Evidence That Postoperative Fibrinolytic Shutdown Is Mediated By Plasma Factors That Stimulate Endothelial Cell Type I Plasminogen Activator Inhibitor Biosynthesis

By Jeannine Kassis, Jack Hirsh, and Thomas J. Podor

Postoperative fibrinolytic shutdown has been attributed to an increase in plasma levels of type I plasminogen activator inhibitor (PAI-1) activity and may contribute to postoperative venous thrombosis. The purpose of this study was to determine whether the postoperative increase in PAI-1 is contributed to by a plasma mediator(s) that stimulates PAI-1 synthesis and secretion by vascular endothelium. Plasma samples collected from patients (N = 11) before and after surgery for total hip replacement were (1) assayed for endogenous plasma PAL1 antigen and activity, and (2) incubated with cultured human umbilical vein endothelial cells (HUVECs) and PAI-1 antigen and activity measured in the conditioned medium (CM). Eighteen hours after surgery, endogenous plasma levels of PAI-1 antigen and activity were increased by 225% (P = .003) and 190% (P = .04), respectively over the preoperative values. In addition, compared with preoperative plasma, postoperative plasma increased HUVEC secretion of PAI-1 antigen and activity by 99% (P = .001) and 66% (P = .002), respectively. This increase in HUVEC PAI-1 secretion reflects an increase in PAI-1 mRNA expression and protein biosynthesis as confirmed by metabolic radiolabeling, immunoprecipitation, and Northern blot analysis. Ultrafiltration experiments indicate that the postoperative plasma mediator(s) that stimulates HUVEC PAI-1 biosynthesis is in a molecular weight (MW) range of approximately 30 to 100 Kd. Heat treatment (56°C; 30 minutes) of postoperative plasma abolished the induction of HUVEC PAI-1 production. Enzyme-linked immunosorbent assay and immunoneutralization experiments indicate that tumor necrosis factor-α (TNFα) and interleukin-1α (IL-1α) do not contribute to the postoperative plasma effect on HUVEC PAI-1 synthesis. These observations demonstrate that postoperative patient plasma contains a factor(s) that may stimulate endothelial cell PAI-1 biosynthesis in vivo and thus mediate postoperative fibrinolytic shutdown.

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MATERIALS AND METHODS

Reagents. Reagents were obtained as follows: fetal calf serum (FCS), trypsin, penicillin, streptomycin, medium 199 (M199), and methionine-free Dulbecco’s media (GIBCO/BRL, Burlington, Ontario, Canada); tissue culture plasticware and 96-well microtiter plates (Costar, Toronto, Ontario, Canada); endothelial cell growth factor (Boehringer-Mannheim, Montreal, Quebec, Canada); bacterial LPS (Escherichia coli, 055:B5), porcine intestinal heparin, bovine serum albumin (BSA), TRIS-Base, caprylic acid, agarose, parafomaldehyde, iodoacetamide, β-mercaptoethanol, trichloro-acetic acid (TCA), and phenyl-methyl-sulfonyl-fluoride (PMSF) (Sigma Chemical Co, St Louis, MO); aprotinin (Miles Labs, Elkhart, Indiana, IN); enzyme-linked immunosorbent assay (ELISA) kits for TNFα and IL-1α antigen, natural human TNFα and IL-1α,

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rabbit polyclonal antihuman TNFα, and antihuman IL-1α IgG (Endogen Inc, Boston, MA).

Plasma preparation. Blood samples were obtained from patients who had undergone total hip replacement surgery. Heparinized and citrated blood was collected preoperatively and 3, 8, 18, and 42 hours postoperatively. All patients received prophylactic heparin starting after the 18-hour postoperative blood sample was drawn. Platelet-poor plasma was prepared by double centrifugation of blood at 3,000g at 4°C for 15 minutes. Aliquots were kept at −70°C until assayed. Heat-treated plasma was prepared by incubating samples in a water bath maintained at 50°C for 30 minutes.

