Fibrin Fragment D-Dimer and Fibrinogen Bβ Peptides in Plasma as Markers of Clot Lysis During Thrombolytic Therapy in Acute Myocardial Infarction

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The validity of markers in plasma of in vitro thrombolysis was investigated in 12 patients with extensive fibrinogen breakdown (>80%, group 1) and in 12 patients with minimal breakdown (<20%, group 2). The patients were treated with 100 mg of recombinant tissue-type plasminogen activator (rt-PA) in the "Thrombolysis in Myocardial Infarction II" (TIMI II) trial. Cross-linked fibrin degradation product levels were measured with two variant enzyme-linked immunosorbent assays (ELISAs), both using a fibrin fragment D-dimer specific capture antibody. In one instance, a tag antibody was used that cross-reacts with fibrinogen (pan-specific tag ELISA); in the other, the tag antibody was specific for fibrin fragment D (fibrin-specific tag ELISA). Apparent concentrations of cross-linked fibrin degradation products at baseline were within normal limits with both assays in most patients. At 8 hours after rt-PA infusion, the measured cross-linked fibrin degradation products were increased about twofold to fourfold in group 2 with both assays. However, in group 1, levels were significantly higher with the pan-specific tag ELISA (5.8 ± 4.2 μg/mL) compared with the fibrin-specific tag ELISA (1.5 ± 1.3 μg/mL). This observation was most likely a result of detection of fibrinogen degradation products in the pan-specific ELISA. Apparent levels of fibrinopeptide Bβ1-42, a marker of fragment X formation, increased during thrombolysis from 4.2 ± 2.8 pmol/mL to 2,000 ± 230 pmol/mL in group 1 and from 4.1 ± 2.1 pmol/mL to 300 ± 43 pmol/mL in group 2, and were correlated significantly with the extent of fibrinogen breakdown ($r = -0.8$). Fibrinopeptide β15-42 levels increased from 4.3 ± 3 pmol/mL to 70 ± 19 pmol/mL in group 1, but did not increase in group 2. The apparent increase in group 1 could be explained by cross-reactivity of fibrinopeptide Bβ1-42 in the fibrinopeptide β15-42 assay. We conclude that cross-linked fibrin degradation product levels as measured with a pan-specific tag ELISA and fibrinopeptide β15-42 levels as measured with certain monoclonal antibody-based ELISA are influenced by the extent of fibrinogen degradation. Fibrinopeptide Bβ1-42 is a marker specific for fibrinogen breakdown. Cross-linked fibrin degradation product levels, measured with a fibrin-specific tag ELISA, appear to be markers specific for thrombolysis. Consequently, assays similar to the fibrin-specific tag ELISA may provide more accurate information when correlated with clinical endpoints.

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LABORATORY ASSESSMENTS of the effects of thrombolytic therapy in acute myocardial infarction (AMI) have largely focused on the intensity and duration of the plasmin-induced coagulation defect in plasma, the so-called "lytic state." Most studies have evaluated levels of fibrinogen, fibrin(ogen) degradation products (FDPs), plasminogen, and in some cases α-2-antiplasmin.[1-12] In the case of streptokinase therapy, this approach reflected the understanding that therapeutic efficacy was directly related to the generation of the lytic state.[1,4] The advent of more fibrin-selective fibrinolytic agents, such as recombinant tissue-type plasminogen activator (rt-PA) and single-chain urokinase-type plasminogen activator (scu-PA), promised effective thrombolysis with less generalized plasminogen activation and coagulation breakdown.[3,5] Indeed, rt-PA has proven to be an effective thrombolytic agent in numerous studies, but still promotes a variable, dose-related degree of fibrinogen breakdown.[6-8,11] At the 150-mg dose used in the early phase of the "Thrombosis in Myocardial Infarction II" (TIMI II) trial and in the TAMI I trials, up to one third of the patients had nadir fibrinogen levels less than 50 mg/dL, whereas, at the presently approved dose of 100 mg, only 5% of patients had nadir fibrinogen levels in this range.[7,10,11] TIMI I, TIMI II, and the TAMI trials have all demonstrated a weak but highly significant association between bleeding complications and the intensity of plasmin-mediated fibrinogen breakdown.[8,9,11] By contrast, cardiac endpoints, except for reduced risk of coronary reocclusion, have shown little correlation with alterations of hemostatic parameters.[6]

The balance between thrombin activity and plasmin activity is an important factor in the dynamic process regulating clot formation and dissolution.[13] Recently, a number of studies have addressed this balance in the setting of thrombolytic therapy for AMI. These studies have applied assays for specific proteolytic products of thrombin and plasmin[14-18] with the intent of evaluating their clinical utility as laboratory markers.[18-28] Figure 1 illustrates the markers of plasmin cleavage of fibrinogen and fibrin that are specifically determined in these assays.

Elms et al[17] have developed a sandwich enzyme-linked immunosorbent assay (ELISA) for cross-linked fibrin degradation products based on capture by a monoclonal antibody (MoAb) directed against a neoantigen on the γ-γ cross-link in fragment D-dimer. The signal antibody is directed against the fragment D domain and is not fibrin-specific per se (pan-specific tag ELISA). In contrast, Declerck et al[18] have developed a cross-linked fibrin degradation product sandwich ELISA based on a fibrin fragment D-dimer specific capture MoAb and a fibrin fragment D-specific tag antibody (fibrin-specific tag ELISA). Elevated levels of cross-linked fibrin degradation products have been demonstrated with both assays in a variety of clinical settings such as pulmonary embolism, disseminated intravascular coagulation, deep venous thrombosis, and acute myocardial infarction.[17,18,24]

Fig 1. Schematic representation of markers of thrombin- and plasmin-mediated proteolysis of fibrinogen.

