Quantitation of Venous Clot Lysis With the d-Dimer Immunoassay During Fibrinolytic Therapy Requires Correction for Soluble Fibrin Degradation

Benjamin Brenner, MD, Charles W. Francis, MD, Saara Totterman, MD, Craig M. Kessler, MD, A. Koneti Rao, MD, Ronald Rubin, MD, Hau C. Kwaan, MD, K. Ruben Gabriel, PhD, and Victor J. Marder

Plasma cross-linked fibrin-degradation products were analyzed using a d-dimer (DD) immunoassay in patients with deep vein thrombosis (DVT) or acute myocardial infarction (MI) treated with fibrinolytic therapy, and the results were correlated with clot lysis documented angiographically. In 13 patients with DVT, the mean DD concentration increased 10-fold (1,074±252 to 10,333±1,004 ng/ml) during therapy, but neither the peak level nor the DD concentration integrated over the course of therapy correlated with clot lysis. Since plasma DD can derive from degradation of soluble plasma fibrin as well as from thrombi, the contribution of the former was estimated by in vitro incubation of the pretreatment plasma with plasminogen activator. Subtraction of this value from the measured posttreatment DD concentration provided a “corrected” level that represented DD originating from lysis of thrombi. This modification resulted in improved correlation of DD levels with clot lysis. The mean corrected peak DD was higher in patients with successful thrombolysis (8,780±1,352 ng/ml) compared with patients without lysis (3,075±589 ng/ml, p<0.001). There was a moderate correlation between the volume of clot lysed and the corrected peak DD (r=0.62) and a higher correlation with the corrected DD integrated over the course of treatment (r=0.97). By contrast, the corrected DD concentrations were near zero in patients treated for MI with or without thrombolytic reperfusion, suggesting that fibrin in small coronary thrombi did not contribute significantly to total plasma DD during therapy. These findings indicate that the elevation in cross-linked fibrin-degradation products during fibrinolytic therapy results from degradation of soluble fibrin as well as from lysis of thrombi. Adjustment of plasma DD concentrations for the contribution from degradation of soluble fibrin offers an approach to noninvasive monitoring of venous clot lysis during fibrinolytic therapy. (Circulation 1990;81:1818–1825)

Venographic assessment of therapeutic response in patients with deep vein thrombosis (DVT) treated with thrombolytic therapy is accurate and quantitative, but its invasive nature makes it unsuitable for routine follow-up. Noninvasive approaches rely on clinical observation or evaluation of venous flow using plethysmography or Doppler ultrasound but may be inaccurate. Clinical changes such as symptomatic improvement or reduction in leg swelling are nonspecific, often reflecting the effects of bed rest, leg elevation, and analgesics rather than a reduction in thrombus size. Venous flow measurements may be insensitive to lysis of calf vein thrombi or incomplete resolution of proximal leg vein clots. A potential alternative
approach to monitoring clot lysis is by measurement of soluble products of fibrin lysis such as D-dimer (DD) in the blood. Because the fibrin matrix of thrombi is cross-linked by factor XIII, plasmin liberates soluble degradation products that are structurally distinct from fibrinogen degradation products and could potentially be used as markers of clot lysis. Application of the DD assay to patients with DVT, pulmonary embolism, or myocardial infarction has shown elevated concentrations at presentation with further elevations during fibrinolytic therapy. However, there has been poor correlation between DD concentration and angiographic improvement, suggesting that circulating cross-linked fibrin-degradation products derived from thrombus dissolution may be masked by lysis of fibrin from other sources.

We have previously suggested that soluble cross-linked fibrin polymers may represent an important source of DD during fibrinolytic therapy. Soluble fibrin containing γ-chain cross-links is found in normal subjects and in increased concentrations in patients presenting with acute myocardial infarction. These fibrin polymers degrade during fibrinolytic therapy and may thereby contribute to elevated DD levels. In the present study, we evaluated the contribution of soluble cross-linked fibrin polymers to DD elevations in patients treated with fibrinolytic therapy for DVT or myocardial infarction. The results indicate that soluble fibrin is an important source of DD during therapy and that analysis of DD levels after adjustment for soluble fibrin degradation may represent a useful approach to noninvasive monitoring of clot lysis.

