Increased Immunoreactivity of Plasma After Fibrinolytic Activation in an Anti-DD ELISA System

Role of Soluble Crosslinked Fibrin Polymers

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After addition of a low concentration of thrombin to normal plasma, a progressive and significant increase in crosslinked fibrin polymers was found by sodium dodecyl sulfate agarose gel electrophoresis, reaching 27% of total fibrinogen and fibrin before gel formation. As measured by enzyme-linked immunosorbent assay with a monoclonal antibody specific for an epitope near the $\gamma\gamma$ crosslink site, increased immunoreactivity of plasma did not occur after adding thrombin despite formation of crosslinked fibrin polymers, which indicates that the antibody does not recognize the epitope in the polymers. Addition of tissue-type plasminogen activator (t-PA) to plasma resulted in a more rapid degradation of fibrin polymers than of fibrinogen, indicating that the fibrin specificity of t-PA is retained with soluble fibrin. Coincident with degradation of plasma crosslinked fibrin polymers, plasma DD immunoreactivity increased 70-fold from $50.3 \pm 4.5$ (mean$\pm$SD) to $3,560 \pm 1,235$ ng/ml. The presence of increased crosslinked fibrin polymers produced by adding thrombin to plasma significantly increased maximum immunoreactivity after t-PA-induced degradation to $18,500 \pm 11,780$ ng/ml. The increase in DD immunoreactivity was dependent on t-PA concentration; no elevation occurred below 0.01 $\mu$g/ml, and maximal increases occurred above 100 $\mu$g/ml. Analysis of gel electrophoretic patterns of thrombin and t-PA-treated plasma samples suggests that the DD reactivity of t-PA-treated plasma is mainly due to degradation of soluble crosslinked fibrin polymers. Our findings indicate that plasmic degradation of soluble fibrin polymers in plasma may be an important source of fragment DD during thrombolytic therapy. (*Circulation* 1989;79: 666–673)

Under physiologic conditions, fibrin formation is localized either intravascularly in the formation of hemostatic plugs or extravascularly in inflammatory foci. However, low concentrations of soluble fibrin circulate, and increased concentrations have been identified in patients with thrombotic disease,1–7 reflecting a systemic effect of thrombin activity that can also be measured by plasma fibrinopeptide levels.8,9 Soluble fibrin also facilitates the thrombin cleavage of factor XIII to a transglutaminase,10 which can form intermolecular covalent crosslinks between $\gamma$ chain pairs of adjacent fibrin monomers.11 Recently, Francis and colleagues7 showed a low concentration of $\gamma$ chain crosslinked fibrin dimer in plasma of normal individuals and a significant increase in patients with acute myocardial infarction.

Pharmacologic stimulation of fibrinolysis in the treatment of thrombotic disease results in a varying degree of degradation of circulating fibrinogen in addition to the desired effect of thrombolysis. Because the $\gamma$ chain isopeptide crosslink in stabilized fibrin renders this portion of the molecule resistant to plasmic degradation,12–14 plasmic degradation products of crosslinked fibrin differ structurally from those of fibrinogen. This difference has been exploited in developing several methods to measure circulating crosslinked fibrin-specific degradation products as potential markers of clot lysis.
These include methods in which specific fibrinogen and fibrin derivatives are identified by electrophoretic migration after selective removal from plasma\textsuperscript{15\textendash}19 or immunologically in gels after electrophoresis of unfractionated plasma.\textsuperscript{20,21} Another approach has used antibodies specific for crosslinked fibrin degradation products in quantitative immunologic assays.

One frequently used antibody is directed toward an epitope near the $\gamma\gamma$ crosslink site present in crosslinked fibrin degradation products such as fragment DD.\textsuperscript{22,23} Because crosslinked fibrin polymers\textsuperscript{17} and crosslinked fibrin degradation products\textsuperscript{12\textendash}14 contain the $\gamma\gamma$ crosslink, both may react with this antibody. In this study, we induced in vitro crosslinked fibrin polymer formation by exposing plasma to low concentrations of thrombin, and we compared the reactivity of soluble fibrin polymers with their plasmic degradation products in an enzyme-linked immunosorbent assay (ELISA) with a monoclonal antibody reactive with the $\gamma$ chain crosslink. The changes in immunoreactivity with thrombin-induced formation of fibrin polymers and with fibrinolytic degradation affect the interpretation of studies of fibrin and fibrinogen degradation products during thrombolytic therapy.

