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Research Article

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Quantitation of Fragment X Formation during Thrombolytic Therapy with Streptokinase and Tissue Plasminogen Activator

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Abstract

We have determined the extent of fragment X formation during thrombolytic therapy by integration over time of the plasma fibrinopeptide Bβ1-42 concentration. This peptide is quantitatively released when fragment X is formed by plasmin action on fibrinogen or fibrin I. In response to streptokinase (SK) and rt-PA, 264±54 and 95±12 mg/dl respectively of fibrinogen was converted to fragment X. By immunoblotting, fragment X was demonstrated as early as 5 min after SK and 30 min after rt-PA, and was still evident 24 h after treatment. Patients treated with SK showed extensive further plasmin degradation of fragment X to fragments Y and D. Thus fragment X concentrations tend to be more similar in the two groups than would be expected from the extent of fibrinogen breakdown. Fragment X forms clots, but these have lower tensile strength and are more susceptible to further plasmin lysis than clots of fibrin. Thus the similar bleeding observed in the two treatment groups might be a reflection of their similar plasma fragment X concentrations.

Introduction

Acute myocardial infarction is associated with and may be caused by coronary artery thrombosis (1–3). Studies in animals and man have shown that the myocardium can survive a period of ischemia and that myocardial salvage can occur if myocardial reperfusion is accomplished within a few hours of the development of coronary occlusion (4–8). These observations have led to the use of thrombolytic agents as means of restoring blood flow in patients with acute myocardial infarction. Streptokinase (SK)[1] has proved to be an effective thrombolytic agent (2, 3, 5–10), however, therapy with SK results in plasmin activation in circulating blood and a high incidence of bleeding complications (9, 10). It was thought that the hemorrhagic diathesis produced by thrombolytic agents might be related to free plasmin being produced in the circulating blood, resulting in a reduction in plasma fibrinogen concentration. However, it has become clear that the hemorrhagic tendency does not simply parallel the reduction in clottable fibrinogen concentration (11). This implies that other factors, such as the presence of fibrinogen degradation fragments, might play a role (12).

Tissue plasminogen activator (t-PA) has recently become available for use as a thrombolytic agent (13, 14). This material can now be produced by genetic engineering (15) and it is designated recombinant t-PA (rt-PA) to distinguish it from the native material. t-PA differs from SK in that it is a relatively clot-specific activator of plasminogen, converting plasminogen to plasmin most efficiently in the presence of fibrin (16). A major impetus toward developing rt-PA as a therapeutic agent was the expectation that similar fibrin selectivity would be seen in vivo. This would lead to minimal production of plasmin in the circulating blood and a minimal decrease in plasma fibrinogen, and it was hoped that this would translate into lower risk of hemorrhage. However, calculations based on the reported kinetic parameters of plasminogen activation by t-PA without fibrin acceleration suggested that significant concentrations of plasmin may occur in the blood at high rates of rt-PA infusion (17).

During plasmin-mediated fibrinogen and fibrin proteolysis, the earliest cleavages result in the formation of fragment X (18). This protein will form clots in response to thrombin action, as does fibrinogen (19). However, clots formed from fragment X have lower tensile strength and are more susceptible to plasmin-mediated lysis (20–22). Furthermore, clots made from fragment X incorporate Glu-plasminogen during the polymerization phase (23). Clots composed of fibrin show no such Glu-plasminogen binding. Thus, clots formed during and immediately following thrombolytic drug administration may contain a substantial amount of fragment X and these clots may not be adequate to prevent subsequent bleeding. Direct quantitation of fragment X concentration is not currently possible. However in vivo production of fragment X can be monitored by the plasma concentration of the specific 42 amino acid peptide (Bβ1-42) released from the amino end of the Bβ chain (24).