Methods

Patients

Patients with DVT included nine men and four women with symptoms of venous thrombosis for less than 7 days who were enrolled in an open, rising-dose, safety, and dose-ranging study of recombinant tissue-type plasminogen activator (rt-PA) (Smith, Kline & French, Philadelphia). Men and postmenopausal or surgically sterilized women between the ages 18 and 75 years were eligible for enrollment if they had a venographically demonstrated DVT involving popliteal, femoral, iliac, or axillary veins. Patients were excluded if they had surgery within 10 days, severe hypertension, abnormal renal function precluding contrast venography, recent gastrointestinal bleeding, significant central nervous system vascular disease, or abnormal hemostasis. After obtaining informed consent, treatment was begun with rt-PA at 4 µg/kg/min i.v. during 4 hours (five patients), 22 hours (three patients), or 34 hours (five patients). The total rt-PA dose was 55±3 mg for the short, 121±8 mg for the intermediate, and 181±11 mg for the long regimen. Plasma fibrinogen concentrations were monitored during therapy, and intravenous heparin administered concurrently with rt-PA in a dose to prolong the aPTT to twice control values if the fibrinogen concentration did not fall by 25% or more.

The 27 patients with myocardial infarction were part of a randomized double-blind trial of 373 patients comparing therapy with intravenous APSAC (Beecham Laboratories, Bristol, Tennessee) to intravenous streptokinase (Hoechst-Roussel, Somerville, New Jersey). Requirements for enrollment included continuous ischemic chest pain for at least 20 minutes beginning less than 4 hours from beginning therapy, ST elevation of 0.1 mV or more in one or more standard leads, or 0.2 mV in precordial leads and age less than 75 years. Patients were excluded if they had an excessive risk of bleeding, cardiogenic shock, streptokinase therapy within the previous 6 months, prosthetic valve, or dilated cardiomyopathy. Women of child-bearing potential were also excluded. After giving informed consent, patients were treated with either 30 units APSAC i.v. given by bolus injection during 2–5 minutes or with 1.5×106 units streptokinase by continuous intravenous infusion during 60 minutes. Heparin was administered as an intravenous bolus of 5,000–10,000 units before catheter insertion. The subset of patients analyzed in this study were chosen randomly from those who had adequate blood sampling and angiography.

Venography and Angiography

Ascending venography was performed before and 6–16 hours after termination of the rt-PA infusion. All venograms were evaluated by one radiologist (S.T.) without knowledge of the DD assay results. Venous clots were diagnosed when a constant intraluminal filling defect was present in more than one projection or when the contrast media did not opacify the deep venous system above the knee despite adequate venographic technique. Clot lysis was quantitated by comparison of the pretreatment and posttreatment venograms by a modification of the technique of Marder et al., in which the volume (ml) of lyed clot was calculated by multiplying the cross-sectional area of the recanalized vessel by its length. Coronary artery patency was determined by angiography at 90–240 minutes after the start of fibrinolytic therapy using the Sones or Judkins technique to determine the degree of stenosis and patency of the infarct-related vessel. The flow was graded according to TIMI criteria, in which grade 0 or 1 was considered occlusion and grade 2 or 3 was termed “patency.”

Blood Samples

Venous blood samples from DVT patients were obtained with a 19-gauge needle before and 1, 2, 6, 12, 36, 48, and 72 hours after starting therapy; samples from patients with acute myocardial infarction were obtained before and 90 minutes after the start of fibrinolytic therapy. Samples were collected into sodium citrate (0.4% final concentration) without inhibitor or with aprotinin (Mobay Chemical, New York) (200 KIU/ml), placed immediately on ice, centrifuged within 1 hour at 3,000 rpm for 20 minutes, and
frozen at $-35^\circ$ C. Citrate plasma samples with aprotinin were used for all studies unless otherwise specified.

**In Vitro Digests**

Pretreatment citrate plasma samples without inhibitor were incubated with 200 $\mu$g/ml rt-PA (100,000 IU/mg) (Burroughs Wellcome, Research Triangle Park, North Carolina) for 20 minutes at $37^\circ$ C, after which further degradation was inhibited by addition of aprotinin to 300 KIU/ml and immediate freezing at $-70^\circ$ C.