Cell culture. HUVECs were isolated by collagenase digestion from umbilical cord segments as described, and grown in primary culture on 25-cm² tissue culture flasks using M199 containing 20% fetal calf serum (FCS), endothelial cell growth factor (20 mg/mL), porcine intestinal heparin (90 mg/mL), penicillin (100 U/mL), and streptomycin (100 mg/mL). Before each experiment, confluent second- or third-passage HUVECs in 24-well tissue culture plates were washed with phosphate-buffered saline (PBS) and then incubated for up to 16 hours with 5% heparinized patient plasma in M199. The conditioned medium (CM) was collected from duplicate wells, centrifuged at 2,000g for 5 minutes to remove cell debris, and frozen at −70°C until assayed. For metabolic radiolabeling experiments, HUVECs in 60-mm dishes were incubated for 6 hours with 3 mL of methionine-free Dulbecco's media containing 100 μg/mL L-[35S]methionine and 2.5% heparinized patient or normal pooled plasma (NPP). CM was centrifuged and aliquots stored at −70°C until assayed.

Quantitation of radiolabeled HUVECs PAI-1. Immunoprecipitation of radiolabeled PAI-1 was performed as described. Briefly, 500 μL of CM samples were precleared with normal rabbit IgG and protein-A-Sepharose (1:10 wt/vol; 50 μL) for 2 hours at 4°C. After centrifuging at 2,000g for 5 minutes, the supernatants were incubated overnight at 4°C with either normal rabbit IgG or rabbit antihuman PAI-1 IgG. The IgG fraction was isolated from rabbit serum by caprylic acid precipitation as described. The IgG complexes were precipitated with protein-A-Sepharose (1:10 wt/vol), the beads washed twice with washing buffer (0.01 mol/L Tris-HCl pH 8.0 containing 0.14 mol/L NaCl, 0.5% Triton X-100, 1 mmol/L PMSF, 5 mmol/L iodoacetamide, 0.2 U/mL aprotinin) and then once with washing buffer containing 0.1% sodium dodecyl sulfate (SDS). The beads were suspended in 30 μL of Laemmli sample buffer (2% SDS, 10% glycerol, 0.5 mg/mL bromophenol blue, 0.125 mol/L Tris-HCl, pH 6.8 and 1% β-mercaptoethanol), boiled, and then fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 4% stacking and 9% separating gels (20°C, 16 hours, 50 V). Gels were treated with Amplify for 1 hour, dried under vacuum at 80°C, and exposed to Kodak XAR5 film (Eastman Kodak, Rochester, NY). The bands corresponding to purified PAI-1 were cut out of the dried gels and quantitated by β-counting. Trichloroacetic acid (TCA) precipitation of total 35S-methionine incorporation into biosynthetically radiolabeled proteins was determined in the CM and Triton X-100 soluble cell extracts as described.

PAI-1 activity assay. Active PAI-1 antigen was determined in the citrated plasma and HUVEC CM by a immunoradiometric assay as previously described. Briefly, 96-well poly styrene microtitre plates were coated overnight at 4°C with purified recombinant t-PA in PBS (50 μL/well; 5 μg/mL). Nonspecific protein binding sites were blocked with blocking buffer (PBS containing 3% BSA, 0.05% Tween-20, and 0.02% NaN3). The wells were washed with SPRIA buffer (PBS containing 0.1% BSA, 0.05% Tween-20, and 0.05% NaN3), and then incubated for 2 hours at 37°C with samples diluted in IRMA buffer (PBS containing 3% BSA, 0.1% Tween-80, aprotinin 20 U/mL, 5 mmol/L EDTA, and 0.05% NaN3). After washing, the bound PAI-1 was detected by incubating the wells for 1 hour at 37°C with rabbit antihuman PAI-1 IgG (1 μg/mL in IRMA buffer) followed by incubation for 1 hour at 37°C with 125I-goat antirabbit IgG (5 x 10⁶ cpm/well). The washed wells were cut out and the bound radioactivity determined by γ-counting. Results are expressed relative to a human recombinant human PAI-1 standard (DuPont-Merck Pharmaceutical) diluted in PAI-1-depleted normal citrated human plasma in which 1 U activity corresponds to 10 ng of antigen as determined by titration with t-PA using the Spectrolyse assay (Biopool, Inter-Haematol Inc, Hamilton, Ontario, Canada).

