**Background**—Endothelial dysfunction is emerging as a common denominator for diverse and highly prevalent cardiovascular diseases. Increased level of plasminogen activator inhibitor-1 (PAI-1) and procoagulant activity have been recognized as hallmarks of endothelial dysfunction. This study was aimed at investigating cellular actions of PAI-1 and a potential link between PAI-1 and procoagulant state.

**Methods and Results**—Human umbilical vein endothelial cells treated with PAI-1 were subjected to laser confocal fluorescence microscopy, immunoprecipitation and Western blotting, and FACS analysis for isolation and identification of endothelial microparticles. PAI-1 treatment resulted in a reduced expression of uPAR, its colocalization with caveolin, and the concomitant increase of uPAR abundance in the culture medium. FACS analysis revealed that PAI-1 rapidly and dose-dependently increased the number of endothelial microparticles expressing uPAR and \( \alpha_\beta_3 \) integrin. This process was attenuated by pretreatment with neutralizing anti-uPAR antibodies. PAI-1 knockout mice showed a significantly decreased number of circulating endothelial microparticles than wild-type mice; however, PAI-1–deficient animals responded to infusion of PAI-1 with a more pronounced rise in the number of microparticles. PAI-1 treatment increased the number of microparticles stained with Annexin V, evidence for the expression of anionic phospholipids. This was accompanied by the accelerated generation of thrombin.

**Conclusions**—The data disclose a novel effect of PAI-1 to dose-dependently promote formation of endothelial microparticles with the reduced transmembrane asymmetry of phospholipids. This phenomenon may be responsible for the observed increase in in vitro thrombin generation. These findings could potentially link these hallmarks of endothelial dysfunction—elevated levels of PAI-1 and propensity toward thrombosis. (*Circulation*. 2002;106:2372-2378.)

**Key Words:** endothelium • urokinase • thrombosis
with 1% BSA. Cells were incubated with primary antibodies for 1 hour and, after washing, exposed for 30 minutes to appropriate secondary antibodies. Preparations were analyzed using a laser confocal microscope (Odyssey) and Metamorph image analysis software (Universal Imaging).

Western Blot Analysis
Cells were washed with ice-cold PBS and lysed using a buffer of the following composition: Tris 10 mmol/L, pH 7.5; NaCl 150 mmol/L; EDTA 1 mmol/L; Triton X-100 1%; Nonidet P-40 0.5%; and sodium orthovanadate 1 mmol/L; supplemented with Complete protease inhibitor mixture (Roche Diagnostic Corp) on ice for 30 minutes. Lysates were clarified, the concentration of protein was determined (BCA kit), and after 4 minutes of boiling, the lysates were subjected to electrophoretic separation on 4% to 20% SDS-polyacrylamide gel (Novex). Proteins were transferred to Immobilon-P membrane (Millipore), and blots were incubated in a blocking buffer (1% BSA in 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.1% Tween 20) for 30 minutes and reacted with primary antibodies, followed by the appropriate secondary antibodies, and developed using a Supersignal West Pico chemiluminescence kit (Pierce). Band density was quantitated using an NIH Scion Image for Windows (Scion Corporation).

For immunoprecipitation, HUVECs were washed with ice-cold PBS containing 1 mmol/L sodium orthovanadate, harvested, and lysed on ice for 30 minutes in TNE buffer (Tris-HCl 10 mmol/L, pH 7.5; EDTA 1 mmol/L; NaCl 150 mmol/L; NP-40 1%; sodium orthovanadate 1 mmol/L; Complete protease inhibitor mixture). Lysates were clarified, and the protein concentration was determined (BCA kit). The lysates were precleared by adding 10 μL of protein A/G-agarose beads (Santa Cruz), and 500 μg of lysate proteins were incubated with 5 μg of a specific antibody overnight at 4°C, followed by addition of 20 μL/mL protein A/G-agarose for 2 hours at 4°C. The beads were washed 4 times with lysis buffer, solubilized in SDS-PAGE ×2 sample buffer, and boiled for 4 minutes. Proteins were resolved by SDS-PAGE, transferred onto PVDF membrane, blocked, and incubated with indicated antibodies.