**DD ELISA**

Cross-linked fibrin-degradation products were measured with an ELISA (Dimertest, American Diagnostica, Greenwich, Connecticut) using plates precoated with monoclonal antibody DD/3B6. Results were calculated using a standard curve prepared with purified DD (provided by the manufacturer) at concentrations from 40 to 5,000 ng/ml. Time-integrated DD values were expressed in arbitrary graph units by plotting DD concentrations versus time on arithmetic graph paper, including all measurements from the beginning of therapy to the second venogram.

**Statistical Analysis**

Student’s $t$ test and linear regression analysis were performed with standard computer software. Classification methods were based on normal distribution theory. For classification of patients into groups with or without lysis, a cutoff between groups was chosen midway between the sample means of patients with lysis or without lysis on a $\ln (x+0.5)$ scale. Estimates were corrected for overoptimism, and confidence intervals were calculated by bootstrap methods. A total of 1,000 subsamples (with replacement) of 13 of the 13 patients were used, recalculating the classification characteristics for each subsample and using these results to obtain the sampling distribution of the estimated characteristics.

**Results**

Pretreatment DD concentrations were increased in the DVT patients ($1,074\pm252$ ng/ml) compared with normal ambulatory adults ($75\pm3$ ng/ml). After initiation of rt-PA therapy, plasma DD levels rose rapidly to $6,112\pm1,001$ ng/ml at 2 hours, then more slowly during the next 4 hours to a mean of $10,333\pm1,004$ ng/ml at 6 hours. After rt-PA was stopped, DD concentrations gradually decreased to $5,314\pm1,293$ ng/ml at 48 hours and eventually reached the pretreatment levels at 72 hours.

Peak DD concentrations observed during rt-PA therapy ranged from 7,000 to 20,000 ng/ml (Table 1). To obtain an estimate of the contribution of soluble fibrin to DD levels during therapy, the pretreatment plasma samples were incubated in vitro with rt-PA. This resulted in an approximately sixfold increase in DD levels to a mean of $7,030\pm986$ ng/ml. For each plasma sample, a corrected DD was calculated by subtracting the "induced" DD concentration of the in vitro digest of the pretreatment sample (representing lysis of soluble plasma fibrin) from the DD concentration measured in the postthrombotic therapy samples (Table 1). To adjust for different durations of therapy, an integrated DD was calculated as the area under the curve connecting the DD concentrations measured from the beginning of therapy until the posttreatment venogram. To correct for the contribution of soluble fibrin degradation, the integrated area for each patient was divided by a horizontal line at the level of "induced" DD concentration. The corrected integrated area was obtained.

**Table 1. Observed and “Corrected” Plasma D-Dimer Concentrations After Fibrinolytic Therapy**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Volume of lyst clot (ml)</th>
<th>Peak concentration (ng/ml)</th>
<th>Total integrated amount (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Induced</td>
<td>Corrected</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>13,500</td>
<td>8,250</td>
</tr>
<tr>
<td>2</td>
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<td>10,000</td>
<td>8,050</td>
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<tr>
<td>3</td>
<td>30</td>
<td>20,000</td>
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<tr>
<td>4</td>
<td>4</td>
<td>11,500</td>
<td>9,000</td>
</tr>
<tr>
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<td>4</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>0</td>
<td>11,000</td>
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<td>11,000</td>
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</tr>
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<td>15</td>
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<tr>
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<td>3,200</td>
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</table>

Peak observed D-dimer (DD) concentrations noted during therapy with recombinant tissue-type plasminogen activator (rt-PA). "Induced" DD concentration represents the total amount measured after in vitro incubation of pretreatment plasma with rt-PA, including that amount detected before rt-PA incubation (1,074±252 ng/ml) (see "Methods"). "Corrected" concentrations of peak DD are obtained by subtracting the induced values from observed peak values, and corrected integrated DD values are obtained as indicated in Figure 1 and the text.
by subtraction of the area below the line, representing the contribution from soluble fibrin lysis, from the total integrated area (Figure 1).

