**New Methods**

**Characterization In Vivo of the Fibrin Specificity of Activators of the Fibrinolytic System**

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Development of appropriate clinical dose regimens of individual plasminogen activators such as tissue-type plasminogen activator (t-PA) has generally relied primarily on nonpharmacological endpoints such as angiographically documented clot lysis. The recent availability of monoclonal antibodies that differentiate products of plasmin lysis of fibrin from those of lysis of fibrinogen should permit delineation of the relative fibrin specificity of different plasminogen activators or of different doses of the same activator in vivo. Thus, their use should accelerate and facilitate development of implementation of optimal dose regimens for diverse activators and combinations of activators. The present study was designed to determine whether assay of such markers effectively differentiates effects of two doses of t-PA, each of which are comparably effective in opening infarct-related arteries, in patients studied at the Washington University Clinical Unit of the National Institutes of Health–sponsored Thrombolysis in Myocardial Infarction Trial. The extent of lysis of fibrin and of lysis of fibrinogen by plasmin resulting from administration of t-PA was evaluated in 19 patients given 150 mg t-PA over 6 hours and 17 given 100 mg over the same interval by assay of serially obtained plasma samples for crosslinked fibrin degradation products (XL-FDP) and Bβ1-42, a peptide released when fibrinogen is degraded to fragment X by plasmin. XL-FDP were markedly elevated after 6-hour infusions of both doses of t-PA. However, elevations were not more with the higher dose [peak value, 4,321 ± 986 ng/ml (± SEM)] compared with the lower dose (3,397 ± 1,096 ng/ml) (p = NS). Thus, the extent of lysis of fibrin appeared to be comparable. In contrast, lysis of fibrinogen reflected by elevated concentrations in plasma of Bβ1-42 2–4 hours after onset of infusion of t-PA was significantly more after administration of 150 mg (423 ± 100 pmol/ml) compared with 100 mg (166 ± 37 pmol/ml) (p<0.05), which is indicative of more intense fibrinogenolysis with the higher dose. Thus, serial assay of XL-FDP and Bβ1-42 differentiated the fibrin specificity of the two doses of t-PA tested and should prove useful in developing dose regimens for individual activators and combinations of activators of plasminogen with distinctive pharmacological properties. (Circulation 1988;78:592–597)

Treatment with thrombolytic agents favorably alters the prognosis of patients with acute myocardial infarction.1–3 Accordingly, potentially clot-selective fibrinolytic agents such as single-chain urokinase (scu-PA) and tissue-type plasminogen activator (t-PA) have been undergoing vigorous development.3–7 Such agents preferentially activate plasminogen bound to fibrin resulting in clot lysis with only modest activation of circulating plasminogen and, hence, only modest degradation of fibrinogen at therapeutically effective doses. High doses increase the extent of degradation of fibrinogen.8,9

Development of specific dose regimens for diverse activators and combinations of activators of plasminogen has generally been based on nonpharmacological endpoints such as angiographically documented clot lysis. However, assessment of the extent of lysis of fibrinogen compared with lysis of fibrin would be helpful in evaluating and comparing diverse doses of individual activators independent
of the confounding effects of ongoing thrombosis\textsuperscript{10} and the extent of underlying vascular stenoses\textsuperscript{11} that may affect angiographic endpoints.