Experimental Animals
Studies were carried out in wild-type (C57BL/6J) and PAI-1 knockout (C57BL/6J Pph1tm1) mice obtained from Jackson Laboratory (Bar Harbor, Maine). Mice were anesthetized with Inactin 100 mg/kg and placed on a temperature-regulated table (37°C), and a polyethylene catheter (PE-10, Portex) was inserted into the right jugular vein. Either 50 μL of PAI-1 solution (10 μg/kg)15,16 or isotonic saline was then administered intravenously to wild-type and PAI-1 knockout mice. One hour later, 1- to 2-mL blood samples were drawn from the left ventricle into tubes containing 0.129 mol/L tri-sodium citrate.

Preparation of Microparticles From Cultured Endothelial Cells and From Plasma
The culture medium of HUVECs was analyzed using a modified protocol by Combes et al.13 Two hours before PAI-1 treatment, culture medium was changed to the serum-free and growth factor-free EBM-2 medium. HUVECs were incubated with recombinant PAI-1 at the concentration 1 to 10 ng/mL for 1 to 3 hours. Culture media were collected and cleared from cells and cell debris by centrifugation at 5000 g for 10 minutes. Supernatant was immediately used for microparticle immunolabeling followed by FACS analysis or laser confocal microscopy. The previously described protocols13,14 were modified by omitting the additional ultracentrifugation at 100 000g for 2 hours because of the generation of conglomerated microparticles.
For platelet-free plasma preparation, microparticles were extracted from whole plasma by 2 sequential centrifugations for 10 minutes at 15,000g, as previously described.\(^{13,17}\) Supernatant containing microparticles was immediately used to assay the procoagulant activity and for immunolabeling followed by FACS analysis.

**Immunolabeling of Microparticles**

In general, 200 \(\mu\)L of microparticle-containing suspension was incubated with primary antibodies for 2 hours at room temperature followed by secondary fluorescently labeled antibodies for 30 minutes at room temperature.\(^{13,17}\) Staining of microparticles with Annexin V was performed according to manufacturer’s protocol.\(^{18}\)

**Flow Cytometry Analysis of Microparticles**

Suspensions containing microparticles were analyzed on a Becton-Dickinson FACScan flowcytometer (Becton-Dickinson). Gating parameters were defined using 1-\(\mu\)m latex beads and negative controls. Microparticles were defined using forward-scatter analysis. Endothelial-derived microparticles in blood samples were enumerated using \(\alpha_\beta\) labeling.\(^{17}\) Simultest \(\gamma_1/\gamma_2\) (IgG1/IgG2) (Becton Dickinson) staining with FITC and PE conjugates or murine monoclonal IgG1 and IgG2 antibodies to human cell-surface antigen were used as negative controls. The time necessary for counting 2000 events was measured in duplicate by the chromogenic assay of 20-\(\mu\)L samples of the incubation mixtures in the presence of 0.3 nmol/L D-Phe-L-Pro-L-Arg-p-nitroanilide (Chromozym-TH, Roche Diagnostics). The chromogenic assay was standardized with pure human \(\alpha\)-thrombin, 20 nmol/L. Activities were expressed as the rate of absorbance change in the chromogenic assay, which is proportional to thrombin concentration. Statistical analysis was performed using 2-tailed \(t\) test or ANOVA followed by Tukey posttest, with \(P<0.05\) considered statistically significant. All values are presented as mean±SEM.

**Results**

**PAI-1 Affects the Expression and Localization of uPAR to Caveolae**

Double staining of HUVECs with antibodies to caveolin-1 and uPAR demonstrated that both were expressed in a punctated pattern and showed 36% colocalization in control cells (Figures 1A and 1B). In contrast, treatment of HUVECs with PAI-1 (10 ng/mL for 3 hours) resulted in a dramatic dissociation of uPAR and caveolin-1 staining (12% colocalization; \(P<0.05\)).