We carried out an adjunct study of fragment X formation in patients enrolled in the thrombolysis in myocardial infarction (TIMI) trial at this institution (10). Using a sensitive and specific radioimmunoassay procedure for fibrinopeptide Bβ1-42 (24), we have quantitated fragment X formation during thrombolytic therapy. To verify the formation of fragment X we directly visualized the fibrinogen degradation products in both plasma and serum by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The total amount of nonclottable fibrinogen and fibrin degradation products appearing in serum was determined as fibrinogen-related antigen (FRA) by radioimmunoassay. The data demonstrate substantial fragment X formation during therapy with either streptokinase or rt-PA and support the hypothesis that bleeding during throm-

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1. *Abbreviations used in this paper:* FRA, fibrinogen-related antigen; rt-PA, recombinant tissue plasminogen activator; SK, streptokinase; THAT, anticoagulant containing trasylol, heparin, adenosine, and theophylline; TIMI, thrombolysis in myocardial infarction; t-PA, tissue plasminogen activator.

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bolytic drug administration is, at least in part, related to fragment X formation.

Methods

All patients enrolled in phase I of the TIMI trial at Columbia Presbyterian Medical Center were considered eligible for the adjunct study of fragment X formation. Patients were admitted to the TIMI trial within 7 h of the onset of chest pain, and drug infusion was started within 9 h of the onset of chest pain (10). 11 patients received rt-PA and 10 patients received streptokinase. All subjects received an intravenous bolus dose of 5,000 U of heparin.

SK was given in a dose of 1,500,000 U as a constant infusion over 60 min. A total dose of 80 mg of rt-PA was given over 3 h, using two different infusion rates: 40 mg/h for the first hour and then 20 mg/h for two hours. Anticoagulation was maintained by the continuous intravenous infusion of 1,000 U/h of heparin begun 3 h after the administration of the bolus dose. All catheters were regularly flushed with saline containing 1 U/ml heparin.

A heparin-bonded catheter lying in either the pulmonary artery or the right atrium was used to draw blood. Samples were collected immediately before starting the infusion, at intervals during the 3-h infusion, then 2 and 24 h after infusion. At each time-point 9 ml of blood was withdrawn and added to 1.0 ml of THAT anticoagulant containing trasylol (1,000 U/ml), heparin (1,400 U/ml), adenosine (10 mM), and theophylline (20 nM). Samples were placed on melting ice immediately after collection and held for not more than 60 min before further processing. Each sample was centrifuged at 3,000 *g* for 15 min, the plasma was transferred to another tube and centrifuged at 40,000 *g* for 20 min to remove platelets. In preparation for assay of fibrinopeptide Bβ1-42, fibrinogen and high molecular weight degradation products were removed by ethanol precipitation at 75% vol/vol final concentration. Excess plasma was stored at −80°C.

Serum for FRA measurement and immunoblotting studies was prepared by mixing plasma with an equal volume of a solution containing ε-aminocaproic acid (100 mM), protamine sulfate (25 μg/ml), Na_2EDTA (10 mM), and thrombin (10 U/ml). The mixture was allowed to clot undisturbed at 37°C for 30 min and the serum harvested.

Each blood sample was assayed for the plasma concentration of fibrinopeptide Bβ1-42 and each plasma-derived serum was assayed for the concentration of fibrinogen-related antigen. In each case a double antibody radioimmunoassay procedure was used (25). Specific rabbit antibody to fibrinopeptide Bβ1-42 was produced in this laboratory (26). The anti-fibrinogen serum, also from rabbit, was obtained from Cappell Laboratories, Cochranville, PA. The anti-fibrinogen antibody was characterized by immunoblotting studies using plasmin-digested pure human fibrinogen. Antibodies of known specificity to fragments D and E and a series of monoclonal antibodies with specificities toward the carboxy terminus of the Aα chain were used for comparison. The anti-fibrinogen was found to react strongly with fragment D, weakly with the carboxy terminal fragments of the Aα chain, and not at all with fragment E. Second antibody, goat anti-rabbit Ig, was obtained from Miles Laboratories Inc., Naperville, IL. Radiolabeling was carried out by the chloramine-T method as described by Greenwood et al. (27). Native human fibrinopeptide Bβ1-42 isolated by high-performance liquid chromatography and purified human fibrinogen were used to calibrate these assays. Plasmin digestion of purified fibrinogen had little effect on its immunoreactivity in the fibrinogen assay.

