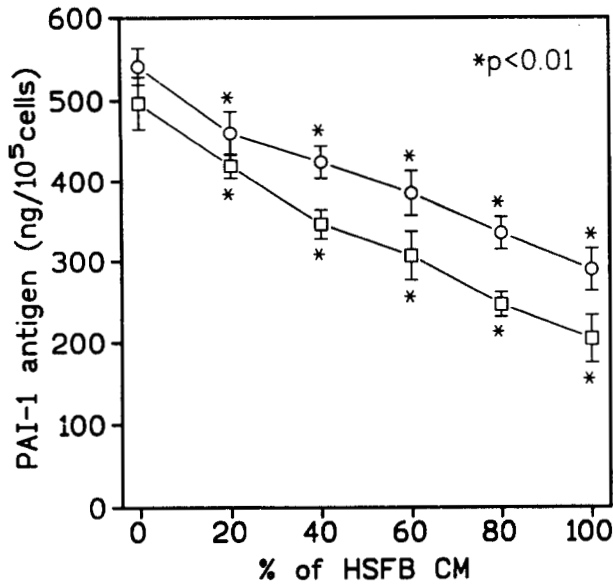
To investigate whether HSFBs produce a soluble factor responsible for downregulation of PAI-1 production by HUVECs, CM from HSFBs were prepared as described in Materials and Methods. When HUVECs were incubated for 24 hours with such CM obtained from HSFBs from five different donors, a significant decrease in PAI-1 antigen produced by HUVECs was observed as compared with the amount of PAI-1 antigen produced by HUVECs incubated with fresh medium (Fig 2A). Incubation of HUVECs with CM from FBs did not influence EC morphology as determined by light microscopy. Furthermore, EC viability was not affected by treatment with HSFb-CM as judged by Trypan-Blue exclusion (data not shown). The decrease in PAI-1 antigen production was also reflected at the level of PAI-1-specific mRNA in HUVECs as evidenced by Northern blotting (Fig 2B). As determined by densitometry, the levels of PAI-1-specific mRNA in HUVECs incubated with conditioned medium from HSFBs were reduced to levels between 24% and 42% (2.2-kb transcript) and to levels between 37% and 55% (3.2-kb transcript) of mRNA levels of the respec-

tive PAI-1 transcript in HUVECs incubated under control conditions.

In control experiments, in which HUVEC monolayers were incubated with fresh media or with HUVEC-CM, no reduction of PAI-1 antigen production was observed (PAI-1 antigen: HUVECs + M199,  $477 \pm 32$  ng/10<sup>5</sup> cells/24 h;



**Fig 2.** Effect of HSFb-CM on PAI-1 expression in HUVECs. (A) PAI-1 antigen. Confluent monolayers of HUVECs were incubated with HSFb-CM derived from five different donors (donors A, B, C, D, and E) and with M199 as a control for 24 hours. To all CM and the control medium, SCS was added to give a final concentration of 4%. Supernatants from such treated cells were harvested, and PAI-1 antigen was determined as described in Materials and Methods. HSFb-derived PAI-1 antigen present in the respective HSFb-CM was determined simultaneously and subtracted from each value. PAI-1 values are expressed as the percentage of PAI-1 produced by HUVECs under control conditions and represent mean values of three independent experiments, each performed in triplicate. Values are given as mean values ± SD. (B) Northern blotting. Confluent monolayers of HUVECs grown in petri dishes were incubated either with HSFb-CM from five different donors (Donors A, B, C, D and E) or with M199 containing 0.1% BSA as a control for 6 hours. Total RNA was extracted, and levels of PAI-1-specific mRNA were determined by Northern blot analysis using a human PAI-1 cDNA probe as described in Materials and Methods. Specific mRNA expression for GAPDH was determined and used as a control to account for uneven loading. \*,  $P < .01$  as compared with control values.