PAI-1 antigen assay. Total PAI-1 antigen in citrated plasma and HUVEC CM was determined by a competitive radioimmunossay as previously described. Briefly, 96-well poly styrene microtitre plates were coated overnight at 4°C with PAI-1-diluted in PBS (300 ng/mL, 50 μL/well). Nonspecific protein binding sites were blocked with blocking buffer. After washing, the wells were incubated for 2 hours at 37°C with samples that were preincubated overnight with rabbit antihuman PAI-1 IgG in IRMA buffer (1 μg/mL final dilution). After washing, the bound anti-PAI-1 IgG was detected by incubating wells for 1 hour at 37°C with 125I-goat antirabbit IgG (5 x 10⁶ cpm/well). The washed wells were cut out and the bound radioactivity determined by γ-counting.

Analysis for TNFα and IL-1α. TNFα and IL-1α antigen levels were determined in citrated plasma by ELISA according to the manufacturer's instructions. For antibody neutralization experiments, HUVECs were incubated for 6 hours with 5% preoperative or postoperative plasma containing rabbit antihuman TNFα, anti-IL-1α, or preimmune IgG fraction (1 to 10 μg/mL). Controls included HUVECs incubated with patient plasma containing TNFα (5 U/mL) or IL-1α (2.5 U/mL) in the presence or absence of neutralizing or preimmune rabbit IgG. CM samples were collected and analyzed for PAI-1 antigen as described above.

Northern blot analysis. HUVECs in 25-cm² tissue culture flasks were incubated for 6 hours with 5% preoperative or postoperative heparinized plasma and the total RNA isolated by acid guanidium isothiocyanate-phenol-chloroform method as described. Ten mi-

Fig 1. Time course of endogenous plasma PAI-1 levels in two patients after elective hip surgery. Plasma samples were obtained from two patients (patient 1, ♦; patient 2, ♂) at various times after surgery and analyzed for the levels of (A) PAI-1 antigen and (B) PAI-1 activity, as described in Materials and Methods. Results are expressed as mean (±SD) of three separate experiments.
programs of RNA determined by spectrophotometry was loaded onto a 1% agarose-formaldehyde gel. RNA was then transferred to a Biotrans membrane by standard capillary blotting technique. The RNA was cross-linked to the membrane by UV illumination and hybridized with a PAI-1 cDNA probe (courtesy of Dr D. Ginsberg, University of Michigan). The probe was labeled with 32P-[dCTP] nick-translated to a specific activity > 10^6 disintegrations per minute/µg DNA. After hybridization, the membrane was washed and subsequently exposed at ~80°C to a Kodak XAR5 film and analyzed by densitometric scanning.

Centrifugal ultrafiltration of plasma. Preoperative and postoperative plasma samples were subjected to differential ultrafiltration using various MW-cutoff Centricon microconcentrators (Amicon, Oakville, Ontario, Canada). Samples were centrifuged for 1 hour at 5,000g for the 10 and 30 kilodalton MW-cutoffs, or 50 minutes at 1,000g for the 100-Kd MW-cutoffs. HUVECs were incubated for 6 hours with plasma retentates or filtrates (final concentration 3 mg/mL) and the CM collected for analysis of PAI-1 antigen as described above. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (Pierce Chemicals, Rockford, IL).

Statistical analysis. The results are expressed as mean and standard deviations of three separate experiments. Paired t-test was used to test the difference between preoperative values and postoperative values.