Kudryk et al[14,15] and Weitz et al[16] have developed assays for the plasmin derived fibrinopeptide Bβ1-42 from fibrinogen and fibrinopeptide β15-42 from fibrin II, based on specific MoAbs. Eisenberg et al,[26] Lawler et al,[28] and Owen et al[22] have demonstrated marked elevation in fibrinopeptide Bβ1-42 levels early in the course of rt-PA therapy, most likely resulting from generation of fibrinogen fragment X. Indeed, Owen et al[22] and Mentzer et al[29] have demonstrated the presence of early and persistent fragment X in plasma from patients undergoing fibrinolytic therapy for acute myocardial infarction. The levels of both fibrinopeptide Bβ1-42 and fibrinopeptide β15-42 have been shown to be elevated not only during infusion of rt-PA, but also for a number of hours after the infusion. Because both peptides have plasma half-lives of 10 to 20 minutes, this has been interpreted to indicate that there is continued lysis of both fibrin I and II.[22,26,27]

Because the extent of fibrinogen breakdown may affect the accuracy of these assays, the present study was performed to evaluate the behavior of the fibrin fragment D-dimer and fibrinogen derived Bβ peptide assays in two well-defined subgroups of patients treated with 100 mg of rt-PA in phase II of the TIMI trial, characterized by marked and minimal fibrinogen degradation, respectively.

MATERIALS AND METHODS

Patient selection and treatment protocol. Patients eligible for the present study were admitted according to the TIMI II protocol; detailed selection and exclusion criteria are described elsewhere.[30] Patients were treated within 4 hours of the onset of chest pain. The infusion dose of rt-PA (Genentech, South San Francisco, CA) in the patients included in this study was 100 mg over a 6-hour period: 60 mg in the first hour, 20 mg in the second hour, and 5 mg in each of the additional 4 hours. All patients were administered an initial 5,000 U bolus of heparin intravenously (IV) at the initiation of rt-PA infusion, and then sufficient continuous IV heparin (usually at the rate of approximately 1,000 U/h) to maintain the partial thromboplastin time (PTT) at 1.5 to 2.0 times the control value up until the fifth day of hospitalization.

Sample collection. Blood samples were drawn at four timepoints: baseline (preinfusion), 50 minutes, 300 minutes, and 480 minutes after the start of the infusion. The collection tubes used in the present study, unless otherwise noted, contained either a mixture of D-Phe-Pro-Arg-chloromethylketone (FPR-ck, 2×10^{-5} mol/L final concentration), aprotinin (150 KIU/mL final concentration), and ethylenediamine tetra-acetate (EDTA, 4.5 mmol/L final concentration), or a mixture of FPR-ck and citrate (0.01 mol/L final concentration). Collection tubes contained dry reagents and were

evacuated to increase shelf life and ease of use. These tubes were prepared with the assistance of the Terumo Medical Corp (Elkton, MD). After collection, tubes were gently inverted for 30 seconds, placed on melting ice, and then centrifuged within 60 minutes. Supernatant plasma was then transferred to another tube and frozen immediately at −20°C for storage. Samples were shipped frozen on dry ice from the field centers to the University of Vermont (Burlington). Additional processing at the University of Vermont included high speed centrifugation to remove residual platelet debris, aliquoting, and repository storage at −135°C. Preliminary studies have shown that these collection tubes and procedures minimize rt-PA–induced in vitro artifact.[31]

Selection criteria for patient samples. In this study, plasma samples from 24 patients were selected on the basis of the fibrinogen concentration in response to rt-PA infusion as determined 8 hours after the start of the rt-PA infusion. Twelve patients with marked fibrinogen breakdown, defined as a decrease of fibrinogen to below 20% of the preinfusion level (group 1), and 12 patients with minimal fibrinogen breakdown, defined as a residual fibrinogen level ≥80% of the preinfusion level (group 2), were selected.

Assay protocols. Fibrinogen levels were determined by the method of Clauss[32] as modified for a semi-automated fibrometer (BBL, Hunt Valley, MD). This assay, which measures the rate of fibrin formation after addition of excess thrombin, was performed with FPR-ck/citrate samples. Preliminary studies indicated that a 45-minute preincubation of the test plasma at 37°C was necessary and sufficient to remove the antithrombin effect of active FPR-ck, thus allowing accurate estimation of the fibrinogen levels from the clotting rate. These studies included matched samples from the same patients drawn into Aprotinin (150 KIU/mL)/citrate. These matched samples were not incubated and measured identical levels of fibrinogen. Heparin at plasma concentrations up to 10 U/mL has no effect on this assay.[10] We have determined a normal range for this assay of 170 to 410 mg/dL plasma.

FDPs were determined by the "tanned red cell hemagglutination inhibition immunoassay" (TRCHII).[33] In the setting of thrombolytic therapy, this method primarily measures degradation products of fibrinogen. For this assay, the preincubated FPR-ck/citrate samples were used. In addition, the plasma samples were treated with protamine sulfate (12 μg/mL) during the clotting step to eliminate heparin effect. Clotting was performed with 60 U/mL thrombin and 2,000 KIU/mL aprotinin overnight at 37°C.