\section*{Methods}

\subsection*{Proteins}

Human fibrinogen (grade L) was purchased from Helena Laboratories (Beaumont, Texas) and was 93\% clottable. Fibrinogen concentration was determined by measuring optical density at 280 nm with an extinction coefficient of 15.1.\textsuperscript{24} Human thrombin (3,200 NIH units/mg) was purchased from Calbiochem-Behring (LaJolla, California), hirudin and bovine serum albumin were from Sigma Chemical (St. Louis, Missouri), aprotinin was from Mobay Chemical (New York, New York), and tissue plasminogen activator (100,000 IU/mg) was a gift from Burroughs Wellcome (Research Triangle Park, North Carolina). Streptokinase (Streptase) was obtained from Hoechst-Roussel Pharmaceuticals (Somerville, New Jersey) and urokinase (Abbokinase) was from Abbott Laboratories (North Chicago, Illinois). Goat anti-human fibrinogen immunoglobulin G was obtained from Cappel Laboratories (Cochranville, Pennsylvania). Purified factor XIII concentrate (Fibrogamin), prepared from human placenta, was kindly provided by Hoechst-Roussel Pharmaceuticals. Factor XIII was assayed by dansyl cadaverine incorporation in casein,\textsuperscript{25} and the activity was expressed in relation to pooled normal plasma defined as 100\%.

\subsection*{Radiolabeling}

Fibrinogen was labeled with the iodogen technique\textsuperscript{20} to a specific activity of 50 $\mu$Ci/mg with 0.02 mg iodogen and 100 $\mu$Ci $^{125I}$/ml fibrinogen (20 mg/ml). Labeled fibrinogen was 93\% clottable and showed the same electrophoretic mobility on sodium dodecyl sulfate (SDS), 7\% polyacrylamide gel electrophoresis after disulfide bond reduction. Goat anti-human fibrinogen immunoglobulin G was radiiodinated to a specific activity of 0.4 mCi/mg with the lactoperoxidase method.\textsuperscript{27} Bound and free iodine were separated after labeling by chromatography on Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, New Jersey).

\subsection*{Preparation of Fibrin Polymers}

Fibrinogen was dissolved to a final concentration of 10 mg/ml, CaCl$_2$ was added to a final concentration of 0.025 mol/l, and factor XIII was added to a concentration of 12 units/ml. Thrombin was then added to a final concentration of 0.0025 units/ml, the solution was incubated at 37° C for intervals of 10\textendash}45 minutes, and the thrombin activity was inhibited by addition of hirudin to a final concentration of 2 units/ml.

\subsection*{Blood Samples}

After informed consent was obtained, blood was collected from normal volunteers or patients with deep vein thrombosis and added to sodium citrate (0.4\% final concentration), and plasma was separated from red cells after centrifugation at 3,500g for 15 minutes at 4° C. Plasma from a patient with severe factor XIII deficiency was purchased from George King Biologicals (Overland Park, Kansas).

\subsection*{Electrophoretic Analysis}

Fibrinogen and fibrin derivatives were identified in plasma after SDS 2\% agarose electrophoresis.\textsuperscript{20} Plasma was prepared for electrophoresis by diluting 1:20 in 0.01 M phosphate buffer, pH 7, containing 1.7\% SDS and by heating at 100° C for 5 minutes or 60° C for 1 hour. Samples of 10 $\mu$l were electrophoresed at 150 V on a flat bed electrophoretic apparatus (Pharmacia Fine Chemicals) with cooling to 10° C. Electrophoresis was continued until the tracking dye had migrated 10 cm.

