A Lifelong Bleeding Disorder Associated With a Deficiency of Plasminogen Activator Inhibitor Type 1

By Jocelyne Diéval, Geneviève Nguyen, Sylvie Gross, Jacques Delobel, and Egbert K.O. Kruithof

A 36-year-old patient was investigated for a lifelong history of epistaxis and delayed bleeding after minor surgeries. Deficiencies or abnormalities of the coagulation system, of platelet function, or of factor XIII and α2-antiplasmin were excluded. Consistently, however, over a period of 7 years, a high basal euglobulin fibrinolytic activity was observed that was characterized by a high tissue-type plasminogen activator (t-PA) activity, normal t-PA antigen, and undetectable plasminogen activator inhibitor type-1 (PAI-1) antigen and activity. The high specific activity of t-PA (640,000 IU/mg) and the minimal amounts of t-PA/PAI-1 complexes detected by fibrin zymography suggest that in this patient all t-PA was active. This is in striking contrast to normal plasma, where the majority of t-PA is complexed to PAI-1. Thus, in this patient, a severe deficiency of PAI-1 is associated with a delayed type bleeding tendency. Our observation underscores the importance of plasma PAI-1 for the stabilization of the hemostatic plug.

Patient. The patient, born in 1953, first presented in 1982 for an important hemorrhage after meniscectomy. He had a lifelong history of epistaxis and delayed bleeding after surgery or trauma (eg, after an amygdalectomy, an appendectomy, and a head injury). He also experienced a hematuria 2 days after a renal contusion. After two dental extractions, bleeding stopped rapidly but resumed 24 hours later, and, on one occasion, dental extraction was complicated by a sublingual hematoma. None of these hemorrhagic episodes required blood transfusion, and on all occasions bleeding stopped spontaneously. Physical examination of the patient showed no particular abnormalities. He had a normal cardiovascular function and no hepatosplenomegaly or clinical evidence of liver disease. After the diagnosis of a hyperfibrinolytic state was made, the patient was treated with epsilon aminocaproic acid before other dental extractions, and in contrast to previous minor traumas, no delayed bleeding was noted. The patient has given written consent to participate in the study.