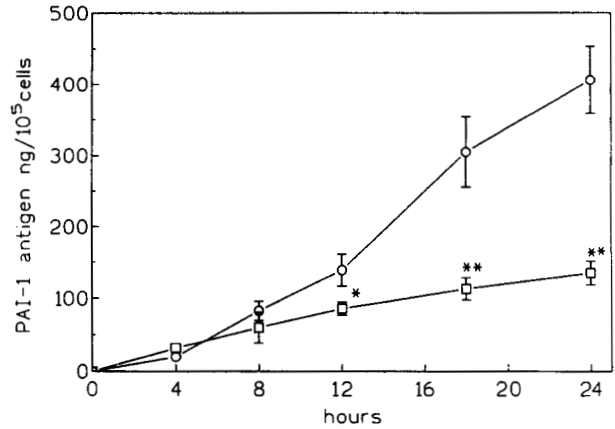
**Fig 3. Dose dependence of PAI-1 antigen production by HUVECs and HSMECs after incubation with HSFb-CM.** Confluent monolayers of HUVECs (□) and HSMECs (○) were incubated for 24 hours with increasing doses of HSFb-CM containing 4% SCS. As a control, HUVECs and HSMECs were incubated with M199 containing 4% SCS. PAI-1 antigen in supernatants of these cells was determined as described in Materials and Methods. PAI-1 present in the HSFb-CM was determined simultaneously and subtracted from each value. PAI-1 antigen is expressed in nanograms per 10<sup>5</sup> cells per 24 hours. The results are mean values of three experiments, each performed in triplicate. Values are given as mean values ± SD. \*, *P* < .01 as compared with control values.

HUVECs + HUVEC-CM, 487 ± 21 ng/10<sup>5</sup> cells/24 h; *P* > .5). Also, when HSFb were incubated with HUVEC-CM, no change in PAI-1 antigen production by FBs was observed (PAI-1 antigen: HSFb + M199, 275 ± 26 ng/10<sup>5</sup> cells/24 h; HSFb + HUVEC-CM, 258 ± 28 ng/10<sup>5</sup> cells/24 h; *P* > .5).

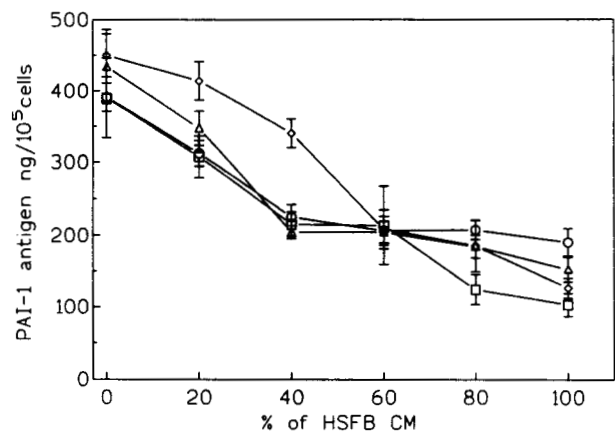
To analyze also whether ECs from a different origin respond to HSFb-CM in a similar way as did HUVECs, HUVECs and HSMECs were incubated with different concentrations of such HSFb-CM. As can be seen from Fig 3, HSFb-CM in concentrations between 20% and 100% significantly decreased PAI-1 production by HUVECs and HSMECs, respectively. For these two types of ECs, the dose-response curves were parallel, indicating a similar reactivity of these cells towards the PAI-1 downregulatory activity in HSFb-CM.

Figure 4 shows a time course of PAI-1 production by HUVECs incubated with and without CM from HSFb. As can be seen, a significant decrease in PAI-1 antigen secreted by such treated HUVECs was observed from 12 hours on.

To address the question of whether this effect was dependent on the addition of serum to the HSFb-CM used, 0.1% bovine serum albumin (BSA), 1% BSA, or ITS (5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite) was added to serum-free CM obtained from HSFb as described in Materials and Methods. The effect of such supplemented media on PAI-1 production by HUVECs was com-



**Fig 4. Time course of PAI-1 antigen production by HUVECs after incubation with HSFb-CM.** Confluent monolayers of HUVECs were incubated with HSFb-CM containing 4% SCS (□) or with M199 containing 4% SCS as a control (○). At the indicated time points, supernatants of these cells were harvested and processed as described in Materials and Methods. PAI-1 antigen was determined in these samples as described in Materials and Methods. PAI-1 antigen present in the HSFb-CM was determined simultaneously and subtracted from each value. PAI-1 antigen is expressed in nanograms per 10<sup>5</sup> cells per 24 hours. The results are mean values of three experiments, each performed in triplicate. Values are given as mean values ± SD. \*, *P* < .01; \*\*, *P* < .001 as compared with control values, respectively.