Cross-linked FDPs were measured using two different ELISAs on samples drawn into the FPR-ck/aprotinin/EDTA anticoagulant. The first, a commercially available kit based on the assay of Elms et al,[17] was the Dimertest (American Diagnostica, New York, NY), herein referred to as "pan-specific tag ELISA," performed in essence as described by the manufacturer. An MoAb specific for fibrin fragments containing the fragment D-dimer structure was precoated onto a 96-well microtiter plate. After washing the pre-

coated plate, 25 µL of sample or standard was placed into a well containing 100 µL of phosphate-buffered saline with 0.1% Tween-20 (PBS-Tween) and the mixture was incubated for 1 hour at room temperature. After washing with PBS-Tween, the signal antibody, a horseradish peroxidase-conjugated mouse MoAb directed at fragment D of fibrinogen (Fig 1) was added and the mixture was further incubated for 1 hour at room temperature. After washing with PBS-Tween, the provided peroxidase substrate, 2,2-azinobis-3-ethylenebenzthiazoline sulfonic acid, was added and allowed to react for 20 minutes. The reaction was terminated with the addition of 50 µL/well of the provided stopping-reagent, and the absorbance was read at 405 nm in an automated plate reader (Bio-Tek, Winooski, VT). Assay values in healthy individuals, according to the product insert sheet, are less than 0.3 µg/mL. Data were calculated, stored, and analyzed using the TiterCalc Software Program (Hewlett-Packard, Palo Alto, CA).

The second assay for cross-linked fibrin degradation products, herein referred to as the "fibrin-specific tag ELISA," was used and performed as described by Declerck et al.[18] This ELISA is based on two fibrin-specific MoAbs: a capture antibody specific for cross-linked fibrin fragment D-dimer (MA-15C5), and a tag antibody specific for fragment D of cross-linked or non–cross-linked fibrin (MA-8D3). Polystyrene microtiter plates (Costar, Cambridge, UK) were coated with 200 µL of MA-15C5 diluted to 32 µg/mL for 48 hours at 4°C in 0.04 mol/L phosphate buffer, pH 7.4, containing 0.13 mol/L NaCl (PBS). The plates were then decanted and blocked with 10 µg/mL bovine serum albumin (BSA) for 2 hours at 22°C. The plates were then washed with PBS and stored at −20°C in a solution of 10 g mannitol and 20 g saccharose per liter. Plates were washed with PBS before use.

Patient samples were diluted into PBS, Tween 80 (0.002%), 0.5 mmol/L EDTA, 10% BSA (assay buffer) pipetted into the wells, and incubated for 16 hours at 4°C. The wells were then washed three times in PBS, Tween 80 (0.002%); 180 µL of the tag antibody MA-8D3 (0.3 µg/mL in assay buffer; previously conjugated with horseradish peroxidase [HRP]) was added and incubated for 2 hours at 22°C. The plates were then washed three times and 150 µL of 0.1 mol/L citrate, 0.2 mol/L Na-phosphate, pH 5.0 containing 200 µg/mL o-phenylenediamine, and 0.003% hydrogen peroxide was added and incubated at 22°C for 1 hour. The reaction was stopped with 50 µL, 4 mol/L sulfuric acid and absorbance was measured at 492 nmol/L using a multiskan spectrophotometer (Titertek, Flow Laboratories, Irvine, Scotland). Standard curves were constructed from purified fragment D-dimer, diluted between 100 and 3 ng/mL in assay buffer. The level of fragment D-dimer in plasma from healthy individuals, using this assay, is reported to be less than 0.37 µg/mL.[18] To calibrate the cross-linked fibrin degradation product assays relative to each other, purified fragment D-dimer provided in the pan-specific tag ELISA was run in both assay systems.

Fibrinogen-derived Bβ1-42 and fibrin-derived β15-42 peptide levels were determined by ELISA (New York Blood Center, New York, NY) on samples drawn into the FPR-ck/aprotinin/EDTA anticoagulant. For these assays, centrifugal ultrafiltration was used to separate peptide material in the sample from intact fibrinogen or higher molecular weight FDPs, which cross-react in the assay. Plasma samples (0.25 mL) were diluted 1:1 with PBS, placed in Centricon 30 ultrafiltration tubes (Amicon, Danvers, MA), and centrifuged at 4,000g for 30 minutes at 4°C. The ultrafiltrate was then collected and stored at −70°C until assayed. No changes in reactivity were noted with repeated freezing and thawing for up to 5 times. To assess recovery, normal control plasmas were spiked to 73 pmol/mL and 80 pmol/mL with fibrinopeptide Bβ1-42 and fibrinopeptide β15-42, respectively. Peptide separation steps were performed as described and average recoveries were found to be

approximately 80% for the fibrinopeptide Bβ1-42 assay and 70% for the fibrinopeptide β15-42 assay.

The fibrinopeptide β15-42 assay was run according to the manufacturer's instructions. Briefly, solubilized fibrin monomer was coated overnight at 4°C onto vinyl 96-well microtiter plates (Falcon no. 3912, Microtest III). After washing and blocking, a preincubated mixture of sample or standard and murine MoAb directed against fibrinopeptide β15-42 was added. After incubation for 45 minutes at room temperature, the plate was washed and secondary antibody, HRP-conjugated antimouse immunoglobulin G (IgG), was added. After incubation for 45 minutes at room temperature, the plate was washed and a substrate solution of ortho-phenylenediamine (Sigma, St Louis, MO) was added, incubated for 20 minutes and the reaction stopped by adding 4 mol/L sulfuric acid. The absorbance was read at 490 nm and the data analyzed with the TiterCalc Program. The concentration of peptide in the sample was inversely proportional to the amount of color development. In our hands, the dynamic range of the assay was 1.25 to 80.0 pmol/mL. With this procedure, we determined the level of fibrinopeptide β15-42 in healthy volunteers to be 4.8 ± 1.8 pmol/mL (n = 8).

