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 breast carcinoma and secretion by vascular endothelium. Plasma samples collected from patients (N = 11) before and after surgery for breast carcinoma 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, paraformaldehyde, iodoacetamide, β-mercaptoethanol, trichloroacetic acid (TCA), and phenyl-methyl-sulfonyl-fluoride (PMSF) (Sigma Chemical Co, St Louis, MO); aprotinin (Miles Labs, Etohioke, Ontario, Canada); protein-A-sepharose (Pharmacia Chemicals, Piscatway, NJ); [35S]-methionine (1,100 Ci/mmol), [3P]dCTP (10 mCi/mL), and Amplify (Amersham Chemicals, Oakville, Ontario, Canada); normal rabbit serum (Cedarlane Laboratories Ltd, Hornby, Ontario, Canada); Biotrans membrane (ICN Biomedicals, St Laurent, Quebec, Canada); recombinant human PAI-1 was provided courtesy of Dr T. Reilly (DuPont-Merck Pharmaceutical, Wilmington, DE); recombinant type I plasminogen activator (t-PA) (E.I. Lilly Pharmaceutical, Indianapolis, IN); enzyme-linked immunosorbent assay (ELISA) kits for TNFα and IL-1α antigen, natural human TNFα and IL-1α,
POSTOPERATIVE PLASMA INDUCES PAI-1 SYNTHESIS

rabbit polyclonal antihuman TNFa, 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-hours 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 μg/mL), porcine intestinal heparin (90 μg/mL), penicillin (100 U/mL), and streptomycin (100 μg/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 drawn. Platelet-poor plasma was prepared by double centrifugation of heparin starting after the 18-hours postoperative blood sample was drawn. Pooled plasma (NPP). CM was centrifuged and aliquots stored at −70°C until assayed. Heat-treated plasma was prepared 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 unit 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 radioimmunoassay as previously described. Briefly, 96-well polystyrene microtiter plates were coated overnight at 4°C with PAI-1–depleted normal citrated human plasma in which 1 μg/mL final dilution. 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 TNFa and IL-1α. TNFa 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 TNFa, anti-IL-1α, or preimmune IgG fraction (1 to 10 μg/mL). Controls included HUVECs incubated with patient plasma containing TNFa (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-
programs of RNA determined by spectrophotometry was loaded onto a 1% agarose-formaldehyde gel. RNA was then transferred to a Bioretrans 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 XARS 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 retenates 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 as measured in the CM was 225% (P = .003) and 190% (P = .001), 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 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 |
|----------------------------------|------------------|------------------|------------------|------------------|------------------|
| No. of patients                  | 11               | Age median (range) | 72 (58-90)       | Sex (M/F)        | 5/6               |
| Reason for surgery              |                  | Sex (M/F)         |                  | Reason for surgery | Osteoarthritis | Aseptic necrosis |
| Other medications               |                  |                  |                  | Other medications | Nonsteroidal anti-inflammatory drugs | Steroids | Anti-hypertension drugs | Diagnosis of postoperative deep venous thrombosis |
|                                 |                  |                  |                  |                  | 8                 | 0               | 2               | 2               |

**Metabolic radiolabeling and immunoprecipitation of HUVEC PAI-1.** To establish whether the increase of PAI-1 in HUVECs 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) increase in PAI-1 mRNA compared to preoperative plasma.

**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 Antigen (ng/mL)</th>
<th>Mean % Increment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>PAI-1 Ag</td>
<td>37 ± 6</td>
<td>225 ± 44</td>
</tr>
<tr>
<td></td>
<td>PAI-1 Act</td>
<td>23 ± 4</td>
<td>190 ± 67</td>
</tr>
<tr>
<td>HUVEC CM</td>
<td>PAI-1 Ag</td>
<td>1,270 ± 259</td>
<td>99 ± 19</td>
</tr>
<tr>
<td></td>
<td>PAI-1 Act</td>
<td>337 ± 66</td>
<td>66 ± 24</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.