Hemostasis tests. Blood was collected at 10 AM and 4 PM, after a 15-minute rest period and anticoagulated with 0.13 mol/L of sodium citrate (9 vol/1 vol). Platelet-poor plasma was obtained after 15 minutes of centrifugation at 3,500g. For the study of the fibrinolytic parameters, blood was drawn before and after a 10-minute venous stasis (cuff pressure between the systolic and the diastolic pressures), and immediately cooled in melting ice. The coagulation tests and the platelet function tests were performed by standard techniques. Plasminogen antigen was measured by radial immunodiffusion. Plasminogen and α2-antiplasmin activities were measured by spectrophotometric assays (Behring, Marburg, Germany), and plasmin–α2-antiplasmin complexes by crossed immunoelectrophoresis. Factor XIII activity was measured by a coagulation assay (Behring) and fibrin degradation products by a latex immunoassay (D-dimer test; Diagnostica Stago, Asnières, France). β-Thromboglobulin was measured by enzyme-linked immunosorbent assay (ELISA) (Asserachrom βTG, Diagnostica Stago). Euglobulin precipitation was performed by diluting the plasma 10-fold in cold distilled water and adjusting the pH to 5.9 with 0.25% acetic acid. The euglobulins were dissolved in 0.1 mol/L Tris HCl buffer, pH 7.5 and their fibrinolytic activity measured by the euglobulin clot lysis time assay (ECLT). t-PA antigen was measured by ELISA (Tint Elize t-PA, Biopool, Umeå, Sweden) and t-PA activity with a chromogenic assay (Spectrolyse Fibrin; Biopool). PAI-1 antigen was measured by radioimmunoassay (RIA) and ELISA (Tint Elize PAI-1; Biopool), and PAI activity with a chromogenic substrate assay (Spectrolyse Fibrin; Biopool). Fibrinogen concentration was determined by a chromogenic method (Spectrolyse Fibrin; Biopool). Fibrinogen concentration was determined by a chromogenic method (Spectrolyse Fibrin; Biopool).
agrose underlay after electrophoresis of the euglobulins on a 7.5% sodium dodecyl sulfate-polyacrylamide gel. To identify the bands on the zymography, the euglobulins were incubated with rabbit antisera to t-PA, PAI-1, u-PA, or α-2-antiplasmin, for 2 hours at 37°C, and the immune complexes precipitated using cellulose coated with donkey antirabbit antibodies (Sac antisera to t-PA, PAI-1, u-PA, or cy-2-antiplasmin, for 2 hours at 37°C, on the zymography, the euglobulins were incubated with rabbit antisera against human α-2-antiplasmin. Because platelets also contain PAI-1, we tried to estimate the contribution of platelets to the PAI-1 measured in the patient plasma and the low PAI-1 antigen levels make it likely that in the patient plasma all PAI-1, if any, is complexed to t-PA. A very sensitive technique by which low amounts of t-PA/PAI-1 complexes can be visualized is fibrin-zymography. To clarify the cause of the high fibrinolytic activity we measured specifically plasma levels of t-PA and PAI-1. In plasma collected at rest, t-PA antigen was normal, 5 ng/mL, but t-PA activity was elevated (3.2 IU/mL). From these results we calculated a specific activity for t-PA at rest of 640,000 IU/mg, a value similar to that given for the International t-PA Standard (83/517, National Institute for Biological Standards and Controls, South Mims, Herts, England): 600,000 IU/mg. These results suggest that, in this patient, all the circulating t-PA was in a free active form, in contrast to normal healthy people where the majority of t-PA at rest is complexed to PAI-1. In agreement with this finding, PAI-1 antigen levels measured by RIA and ELISA as well as PAI activity were below the limits of detection (antigen <6 ng/mL and <2 ng/mL, respectively; and activity <1 U/mL) at two different times of day, 10 AM and 4 PM (Table 2). In contrast to plasma, serum PAI-1 as measured by RIA (270 ng/mL) was within the normal range (70 to 330 ng/mL) and the difference between serum and plasma PAI-1 activity was 7.8 U/mL, a value close to the median obtained for three healthy individuals of the same age (9.2 U/mL), indicating that the PAI-1 pool in platelets and the release of PAI-1 from platelets were normal. The patient had a good response to venous stasis: t-PA antigen increased to 43 ng/mL and t-PA activity to 20.7 IU/mL, somewhat higher than the upper limit of the normal range. The high specific activity of t-PA in the patient plasma and the low PAI-1 antigen levels make it likely that in the patient plasma all PAI-1, if any, is complexed to t-PA. A very sensitive technique by which low amounts of t-PA/PAI-1 complexes can be visualized is fibrin-zymography. To clarify the cause of the high fibrinolytic activity we measured specifically plasma levels of t-PA and PAI-1. In plasma collected at rest, t-PA antigen was normal, 5 ng/mL, but t-PA activity was elevated (3.2 IU/mL). From these results we calculated a specific activity for t-PA at rest of 640,000 IU/mg, a value similar to that given for the International t-PA Standard (83/517, National Institute for Biological Standards and Controls, South Mims, Herts, England): 600,000 IU/mg. These results suggest that, in this patient, all the circulating t-PA was in a free active form, in contrast to normal healthy people where the majority of t-PA at rest is complexed to PAI-1. In agreement with this finding, PAI-1 antigen levels measured by RIA and ELISA as well as PAI activity were below the limits of detection (antigen <6 ng/mL and <2 ng/mL, respectively; and activity <1 U/mL) at two different times of day, 10 AM and 4 PM (Table 2). In contrast to plasma, serum PAI-1 as measured by RIA (270 ng/mL) was within the normal range (70 to 330 ng/mL) and the difference between serum and plasma PAI-1 activity was 7.8 U/mL, a value close to the median obtained for three healthy individuals of the same age (9.2 U/mL), indicating that the

RESULTS
To identify the cause of recurrent bleeding in our patient, we have undertaken on several occasions, over an interval of 7 years, a detailed analysis of the different hemostasis subsystems. On all occasions, bleeding time, all coagulation tests (including factor XIII), and platelet function tests were within the normal range, except for fibrinogen that was at the lower limit of the normal range (Table 1).