Fibrinopeptide Bβ1-42 levels were also determined by ELISA (New York Blood Center) methodology similar to that for fibrinopeptide β15-42. The assay was run according to the instructions provided with slight modifications of incubation times. Fibrinogen coating and ultrafiltration were performed as described previously. After washing and blocking the assay plate, a preincubated solution containing sample and mouse anti-fibrinopeptide Bβ1-42 MoAb was added and incubated for 20 minutes. After washing, HRP-conjugated antimouse IgG was added and incubated for 20 minutes. The remainder of the procedure was identical to that of the fibrinopeptide β15-42. The dynamic range of this assay was 4.6 to 73 pmol/mL. The level of fibrinopeptide Bβ1-42 in healthy volunteers was 5.9 ± 2.9 pmol/mL (n = 10).

t-PA antigen levels were determined by the method of Holvoet et al,[34] with slight modification for adaption to the Hewlett-Packard Microassay Robotic System. This assay is a two-site ELISA using three different MoAbs directed against t-PA. The plasma normal range for this assay is 5 ng/mL to 20 ng/mL.[34]

Plasminogen assays were performed using the chromogenic substrate Spectrozyme PL, (American Diagnostica) to measure the amount of plasmin present after direct activation with 3,500 U/mL streptokinase (Sigma). The assay was done in microtiter plates and was adapted to the Hewlett-Packard Microassay Robotic System. We have determined the normal range (mean ± 2 SD) for the assay on plasma to be 75% to 125% of a normal standard plasma.

Preparation of "lysed" control plasmas. Normal human plasma, batch NHP-N9, was prepared as previously described.[35] rt-PA (Genentech) was added to 5.0 mL of NHP-N9 to a final concentration of 25 µg/mL. This was then incubated in a 37°C water bath for 18 hours to ensure complete plasmin degradation of fibrinogen. After the incubation period, aprotinin (Bayer, Leverkusen, West Germany) was added to a final concentration of 1,000 KIU/mL to neutralize residual plasmin. The plasma was then aliquoted and stored at −70°C until further use. Alternatively, streptokinase (Sigma) was added to 5.0 mL NHP-N9 to a final concentration of 2,000 U/mL. The mixture was incubated, treated with aprotinin, aliquoted, and stored at −70°C, as described above.

Statistical methods. Statistical analysis was performed using Systat software, version 4.0 (Systat, Inc, Evanston, IL). Basic statistics include mean, standard deviation, standard error of the mean, variance, and coefficient of variation. Correlations and *P* values were calculated in the Multivariate General Linear Hypothesis module using a best fit least square line. "r" is the simple Pearson correlation coefficient. For the FDP results, because the standard deviations were large and raised the possibility of a non-normal

distribution, we also analyzed correlation using a nonparametric rank correlation test, with "r" in this case being the Spearman correlation coefficient.

RESULTS

Routine assay results. t-PA antigen, fibrinogen, FDP, and plasminogen levels were determined in plasma samples of the 24 patients at baseline and 50 minutes, 5 hours, and 8 hours after the start of the rt-PA infusion. Group 1, with marked fibrinogen breakdown, and group 2, with minimal fibrinogen breakdown, are clearly distinguished (Figs 2A and 2B). The peak plasma levels of rt-PA antigen at the 50-minute timepoint were 3.7 ± 1.5 μg/mL and 1.2 ± 0.5 μg/mL for group 1 and group 2, respectively. rt-PA antigen levels subsequently decreased to 69 ng/mL and 71 ng/mL, respectively, at 8 hours. Initial fibrinogen levels were 333 ± 78 mg/dL and 284 ± 55 mg/dL in group 1 and group 2, respectively. The nadir fibrinogen levels, 8 hours post rt-PA infusion, were 51 ± 22 mg/dL for group 1 and 260 ± 44 mg/dL for group 2. As anticipated, the peak FDP levels at 8 hours postinfusion were inversely proportional to the plasma fibrinogen concentrations, and were 1,558 ± 1,100 μg/mL in group 1 and 53 ± 31 μg/mL in group 2. The mean nadir plasminogen level at the 8-hour timepoint was 41% ± 4% of normal in group 1 versus 72% ± 4% in group 2.

Evaluation of assays for cross-linked fibrin degradation products. Preinfusion levels of cross-linked fibrin degradation products measured with the pan-specific tag ELISA (Fig 3) were within the normal range (less than 0.3 μg/mL) of the assay in all but one patient (0.6 μg/mL), but increased post rt-PA infusion in both groups, with peak levels occurring at the 5-hour timepoint. The 8-hour level of group 1 at 5.8 ± 4.2 μg/mL was more than five times higher than that
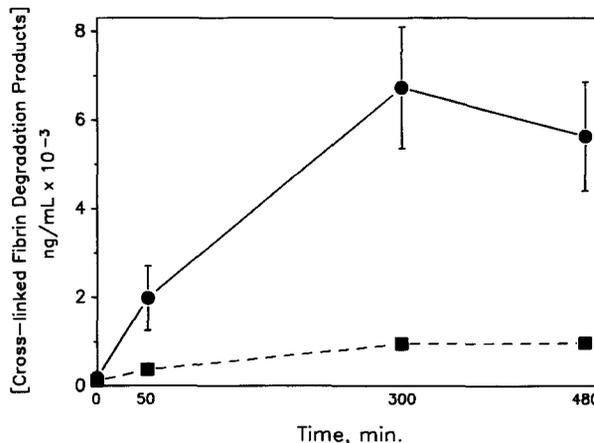