The extent of conversion of fibrinogen to fragment X during the 5 h following the initiation of thrombolytic drug administration was determined by piecewise integration of the plasma fibrinopeptide Bβ1-42 vs. time. The half disappearance time was determined from the rapid clearance phase in the patients treated with SK. Estimates of the reliability of these calculations were made by Monte Carlo simulation (28). The observed mean fibrinopeptide Bβ1-42 concentrations were replaced by values randomly drawn from populations defined by the observed mean and SEM at each time-point. The area under the curve was estimated by piecewise integration and a value calculated for the amount of fragment X formed. This random replacement procedure was carried out 10,000 times, and the SD of the results was used as an estimate of the variability of the calculated cumulative fragment X formation.

The extent of fibrinopeptide Bβ1-42 release occurring ex vivo in samples collected from patients who received rt-PA was determined by addition of rt-PA to normal plasma. The rt-PA used in this experiment was obtained from Genentech Inc., San Francisco, CA. Normal plasma was prepared from blood collected into the THAT anticoagulant, and sufficient rt-PA was added to raise the plasma concentration to 1,000 ng/ml. The mixture was incubated in melting ice. At intervals over 2 h aliquots were removed and the reaction was terminated by precipitation of fibrinogen by the addition of 3 vol of ethanol. These samples were then centrifuged, dried, and assayed for fibrinopeptide Bβ1-42 in the same way as the study samples. In a separate experiment, a range of concentrations of rt-PA in normal plasma was allowed to incubate on melting ice for 1 h before terminating the reaction and processing as above.

Immunoblot analysis of the plasma and serum fibrinogen-related antigen was carried out using the same rabbit anti-human fibrinogen as was used for measurement of FRA. Plasma or serum was diluted 1/6 in 2% SDS and heated to 95°C for 5 min. 5 μl of dilute sample was applied to a 7.5% polyacrylamide gel and electrophoresis was performed using a modified Laemmli discontinuous buffer system. Proteins were electrophoretically transferred to nitrocellulose membranes. After blocking in 5% wt/vol fat-free powdered milk in saline, the membranes were incubated for 48 h in a mixture of 1/4,000 dilution of the same rabbit anti-human fibrinogen used for radioimmunoassay. In separate experiments rabbit anti-human fragment E (Cappel Laboratories, Cochranville, PA) was used to demonstrate the location fragment E which is not recognized by this anti-fibrinogen antibody. Bands containing these fibrinogen-related antigens were identified by incubation with horseradish peroxidase-conjugated goat anti-rabbit Ig and subsequent reaction with chloronaphthol and hydrogen peroxide.

For each patient, concentration of t-PA antigen was determined in the 60-min blood sample using the ELISA kit marketed by American Diagnostica Inc., Greenwich, CT.

Assays for fibrinopeptide Bβ1-42 and FRA were performed without knowledge of either the clinical result of thrombolytic therapy or the identity of the active agent, and the data from these measurements were withheld from the primary TIMI physicians. The identity of the active agent employed in each patient was not revealed by the TIMI coordinating center until after the study was completed, and both clinicians and laboratory personnel were blind to the results of the t-PA antigen assays.

Results

The serum concentrations of FRA have been pooled according to drug received and time relative to the beginning of drug infusion and are plotted in Fig. 1. After SK administration, the FRA rose rapidly to 233 mg/dl at 60 min and remained at approximately that level through 150 min before beginning a gradual decrease. In contrast, the serum concentration of FRA rose less and more slowly after rt-PA administration, reaching a level of 120 mg/dl at 150–180 min the conclusion of the drug infusion. 2 h after the completion of rt-PA infusion the serum FRA concentration remained elevated at 109 mg/dl. The mean initial plasma fibrinogen concentration measured by the same technique was 349 mg/dl (range, 213–556 mg/dl) in those who received SK and 352 mg/dl (range, 186–508 mg/dl) in those who received rt-PA. Thus, patients who were treated with SK had maximum levels of serum FRA corresponding to the proteolysis of two-thirds of the circulating fibrinogen. Patients who received rt-PA had lower serum levels of FRA, corresponding to the proteolysis of approximately one-third of the circulating fibrinogen.