**Communoprecipitation of uPAR and Caveolin**

Western blot analysis of anti-caveolin-1 immunoprecipitates demonstrated that PAI-1 treatment (10 ng/mL for 3 hours)
resulted in >3-fold decrease in the coprecipitated uPAR (Figure 2). The concentration of uPAR in the culture medium conditioned by PAI-treated HUVECs increased >2-fold (Figure 3). The observed decline in uPAR expression by HUVECs treated with PAI-1 and the concomitant appearance of uPAR in the culture medium alluded to the possibility of uPAR cleavage and formation of soluble uPAR or, alternatively, its loss from the cell surface through PAI-1–induced formation of microparticles, the recently described 1-µm vesicles shed from endothelial cells.20

Analysis of Microparticles in HUVEC Cultures
The culture medium from unstimulated HUVECs was subjected to FACS analysis, which revealed 15.18±4.54×10^3 microparticles/10^6 cells. Most microparticles were stained with antibodies against uPAR or αβ3 integrin.12 Application of 10 ng/mL PAI-1 (a pathologically relevant concentration) for 1 to 3 hours significantly increased the number of microparticles harvested from the HUVEC-conditioned culture medium, with the concomitant almost 2-fold increase in the number of microparticles expressing immunodetectable uPAR or αβ3 integrin receptor (Figure 4). Lower (physiologically relevant) concentrations of PAI-1 were ineffective, and no significant differences were noted in microparticle formation between 1 and 3 hours of exposure. These data provide the first evidence that PAI-1 promotes microparticle formation in vitro.

To evaluate the participation and contribution of uPAR to the observed phenomena, HUVECs were pretreated with neutralizing anti-uPAR antibodies (50 µg/mL) 10 minutes before the application of PAI-1. In control series, HUVECs were treated with antibody alone, or the neutralizing antibodies (50 µg/mL) were added 10 minutes after application of PAI-1. As shown in Figure 4C, only antibody pretreatment significantly suppressed the number of microparticles produced after addition of PAI-1. These data suggest that the expression of uPAR, together with supraphysiological concentrations of PAI-1, is required for endothelial microparticle formation.

Endothelial Microparticles in PAI-1 Knockout Mice
To examine the in vivo relevance of these findings, experiments were performed in PAI-1−/− mice before and 1 hour after injection of 10 µg/kg PAI-1. Wild-type mice were subjected to the same protocol. FACS analysis demonstrated that the number of microparticles was significantly decreased under basal conditions in PAI-1−/− mice (Figure 5A). Administration of PAI-1 resulted in an insignificant elevation of the number of circulating αβ3-immunodetectable12,13 endothelial microparticles in wild-type and PAI-1−/− animals. However, the number of circulating microparticles expressing uPAR after PAI-1 injection to PAI-1−/− mice increased 4-fold (Figures 5B and 5C). The number of circulating microparticles expressing both epitopes followed the pattern observed for uPAR alone. Hence, the studies performed in PAI-1−/− deficient mice showed that baseline number of endothelial microparticles was significantly lower, whereas responses to administration of PAI-1 were significantly higher than in wild-type animals.

Procoagulant Activity of Endothelial Microparticles
It has been suggested that microparticles are shed from apoptotic cells.21 Annexin V binding by microparticles harvested from the culture media conditioned by unstimulated HUVECs showed that 36±4.7% of them bound the fluorophore (Figure 6A). This proportion increased to 43.4±5.2% and 43.4±4.4% 1 and 3 hours after 10 ng/mL PAI-1 (P<0.05 compared with control, but no statistical difference between 1
and 3 hours). Because the observed increase in the proportion of microparticles exteriorizing phosphatidylserine occurred after treatment with PAI-1 and because this phospholipid is a known cofactor in thrombin activation, we next addressed the possibility of a procoagulant action of endothelial microparticles. Specifically, we determined in vitro activity of microparticles in thrombin generation assay, which totally depends on the expression of anionic phospholipids. As shown in Figure 6B, equivalent amounts of endothelial microparticles obtained from untreated (control) and 10 ng/mL PAI-1–treated HUVECs resulted in a significantly increased rate of thrombin generation.