Western blotting was done with a modified method of Towbin and colleagues,\textsuperscript{28} with a Transblot Cell (BioRad Laboratories, Richmond, California). Transfer to nitrocellulose paper (pore size, 0.2 $\mu$m; Schleicher and Schuell, Keene, New Hampshire) was done in 0.01 M Tris hydrochloric buffer, pH 8.3, containing 0.096 M glycine and 20\% (v/v) methanol at 60 V for 3 hours at 20° C. The nitrocellulose paper was incubated at 3° C for 24 hours in 0.05 M phosphate buffer, pH 7.3, containing 0.15 M NaCl and 3\% (w/v) bovine serum albumin. This was removed, and the paper was incubated for 2 hours at 20° C with gentle rocking with 50 ml 0.05 M phosphate buffer, pH 7.3, containing 0.15 M NaCl, 3\% (w/v) bovine serum albumin, 0.05\% (v/v) Tween 20, and 2 $\times$ 10$^{-5}$ $\mu$Ci $^{125I}$-labeled goat anti-human fibrinogen immunoglobulin G. The nitrocellulose paper was then washed; each wash consisted of 6 volumes of 40 ml 0.05 M phosphate buffer, pH 7.5, contain-
ing 0.15 M NaCl and 0.05 (v/v) Tween 20 and was done by gentle agitation on a platform shaker for 10 minutes. After drying, the paper was processed for autoradiography with Kodak X-Omat film (Eastman Kodak, Rochester, New York) with exposure times up to 72 hours.

Enzyme-Linked Immunosorbent Assay

Crosslinked fibrin degradation products were measured with an ELISA (Dimer Test, American Diagnostica, Greenwich, Connecticut) by a monoclonal antibody (DD/3B6) reactive with a site near the factor XIIIa–mediated crosslink.22,23 Precalculated plates were used, and results were calculated with a standard curve prepared from purified fragment DD provided by the manufacturer at concentrations from 40 to 5,000 ng/ml. Plasma was assayed undiluted before incubation with t-PA and at 1:5 dilution after incubation.

Statistics

Statistical analysis was performed with the Student's t test.

Results

Addition of a low concentration of thrombin to normal plasma (0.025 units/ml final concentration) resulted in a progressive increase in crosslinked fibrin polymers before clot formation as shown in the autoradiogram (Figure 1). Before thrombin addition (time 0), bands corresponding to fibrin dimer and a trace of trimer were present in the 125I-labeled fibrinogen. After thrombin addition, but before clot formation, the dimer band became more prominent, and larger polymers formed. The greatest change occurred in the last sample before clotting (at 0.92 clotting time), when up to six polymers could be seen and when some protein failed to enter the 2% agarose gel. The use of 125I-labeled fibrinogen allowed accurate quantification of fibrin polymer formation by slicing the fixed gel after staining and electrophoresis and then by counting individual slices to determine the proportion of each polymer band (Figure 2). Before thrombin was added, 8% of the radiolabel migrated as crosslinked dimer, and 8.2% migrated as dimer and larger polymers. The proportion of crosslinked polymer increased progressively

![Figure 1. Autoradiogram of formation of crosslinked fibrin polymers in plasma after addition of thrombin. Pooled normal citrated plasma containing 125I-labeled fibrinogen was incubated at 37° C with thrombin (0.025 units/ml final concentration) and CaCl2 (10 mM final concentration). At intervals, aliquots were withdrawn and prepared for electrophoresis by diluting 1:20 in sodium dodecyl sulfate containing divalent cation and by heating at 60° C for 1 hour. Samples of 10 μl were electrophoresed, and the autoradiogram was prepared after drying with an exposure time of 3 days. Time/clotting time is the time at which the sample was withdrawn divided by the observed clotting time, which was 34 minutes. Arrow indicates the location of application wells.](image)

![Figure 2. Plot of increase in crosslinked fibrin polymers in plasma after thrombin addition relative to clotting time. Thrombin (0.025–0.1 units/ml, final) and CaCl2 (10 mM final) were added to pooled normal plasma containing 125I-labeled fibrin. Aliquots were withdrawn at intervals before clot formation during incubation at 37° C and prepared for electrophoresis. After staining with Coomassie blue, bands corresponding to monomer, dimer, and larger polymers were cut from the gel and counted. Time/clotting time is the time of withdrawal of the sample divided by the final clotting time. Data are mean ± SEM of five experiments. Statistically significant increases in comparison with time 0 values: *p < 0.05; **p < 0.01; ***p < 0.005](image)
after thrombin was added, with a peak of 20% dimer and 27% total crosslinked polymer present just before visible fibrin formation. A statistically significant increase in dimer concentration occurred by 0.5 clotting time, and a significant increase in total crosslinked polymer concentration occurred at 0.25 clotting time. Polymers larger than dimer were most abundant immediately before visible clot formation at 0.9 clotting time when they represented 7% of the total. When the same experiment was done with plasma anticoagulated with ethylenediaminetetraacetic acid (0.1% final concentration) and without added calcium chloride, no increase in polymer formation occurred.