On all five occasions over a 7-year period, basal fibrinolytic activity was elevated, as measured by the ECLT (between 80 and 105 minutes, normal >140 minutes). However, no evidence for systemic fibrinogenolysis was noted: the levels of α-2-antiplasmin, plasminogen, and fibrin degradation products were normal, and no α-2-antiplasmin-plasmin complexes were detected by crossed immunoelectrophoresis. To clarify the cause of the high fibrinolytic activity we measured specifically plasma levels of t-PA and PAI-1. In plasma collected at rest, t-PA antigen was normal, 5 ng/mL, but t-PA activity was elevated (3.2 IU/mL). From these results we calculated a specific activity for t-PA at rest of 640,000 IU/mg, a value similar to that given for the International t-PA Standard (83/517, National Institute for Biological Standards and Controls, South Mims, Herts, England): 600,000 IU/mg. These results suggest that, in this patient, all the circulating t-PA was in a free active form, in contrast to normal healthy people where the majority of t-PA at rest is complexed to PAI-1. In agreement with this finding, PAI-1 antigen levels measured by RIA and ELISA as well as PAI activity were below the limits of detection (antigen <6 ng/mL and <2 ng/mL, respectively; and activity <1 U/mL) at two different times of day, 10 AM and 4 PM (Table 2). In contrast to plasma, serum PAI-1 as measured by RIA (270 ng/mL) was within the normal range (70 to 330 ng/mL) and the difference between serum and plasma PAI-1 activity was 7.8 U/mL, a value close to the median obtained for three healthy individuals of the same age (9.2 U/mL), indicating that the

Table 1. Coagulation Parameters in the Patient With Bleeding and Deficient PAI-1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time (Ivy method, min)</td>
<td>5</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Platelet count (G/L)</td>
<td>2.3</td>
<td>1.5-4</td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
<td>12</td>
<td>11-13</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>34</td>
<td>30-37</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>1.7</td>
<td>1.8-4</td>
</tr>
<tr>
<td>Thrombin time (s)</td>
<td>17</td>
<td>16-20</td>
</tr>
<tr>
<td>Factor XIII (IU/mL)</td>
<td>1.0</td>
<td>0.7-1.5</td>
</tr>
</tbody>
</table>

Abbreviation: APTT, activated partial thromboplastin time.

Table 2. Fibrinolytic Parameters in the Patient With Bleeding and Deficient PAI-1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before stasis</th>
<th>After stasis</th>
<th>Patient</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECLT (min)</td>
<td>80-105</td>
<td>&gt;140</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-PA antigen (ng/mL)</td>
<td>5</td>
<td>3-13</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>t-PA activity (IU/mL)</td>
<td>3.2</td>
<td>0.2-2</td>
<td>10-15</td>
<td>10-90</td>
</tr>
<tr>
<td>PAI-1Ag (ng/mL) ELISA</td>
<td>&lt;2*</td>
<td>4-43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RI A</td>
<td>&lt;6</td>
<td>12.5-39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-2-antiplasmin (%)</td>
<td>100</td>
<td>80-120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasminogen antigen (g/L)</td>
<td>0.1</td>
<td>0.1-0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>activity (%)</td>
<td>80</td>
<td>80-120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrin degradation</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>products (μg/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Lower limit of detection for PAI-1 Ag by ELISA method is 2 ng/mL, and by RIA (t) is 6 ng/mL.
†The detection limit for PAI activity is 1 U/mL. The patient PAI-1 antigen and activity were measured at 10 AM and 4 PM.
94----
94--
67----
67--

Fig 1. Zymography of the euglobulins of the patient with PAI-1 deficiency. Euglobulin precipitates obtained from 1 mL of citrated plasma were dissolved in 250 μL of 0.1 mol/L Tris-HCl buffer pH 7.5, resulting in a fourfold concentrated euglobulin fraction. Further dilutions of the euglobulins were made in the same buffer. The different dilutions of the euglobulins were analyzed by electrophoresis, followed by zymography on a plasminogen-rich fibrin agarose underlay. The zymographies were incubated for 48 hours at 37°C. (Lanes A through C) Patient euglobulins concentrated fourfold (A), twofold (B), and unconcentrated (C). (Lanes D through G) Euglobulins from a control subject with low PAI-1 antigen and activity (3.5 ng/mL, twofold (D), uncentroncented (E), concentrated twofold (F), and concentrated fourfold (G). The molecular weight markers are given in kilodaltons. The control (t-PA: Ag = 6.0 ng/mL) showed mainly a 110-Kd band, whereas the patient (t-PA: Ag = 5.2 ng/mL) showed mainly a band at 67 Kd.

