Residual Plasminogen Activator Inhibitor Activity After Venous Stasis as a Criterion for Hypofibrinolysis: A Study in 83 Patients With Confirmed Deep Vein Thrombosis

By Geneviève Nguyen, Marie Hélène Horellou, Egbert K.O. Kruthof, Jacqueline Conard, and Michel M. Samama

In eighty-three patients with confirmed deep vein thrombosis, the fibrinolytic system was studied before and after a 10-minute venous occlusion. Blood was collected at least 3 months after the last acute episode, and PAI-1 antigen and activity, as well as tissue-type plasminogen activator (t-PA) antigen, urokinase-type plasminogen activator (u-PA) antigen, and fibrinolytic activity were measured in these samples. During venous stasis, plasminogen activator inhibitor (PAI) activity decreased in almost all patients 3 months after the last acute episode. and PAI-i antigen (P < 0.001, Wilcoxon signed-rank test). Because PAI-1 antigen increased from a median value of 16 to 19.2 ng/mL (P < 0.001), the decline in PAI activity was attributed to an increase in PAI-1 antigen from a median value of 10 to 21.7 ng/mL (P < 0.001). Neutralization of PAI activity thus reflects the patient's capacity to overcome basal inhibitory potential through t-PA release. Based on residual PAI activity after 10-minute stasis, patients were classified as good or bad responders (PAI activity below detection limit, ie, ≤1.0 and >1.0 U/mL, respectively). Good responders had a significantly higher fibrinolytic response after stasis than bad responders (median euglobulin clot lysis time 60 v 180 minutes; dilute whole blood clot lysis time 60 v 120 minutes; fibrinolytic activity on fibrin plates 7.7 v 0 U/mL). Furthermore, good responders, as compared with bad responders, had higher t-PA release (median 16.5 v 11.5 ng/mL), lower basal PAI activity (median 4.8 v 11.2 U/mL), and lower basal PAI-1 (median 11 v 21 ng/mL) and u-PA antigen (median 7.9 v 9.0 ng/mL, P < 0.02). Hypofibrinolysis, as defined by the inability of released t-PA to overcome basal inhibitory potential, was observed in 45 of 83 patients (54%) and resulted either from an insufficient release of t-PA or from an increased basal PAI activity.

Blood was collected between 8 and 10 AM. Part of the blood was anticoagulated with potassium EDTA (final concentration 5 mmol/L), and the remainder with citrate (1 vol 0.11 mol/L citrate, pH 4.5, added to 9 vol blood). EDTA samples were immediately cooled on ice and platelet-poor plasma (PPP) was obtained by a 10-minute centrifugation (3,500 g, 4°C) followed by a second centrifugation in an Eppendorf centrifuge (2 minutes, 10,000 rpm, 4°C). Aliquots were stored at -30°C. For most fibrinolytic assays, except for the clot lysis time assays, blood anticoagulated with EDTA was used. Euglobulin fractions were obtained by acidification (pH 5.9) of a tenfold diluted plasma, and fibrinolytic activity (EFA) was measured on bovine plasminogen-rich fibrin plates. Activity was expressed in t-PA equivalent units by reference to a standard curve of purified t-PA calibrated in the fibrin plate assay against the international reference preparation of t-PA (t-PA 83/5 17, National Institute of Biological Standards and Controls, London). The euglobulin clot lysis time (ECLT, plasma diluted 15-fold, pH 5.9) and the diluted whole blood clot lysis time (DWBLT) assays were performed as previously described. PAI activity was assayed by a titration method as described by Verheijen and colleagues. A T cap III activity was measured by a chromogenic substrate assay (Coatest AT III, Kabi, Sweden), and protein C activity was measured by a clotting method according to Francis.

Antigen Determinations

t-PA was measured by an enzyme-linked immunosorbent assay (ELISA) method (Imulys 5, Biopool, Umeå, Sweden). PAI-1 and...

MATERIALS AND METHODS

The study group comprised 83 consecutive patients, 43 females and 40 males, with deep vein thrombosis and/or pulmonary embolism, confirmed by phlebography or pulmonary angiography. All patients were investigated at least 3 months after the last acute episode (range 4 to 372 months). For each patient, the anticoagulant treatment at the time of blood sampling was recorded. A control group of 10 apparently healthy volunteers was included (6 females, 4 males). Each patient and volunteer was studied before and after a 10-minute venous occlusion.
u-PA were measured by radioimmunoassay (RIA).\textsuperscript{17,18} Antithrombin III (AT III) was determined by radial immunodiffusion using partigen plates (Behring, Marburg, FRG). Protein C and protein S were measured by ELISA techniques (Asserachrom Protein C and Protein S, Diagnostica Stago, France).