Fig 3. Comparison of fibrin fragment D-dimer levels, as measured by the pan-specific tag ELISA in the groups with marked (●) or limited (■) fibrinogen breakdown during rt-PA infusion. Sample times are 0, 50, 300, and 480 minutes after the start of the rt-PA infusion (n = 12 in both groups).

observed for group 2 at 0.98 ± 0.45 μg/mL. Preinfusion levels of cross-linked fibrin degradation products reported with the fibrin-specific tag ELISA were 0.18 ± 0.16 μg/mL in group 1 and 0.12 ± 0.06 μg/mL in group 2, and increased to 1.5 ± 1.3 μg/mL in group 1 and to 0.5 ± 0.35 μg/mL in group 2 at 8 hours.

The magnitude of the 8-hour values seemed to indicate that the pan-specific tag ELISA reflected fibrinogenolysis. Measurement of cross-linked fibrin degradation products in plasma after in vitro activation of the plasma fibrinogenolytic system with rt-PA or streptokinase with the pan-specific tag
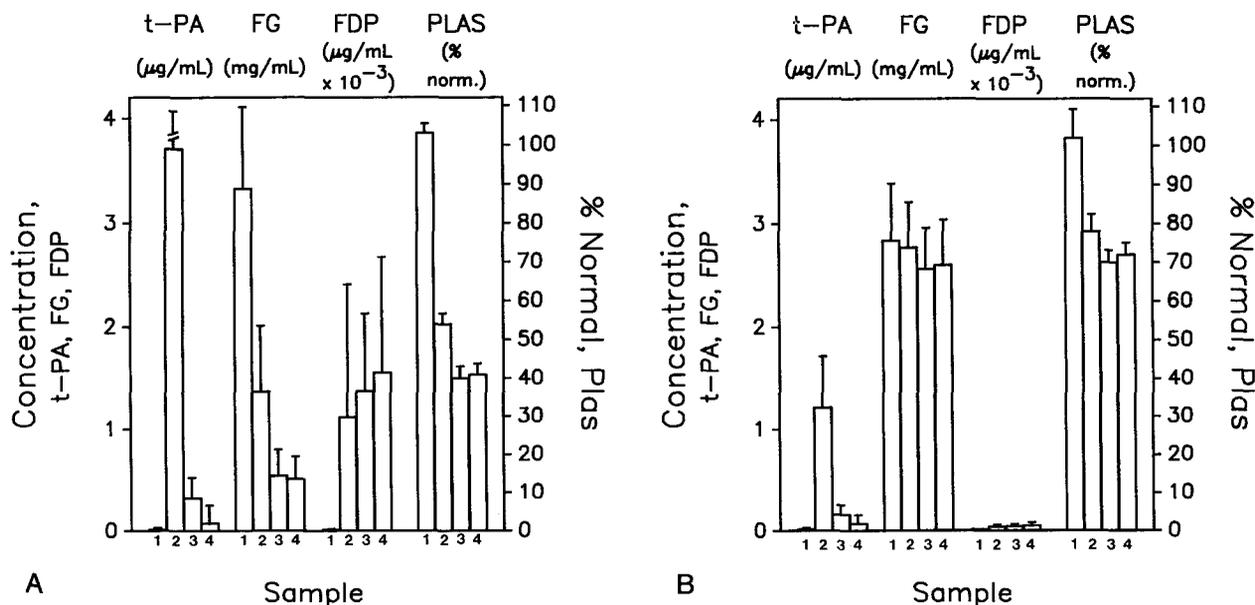

Fig 2. Changes in laboratory parameters of fibrinolysis during thrombolytic therapy with rt-PA in patients with marked (A) or limited (B) fibrinogen breakdown as defined by residual fibrinogen determination. t-PA, t-PA antigen; FG, fibrinogen; FDP, fibrin(ogen) degradation products; PLAS, plasminogen. Values are means and standard deviation. Numbers on horizontal axis refer to sample number: 1, pre-infusion; 2, 50 minutes; 3, 300 minutes; 4, 480 minutes after the start of the rt-PA infusion.

ELISA yielded values in the range of 1.2 μg/mL. Progressive additions of rt-PA–activated plasma into a constant volume of normal plasma pool (N-9) (repeated three times) demonstrated an increasing cross-linked fibrin degradation product signal in the pan-specific tag ELISA that reached a plateau between 1.2 and 1.5 μg/mL (Fig 4). However, as shown, the fibrin-specific tag ELISA did not measure an increase in fragment D-dimer levels in this in vitro experiment. In fact, a slight decrease was observed. This has also been reported by Declerck et al.[18] The cause of this small decrease is unknown at this time.

The results obtained for the fibrin-specific tag ELISA and the pan-specific tag ELISA were correlated for group 1 (n = 12) and group 2 (n = 11, with exclusion of one value that was greater than 3 SDs from the mean of the rest), respectively. The correlation between the two assays was r = .83 for group 1 and r = .84 for group 2. The slopes of the regression lines were quite different. The regression line for group 1 had a slope of 2.6 and the regression line for group 2 had a slope of 0.96.