After therapy, repeat venograms showed substantial response in five patients, with lysis of 15–45 ml of clot, minimal lysis in three patients (3–4 ml), and no lysis in five patients (Table 1). Peak and integrated DD levels before and after correction for soluble fibrin lysis were compared in patients with substantial, minimal, or no clot lysis (Figure 2). Measured peak DD levels did not differ in patients with or without lysis (Figure 2A, left). However, after correction for the contribution of soluble fibrin degradation, the DD concentrations were higher in patients with lysis compared with patients with minimal or no lysis (8,780±1,352 vs. 3,075±589 ng/ml; p<0.001) (Figure 2A, right). The time-integrated DD levels were higher in patients with substantial lysis, but values overlapped with those of patients whose thrombi did not lyse (Figure 2B, left), and mean values were not significantly different (43.6±6.3 vs. 22±6.7 units; p=0.06). However, the difference was more striking when the corrected time-integrated DD values were compared (24.7±5.7 vs. 2.2±0.5; p<0.0005) (Figure 2B, right). All five patients with lysis had a corrected value of 9.5 or more, and all eight without lysis had a value of 4.5 or less, suggesting that the corrected integrated DD could be used to classify patients into those with or without substantial clot lysis. Using an integrated corrected DD of 6.6 as a cutoff between patients with or without substantial lysis, statistical analysis suggests a median estimate of 0.055 for the probability of misclassification, with a 95% confidence interval of 0.00–0.08, indicating a high degree of confidence that no more than 8% of patients would be misclassified.

Correlations of DD concentrations with each patient’s venographic response (ml of lysed clot) are shown in Figure 3. While there was no correlation between measured peak DD concentration and clot lysis, a moderate correlation (r=0.62) existed between the corrected peak DD value and volume of lysed clot (Figure 3A). Similarly, the correlation demonstrated between the measured time-integrated DD and clot lysis (r=0.47) was considerably improved after correction for DD derived from soluble fibrin degradation (r=0.97) (Figure 3B).
For comparison, a similar analysis was performed on samples obtained from 27 patients with myocardial infarction who received fibrinolytic therapy. Of this group, 21 showed substantial patency of the infarct-related artery (TIMI grade 2 or 3) at 2.4±0.9 (±SD) hours after treatment, whereas six still showed vascular occlusion (grade 0 or 1). The corrected postinfusion DD concentration in patients with (−120±134 ng/ml) or without (−213±413 ng/ml) patent coronary arteries did not differ significantly. Both were close to zero, indicating that there was no significant increase in DD resulting from lysis of the small coronary artery thrombus (Figure 4).

Discussion

These results demonstrate a correlation between the amount of venous thrombus lysed during thrombolytic treatment and the increase in plasma concentra-
The pretreatment sample of each patient's plasma was incubated with rt-PA in vitro to determine the amount of DD that could derive from soluble fibrin. The high rt-PA concentration (200 μg/ml) used to treat pretreatment plasma results in maximum elevation of DD concentration in vitro, with preferential degradation of soluble fibrin polymers compared to fibrinogen. The DD potentially derived from soluble fibrin was subtracted from the measured DD concentration in posttreatment plasma samples to provide corrected DD concentrations (Figure 1). Elevated corrected DD levels were interpreted as deriving from lysis of thrombi and were correlated with angiographic findings. Three technical considerations impact on this approach. The first concerns the specificity of the DD assay for cross-linked fibrin-degradation products rather than fibrinogen derivatives. The reaction of monoclonal antibody DD3B6 with fibrinogen fragment D in Western blots has raised questions about its specificity for cross-linked fibrin derivatives. Also, the panspecific antifibrinogen secondary antibody could theoretically react with fibrinogen-degradation products captured in association with cross-linked fibrin derivatives such as DD. However, no appreciable crossreactivity with fibrinogen degradation products has been found with the ELISA or latex agglutination assays. Second, the formation of soluble cross-linked fibrin polymers during storage would increase the in vitro correction and mask DD derived from thrombolysis. If this occurred, the corrected levels in patients without clot lysis should be negative; this was not seen in patients with DVT, and values in patients with myocardial infarction were within experimental error of zero. Last, fibrinolysis occurring in vitro would also interfere with the results, causing excessively elevated DD levels, but the use of aprotinin effectively inhibits plasmin action after sample collection.