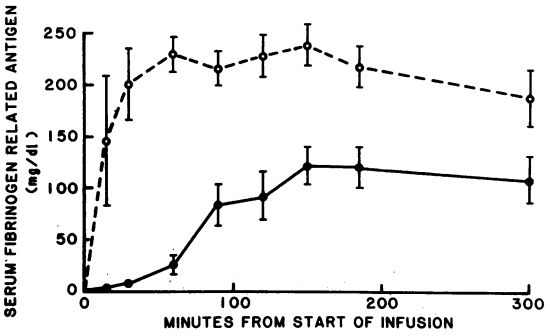

Figure 1. FRA in serum (- - o - -) are for patients who received SK and (— • —) are for those who received rt-PA. Concentrations are given as the amount of fibrinogen that would give the same immunoreactivity in the assay. Each point is shown as the mean±1 SE of the mean.

The mean plasma concentration of t-PA in patients receiving rt-PA was 996 ng/ml (range, 719–1329 ng/ml) 60 min after starting the infusion. Assuming a volume of distribution of ~ 2,500 ml (plasma volume) and that steady-state had been reached, this would correspond to a plasma half disappearance time of ~ 3 min for rt-PA.

The addition of rt-PA to normal plasma collected into THAT anticoagulant showed that the rate of production of fibrinopeptide Bβ1-42 was a linear function of both the rt-PA concentration and the duration of incubation on melting ice. The rate of in vitro release of Bβ1-42 was found to be 111±12 pmol Bβ1-42/μg rt-PA/h/ml plasma. This equation was used to correct the observed Bβ1-42 levels, in patients who received rt-PA, for ex vivo plasmin action. The known time delays from collection to fibrinogen precipitation were used together with the concentration of rt-PA at the different times. The concentration of rt-PA in plasma at each time point was calculated from the rate of infusion and the clearance rate of rt-PA. From 15 to 60 min the concentration was calculated as 996 ng/ml, 498 ng/ml for the time-points 90 to 150 min, 132 ng/ml at 185 min, and at baseline by 300 min.

The effect of SK or rt-PA infusion on the plasma concentration of fibrinopeptide Bβ1-42, an index of fragment X formation, is shown in Fig. 2. The levels are shown, using a logarithmic scale, as geometric means±the standard error of the geometric mean. SK produced a rapid increase in Bβ1-42 concentration to a peak value of 9,027 nM at 30 min. A rapid decrease in Bβ1-42 concentration was noted over the latter half of the SK infusion. A more gradual increase in Bβ1-42 occurred during rt-PA infusion, reaching a level of 948 nM at 60 min and rising to 1,085 nM at 150 min. Preinfusion Bβ1-42 levels were slightly increased above the normal level of 1–4 nM, at 7.2 nM and 5.2 nM respectively in patients who received SK and rt-PA.

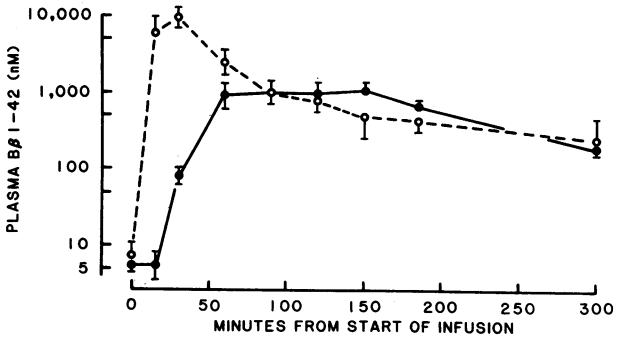

Figure 2. Plasma concentrations of fibrinopeptide Bβ1-42. (- - o - -) represent patients who received SK and (— • —) for those who received rt-PA. Each point is shown as the mean±1 SE of the mean.

The half disappearance time of fibrinopeptide Bβ1-42 from plasma was determined using data from patients who received SK. The observed decrease in the plasma concentration of the peptide, from 30 to 90 min after beginning the infusion, was well described (r^2 = 0.92) by a single exponential curve with half disappearance time of 24 min. This corresponds to a fractional clearance rate of 0.029 min^{-1}.