We have recently used monoclonal antibodies specific for the products of the degradation of crosslinked fibrin (XL-FDP)\textsuperscript{12-14} to compare the persistence of lysis of fibrin induced by t-PA with that induced by streptokinase.\textsuperscript{15} Observations in a small number of patients suggested that higher doses of t-PA may not have augmented lysis of crosslinked fibrin. In the present study, we evaluated the usefulness of specific markers of lysis of crosslinked fibrin in assessing the response to plasminogen activators in patients studied at Washington University given two different doses of t-PA during the course of the Thrombolysis in Myocardial Infarction Trial (TIMI). This study was not undertaken to evaluate the relative efficacy of the two doses of t-PA on infarct-related artery patency. Of note, however, is that both the 100-mg and 150-mg regimens elicit lysis after 90 minutes in approximately the same percentages of infarct-related arteries judging from results in the TIMI trial as a whole.\textsuperscript{9,16} Concentrations in plasma of XL-FDP and B1\textsubscript{1}-42, a fibrinopeptide released only when fibrinogen or fibrin I is degraded to fragment X by plasmin (Figure 1),\textsuperscript{17-19} were measured in response to administration of 150 and 100 mg of t-PA given over 6 hours in decremental infusions (90, 20, 10, 10, 10, and 10 mg/hr or 60, 20, 5, 5, 5, and 5 mg/hr, respectively). Ten percent of the initial 1-hour dose was given as a bolus. The results indicate that assay of biochemical markers of lysis of fibrin compared with lysis of fibrinogen facilitates characterization of relative fibrin specificity of the two different dose regimens of t-PA and suggest that such assays will facilitate development and implementation of optimal dose regimens of diverse activators and combinations of activators of the fibrinolytic system.

Materials and Methods

Patient Selection

Patients entered into Phases IB, IC, and II of TIMI studied at the Washington University and Barnes Hospital TIMI Clinical Unit comprised the study group. Among others, inclusion criteria for entry into these phases of the TIMI trial were being less than 76 years old and having at least a 0.1-mV ST-segment elevation in two anatomically continuously leads on the admission electrocardiogram. For patients entered into TIMI IB and IC, the infusion of t-PA was initiated within 7 hours of the onset of symptoms of myocardial ischemia and immediately after coronary occlusion had been documented by angiography. For patients entered into Phase II, i.v. t-PA was administered beginning within 4 hours after the onset of symptoms. Patients in Phase II did not undergo coronary angiography before administration of t-PA. Among others, exclusion criteria for these phases of the TIMI trial\textsuperscript{7} were conventional contraindications to the administration of any thrombolytic agent.

Heparin (5,000 IU i.v.) was given to all patients studied as a bolus before infusion of t-PA followed by a continuous infusion titrated to maintain the activated partial thromboplastin time at one and one half to two times that of normal. All patients were treated with lidocaine prophylactically to prevent or attenuate malignant ventricular arrhythmias.

Acquisition of Blood Samples

Initial plasma samples assayed for crosslinked fibrin degradation products and B1\textsubscript{1}-42 were collected by venipuncture; subsequent samples were drawn through a large-bore heparin lock (18 gauge). The initial sample was obtained immediately on admission to the emergency department (usually less than 60 minutes). Samples were obtained at 4 and 6 hours after the initiation of t-PA, during the infusion. Additional samples were available in some patients 2 hours after the initiation of the infusion. Subsequent samples were collected 2 and 6-12 hours after the infusion of t-PA was completed.

Blood was drawn into collection tubes containing EDTA, aprotinin (200 KIU final concentration), and D-phenylalanyl-L-prolyl-L-arginine-chloromethyl ketone (PPACK) at final concentrations of more than 5 \textmu M, shown previously to prevent lysis of fibrinogen in vitro by t-PA and to inhibit thrombin.\textsuperscript{20-22} To ensure stability of the PPACK, the anticoagulant solution pH was 4.5. Samples for fibrinogen were collected as part of the clinical coagulation profile into citrate tubes. Blood samples were cooled rapidly to 4°C and centrifuged at 1,500g for 30 minutes. Plasma was separated and frozen at \(-20°C\) for no more than 24 hours and stored at \(-70°C\) until assayed. Samples were thawed only at the time of assay to prevent loss of activity of PPACK, except for a few samples requiring a second dilution.

Assay of Crosslinked Fibrin Degradation Products

XL-FDP were assayed by ELISA (American Diagnostics, Greenwich, Connecticut) with specific monoclonal antibodies as previously described.\textsuperscript{12-14} Briefly, a monoclonal antibody (DD3B6) specific for fibrin crosslinked between D regions (D dimer) was coated onto a 96-well microtiter plate (Immunlon 2, Dynatech). A 20-\textmu l aliquot of plasma diluted at least 1:5 was added to the microtiter plate and incubated for 1 hour. The plate was washed twice. A second horseradish peroxidase conjugated antibody, specific for an epitope in the D region of fibrinogen, was added and incubated for 1 hour. After the plate had been washed, substrate \([2,2'\text{-}azino-di(3-ethylbenzthiazoline sulfonate)]\) (ATBS, Kirkegaard and Perry, Gaithersburg, Maryland) was added and absorbance determined with an automated microtiter plate reader (Bio Rad Laboratories, Richmond, California) and the use of conventional software (MacReader, Bio Rad). Samples