The linear correlation between the two cross-linked fibrin degradation product assays (8-hour time points) and the routine assays used to characterize the plasmin-induced hemostatic defect (peak rt-PA, FDP; nadir fibrinogen, plasminogen) are illustrated in Table 1. The fibrin-specific tag ELISA was correlated only with FDP (r = .69; P = .001). The pan-specific tag ELISA demonstrated a stronger correlation with FDP (r = .89; P = < .001), and significant correlations with t-PA (r = .67; P = .001) and nadir fibrinogen (r = −.53; P = .008). Neither assay correlated with the nadir for plasminogen. Rank correlation analysis supported the association of FDP with the fibrin-specific tag ELISA (r = .45; P = .029) and the pan-specific tag ELISA (0.80, P < .0001), with the latter association being considerably stronger.

Fibrinopeptide Bβ1-42 and fibrinopeptide β15-42 assays. Levels of fibrinopeptide Bβ1-42 and fibrinopeptide β15-42 were also determined for both groups at all timepoints, and these values are shown in Figs 5 and 6. Preinfusion levels of fibrinopeptide Bβ1-42 were 4.2 ± 2.8 pmol/mL and 4.1 ± 2.1

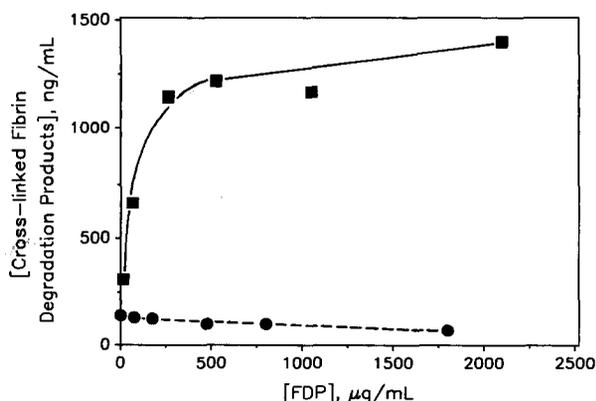pmol/mL in groups 1 and 2, respectively, both within the normal range of the assay. A marked increase in fibrinopeptide Bβ1-42 was seen in both groups at the 50-minute timepoint with levels of 2,000 ± 230 pmol/mL in group 1 and 300 ± 43 pmol/mL in group 2. By the 5-hour timepoint, there was a marked decrease in fibrinopeptide Bβ1-42 levels; however, elevated levels of the peptide persisted to the 8-hour timepoint. Fibrinopeptide Bβ1-42 levels at 50 minutes correlated closely to the nadir (8-hour) fibrinogen (r = −.80; P ≤ .001) and peak (8-hour) FDP (r = .76; P ≤ .001) levels. The rank order correlation between peak FDP and (50 min) Bβ1-42 was r = .84; P < .001.

The preinfusion levels of fibrinopeptide β15-42 were 4.3 ± 3 pmol/mL for group 1 and 5.2 ± 3.6 pmol/mL for group 2. In contrast to the increase in fibrinopeptide Bβ1-42 seen in both groups at the 50-minute timepoint, fibrinopeptide Bβ15-42 values did not increase appreciably from the baseline level in group 2. However, in group 1, fibrinopeptide Bβ15-42 levels increased to 70 ± 19 pmol/mL at the 50-minute timepoint, decreased to 8 pmol/mL at the 5-hour timepoint, and returned to baseline by 8 hours.

Because of the relatively high concentrations of fibrinopeptide Bβ1-42 generated at the 50-minute timepoint in group 1 with extensive fibrinogen breakdown, we evaluated the potential immunoreactivity of this peptide in the fibrinopeptide β15-42 assay. As shown in Table 2, addition of purified fibrinopeptide Bβ1-42 standard (New York Blood Center) to the fibrinopeptide β15-42 assay demonstrated a 3% cross-reactivity. Similarly, activation of the fibrinolytic system in

Table 1. Correlations Between Routine Assays and Assays of Cross-Linked FDP

		t-PA 50 min	Fibrinogen 8 h	FDP 8 h	Plasminogen 5 h
Pan-specific	r	.665	−.531	.890	.161
tag ELISA	P	.001	.008	<.001	.497
Fibrin-specific	r	.331	−.333	.694	.018
tag ELISA	P	.114	.120	<.001	.941

Fig 4. Correlation between FDP and fibrin fragment D-dimer levels measured with the pan-specific tag ELISA (■) and with the fibrin-specific tag ELISA (●) in mixtures of normal and rt-PA (25 μg/mL) activated plasma.
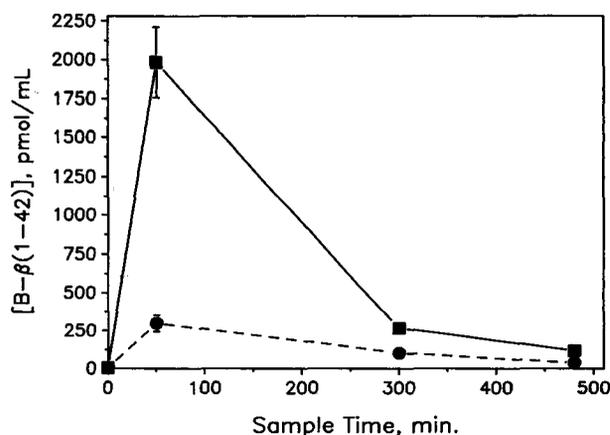
Fig 5. Fibrinopeptide Bβ1-42 levels in the patient groups with marked (■) versus limited (●) fibrinogen breakdown. Peak values occur at 50 minutes for both groups.
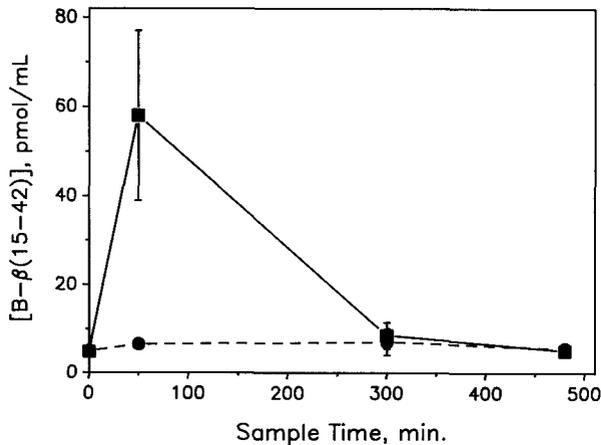
Fig 6. Comparison of fibrinopeptide β15-42 levels in the patient groups with marked (■) versus limited (●) fibrinogen breakdown.