Discussion

Diverse cardiovascular and thromboembolic diseases, accompanied by endothelial dysfunction, are characterized by elevated levels of PAI-1 and procoagulant state. This serpin is released from stimulated platelets or synthesized by endothelial cells and inhibits both plasminogen activators. Urokinase binds to a glycosylphosphatidylinositol-anchored receptor (uPAR) expressed on the surface of endothelial cells and, on stoichiometric PAI-1 binding, this complex undergoes rapid endocytosis and degradation, as demonstrated by Conese and Blasi. Based on these properties to inhibit both plasminogen activators and, consequently, formation of plasmin, PAI-1 has been traditionally considered an inhibitor of fibrinolytic cascade with no apparent effect on coagulation. Studies presented herein describe an unexpected finding—supraphysiological concentrations of PAI-1, usually encountered in patients with endothelial dysfunction, promote formation of endothelial microparticles and exert a procoagulant effect.

Formation of microparticles is a common mechanism of membrane shedding by activated cells. Circulating formed elements, like platelets and leukocytes, have been shown to constitutively shed microparticles, the process that is enhanced during cardiopulmonary bypass, unstable angina and lacunar infarcts, or diabetes mellitus and...
contributes to procoagulant state in these and other conditions. Formation of microparticles by endothelial cells has been documented in patients with lupus anticoagulant13 and acute coronary syndromes.32 Scanning electron microscopy provided dramatic images of the vesicle-shedding process.33 The fact that PAI-1 is capable of promoting formation of endothelial microparticles is a novel observation. Not only did PAI-1 increase the number of endothelial microparticles in vitro, but also the number of endothelial microparticles was 3 times lower in PAI-1-deficient mice, whereas administration of PAI-1 to these animals resulted in a rapid rise in the number of circulating endothelial microparticles. Collectively, these observations strongly implicate PAI-1 as one of the promoters of microparticle formation by endothelial cells. This process, however, requires supraphysiological concentrations of PAI-1, similar to those found in patients with various manifestations of endothelial cell dysfunction, and the expression of a functional uPAR.

As judged from the proportion of microparticles stained with annexin V, only about one third of them express anionic surface phospholipid on the outer membrane leaflet, suggesting that the bulk (≈70%) are derived from areas of the cell membrane that have maintained asymmetric phospholipid distribution. However, PAI-1 treatment of endothelial cells leads to the formation of microparticles displaying a significantly larger proportion of externalized phosphatidyl-serine. This anionic phospholipid is among the most potent promoters of the tissue factor–induced activation of factor VIIa and coagulation cascade leading to generation of thrombin.34 Based on these data, we explored the possibility of PAI-1–induced procoagulant activity of endothelial microparticles. Indeed, the rate of in vitro thrombin generation was accelerated significantly by the equivalent number of microparticles obtained from the culture medium conditioned by HUVECs pretreated with PAI-1. In vivo attribution of this phenomenon to endothelial microparticles is difficult to obtain because of the substantial number of platelet microparticles that obscure the measurements. Nonetheless, the data presented strongly suggest the possibility of a vicious circle of thrombin and PAI-1 generation. According to the proposed scenario, increased levels of PAI-1 might serve as an initiator of a process resulting in the formation of endothelial microparticles with perturbed asymmetry of anionic phospholipids and procoagulant activity, in turn accelerating the rate of thrombin formation. Furthermore, it is known that thrombin stimulates PAI-1 synthesis.35 Collectively, these data close the circle, thus potentially providing a missing link between the characteristically elevated PAI-1 levels and procoagulant state, both observed in diverse syndromes manifesting as endothelial cell dysfunction.

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References


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