Plasmic degradation of soluble crosslinked fibrin polymers was monitored electrophoretically after t-PA was added to normal plasma to which a preparation of ^125^I-labeled fibrin polymer had been added (Figure 3). At 16 \( \mu g/ml \) t-PA, the dimer band was fainter at 3 hours, and the dimer and monomer showed an increased migration consistent with conversion primarily to fragments XX and X, respectively. At 55 and 100 \( \mu g/ml \) t-PA, fibrinogen was degraded to fragment X within 3 hours but showed little cleavage to smaller fragments Y, D, and E. In contrast to the plasmic resistance of fibrinogen and fragment X, the crosslinked fibrin dimer degraded rapidly. At 55 and 100 \( \mu g/ml \) t-PA, fibrin dimer degraded more completely by 1 hour with little dimer or fragments larger than fibrinogen remaining at 3 hours.

Because plasma crosslinked fibrin polymers increase after thrombin exposure and because plasmic degradation of polymers produces fragment DD, we determined the effect of increased fibrin polymers on the apparent D dimer concentration by measuring D dimer after incubation of plasma with a plasminogen activator (t-PA, streptokinase, or urokinase), with or without prior thrombin treatment. The effect of varying concentrations of t-PA on DD immunoreactivity in untreated plasma is shown in Figure 4. With a 1-hour incubation at 37°C, immunoreactivity increased at a t-PA concentration of 0.01 \( \mu g/ml \), increasing markedly above 1 \( \mu g/ml \) and leveled off at 100 \( \mu g/ml \). Plasma incubated with 5 \( \mu g/ml \) t-PA or 500 units/ml streptokinase or urokinase underwent changes in DD immunoreactivity with enzyme concentrations that are

**Figure 3.** Autoradiogram of tissue-type plasminogen activator-induced degradation of fibrinogen and crosslinked fibrin polymers in plasma. Tissue-type plasminogen activator at concentrations of 16, 55, and 100 \( \mu g/ml \) was added to pooled normal plasma containing an ^125^I-labeled preparation of crosslinked fibrin polymers (see "Methods"). During incubation at 37°C, aliquots were withdrawn at the indicated times and were prepared for electrophoresis. After staining and drying, the autoradiogram was exposed for 6 days. The 2% agarose gel system has limited resolution of fragments smaller than fibrinogen. Fragments Y and DD overlap and cannot be resolved, and Fragment E migrates with degradation products of the Aa chain.

**Figure 4.** Plot of plasma D-dimer immunoreactivity after in vitro incubation with varying concentrations of tissue-type plasminogen activator. Pooled normal plasma was incubated with tissue-type plasminogen activator for 1 hour at 37°C, aprotinin (200 units/ml) was added to inhibit further plasmin action, and the D-dimer concentration was measured by enzyme-linked immunosorbent assay. Values are the means of the two determinations.
FIGURE 5. Plot of plasma immunoreactivity in a D-dimer enzyme-linked immunosorbent assay (ELISA) before and after treatment with tissue-type plasminogen activator (t-PA), streptokinase (SK), or urokinase (UK). Citrated plasma was collected from 10 normal individuals and D-dimer concentration was measured by ELISA before (left) and after incubation for 1 hour at 37°C with t-PA (5 μg/ml), SK (500 IU/ml); or UK (500 IU/ml). Mean ± SE is indicated by the brackets.

achievable in vivo (Figure 5). After a 1-hour incubation with 5 μg/ml t-PA, the DD reactivity increased from 62 ± 6.2 to 893 ± 226 ng/ml, consistent with the results in Figure 4, whereas DD reactivities of 3,140 ± 481 and 2,585 ± 818 ng/ml were obtained with streptokinase and urokinase, respectively.

Further experiments examined the change in DD immunoreactivity with fibrinolytic activation after exposure of plasma to thrombin. There was no change after incubation of plasma with thrombin only (0.05 units/ml final concentration) for 20 minutes at 25°C (Figure 6). However, the DD concentration increased markedly to 3,560 ± 1,235 ng/ml after incubation with a high t-PA concentration (200 μg/ml) to maximize the increase. After t-PA incubation with thrombin-exposed plasma, an even larger increase in DD concentration occurred to 18,580 ± 11,780 ng/ml. With factor XIII-deficient plasma substrate, no increase occurred in DD reactivity of thrombin-treated plasma after t-PA incubation (not shown), which confirms the central role for factor XIII crosslinking.