RESULTS

Time course of plasma PAI-1 activity and antigen in relation to surgery. Levels of plasma PAI-1 antigen and activity measured in two patients preoperatively and postoperatively are shown in Fig 1. Peak levels for both PAI-1 antigen (Fig 1A) and activity (Fig 1B) were observed at 18 hours postoperatively. We then determined the preoperative and 18 hours postoperative plasma PAI-1 antigen and activity in 11 patients who had undergone elective hip replacement surgery (Table 1). The mean increment of PAI-1 antigen and activity in postoperative plasma was 225% (P = .003) and 190% (P = .04), respectively (Table 2). To establish whether the observed increase in plasma PAI-1 was a result of secretion or synthesis of PAI-1 by endothelial cells in response to a circulating factor(s), HUVECs were incubated with various doses preoperative and postoperative plasma and PAI-1 levels determined in the CM. The incubation of HUVECs with 18-hour postoperative plasma resulted in a dose-dependent (Fig 2) increase in PAI-1 antigen and activity in the CM by 99% (P = .001) and 66% (P = .002), respectively (Table 2).

Table 1. Characteristics of Patients Undergoing Elective Total Hip Replacement Surgery

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age median (range)</td>
<td>72 (58-90)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>5/6</td>
</tr>
<tr>
<td>Reason for surgery</td>
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</tr>
<tr>
<td>Osteoarthritis</td>
<td>10</td>
</tr>
<tr>
<td>Aseptic necrosis</td>
<td>1</td>
</tr>
<tr>
<td>Other medications</td>
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</tr>
<tr>
<td>Nonsteroidal anti-inflammatory drugs</td>
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</tr>
<tr>
<td>Steroids</td>
<td>0</td>
</tr>
<tr>
<td>Anti-hypertension drugs</td>
<td>2</td>
</tr>
<tr>
<td>Diagnosis of postoperative deep venous thrombosis</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. PAI-1 Antigen and Activity Levels in Patient Plasma and in Plasma-treated HUVEC CM

<table>
<thead>
<tr>
<th>Sample</th>
<th>PAI-1 Ag (µg/mL)</th>
<th>PAI-1 Act (U/mL)</th>
<th>Mean % Increment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Pre-op</td>
<td>37 ± 6</td>
<td>103 ± 25</td>
<td>225 ± 44</td>
<td>P = .003</td>
</tr>
<tr>
<td>Plasma Post-op</td>
<td>23 ± 4</td>
<td>57 ± 8</td>
<td>190 ± 67</td>
<td>P = .04</td>
</tr>
<tr>
<td>HUVEC CM Pre-op</td>
<td>1,270 ± 259</td>
<td>2,169 ± 404</td>
<td>99 ± 19</td>
<td>P = .001</td>
</tr>
<tr>
<td>HUVEC CM Post-op</td>
<td>337 ± 66</td>
<td>548 ± 103</td>
<td>66 ± 24</td>
<td>P = .002</td>
</tr>
</tbody>
</table>

HUVECs were incubated overnight (16 hours) with 5% preoperative or postoperative (18 hours) patient plasma (N = 11) and the PAI-1 antigen and activity in the CM was analyzed as described in Materials and Methods.

Metabolic radiolabeling and immunoprecipitation of HUVEC PAI-1. To establish whether the increase of PAI-1 in HUVEC CM was caused by de novo biosynthesis, HUVECs were metabolically radiolabeled with 35S-methionine for 6 hours in the presence of preoperative or postoperative plasma from various patients and the resulting 35S-labeled PAI-1 was collected by immunoprecipitation and quantitated (Fig 3). Postoperative plasmas increased the amount of 35S-labeled PAI-1 in the CM by an average of 40% (Fig 3, lanes 2 and 4) relative to preoperative plasmas (Fig 3, lanes 1 and 3). This increase in 35S-labeled PAI-1 was relatively specific because total incorporation of 35S-methionine into TCA precipitable protein was decreased to approximately 90% of preoperative plasma-treated cells. Treatment of HUVECs with NPP in the presence or absence of LPS (10 µg/mL) resulted in a 70% relative increase in 35S-labeled PAI-1.