**Fig 5. Effect of serum, BSA, and ITS on PAI-1 modulation in HUVECs by HSFb-CM.** Confluent monolayers of HUVECs were incubated for 24 hours with increasing doses of HSFb-CM containing 4% SCS (◇), 0.1% BSA (□), 1.0% BSA (△), or ITS (○). As a control, HUVECs were incubated with M199 containing the respective supplement at the concentrations given above. PAI-1 antigen in supernatants of these cells was determined as described in Materials and Methods. PAI-1 present in the HSFb-CM was determined simultaneously and subtracted from each value. PAI-1 antigen is expressed in nanograms per 10<sup>5</sup> cells per 24 hours. The results are mean values of three experiments, each performed in triplicate. Values are given as mean values ± SD. The reduction of PAI-1 antigen by all differently supplemented HSFb-CM was significant at concentrations between 40% and 100% (*P* < .01 as compared with the respective control value).

**Table 1. Comparison of Endothelial PAI-1 Downregulation by HSF-B-CM and HPASMC-CM**

	PAI-1 Antigen (ng/10 <sup>5</sup> cells/24 h)
Control	406 ± 47
HSFB-CM	135 ± 6*
HPASMC-CM	158 ± 24*

Confluent monolayers of HUVEC were incubated for 24 hours with HSF-B-CM or HPASMC-CM, both containing 4% SCS. As a control, HUVECs were incubated with M199 containing 4% SCS. PAI-1 antigen in supernatants of these cells was determined as described in Materials and Methods. PAI-1 present in the HSF-B-CM and HPASMC-CM was determined simultaneously and subtracted from each value. PAI-1 antigen is expressed in nanograms per 10<sup>5</sup> cells per 24 hours. The results are mean values of three experiments, each performed in triplicate. Values are given as mean values ± SD.

\*  $P < .001$  as compared with control values.

pared with the PAI-1 reduction achieved with CM from HSF-Bs containing 4% SCS. As shown in Fig 5, these differently supplemented HSF-B-CM reduced PAI-1 production by HUVECs in a similar way.

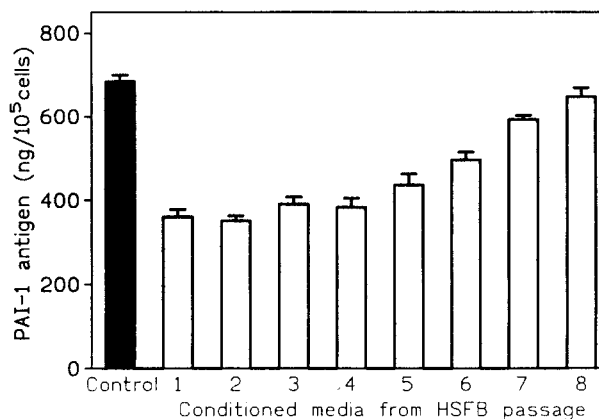
To compare the PAI-1 downregulatory activity of HSF-B-CM with that contained in HPASMCs,<sup>9</sup> HUVECs were incubated with the respective CM for 24 hours. As shown in Table 1, HSF-B-CM decreased PAI-1 production by HUVECs to a similar extent as did CM from HPASMCs.

HSF-B-CM did not affect t-PA antigen production and t-PA-specific mRNA expression in HUVECs. Furthermore, the expression of u-PA- and u-PAR-specific mRNA in HUVECs was not influenced by HSF-B-CM (data not shown).

The PAI-1 downregulatory activity present in CM of HSF-Bs was dependent on the passage of these cells. Whereas CM obtained from HSF-Bs up to passage no. 4 inhibited PAI-1 production in HUVECs by almost 50%, the level of inhibition gradually decreased when CM from HSF-Bs in passages no. 5 to 7 were used. No downregulation of PAI-1 production by HUVECs was observed when CM from HSF-Bs in passage no. 8 were used (Fig 6).

As can be seen from Table 2, boiling of HSF-B-CM for 5 minutes abolished the PAI-1 downregulatory activity as compared with that for fresh HSF-B-CM ( $P < .01$ ), whereas acid treatment or incubation with heparin Sepharose for 24 hours, which was performed to investigate whether the PAI-1 downregulatory activity (similar to that of many growth factors) would show affinity towards heparin, had no effect ( $P > .05$ ). HSF-B-CM subjected to 2 freeze-thaw cycles or incubated at 37°C for 24 hours did not lose its PAI-1 downregulatory activity (data not shown).