All postocclusion values are given after correction for the change of hematocrit (Ht) induced by venous stasis, using the correction factor \((100 - \text{Ht post})/(100 - \text{Ht pre})\). For each patient, the body mass index, weight \((\text{kg})/\text{height squared} \,(\text{m}^2)\), was calculated.\textsuperscript{19} Differences between sets of data were determined by the Mann-Whitney rank-sum test (nonpaired data) and the Wilcoxon signed-rank test (paired data), and differences between the means of the body mass index were determined by Student's \(t\) test.

**RESULTS**

*Effect of Venous Occlusion on PAI Activity in Patients With Thrombotic Disease*

Figure 1 shows the distribution of PAI activity values in 83 patients before and after 10 minutes of venous occlusion. Venous stasis resulted in a significant \((P < .001, \text{Wilcoxon test})\) decrease of PAI activity from a median of 8.2 to 2.9 U/mL. In contrast, PAI-I antigen showed an increase from a median of 16.0 to 19.2 ng/mL \((P < .001)\), after correction for hemococoncentration. The different effect of venous stasis on PAI activity and PAI-I antibody is explained by a twofold increase of t-PA antigen, resulting in a complete depletion of PAI activity in 38 of 83 patients (46%) and a partial depletion in 43 of 83 patients (52%). In two patients, PAI activity increased after stasis (from 6 to 7.3 U/mL and from 6.7 to 8.9 U/mL).

The euglobulin fibrinolytic activity at rest was undetectable in most patients, and increased to a median of 2.5 U/mL, which represents the detection limit in our assay system, we investigated the usefulness of residual PAI activity as a criterion for a bad response to venous occlusion. To this end, on the basis of PAI activity measurements after stasis, patients were arbitrarily divided into good responders, group G, \(n = 38\) (PAI activity poststasis \(\leq 1.0\) U/mL), and bad responders, group B, \(n = 45\) (PAI activity poststasis \(> 1.0\) U/mL).

The two patient groups were comparable with regard to anticoagulant treatment, age, age of first thromboembolic episode, recurrence rate, positive family history, sex ratio, and hematocrit changes during venous stasis; however, patients of group B were significantly overweight as judged by the body mass index (Tables 1 and 2). Six patients had deficiencies in AT III, protein C, and protein S (Table 2).

*Comparison of Fibrinolytic Parameters in Good and Bad Responders*

Fibrinolytic activity after stasis. Fibrinolytic activity after stasis as measured by three different techniques (ECLT, DWBCLT, EFA), was significantly higher in the control group and in group G than in group B (Table 3). In group B after venous stasis, residual PAI activity correlated with the lysis times as measured by ECLT \((r = 0.75, P < .001)\), and DWBCLT \((r = 0.64, P < .001)\), but was not correlated with fibrinolytic activity on fibrin plates \((r = -0.07)\). Approximately 90% of patients with residual PAI activity had abnormally long (>90 minutes) ECLT and DWBCLT.

**Table 1. Comparison of Control Group and Good (G) and Bad (B) Responder Patients for Age, Sex Ratio, and Ht Change During Venous Occlusion Test**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Control</th>
<th>Group G</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>38</td>
<td>45</td>
</tr>
<tr>
<td>Age (yr; mean and range)</td>
<td>36.5</td>
<td>40.1</td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>(22-60)</td>
<td>(21-74)</td>
<td>(20-69)</td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>6</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Correction factor due to Ht change</td>
<td>0.86</td>
<td>0.86</td>
<td>0.88</td>
</tr>
</tbody>
</table>

**Table 2. Comparison of Main Clinical Parameters Between Good (G) and Bad (B) Responder Patients**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Group G</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average age at first thromboembolic episode (yr)</td>
<td>33.2</td>
<td>32.8</td>
</tr>
<tr>
<td>Average delay between last acute thromboembolic episode and analysis (mo)</td>
<td>37</td>
<td>39</td>
</tr>
<tr>
<td>Recurrence rate (%)</td>
<td>45</td>
<td>50</td>
</tr>
<tr>
<td>Positive family history (%)</td>
<td>45</td>
<td>49</td>
</tr>
<tr>
<td>Deficiencies*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT III</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Protein C</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Protein S</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Treatment at time of sampling*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Anti-vitamin K</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>No treatment</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Body mass index (%)†</td>
<td>23.6 ± 4.2</td>
<td>26.5 ± 6.9</td>
</tr>
</tbody>
</table>

*Number of patients.
†Difference of group G v group B: \(P < .05\).
Correction for the presence of PAI-I complexed to t-PA must control group or the good responder group (Table 3). Because circulating t-PA is mainly bound to PAI-I, a ratio of PAI activity and free PAI-I antigen. Estimation of Specific Activity of PAI-I in Plasma

The specific activity of PAI-I in plasma was estimated from the ratio of PAI activity and free PAI-I antigen. Because circulating t-PA is mainly bound to PAI-I, a correction for the presence of PAI-I complexed to t-PA must be made. Assuming a mol wt for t-PA of 67,000 and a mol wt for PAI-I of 52,000, the concentration of t-PA-bound PAI-I equals the concentration of t-PA multiplied by 52/67. Therefore, (free PAI-I) = (total PAI-I) − (t-PA × 52/67) mg/mL. After correction for t-PA-bound PAI-I, and assuming a specific activity for t-PA of 500,000 IU/mg, the median PAI-I specific activity was calculated to be 880,000 U/mg, a value close to the theoretical value of 650,000 U/mg (ie, 500,000 × 67/52).