Northern blot analysis. HUVECs were incubated for 6 hours with preoperative or postoperative plasmas and the total RNA isolated and analyzed for PAI-1 mRNA as described in Materials and Methods. The 18-hour postoperative plasma induced a 5.1-fold (Fig 4, lane 2, patient A)
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Fig 3. Effect of postoperative patient plasma on HUVEC PAI-1 biosynthesis. HUVECs were incubated for 6 hours with 2.5% patient plasma containing 35S-methionine. The 35S-methionine labeled PAI-1 was immunoprecipitated from the CM and quantitated as described in Materials and Methods. (A) Autoradiograms of immunoprecipitated (lanes 1 through 4, anti-PAI-1 IgG; lane 5, normal rabbit IgG) 35S-methionine labeled PAI-1 from HUVECs incubated with: lane 1, preoperative plasma, patient A; lane 2, 18-hour postoperative plasma, patient A; lane 3, preoperative plasma, patient B; lane 4, 18-hour postoperative plasma, patient B; lane 5, NPP plus LPS (10 μg/mL). (B) Mean quantitation of disintegration per minute (dpm) measured with a β counter for each corresponding lane (±SD).

Identification of the soluble mediator(s). Experiments were performed to determine the identity of the soluble mediator(s) in postoperative plasma. Our findings failed to confirm a direct role for TNFa or IL-1α in the stimulation of HUVEC PAI-1 biosynthesis. Plasma antigen levels of TNFa and IL-1α were not elevated 18 hours or 42 hours postoperatively in the seven patients analyzed (Table 3). However, the incubation of HUVECs with either TNFa (5 U/mL) or IL-1α (2.5 U/mL) resulted in an increase in PAI-1 antigen in the CM that was inhibited by specific antibodies to these cytokines (Fig 5). Moreover, incubation of HUVECs with preoperative or postoperative patient plasma containing neutralizing antibodies to TNFa or IL-1α had no effect on secretion of PAI-1 into the CM (Fig 5, A and B).

Characterization of the soluble mediator(s). The preoperative and postoperative plasma samples were subjected to differential centrifugal ultrafiltration and the effects of the retentates and filtrates on HUVEC PAI-1 antigen secretion were examined. Stimulation of HUVEC PAI-1 biosynthesis by postoperative plasma filtrates was only detected after ultrafiltration using 100-Kd MW-cutoff membranes (Fig 6B). In contrast, stimulation of HUVEC PAI-1 biosynthesis by postoperative plasma was retained only by the 10- and 30-Kd MW-cutoff membranes (Fig 6C). Furthermore, treatment of postoperative plasma at 56°C for 30 minutes abolished the induction of HUVEC PAI-1 production (data not shown).

DISCUSSION

In this study, we used cultured HUVECs incubated with preoperative or postoperative plasma to explore the mecha-
nism of the postoperative increase in PAI-1 levels. First, we confirmed the previous observation of an increase in plasma PAI-1 activity and antigen postoperatively.\(^2\),\(^8\),\(^1\),\(^13\) Peak levels appear approximately 18 hours after surgery (Fig 1, Table 2). Secondly, our results from the metabolically radiolabeled immunoprecipitation of PAI-1 (Fig 3) and Northern blot analysis of PAI-1 mRNA expression (Fig 4) confirmed the presence of soluble factors in postoperative plasma that stimulate de novo biosynthesis of PAI-1 by HUVECs. This increase in HUVEC PAI-1 biosynthesis was substantial and comparable with that induced by purified LPS (Fig 4), TNF\(\alpha\), or IL-1\(\alpha\) (Fig 5).