10 normal plasmas in vitro produced an increase in measured fibrinopeptide Bβ1-42 from 4.0 ± 2.3 to 1,910 ± 588 pmol/mL and an apparent increase in fibrinopeptide β15-42 from 3.2 ± 0.37 to 212 ± 49 pmol/mL, which represents 10% of the increase in fibrinopeptide Bβ1-42. Examination of Figs 5 and 6 shows that the elevated mean fibrinopeptide β15-42 level for group 1 was 3% of the mean fibrinopeptide Bβ1-42 level. In addition, examination of individual patient assay values for fibrinopeptide Bβ1-42 versus fibrinopeptide β15-42, for group 1 patients at 50 minutes, showed a close correlation ($r = .77$; $P \leq .001$).

DISCUSSION

The patients evaluated in this study all received 100 mg of rt-PA over 6 hours according to the TIMI-II protocol. Although the TIMI protocol differed slightly from the presently accepted Food and Drug Administration (FDA) approved administration schedule, the dose during the first 2 hours also represented 80% of the total dose in the FDA approved schedule. Thus, the drug administration scheme for TIMI II closely approximates the state of approved clinical practice. For this pilot study on the evaluation of laboratory markers of in vivo clot lysis, patients were chosen to represent the extremes of the plasmin-induced coagulation defect. The relationship between t-PA antigen levels and FDP in the two groups is consistent with the dose-response effect reported in other studies,[5,6] although it is more marked.

We were interested in gaining more information about the intensity and duration of increased fibrin fragment D-dimer

Table 2. Fibrinopeptide Bβ1-42 Cross-Reactivity in the Fibrinopeptide β15-42 Assay

Fibrinopeptide Bβ1-42 Added (pmol/mL)	Fibrinopeptide β15-42 Measured (pmol/mL)	Cross-Reactivity of Fibrinopeptide Bβ1-42 in the Fibrinopeptide β15-42 Assay (%)
950	28	3.0
480	17	3.6
240	8	3.4

Purified fibrinopeptide Bβ1-42 was added to plasma that was assayed in the fibrinopeptide β15-42 ELISA.

and fibrinopeptide β15-42, presumed markers of clot lysis in plasma, and about the interference of fibrinogen breakdown with these assays. The pan-specific tag ELISA for cross-linked fibrin degradation products and the fibrinopeptide β15-42 assays were not originally designed for use during intense thrombolytic therapy, and certain suspected limitations of these assays in this setting were established in this study.

A few limited studies have evaluated cross-linked FDP and fibrin-derived Bβ peptides in patients undergoing thrombolytic therapy, and have found little correlation with clinical outcome other than a direct relationship to the intensity of the fibrinolytic coagulation defect.[19,27] A possible problem limiting the clinical utility of these assays might be associated with a lack of specificity in the presence of varying intensities of fibrinogen breakdown. In TIMI Phase-II, 25% of patients treated with 100 mg rt-PA had mean nadir fibrinogen levels below 50% of the preinfusion value. This level of fibrinogen breakdown could impact significantly on the validity of values determined with pan-specific ELISA. The in vitro plasma degradation studies illustrated in Fig 4 demonstrated a lack of specificity with the pan-specific tag ELISA in the setting of intense, plasmin-induced fibrinogen breakdown. The cross-linked FDP levels achieved after in vitro activation of the fibrinolytic system in clot-free plasma represented a significant proportion of the apparent levels measured in patients with marked fibrin(ogen)olysis (Fig 2). It has been previously demonstrated that fibrinogen and non–cross-linked FDP can bind noncovalently to cross-linked fibrin and its degradation products.[36,37] Thus, non–cross-linked FDP products and fibrinogen are likely to be bound with cross-linked FDP to the capture antibody, and appear to be detected in the second stage of the pan-specific tag ELISA by its non–cross-link specific antibody. This hypothesis is confirmed in additional studies.[38]