**Fig 2.** Dose-dependent increase in PAI-1 antigen measured in the CM after HUVECs were incubated overnight (16 hours) with preoperative and 18-hour postoperative plasma. Each bar represents the mean (±SD) of two different patient plasmas in two separate experiments.
and 1.4-fold (Fig 4, lane 5, patient B) increase in the 3.2-kb and 1.9-fold (Fig 4, lane 2, patient A) and a 1.3-fold (Fig 4, lane 5, patient B) increase in the 2.3-kb PAI-1 mRNA expression relative to preoperative plasma (Fig 4, lanes 1 and 4, patients A and B, respectively) as determined by scanning densitometry. Similarly, 42-hour postoperative plasma induced a 2.7-fold increase in the 3.2-kb and a 1.3-fold increase in the 2.3-kb PAI-1 mRNA expression (Fig 4, lane 3). This increase in HUVEC PAI-1 mRNA expression by postoperative plasma was comparable with that induced by purified LPS (Fig 4, lane 6).

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 TNFα or IL-1α in the stimulation of HUVEC PAI-1 biosynthesis. Plasma antigen levels of TNFα 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 TNFα (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 TNFα 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.2,8-10 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), TNFa, or IL-1a (Fig 5).

Inflammatory mediators such as LPS, TNFa, and transforming growth factor-β (TGF-β) induce tissue-specific PAI-1 gene expression in vivo.32 These inflammatory mediators, particularly LPS, stimulate PAI-1 biosynthesis by endothelial cells in vitro16-22 and in vivo.35 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 nondecoding stimuli such as endotoxin,17,18,20-22 IL-1,16,19,22,34,35 TNF,16,18,20-22,34,36-38 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-1a and TNFa, are produced by activated macrophages and other cells in response to tissue injury.42,43 Our analysis of postoperative plasma indicates that neither TNFa nor IL-1a 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. Lp(a) alters fibrinolysis by competing with plasminogen for binding to endothelial cells46 and by inducing the synthesis of PAI-1.40 Plasma levels Lp(a) have been reported to be elevated in the postoperative period41 and in patients with cancer or postmyocardial infarction.42,46-48 However, Lp(a) is unlikely to be responsible for our observed stimulating activity because its molecular weight is greater than 106 daltons and our ultrafiltration experiments indicate that the molecular weight of the postoperative plasma mediator(s) is between approximately 30 and 100 Kd (Fig 6). Postoperative plasma levels of IL-6, the major regulator of the acute phase response,49-51 were elevated over preoperative levels in six of eight patients analyzed (data not shown). Similar data were reported post-partial pancreatectomy.45 However, recent in vivo32 and in vitro studies with cultured HUVECs21,32 indicate that PAI-1 synthesis is unresponsive to IL-6. The possible role of TGF-β in the postoperative plasma stimulation of endothelial cell PAI-1 synthesis is currently being investigated.

Perturbation of human and bovine endothelial cells in culture results in an increased synthesis16-19,21,22 and cell surface expression30 of PAI-1, tissue factor35,36 and a decreased synthesis and cell surface expression of thrombomodulin.37 In addition, perturbed endothelial cells synthesize and secrete IL-653 and IL-1a,44 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

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**Table 3. Concentrations of TNFα and IL-1α Antigen in Preoperative and Postoperative Patient Plasmas**

<table>
<thead>
<tr>
<th>Cytokine Assay</th>
<th>Hours After Surgery</th>
<th>NPP</th>
</tr>
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<tbody>
<tr>
<td>TNFα (pg/mL)</td>
<td>0</td>
<td>31 ± 7</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>29 ± 8</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>30 ± 7</td>
</tr>
<tr>
<td>IL-1α (pg/mL)</td>
<td>0</td>
<td>213 ± 63</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>198 ± 45</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>183 ± 16</td>
</tr>
</tbody>
</table>

Preoperative and postoperative patients' plasmas (N = 7) were analyzed for TNFα and IL-1α antigen by specific ELISAs as described in Materials and Methods.

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![Fig 5. Immunoneutralization analysis of TNFα or IL-1α in postoperative plasma. (A and B) HUVECs were incubated for 6 hours with preoperative (B) or postoperative (B) plasma in the presence (+) or absence of purified TNFα (5 U/mL) or IL-1α (2.5 U/mL) and/or rabbit anti-TNFα, anti-IL-1α, or normal rabbit IgG (NRRG; 10 μg/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.](http://www.bloodjournal.org)
HUEVCI PAI-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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