Fig 2. Identification of the lysis bands in the zymography of the patient. Euglobulins were incubated for 2 hours at 37°C with rabbit antiserum to human PAI-1. t-PA, u-PA, and α-2-antiplasmin. The immune complexes were precipitated using cellulose coated with donkey antirabbit antibodies. After centrifugation, the supernatants were analyzed by sodium dodecyl sulfate 7.5% polyacrylamide gel electrophoresis followed by zymography for 96 hours. (Lane A), Rabbit gamma globulins; (lane B), anti-α-2-antiplasmin; (lane C), anti-u-PA; (lane D), anti-t-PA; (lane E), anti-PAI-1; (lane F), no antisera. The molecular mass markers are given in kilodaltons. The decrease in the intensity of the 67-Kd band in lane D only, and of the 110-Kd band in lanes D and E identifies the 67-Kd band as free t-PA and the 110-Kd band as a t-PA/PAI-1 complex.

DISCUSSION

Deficiencies of the fibrinolytic system sometimes may be responsible for severe, lifelong bleeding disorders. The hemorrhagic consequences of deficiencies of α-2-antiplasmin and of factor XIII, the factor that cross-links α-2-antiplasmin to fibrin and renders the fibrin more resistant to fibrinolysis, are well known, whereas those of PAI-1 deficiencies still need to be established. In typical hemorrhagic states caused by deficiencies in α-2-antiplasmin and of factor XIII, platelet activation and aggregation are not affected, and fibrin polymerization takes place normally, but the fibrin is more sensitive to the action of plasmin because the fibrin is not stabilized by factor XIII and no α-2-antiplasmin is adsorbed on the clot. Therefore, primary hemostasis explored by the bleeding time is normal as in our patient, but the clot dissolution by physiologic fibrinolysis is accelerated leading to sometimes excessive and prolonged bleeding after a delay of up to several days. The characteristics of hyperfibrinolytic bleeding (ie, a normal primary hemostasis followed, after a delay of up to several days, by sometimes excessive and prolonged bleeding) were also observed in our patient. The diagnosis of constitutive hyperfibrinolysis because of a deficiency of plasma PAI-1 is based on the following observations: (1) The patient has a lifelong history of delayed bleeding after minor trauma and we were unable to detect any abnormality of coagulation or platelet function. The slight reduction of fibrinogen is clearly insufficient to account for the bleeding tendency. (2) A high fibrinolytic activity was measured on five occasions over a period of 7 years. (3) Both PAI-1 antigen and PAI-1 activity were undetectable in the patient’s plasma. However, PAI-1 levels in serum were normal, indicating that the deficiency is limited to the plasma compartment. (4) A possible inversion of the diurnal variation of PAI-1 was excluded. (5) The specific activity of t-PA in the patient at rest was 640,000 IU/mg, a value similar to the international t-PA standard. This result in combination with that of fibrin-zymography demonstrates that in the patient plasma all t-PA is active. In contrast, in similarly collected plasmas of patients and controls (ie, resting, morning plasma and no precaution taken to prevent the in vitro reaction of t-PA with PAI-1) the specific activity of t-PA is very low and the majority of t-PA is complexed to PAI-1. (6) Treatment with EACA, the treatment of choice in hyperfibrinolytic bleeding, completely prevented bleeding after dental extractions, whereas on previous occasions delayed bleeding occurred. Several reports of hyperfibrinolytic bleeding because of uncontrolled t-PA activity have appeared in recent years. Booth et al observed a bleeding disorder and increased fibrinolysis in amyloidosis. In recent preliminary reports, excessive fibrinolytic activity associated with delayed bleeding was attributed to high t-PA antigen levels, but normal PAI-1, in one patient and to a monoclonal, amyloidogenic autoantibody to PAI-1 in another.
In a 76-year-old patient with a lifelong bleeding tendency, high fibrinolytic activity was attributed to a functionally abnormal PAI-1. Our patient represents the first case of a quantitative deficiency of plasma PAI-1. Traces of PAI-1 were detected in the patient plasma, but they may have been derived from the patient platelets. Indeed, the patient plasma contained 23 ng/mL of β-thromboglobulin and because the platelets of the patient contained normal amounts of PAI-1, we can estimate the amount of platelet PAI-1 that should be present in the plasma. Using a ratio of PAI-1/β-thromboglobulin of 0.01 to 0.02, we may estimate that between 0.2 and 0.5 ng/mL of PAI-1 in the plasma is platelet derived. Because the concentration of PAI-1 detected in the plasma of our patient was much lower than 2 ng/mL, it is even possible that all the PAI-1 detected in the plasma is platelet derived. The observation of normal levels of PAI-1 antigen and activity in the serum suggests that there are no major abnormalities of the PAI-1 gene. Because platelet PAI-1 is stored intracellularly, it is possible that the deficiency is at the level of secretion, as has been described for the Z variant of α-1-antitrypsin deficiency. It is also possible that the tissue-specific expression of PAI-1 in the cells contributing to plasma PAI-1 (endothelial cells and possibly also hepatocytes) is abnormal.