When HSF-B-CM was subjected to gel filtration on Sephacryl S-200 HP, the PAI-1 downregulatory activity eluted as a single peak corresponding to a molecular mass of approximately 5 kD (Fig 7A). Neither HSF-B-CM nor the PAI-1 downregulatory activity obtained after gel filtration contained detectable amounts of u-PA and t-PA (data not shown). A similar elution profile was observed when HPASMC-CM was subjected to gel filtration under the same conditions. Again, the PAI-1 downregulatory activity was



**Fig 6. Passage dependence of PAI-1 downregulatory activity in HSF-B-CM.** Serum-free CM from HSF-Bs (passages no. 1 to 8) were collected. To these CM and to the control media, SCS was added to give a final concentration of 4%. Confluent monolayers of HUVECs were incubated with the respective media for 24 hours. PAI-1 antigen in supernatants of these cells was determined as described in Materials and Methods. PAI-1 present in the HSF-B-CM was determined simultaneously and subtracted from each value. PAI-1 antigen is expressed in nanograms per 10<sup>5</sup> cells per 24 hours. The results are mean values of three experiments, each performed in triplicate. Values are given as mean values ± SD.

eluted corresponding to a molecular mass of around 5 kD (Fig 7B).

## DISCUSSION

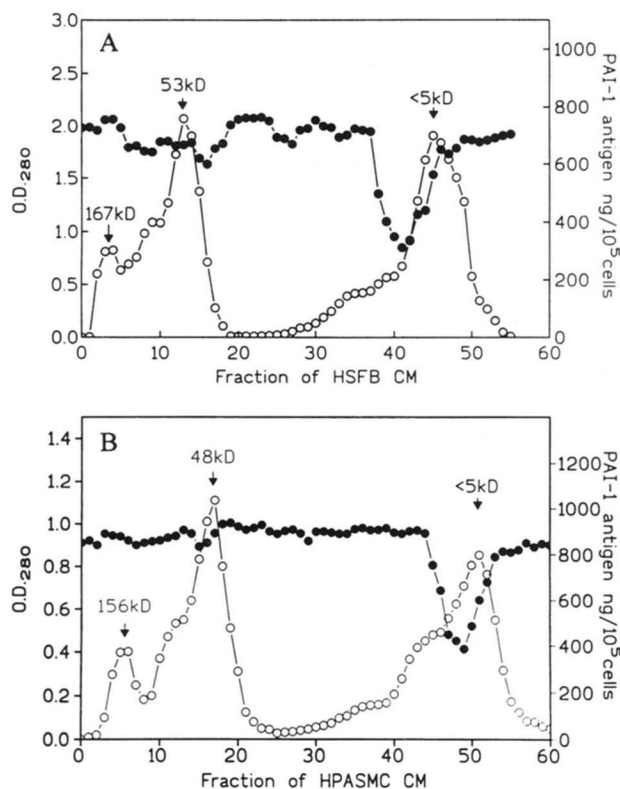
We have shown recently that PAI-1 expression in human ECs in culture is downregulated by CM from HPASMCs.<sup>9</sup> In this study, we show that HSF-Bs can decrease PAI-1 production by human macrovascular and microvascular ECs to a similar extent. This effect did not result from cytotoxicity, as shown by unchanged cell viability determined by Trypan-Blue exclusion, and was not because of media exhaustion

**Table 2. Characterization of PAI-1 Downregulatory Activity in HSF-B-CM**

	PAI-1 Antigen
Control	100 ± 6.2
HSFB-CM, untreated	59.5 ± 5.9*
HSFB-CM, boiled	92.0 ± 12.0
HSFB-CM, acid-treated	50.0 ± 7.9*
HSFB-CM, treated with heparin-Sepharose	55.8 ± 7.9*

Serum-free CM obtained from HSF-Bs was boiled for 5 minutes, was acidified to pH 3.0 for 1 hour and readjusted back to pH 7.4, and was incubated with heparin-Sepharose for 24 hours at 4°C. To all these CM and control medium, SCS was added to give a final concentration of 4%. Confluent monolayers of HUVECs were incubated for 24 hours with such treated or untreated HSF-B-CM or with fresh medium as a control. PAI-1 antigen was determined in the supernatant of these cells as described in Materials and Methods. PAI-1 present in the HSF-B-CM was determined simultaneously and subtracted from each value. Data are expressed as the percentage of control and represent mean ± SD of three independent experiments.

\*  $P < .01$  as compared with control values.