The fractional clearance rate of 0.029 min^{-1} was used to calculate the amount of fragment X formation, by integration of the Bβ1-42 concentrations over time. The results of these calculations are shown in Fig. 3. Bars depict the cumulated amount of fragment X formed by plasmin-mediated proteolysis of fibrinogen during the indicated interval from the start of drug infusion. The values for patients who received SK show a rapid rise, with approximately one-third of the fragment X formation (and thus fibrinogenolytic activity) occurring in the first 30 min. In contrast the curve for patients receiving rt-PA shows a distinct lag, with significant proteolysis becoming evident only in the second hour of the infusion. During the 5 h from the start of the infusion 264 mg/dl (SD, 54 mg/dl) of fibrinogen is converted to fragment X in response to SK infusion, whereas 96 mg/dl (SD, 12 mg/dl) of fragment X is formed in response to rt-PA.

Immunoblots of the FRA in plasma and serum were obtained on all subjects. Representative blots from patients receiving rt-PA or SK are shown in Figs. 4 and 5. The anti-fibrinogen probe, which reacts predominantly with an epitope in the D domain of fibrinogen, was the same anti-fibrinogen serum used to perform the radioimmunoassay for fibrinogen-related antigen, and this allows visualization of the fibrinogen fragments measured as FRA. In the left panel of Fig. 4 it is evident that large amounts of fragments Y and D are formed as early as 15 min after the beginning of the SK infusion. The corresponding immunoblots

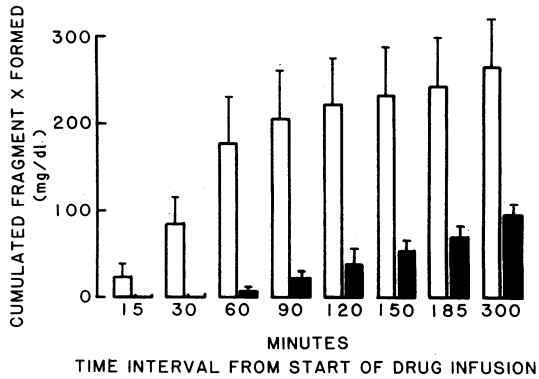

Figure 3. Cumulative fragment X formation during thrombolytic therapy, calculated from the observed fibrinopeptide Bβ1-42 concentrations. *Open bars* are for patients who received SK. *Solid bars* are for those who received rt-PA. Calculated amount of fragment X formed in the interval since start of infusion ± SD of the estimates are shown. Monte Carlo simulation was used to determine the SD of the integrated function.