Inflammatory mediators such as LPS, TNF\(\alpha\), and transforming growth factor-\(\beta\) (TGF-\(\beta\)) induce tissue-specific PAI-1 gene expression in vivo.\(^3\) These inflammatory mediators, particularly LPS, stimulate PAI-1 biosynthesis by endothelial cells in vitro\(^{16,22}\) and in vivo.\(^{33,34}\) The normal intact endothelium is nonthrombogenic and does not activate platelets or blood coagulation factors.\(^2\) In contrast, when endothelial cells are exposed to a number of nonde-nuding stimuli such as endotoxin,\(^{17,18,20,22}\) IL-1,\(^{6,19,22,24,35}\) TNF,\(^{16,18,20,21,24,36,38}\) thrombin,\(^{39}\) or lipoprotein(a) [Lp(a)]\(^{40}\) they acquire thrombogenic properties. The possibility that the observed increase in PAI-1 synthesis was caused by endotoxin contamination of postoperative plasma was ruled out because the ability of postoperative plasma to stimulate PAI-1 synthesis was heat labile. The acute-phase cytokines, IL-1\(\alpha\) and TNF\(\alpha\), are produced by activated macrophages and other cells in response to tissue injury.\(^{42,43}\) Our analysis of postoperative plasma indicates that neither TNF\(\alpha\) nor IL-1\(\alpha\) are elevated in 18-hour or 42-hour postoperative plasma samples (Table 3), nor do they contribute to the observed functional HUVEC stimulatory activity in preoperative or postoperative plasma (Fig 5). There has been one report of an elevation of IL-1 after cardiopulmonary bypass surgery,\(^{44}\) but an effect of other surgical procedures on the plasma levels of this cytokine has not been reported. In addition, a recent study has demonstrated no significant increase in TNF levels in patients undergoing partial pancreatectomy.\(^{45}\) Thrombin generated during blood coagulation is an unlikely candidate for the postoperative plasma stimulating activity because we used heparinized patient plasma that does not express free factor IIa activity.

<table>
<thead>
<tr>
<th>Cytokine Assay</th>
<th>Preoperative</th>
<th>0</th>
<th>18</th>
<th>42</th>
<th>Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF(\alpha) (pg/mL)</td>
<td>31 ± 7</td>
<td>29 ± 8</td>
<td>30 ± 7</td>
<td>35 ± 2</td>
<td></td>
</tr>
<tr>
<td>IL-1(\alpha) (pg/mL)</td>
<td>213 ± 63</td>
<td>198 ± 45</td>
<td>183 ± 16</td>
<td>145 ± 21</td>
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</tr>
</tbody>
</table>

Preoperative and postoperative plasma samples (\(N = 7\)) were analyzed for TNF\(\alpha\) and IL-1\(\alpha\) antigen by specific ELISAs as described in Materials and Methods.

Perturbation of human and bovine endothelial cells in culture results in an increased synthesis\(^{16,19,20,22}\) and cell surface expression\(^{30}\) of PAI-1, tissue factor\(^{36}\) and a decreased synthesis and cell surface expression of thrombomodulin.\(^{37}\) In addition, perturbed endothelial cells synthesize and secrete IL-6\(^{53}\) and IL-1,\(^{54}\) which may further amplify the local and systemic prothrombotic response to trauma. Therefore, it is possible that the mediator(s) in postoperative plasma that we have shown to stimulate...

**Fig 5.** Immunoneutralization analysis of TNF\(\alpha\) or IL-1\(\alpha\) in postoperative plasma. (A and B) HUVECs were incubated for 6 hours with preoperative (B) or postoperative (A) plasma in the presence (+) or absence of purified TNF\(\alpha\) (5 U/mL) or IL-1\(\alpha\) (2.5 U/mL) and/or rabbit anti-TNF\(\alpha\), anti-IL-1\(\alpha\), or normal rabbit IgG (NRG; 10 \(\mu\)g/mL). The CM was collected and assayed for PAI-1 antigen as described in Materials and Methods. Each bar represents the mean (±SD) of two separate experiments.
H U V E C P A I-1 synthesis also upregulates the endothelial cell surface expression of tissue factor as well as downregulating thrombomodulin. It is also possible that these endothelial changes are of major importance in the pathogenesis of postoperative venous thrombosis and other clinical conditions that predispose to venous thrombosis. The identity and functional characterization of the soluble postoperative plasma mediator(s) that induced PAI-1 synthesis remains to be elucidated.

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