were assayed in duplicate. Values were determined with reference to a concurrently run standard curve in each case. The standard for this assay is purified D dimer (MW, 190,000 kDa). Because this antibody recognizes a variety of molecular species of XL-FDP in addition to D dimer, the results are expressed in nanograms per milliliter: 190 ng/ml XL-FDP would be equivalent to 1 pmol/ml D dimer. The interassay variability was 18.6 ± 15.2% (n = 55). The interassay variability for sample values above the normal range (>300 mg/ml) was only 4.2 ± 2.8%.

Measurement of Bβ1-42 Fibrinopeptide

Bβ1-42 fibrinopeptide was measured by ELISA with a specific monoclonal antibody (New York Blood Center, New York). Plasma samples were treated with 75% ethanol (vol:vol). The peptide was recovered by vacuum centrifugation of the supernate (Savant Speedvac, Farmingdale, New York). The dried sample residue was reconstituted to the original volume in 0.15 M NaCl-0.01 M Na₂HPO₄, pH 7.4. A microtiter plate (Costar flatbottom, Bio Rad) was coated with fibrinogen. Sample, control, or standard (180 μl) was added to the plate and incubated for 30 minutes with antibody against Bβ1-42. The antibody recognizes Bβ1-42 and its carboxypeptidase degradation product but does not recognize the fibrin specific degradation product, Bβ15-42. The plate was washed twice. A second horseradish peroxidase conjugated anti-mouse antibody was added and incubated for 30 minutes. After the plate had been washed, substrate for the peroxidase reaction was added, and absorbance was determined. The amount of antibody bound was inversely proportional to the concentration of Bβ1-42 in standards. Samples were assayed in duplicate. Values were determined with reference to a concurrently run standard curve in each case. The standard was purified Bβ1-42 peptide. The assay detected 5 pmol/ml Bβ1-42. Samples with more than 30 pmol/ml were diluted with phosphate-buffered saline. Bβ1-42 was undetectable in samples from 90% of normal control subjects (n = 10). The interassay variability for this assay for samples in the abnormal range was 4.3 ± 3.87.

Statistical Analysis

Results are expressed as mean ± SEM. Log transformation was used for values of Bβ1-42 and for values of XL-FDP. Data were analyzed with a t test (two-tailed) at times previously identified to be those when marked differences in the plasma concentrations of XL-FDP and Bβ1-42 occur with different plasminogen activators.

Results

Thirty-six patients given t-PA were studied. Six TIMI Phase IIB and C patients were treated with t-PA after coronary angiography; two with 100 mg and four with 150 mg. Most of the patients (n = 30) were studied in Phase II and, therefore, did not undergo coronary angiography before being given t-PA. However, results of coronary angiography performed within 48 hours were available in 75% of these subjects. Patients given 100 mg t-PA were similar to those given 150 mg (Table 1).

Extent of Fibrinolysis Reflected by Crosslinked Fibrin Degradation Products

The concentration of XL-FDP in plasma rose promptly after the onset of infusion of t-PA (Figure

FIGURE 1. Schematic representation of the proteolysis of fibrinogen, fibrin II, and crosslinked fibrin induced by plasmin. Bβ1-42 fibrinopeptide is specific for the degradation of fibrinogen or fibrin I monomer degradation. Bβ15-42 is specific for degradation of fibrin II monomer or crosslinked fibrin. Crosslinked fibrin degradation products (XL-FDP) result from lysis of fibrin containing crosslinked D regions.
TABLE 1. Characteristics of Patients Studied

<table>
<thead>
<tr>
<th>Dose of t-PA administered</th>
<th>100 mg</th>
<th>150 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>54.5±2.7</td>
<td>60.2±2.2</td>
</tr>
<tr>
<td>Gender (men:women)</td>
<td>15:2</td>
<td>13:6</td>
</tr>
<tr>
<td>Location of infarction</td>
<td>9 INF, 8 ANT</td>
<td>12 INF, 7 ANT</td>
</tr>
<tr>
<td>Time to treatment* (hr)</td>
<td>2.6±0.3</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>Rate of recanalization</td>
<td>15:17</td>
<td>16:19</td>
</tr>
</tbody>
</table>

t-PA, tissue-type plasminogen activator; INF, inferior and inferoposterior; ANT, anterior and anterolateral.