One would predict that an assay configured with fibrin-specific tag and capture antibodies might, in the setting of marked fibrinogen degradation, measure lower levels of cross-linked FDP than the pan-specific tag ELISA. The different slopes of the regression lines drawn from the correlation studies of the pan-specific tag ELISA and the fibrin-specific tag ELISA support this hypothesis. In group 2, with minimal fibrinogen breakdown, the two assays show a close correlation ($r = .85$; $P = .001$) and the regression line has a slope of approximately 1. In contrast, group 1, with marked fibrinogen breakdown, showed a similar close correlation ($r = .8$, $P = .002$), but the slope of the regression line is 2.6. These data are consistent with the hypothesis that, with increased fibrinogen breakdown, increasing amounts of FDP are bound to the captured cross-linked fibrin degradation products and recognized by the second antibody in the pan-specific tag ELISA. Our experiments with in vitro activation of the fibrinolytic system (Fig 4) suggest that this process is saturable. Presumably, the increasing signal with both assays observed in group 2 is due to increased amounts of initially captured cross-linked fibrin. Group 1 and group 2, in these experiments, represent extremes of fibrinogen breakdown. If our hypothesis is correct, patient samples between these extremes would demonstrate varying degrees of FDP adsorption, leading to unreliable results with the pan-specific

tag ELISA. At the same time, the fibrin-specific tag ELISA does also register substantially increased cross-linked fibrin degradation products in group 1 patients with increased fibrinogenolysis compared to group 2. This seems to indicate that both fibrinolysis and fibrinogenolysis respond to circulating rt-PA concentrations in a dose-dependent manner. It seems likely that at least part of the correlation between the fibrin-specific and pan-specific tag ELISAs may be explained by the parallel occurrence of fibrin and fibrinogen breakdown in vivo.

The lack of correlation between the fibrin-specific ELISA and the routine assays (Table 2), with the exception of FDP in group 1, contrasts with the significant correlation between these assays and the pan-specific tag ELISA. Because the routine assays are primarily measures of the intensity of fibrinogen breakdown, the closer correlation of the pan-specific tag ELISA most likely results from cross-reactivity of this assay system with non–cross-linked FDP. The significant but weaker correlation between the fibrin-specific tag ELISA and FDP levels may be explained by a parallel occurrence of fibrin and fibrinogen breakdown in vivo.

The rapid onset and the duration of fibrinogen breakdown was best reflected by the concentration of the fibrinopeptide Bβ1-42, which has a short half-life ($t\frac{1}{2} = 10$ minutes). The highest fibrinopeptide Bβ1-42 levels were achieved at the 50-minute timepoint during the initial 60-mg infusion. These levels showed a highly significant correlation to nadir fibrinogen and peak FDP levels, and most likely represent fragment X formation. Fibrinopeptide Bβ1-42 levels remained elevated even at 8 hours, 2 hours after the end of the rt-PA infusion. The persistence of fragment X formation and its continued presence in plasma is consistent with the reports of Owen et al[22] and Mentzer et al[29] in the setting of thrombolytic therapy. Fragment X is clottable, forming weak clots,[39] and may therefore play a role in certain clinical outcomes especially hemorrhagic complications.

The fibrinopeptide β15-42 levels were only elevated at the 50-minute and 6-hour timepoints, and only in group 1 with marked fibrinogen breakdown. The addition of purified fibrinopeptide Bβ1-42 to the fibrinopeptide β15-42 assay mixture showed a significant cross-reactivity, and the mean fibrinopeptide β15-42 elevations in patient samples at 50 minutes and 6 hours represented a comparable fraction of the fibrinopeptide Bβ1-42 levels. Furthermore, increased levels of β15-42 were measured after in vitro rt-PA–induced plasmin digestion of clot-free plasma. These data suggest that the apparent elevations in fibrinopeptide β15-42 might be explained by cross-reactivity of fibrinopeptide Bβ1-42 in

the assay. This hypothesis was corroborated by the close correlation of the fibrinopeptide Bβ1-42 and fibrinopeptide β15-42 levels in these patients at each of the time points. Consequently, it appears likely that most of these patients did not have measurable increases in fibrinopeptide β15-42 before, during, or after their rt-PA infusions. The lack of an observed increase in the fibrinopeptide β15-42 differs from the results of other studies.[19,27] The apparent absence of fibrinopeptide β15-42 in patients with intense fibrinogen breakdown and fibrinolysis is puzzling. The peptide may not be readily cleaved or released from fibrin II. Alternatively, relatively low concentrations of peptide would be difficult to detect in the assay system as it is currently configured. Finally, the differences could be attributed in part to plasma processing, as the previous studies used a heat precipitation step to separate the peptide, whereas we used a centrifugal ultrafiltration step.

The major conclusions arising from this study are that caution must be exercised in the interpretation of results of assays of cross-linked FDP and fibrinopeptide β15-42 assays in the setting of marked fibrinogenolysis. The pan-specific tag ELISA for fragment D-dimer measured considerably more cross-linked FDP than the fibrin-specific tag ELISA. It would appear that accurate quantitation of fibrinolysis requires assays designed similarly to the fibrin-specific tag ELISA in the setting of therapeutic thrombolysis. The patients reported in this study appear to have had a dose-dependent, low level of fibrinolysis that was exaggerated by the pan-specific ELISA. Consequently, assays similar to the fibrin-specific tag ELISA may provide more accurate information when correlated with clinical endpoints.

The fibrinopeptide Bβ1-42 assay appears to be a sensitive index of fragment X formation during rt-PA infusion and up to 2 hours after the cessation of the infusion. This latter finding suggests there is persistent fibrinogen or fibrin I breakdown extending well after the clearance of active rt-PA from the circulation. The clinical significance of this persistent fibrin(ogen) breakdown remains to be determined. The absence of an observed elevation of the fibrinopeptide β15-42 was unexpected because this has been described previously. However, the innate sensitivity and cross-reactivity of the assay, together with differences in sample processing, may explain these differences.

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