The changes after thrombin and t-PA were reflected in the gel pattern after Western blotting with antifibrinogen antiserum (Figure 7). Normal plasma showed a heavy fibrinogen and a faint dimer band; after thrombin exposure, more dimer and bands corresponding to five larger crosslinked polymers were present. After incubation of untreated and thrombin-treated plasma with t-PA, bands corresponding to polymers were no longer visible, indicating their degradation to smaller fragments, migrating more rapidly than fibrinogen. Before and after thrombin incubation, the t-PA exposure resulted in degradation of fibrinogen primarily to fragment X.

The t-PA–induced changes in DD immunoreactivity of normal plasma were compared with results in plasma samples from eight patients with venographically confirmed deep venous thrombosis (Table 1). The DD concentration in untreated pathologic samples (before t-PA incubation) was markedly elevated, consistent with physiologic lysis of large thrombi. After in vitro incubation with t-PA, the DD concentration of patient samples was further elevated to 6,643 ± 3,327 ng/ml, which is higher than in normal subjects (3,560 ± 1,235 ng/ml, p < 0.025) and consistent with degradation to DD of a larger amount of fibrin dimer in the patient samples.

Discussion

Although fibrin formation and fibrinolysis are primarily localized processes, they may have systemic effects, and these can become prominent in pathologic conditions such as disseminated intravascular coagulation or when plasminogen activa-
Fibrin Polymers and DD Immunoreactivity

Tors are administered therapeutically. This study shows several effects on plasma fibrinogen resulting from exposure to a low concentration of thrombin or activation of fibrinolysis or both. First, there is a progressive and significant increase in soluble crosslinked fibrin polymers before clot formation in vitro (Figures 1 and 2) resulting from the combined action of thrombin and factor XIII. Second, fibrin dimer-derived fragment XX is degraded more rapidly than fibrinogen-derived fragment X (Figure 3), indicating that the relative fibrin specificity of t-PA, demonstrated with insoluble fibrin, is 

![Fibrinogen Normal Plasma Plasma after Thrombin](image)

normal individuals and patients with stroke or myocardial infarction have been found with fibrinogen affinity chromatography used to purify fibrin molecules containing the gly-pro-arg site exposed by thrombin cleavage. An increase in soluble fibrin in patients with stroke or myocardial infarction has also been inferred by an increase in large early eluting fibrinogen antigen after gel filtration chromatography.

The soluble crosslinked fibrin polymers that we have identified (Figures 1 and 2) require the additional action of factor XIIIa to crosslink the γ chains of fibrin monomers. Several observations support the concept of such coordinated action of thrombin and factor XIIIa during activation of coagulation. During spontaneous clotting of plasma in vitro, cleavage of fibrinopeptides and activation of factor XIII are closely related events. The binding of thrombin and factor XIII to fibrin may explain the increase in thrombin-catalyzed factor XIII by fibrin polymers in plasma. Prior studies provided indirect evidence for circulating crosslinked fibrin polymers by identifying γ chain dimers in extracts of plasma obtained from patients with thrombotic disease. Plasma fibrinopeptide A concentration, elevated in thrombotic diseases, reflects the action of thrombin in cleaving fibrinopeptide A from fibrinogen and is a sensitive marker of thrombin action but an indirect measure of plasma soluble fibrin. A concentration of soluble fibrin in normal plasma of 0.6–0.7 mg/dl and a 10-fold increase after acute myocardial infarction have been found with fibrinogen affinity chromatography used to purify fibrin molecules containing the gly-pro-arg site exposed by thrombin cleavage. An increase in soluble fibrin in patients with stroke or myocardial infarction has also been inferred by an increase in large early eluting fibrinogen antigen after gel filtration chromatography.