Several studies have reported an increase in plasma DD in patients with DVT, perhaps reflecting physiological thrombolysis. Mirshahi et al. found increased plasma concentrations at presentation but no correlation with extent of thrombosis shown venographically. They further observed a decrease in concentration during heparin therapy in patients whose follow-up venogram showed a decrease in thrombus size but persistently high levels in patients with little or no venographic resolution. Elevated concentrations have also been reported at presentation with pulmonary embolism, and Goldhaber et al. found that a DD level of more than 145 ng/ml had a diagnostic sensitivity of 89% but a specificity of only 44%. Limited diagnostic specificity was also found by Greenberg et al. who found elevations of plasma DD in hospitalized patients with numerous conditions including arterial and venous thrombosis, septicemia, carcinoma, and the postoperative state.

Greater elevations in DD levels have been found during fibrinolytic therapy, with concentrations increasing fivefold to 25-fold compared with pretreatment values during therapy for myocardial infarction, DVT, or pulmonary embolism. Despite the consistent increase during lytic therapy, no correlation between the degree of thrombolysis and the elevation in DD levels has been found during treatment for myocardial infarction or DVT. A potential explanation that has been proposed for the lack of correlation is the presence of multiple sources of fibrin that may degrade during therapy. For example, patients with myocardial infarction may have concurrent DVT, left ventricular mural thrombi, and fibrin on atherosclerotic plaques, while patients with DVT may also have clinically unsuspected pulmonary emboli. Whatever the extraneous source of fibrin, it has been of sufficient quantity to obscure the specific detection of thrombus-derived DD, thereby limiting the application of this assay for noninvasive quantitation of therapeutic thrombolysis.
These results support the hypothesis that a major source of cross-linked fibrin-degradation products during fibrinolytic therapy is soluble plasma fibrin. Electrophoretic analyses in normal subjects indicates that approximately 0.8% of total fibrinogen is present as cross-linked fibrin dimer and that this percentage is increased more than fourfold in patients with myocardial infarction. After incubation of plasma with rt-PA in vitro, these polymers are degraded in a manner and extent similar to that induced by fibrinolytic therapy, associated with an increase in DD reactivity from a mean of 1.07±252 to 7.03±986 ng/ml (Table 1). Considering that 29% of cross-linked fibrin dimer is DD, this increase in immuno-reactivity would reflect degradation of approximately 23 µg/ml of fibrin dimer, representing 0.6% of an average plasma fibrinogen concentration (3.5 mg/ml). The DD concentration after in vitro incubation with rt-PA (Table 1) represents the sum of DD present in the plasma before treatment plus that produced by degradation of soluble fibrin. Higher concentrations during therapy may therefore result from lysis of fibrin in thrombi, and this was observed with successful treatment of DVT with a good correlation between the degree of elevation and extent of lysis. DD levels during treatment for myocardial infarction were close to those resulting from digestion of the pretreatment plasma in vitro, suggesting that DD elevations during therapy resulted from degradation of soluble plasma fibrin and consistent with the small size of a coronary thrombus in comparison to a leg vein DVT.

While this method corrects for the contribution of soluble fibrin to elevated plasma DD concentrations during fibrinolytic therapy, there may be additional intravascular or extravascular sites of fibrin deposition. However, the finding of a strong correlation between the DD levels and quantitative venous clot lysis after correction for soluble fibrin degradation suggests that other fibrin deposits do not contribute significantly to the results. Furthermore, the net corrected DD value of close to zero in patients with myocardial infarction argues against the presence of unknown, noncoronary thrombi in these patients. An improved correlation may be possible by mathematically correcting the time-integrated values for clearance of DD from the blood, but this approach is not yet feasible because a multitude of degradation products are present and little information is available on clearance rates during thrombolytic therapy.

This approach offers promise for more accurate noninvasive monitoring of lytic therapy for venous thrombosis, a possibility that would require assessment by a prospective study. If DD levels measured during therapy do not increase above the in vitro digest level of the pretreatment sample, our analysis would suggest that little or no lysis is occurring. On the other hand, if concentrations do exceed the in vitro digest level, the degree of elevation should correlate with the amount of clot lysis. Potentially, the course of therapy over time could be followed by measuring serial DD values and predicting continued clot lysis if the in vivo level remained above that of the in vitro digest, thereby guiding the duration of therapy.

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References


**KEY WORDS** • venous thrombosis • thrombolysis • fibrin • tissue plasminogen activator
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