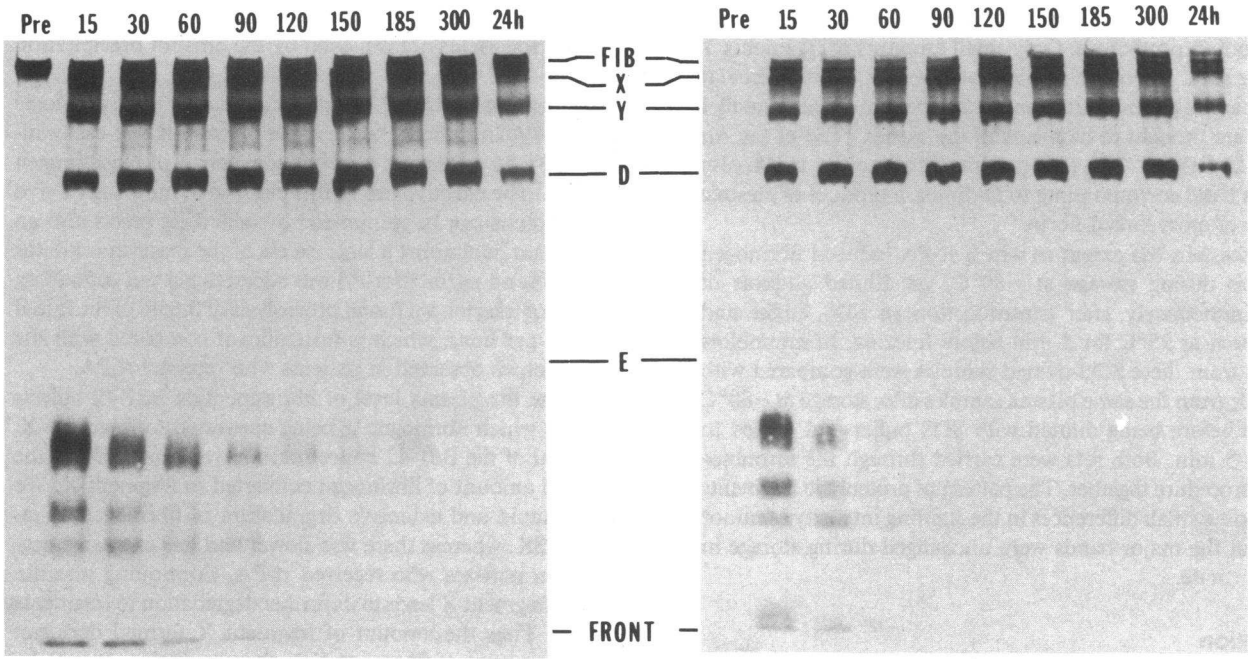

Figure 4. Immunoblots (5–15% PAGE) of FRA in plasma (*left*) and serum (*right*) from a patient who received SK. The figures above each well refer to the time in minutes after the start of drug infusion. The lanes marked *24h* are blood samples collected 24 h after thrombolytic therapy. The bands were identified by apparent molecular weight and comparison with immunoblots of plasmin digests of purified fibrinogen (not shown).