*Interval from the onset of symptoms to initiation of infusion of t-PA.

2). Peak elevations were not seen generally until the infusion of t-PA had been completed (5–12 hours after administration). Although XL-FDP declined after 12 hours, elevations persisted for some time in all patients. There was no difference in the magnitude or temporal profile of elevation of XL-FDP in response to 100 mg compared with 150 mg of t-PA. The number of patients analyzed was small, although the maximum difference at any point was only 28% between groups for XL-FDP differences detectable with a larger sample size cannot be excluded. Elevations of XL-FDP in patients evaluated by coronary angiography before administration of t-PA were similar in response to both doses of t-PA in patients who did not undergo pretreatment angiography compared with those who did (data not shown).

Lysis of Fibrinogen

Degradation of fibrinogen to fragment X after administration of t-PA with consequent elevation of plasma Bβ1-42 was most marked during the first 4 hours after the onset of infusion of t-PA (Figure 3). After the infusion had been completed, plasma Bβ1-42 fell rapidly towards baseline consistent with the short half-life (10–20 minutes) of this peptide in plasma.18 The peak concentration of Bβ1-42 was significantly greater during the 1–4 hour sampling interval with the 150-mg dose (423.4±100.7 pmol/ml, n = 16) compared with the 100-mg dose of t-PA (166.3±37.3 pmol/ml, n = 12, p<0.01). Among the 15 patients from whom samples were obtained 2 hours after onset of administration of t-PA, values were higher after the 150-mg dose (420.2±147.0 pmol/ml, n = 10) than after the 100-mg dose (142.2±63.2 pmol/ml, n = 5, p = 0.13). There was no significant difference between concentrations of Bβ1-42 in plasma after 4 hours of infusion of t-PA at the two doses.

Plasma fibrinogen 4.2±0.3 hours after initiation of infusion of t-PA was significantly decreased compared with values before treatment (Figure 4). Although the decrease was somewhat more marked with the 150-mg compared with the 100-mg dose, the difference was not significant.

Relations Between Fibrinolysis and Fibrinogenolysis

The extent of lysis of crosslinked fibrin reflected by elevations of concentrations of XL-FDP in plasma and the extent of degradation of fibrinogen reflected by elevations of concentrations of Bβ1-42 in plasma during infusion of t-PA were not correlated (r = 0.04). Lysis of crosslinked fibrin was often marked even in the absence of elevation of the concentration in plasma of products of degradation of fibrinogen.

Discussion

The results obtained in the present study indicate that assays of XL-FDP and products of degradation of fibrinogen facilitate characterization of the fibrin specificity of disparate doses of t-PA. Judging from the pharmacodynamics of t-PA, higher doses should increase the extent of degradation of fibrinogen.23 Data reported from TIMI Phase I are consistent with this expectation.9 Measurements of fibrin and fibrinogen degradation products provide information that differs from that available with conventional nonpharmacological endpoints.