Using several techniques, previous studies have found low concentrations of soluble fibrin in normal plasma and increased concentrations in thrombotic disease. Plasma fibrinopeptide A concentration, elevated in thrombotic diseases, reflects the action of thrombin in cleaving fibrinopeptide A from fibrinogen and is a sensitive marker of thrombin action but an indirect measure of plasma soluble fibrin. A concentration of soluble fibrin in normal plasma of 0.6–0.7 mg/dl and a 10-fold increase after acute myocardial infarction have been found with fibrinogen affinity chromatography used to purify fibrin molecules containing the gly-pro-arg site exposed by thrombin cleavage. An increase in soluble fibrin in patients with stroke or myocardial infarction has also been inferred by an increase in large early eluting fibrinogen antigen after gel filtration chromatography.

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Tissue plasminogen activator has a high affinity for fibrin and preferentially activates fibrin-bound plasminogen, which are properties that localize physiologic fibrinolysis and that also form the theoretical basis for the relative fibrin selectivity of t-PA as a therapeutic agent. Soluble fibrin also stimulates activation of plasminogen by t-PA, and this effect has been used as a potential method.

| Table 1. Plasma Concentration of D Dimer in Normal Individuals and Patient Samples Before and After In Vitro Treatment With Tissue-type Plasminogen Activator |
|-----------------------------|---------------|----------------|
| Subjects                      | Before t-PA   | After t-PA*    |
| Normal individuals (n=9)      | 50 ± 5        | 3,560 ± 1,235  |
| Patients with deep vein thrombosis (n=8) | 1,068 ± 716   | 6,643 ± 3,327  |

All values mean ± SD and are ng/ml.

*Final concentration is 200 µg/ml for 1 hour at 37° C.
for measuring soluble fibrin.\cite{44} Our findings show preferential degradation of soluble crosslinked fibrin dimer compared with monomer (Figure 3), the principal difference being the greater resistance to cleavage of the coiled-coil portions of fragment X compared with those in fragment X dimer. The relative resistance of fibrinogen-derived fragment X to degradation by t-PA is supported by the findings of Owens and colleagues,\cite{45} who showed a predominance of fragment X in plasma of patients receiving fibrinolytic therapy with t-PA or streptokinase and little cleavage of X to smaller fragment Y, D, or E in patients who received t-PA. Although the relative affinity of t-PA for solid fibrin compared with soluble crosslinked fibrin polymers is not known, our findings suggest that therapeutic administration of t-PA results in preferential degradation of fibrin clot and soluble crosslinked fibrin polymers.

The degradation of soluble fibrin polymers must be considered in evaluating assays for crosslinked fibrin degradation products, particularly in the setting of fibrinolytic therapy. Although the monoclonal antibody directed against the \( \gamma \) chain crosslink epitope has little reactivity with this site in soluble crosslinked fibrin, plasmin degradation increases immunoreactivity markedly (Figures 4, 5, and 6 and Table 1). With administration of a fibrinolytic agent such as streptokinase, marked degradation of plasma fibrinogen occurs, and concomitant degradation of plasma crosslinked fibrin dimer and polymers should produce an increase in plasma DD reactivity as shown in Figure 5. Because the plasma fibrinogen also decreases with administration of relatively fibrin-specific agents such as t-PA\cite{46,47} and because plasma crosslinked fibrin polymers are also degraded (Figure 7) more rapidly than fibrinogen (Figure 3), an increase in plasma DD would be expected with their use as well.

Administration of t-PA to normal individuals has been reported to increase plasma D dimer levels to 980 ng/ml as measured by an ELISA with antibodies different from those we used.\cite{48} This value is close to our findings with concentrations of t-PA, urokinase, or streptokinase that are achievable in patients (Figure 5). After thrombolytic therapy for acute myocardial infarction, a wide range of D dimer concentrations from 1,500 to 10,732 ng/ml has been reported with various techniques.\cite{48-50} The data presented here (Figures 5 and 6, Table 1) suggest that a significant portion of these elevations also could derive from degradation of plasma soluble crosslinked fibrin polymer. The higher levels of 24,200\cite{51} and 64,000 ng/ml\cite{52} measured after fibrinolytic therapy for deep vein thrombosis and 23,900\cite{52} or up to 100,000 ng/ml for pulmonary embolism\cite{53} are unlikely to originate entirely from plasma soluble fibrin polymers (Table 1), suggesting that the additional crosslinked fibrin degradation products arose from lysis of the thrombus or embolus by successful fibrinolytic therapy.

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