**Fig 7.** Gel filtration profile of HSFB- and HPASMC-derived CM on Sephacryl S-200 HP. CM (450 mL) obtained from HSFBs or CM (250 mL) obtained from HPASMCs were lyophilized, resuspended in distilled water, and dialyzed against PBS. The sample was then separated by gel filtration on a Sephacryl S-200 column. Protein content of the respective fractions was determined and is shown as absorbance at 280 nm (○). The fractions of HSFB-CM were diluted 1:4 (HSFBs) or 1:3 (HPASMCs) with M199. Confluent monolayers of HUVECs were incubated with each diluted fraction containing 4% SCS for 24 hours. PAI-1 antigen production by these cells was determined as described in Materials and Methods and is expressed as nanograms per  $10^5$  cells per 24 hours (●). PAI-1 present in the respective fractions was determined simultaneously and subtracted from each value. Molecular masses of the respective peaks are indicated by arrows. The experiment was repeated twice and similar results were obtained. (A) Gel filtration profile of HSFb-CM. (B) Gel filtration profile of HPASMC-CM.

caused by conditioning, because HUVECs incubated with CM from HUVECs did not show a reduction in PAI-1 production. An increase in PAI-1 clearance caused by clearance of PAI-1-t-PA complexes or PAI-1-u-PA complexes via the very low-density lipoprotein receptor seems unlikely to contribute to the reduction in PAI-1 antigen observed in HUVECs treated with CM from HSFBs, because neither CM obtained from HSFBs nor fractions of these CM obtained after gel filtration contained any detectable amounts of t-PA or u-PA.<sup>17</sup> The possibility that other factors present in the CM influence the clearance of PAI-1 by HUVECs thereby contributing to the observed decrease in PAI-1 can not be ruled out completely. However, the results of Northern blotting experiments provide evidence for a significant reduction of PAI-1 expression in HUVECs treated with CM from HSFBs.

Furthermore, we provide evidence that the PAI-1 downregulatory effect depended on the "age" of the HSFBs used. Similarly, we could show previously that CM harvested from low-passage smooth muscle decreased SMC PAI-1 expression in ECs, whereas CM from senescent SMCs increased PAI-1 production by ECs significantly.<sup>18</sup>

The PAI-1 downregulatory activity present in HSFb-CM was heat-labile and has a molecular mass of approximately 5 kD. The PAI-1 downregulatory activity present in CM of HPASMCs also showed a similar elution profile when subjected to gel filtration, suggesting that the SMC-derived and FB-derived activities are probably very similar. The molecular nature of this downregulatory activity remains unclear. However, FB-derived cytokines and growth factors such as interleukin-1, tumor necrosis factor- $\alpha$ , transforming growth factor- $\beta$ , basic FB growth factor, vascular EC growth factor, or hepatocyte growth factor seem to be highly unlikely candidates because PAI-1 expression in ECs has been shown to be increased or not affected by these factors.<sup>19-21</sup>

FBs in situ are in close contact with ECs, especially in the capillary vasculature that composes the vast majority of the endothelium in vivo. A downregulatory effect of FBs on endothelial PAI-1 expression as shown in this report might help to explain the difference between the low in vivo and the high in vitro levels of PAI-1 in ECs. Furthermore this downregulatory mechanism could be operative during wound-healing and at the capillary site in general, where FBs, by decreasing endothelial PAI-1 production, could render ECs profibrinolytic, thereby facilitating processes requiring an increase in proteolytic activity such as EC migration and proliferation. In this respect, it is also of interest that wounded ECs express increased levels of u-PA and u-PAR.<sup>22,23</sup> Furthermore, one could speculate that, if the PAI-1 downregulatory activity of FBs is also dependent on the number of cell doublings in vivo, then, during the early stages of the wound-healing process, the proteolytic capacity of the ECs is increased by FBs producing PAI-1 downregulatory activity; whereas, at the late stages of this process (when the FBs have undergone numerous cell doublings), such activity is no longer produced and consequently the proteolytic capacity of the ECs decreases. Therefore, processes depending on extracellular proteolysis such as matrix degradation and EC migration and proliferation would be favored during early wound-healing, whereas, at late stages of this event, because of the decrease in proteolytic capacity, matrix deposition would be favored. The importance of the fibrinolytic system for wound-healing has been emphasized by the results of a recent study showing that plasminogen-deficient mice suffer from impaired wound-healing.<sup>24</sup>

In conclusion, our data presented in this study implicate FBs as yet another cell type, aside from SMCs, that is important in the regulation of the fibrinolytic system of ECs. Such regulation of the endothelial proteolytic capacity by FBs, if also operative in vivo, might have an impact on a variety of physiological and pathophysiological processes such as wound-healing, angiogenesis, cell migration and proliferation, and tissue remodelling, all of which require matrix deposition and degradation through controlled extracellular proteolysis.

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