In the present study, samples for Bβ1-42 were collected in tubes containing PPACK to prevent degradation of fibrinogen or fibrin in vitro induced by t-PA in the circulating blood and, hence, in the sample.21,22 Differences between the effects of 150 and 100 mg t-PA were not evident simply from assay of fibrinogen, although there was a trend toward greater fibrinogen lysis with the higher dose. The Ellis assay for fibrinogen was used because it is less sensitive to the inhibitory effects of fibrinogen degradation products than methods that detect clottable fibrinogen. However, the Ellis assay detects large fibrinogen degradation products including fragment X.25 Others have found more extensive fibrinogen lysis after 150 mg t-PA compared with 100 mg when fibrinogen was measured with other methods.9,26 Fibrinogen concen-
tration measured by clotting rate\(^\text{26}\) is lower after 150 mg t-PA than when measured by precipitation methods.\(^\text{9}\) In the present study, fibrinogen assays may have failed to detect differences between the two doses of t-PA in part because of degradation of fibrinogen in vitro in samples used for fibrinogen analysis that were not collected with PPACK. Alternatively, detection of fragment X may have obscured differences. Assay of degradation of fibrinogen to fragment X (reflected by liberation of BB\(\text{1-42}\) into plasma) appears to circumvent such confounding effects. Because of its short half-life, assay of BB\(\text{1-42}\) permits characterization of the duration of fibrinogenolytic activity. This is not possible with measures of \(\alpha-2\)-antiplasmin because plasminogen activation may continue despite depletion of \(\alpha-2\)-antiplasmin.

The extent of degradation of fibrinogen to fragment X in our study is similar to that observed by Owen et al.\(^\text{18}\) who have shown that fragment X is the predominant product of degradation of fibrinogen early after administration of t-PA. In our study, the concentration of BB\(\text{1-42}\) in plasma after administration of t-PA was considerably less after 100 mg than that observed by Owen et al after 80 mg. The difference may be attributable to greater fibrin specificity of the predominantly single-chain preparation of t-PA used in this study compared with the predominantly two-chain preparation used in the study by Owen et al.\(^\text{5,9}\) Alternatively, the difference may have reflected differences in the antibody used to detect BB\(\text{1-42}\) in the two studies.

The assay of XL-FDP for quantification of products of lysis of fibrin used in this study depends on detection of crosslinked gamma chains within the D region of fibrin with a specific monoclonal antibody. Crosslinking is mediated by Factor XIIIa in the presence of calcium. The action of plasmin on crosslinked fibrin produces a variety of degradation products ranging from the smallest product, D dimer, to very large crosslinked fibrin polymers.\(^\text{27-29}\) Thus, the XL-FDP measured in our study compose diverse molecular species. The half-life of XL-FDP in the circulation is unknown though likely to be at least 6 hours judging from the half-life of fragment D. Thus, it is difficult to extrapolate from plasma concentrations of XL-FDP the quantity of clot lysed. In addition, XL-FDP may not be totally specific indexes of clot lysis because some crosslinked fibrin polymers may circulate in the plasma of patients with acute myocardial infarction.\(^\text{30,31}\) If D dimer was the predominant XL-FDP measured, the peak plasma concentrations (approximately 4,000 ng/ml) we observed would be consistent with the degradation of 50 nmol of crosslinked fibrin, assuming a plasma volume of distribution of 2.5 l. This is consistent with detection of lysis of a coronary thrombus. Modest elevation of XL-FDP (<1.700 \(\mu\)g/ml) may be observed after administration of t-PA in the absence of thrombosis.\(^\text{32}\) Nonetheless, assays of lysis of crosslinked fibrin are likely to provide useful criteria reflecting the magnitude of lysis of fibrin induced by t-PA. Because no augmentation of lysis of fibrin was observed with the higher dose of t-PA though products of lysis of fibrinogen were increased, our data are consistent with the appropriateness of the decision that had been made for entirely different reasons to decrease the dose in the TIMI trial.\(^\text{33}\)

Judging from our results and from previous observations, lysis of fibrin persists for many hours after administration of t-PA despite rapid clearance of the activator from the circulation.\(^\text{15,34}\) Our results suggest that measurement of crosslinked fibrin degradation products and of specific products of degradation of fibrinogen such as BB\(\text{1-42}\) will be useful in the pharmacological characterization of specific dose regimens of individual activators and combinations of activators of plasminogen and that comparison of different activators with respect to relative lysis of fibrin and of fibrinogen will be facilitated.

References


Key Words • fibrinolysis • thrombolysis • myocardial infarction • t-PA • fibrinogen degradation products
Characterization in vivo of the fibrin specificity of activators of the fibrinolytic system.

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_Circulation_. 1988;78:592-597
doi: 10.1161/01.CIR.78.3.592

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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