The role of PAI-1 in the stabilization of the primary hemostatic plug is not yet well established. It is known that PAI-1 is the primary inhibitor of t-PA and urokinase in plasma, and that the release of PAI-1 by aggregating platelets may provide a means to prevent premature fibrinolysis and thus contribute to the stabilization of the hemostatic plug in the early stage of hemostasis. In addition, recent studies have shown that PAI-1 binds to the fibrin clot, directly or via the vitronectin that is also secreted by the platelets during their activation, and fibrin-bound PAI-1 was still able to inhibit t-PA and u-PA activities. t-PA/PAI-1 complexes compete with free t-PA for fibrin binding, thus reducing the amount of free t-PA adsorbed on fibrin and, therefore, its fibrinolytic activity. Because platelet PAI-1 content and secretion appear to be normal in our patient, the association of a quantitative deficiency of plasma PAI-1 with a bleeding disorder suggests that plasma PAI-1 is essential for the maintenance of the integrity of the hemostatic plug. It is not known whether the good response to venous stasis of the patient has also contributed to the bleeding tendency.

We conclude that in our patient, the deficiency of plasma PAI-1 associated with a high potential release of t-PA led to uncontrolled t-PA activity on fibrin clots. This observation emphasizes the importance of plasma PAI-1 in the stabilization of the hemostatic plugs, and provides the first documented demonstration of the importance of PAI-1 in the control of the in vivo fibrinolysis.

REFERENCES

of vascular plasminogen activator after venous stasis: Electro-
D: Increased fibrinolysis and amyloidosis. Arch Intern Med 143:
678, 1983
22. Loskutoff DJ, Roegner K, Schleef R, Triplett D: Elevated
PA antigen in a young man with recurrent bleeding problems.
Fibrinolysis 1986, Eighth International Congress on Fibrinolysis,
Vienna, Austria, Abstr 144
23. Bathurst IC, Errington DM, Foreman RC, Judah JD,
Carrell RW: Human Z α1-antitrypsin accumulates intracellularly
and stimulates lysosomal activity when synthetised in the Xenopus
24. Kruithof EKO, Tran-Thang C, Bachmann F: The fast acting
inhibitor of tissue-type plasminogen activator in plasma is also the
25. Erickson LA, Ginsberg MH, Loskutoff DJ: Detection and
partial characterization of an inhibitor of plasminogen activator in
release of a plasminogen activator inhibitor by human platelets.
Thromb Haemost 55:201, 1986
27. Murayama H, Bang NU: Incorporation of plasminogen
activator inhibitor into fibrin, an alternative regulatory pathway for
fibrinolysis. Thromb Haemost 58:1643, 1987 (abstr)
28. Wagner OF, de Vries C, Hohmann C, Veerman H, Pann-
keoek H: Interaction between plasminogen activator inhibitor
type 1 (PAI-1) bound to fibrin and either tissue-type plasminogen
activator (t-PA) or urokinase-type plasminogen activator (u-PA). J
Clin Invest 84:647, 1989
29. Freissner KT, Holzhüter S, Justus C, Müller-Berghaus G:
Identification and partial characterization of platelet-derived plasminogen
A lifelong bleeding disorder associated with a deficiency of plasminogen activator inhibitor type 1

J Dieval, G Nguyen, S Gross, J Delobel and EK Kruithof