Table 4. Differences in t-PA Release in Bad Responders. Characterized by High or Low Basal PAI Activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal PAI Activity (U/mL)</th>
<th>t-PA Antigen Release (ng/mL, median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 38</td>
<td>1.2-17.2 (4.8)</td>
<td>23.2 ± 26.8 (14.5)</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 15</td>
<td>15.7-45.0 (23.4)</td>
<td>20.0 ± 20.8 (17.2)</td>
</tr>
<tr>
<td>n = 15</td>
<td>9.9-15.0 (11.2)</td>
<td>12.2 ± 20.7 (5.9)</td>
</tr>
<tr>
<td>n = 15</td>
<td>3.7-9.0 (6.7)</td>
<td>7.6 ± 3.9* (9.8)</td>
</tr>
</tbody>
</table>

*P < .001. P values were determined with respect to good responders. t-PA antigen release was determined in good responders and in bad responders. The bad responder group was divided into three groups of equal number according to basal PAI activity. Median values are given in parentheses.

Discussion

Deficiencies of the fibrinolytic system are a common finding in patients with a history of thromboembolism. Because fibrinolytic activity is dependent upon numerous profibrinolytic and antifibrinolytic factors, the mechanism of hypofibrinolysis is not well defined at present. For more specific definition of defective fibrinolysis, assay systems that may guide the clinician to diagnose a specific defect must be available.

Recent work has suggested that the poor fibrinolytic
response to venous stasis, found in approximately one third of patients with a history of thromboembolic disease, may be owing to either an excess of PAI-1 or an impaired release of t-PA.\textsuperscript{14} In these studies, however, criteria for defining a poor fibrinolytic response were either absent or based on arbitrary values of fibrinolytic activity after venous stasis. Therefore, the inability of released t-PA to overcome the inhibitory potential of PAI-1 appears to be a more appropriate criterion for poor fibrinolytic response after various stimuli. In the present study, this was defined as a residual PAI activity \(< 1.0\) U/mL, ie, the detection limit for PAI activity in our system, after a 10-minute venous stasis. By using this criterion, we classified patients into two groups, good responders (46\%) and bad responders (54\%). The mean values of the euglobulin fibrinolytic activity in good responders, after venous stasis, were tenfold higher than in bad responders (11.2 and 1.1 U/mL, respectively) and were not significantly different from the control group (8.3 U/mL).

The implication of PAI-1 in an impaired fibrinolytic response is evidenced by the significantly higher basal PAI activity and PAI-1 antigen in bad responders than in good responders. Strikingly, prestasis values of PAI-1 antigen in bad responders were also significantly higher. This finding confirms a previous observation on a correlation between basal t-PA and PAI-1.\textsuperscript{15} The increase in t-PA antigen after stasis in bad responders, however, was significantly lower than in good responders. In our patients, bad responders were overweight, whereas good responders were of almost normal weight. A positive correlation between PAI activity and the body mass index in normal subjects, as well as in obese women was reported previously.\textsuperscript{16} Whether weight normalization in thrombotic patients would reduce PAI-1 concentrations and contribute to a decreased risk of thrombosis is not known at present.

A significantly lower basal u-PA concentration was noted in the good responder group as compared with the control group and the bad responder group. Because PAI activity and t-PA antigen release are normal in the good responder group, the question of whether a low basal u-PA concentration may have contributed to a thrombotic tendency in some of these patients and thus constitute a risk factor for thrombotic disease may be raised.

From PAI activity, PAI-1 antigen, and t-PA determinations, we were able to estimate the specific activity of free PAI-1 (ie, not bound to t-PA). The value of 880,000 U/mg, close to the theoretical value of 650,000 U/mg, suggests that in human plasma PAI-1 is mainly in its active form or bound to t-PA. Our result is in agreement with those of Urdén and co-workers who calculated a mean specific activity for PAI-1 of \(~ 800,000\) U/mg.\textsuperscript{21} This result is also in agreement with PAI-1 being secreted in an active form and having a short in vivo half-life (7 minutes), a period too short for "spontaneous" inactivation (half-life 1 to 2 hours) to play a significant role.\textsuperscript{22,23}

In the present study, the usefulness of residual PAI activity after venous stasis as a criterion yielded a classification comparable to that using clot lysis time assays. However, residual PAI activity more closely reflects the change in the balance of t-PA/PAI-1 during venous occlusion, and suggests that a follow-up study be made to establish whether the defect is due to a high basal PAI-1 or a low release of t-PA. The identification in an individual patient of the cause of poor fibrinolytic response may serve to adapt the treatment, eg, weight reduction in obese patients having high PAI-1.

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