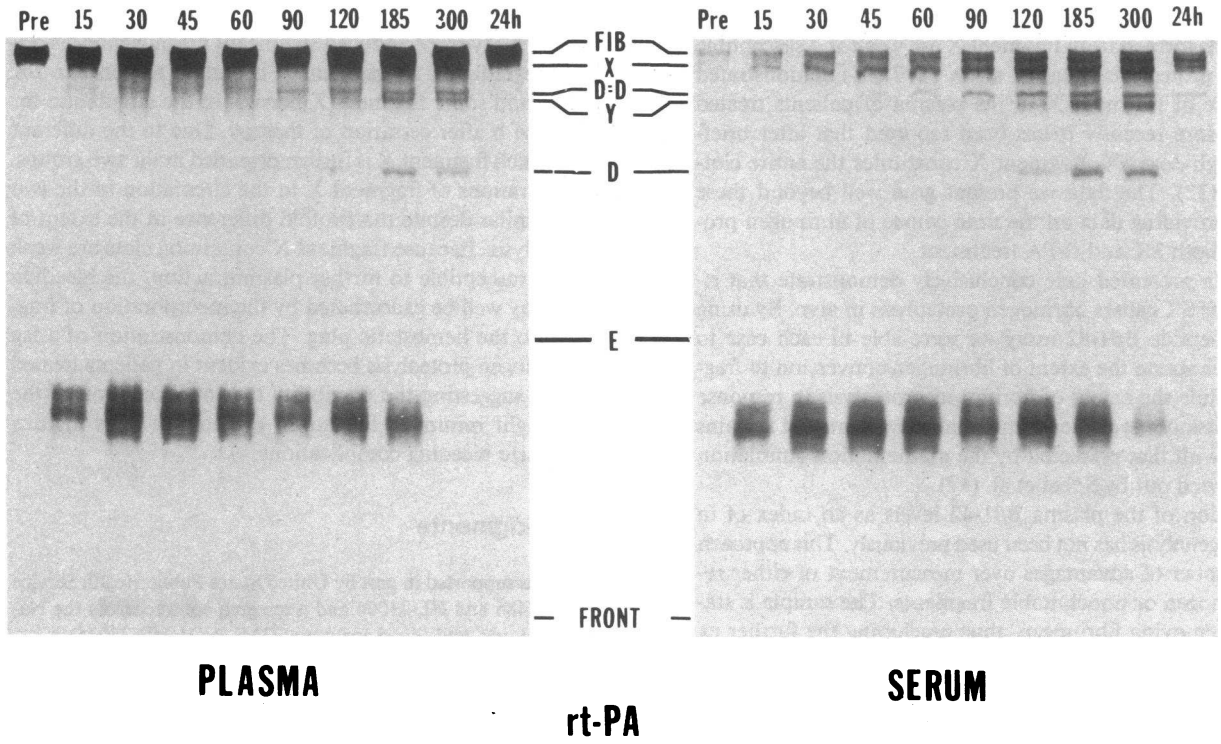

Figure 5. Immunoblots (5–15% PAGE) of FRA in plasma (*left*) and serum (*right*) from a patient who received rt-PA. The figures above each well refer to the time in minutes after the start of drug infusion. The lanes marked *24h* are blood samples collected 24 h after thrombolytic therapy. D dimer is seen 30 min after the start of infusion.

from a patient who received rt-PA (Fig. 5) demonstrate much less fibrinogen proteolysis. Only small amounts of fragments Y and D are seen. A series of peptides of lower apparent molecular weight than fragment E are seen in both patients. These smaller peptides are thought to be pieces of the carboxy end of the Aα chain of fibrinogen (29). This patient, who received rt-PA, also showed a band corresponding to D-dimer, a product of plasmic digestion of cross-linked fibrin.

To ascertain the extent to which rt-PA induced fibrinogen proteolysis during storage at −80°C, we diluted aliquots of plasma immediately after centrifugation in SDS buffer and heated them at 95°C for 5 min before freezing. Immunoblots prepared from these SDS-treated samples were compared with blots made from the same plasma samples after storage at −80°C for 1 mo before being diluted with SDS buffer and heated to 95°C for 5 min. Both sets were carried through the immunoblotting procedure together. The pattern of proteolytic fragments showed some small differences in the staining intensity of minor bands, but the major bands were unchanged during storage in the frozen state.

Discussion

Phase 1 of the TIMI trial demonstrated in a prospective double-blind trial that, in the doses used, rt-PA was more effective than SK in producing coronary artery reperfusion (10). However an unexpected finding was that the frequency of bleeding complications was not different between the two groups. This was true for bleeding at sites of arterial puncture and for sites remote from puncture. It has long been speculated that fibrinogen proteolysis might play a role (30) in the bleeding diathesis, but the precise mechanism remains elusive. Nonclottable fibrinogen degradation products can interfere with fibrin polymerization, whereas clots formed from fragment X are weak and susceptible to plasmin proteolysis. Lane et al. in 1977 (31) demonstrated the presence of fragment X in the plasma of patients treated with SK. More recently it has been reported that after brief-duration high-dose SK, fragment X constitutes the entire clot-table pool (32). The data we present goes well beyond these studies in providing data on the time course of fibrinogen proteolysis, in both SK and rt-PA treatment.

The data presented here conclusively demonstrate that rt-PA as well as SK causes fibrinogen proteolysis in vivo. By using the fibrinopeptide Bβ1-42 assay we were able in each case to precisely quantitate the extent of fibrinogen conversion to fragment X. While the extent of fibrinogen proteolysis in response to rt-PA infusion was greater than initially anticipated, it remains in keeping with that predicted by the mathematical simulation analysis carried out by Sobel et al. (17).

Integration of the plasma Bβ1-42 levels as an index of in vivo fibrinogenolysis has not been used previously. This approach offers a number of advantages over measurement of either residual fibrinogen or nonclottable fragments. The sample is stabilized by removing fibrinogen, thus precluding the further ex vivo generation of fibrinopeptide Bβ1-42 in response to t-PA–induced plasmin generation. The high sensitivity of the assay allows low levels of plasmin action on fibrinogen to be detected, while the clearance of the peptide is sufficiently quick that rapid changes in plasmin activity can be monitored. The specificity of this assay is such that only fibrinopeptide Bβ1-42 is being measured. Smaller fragments such as fibrinopeptides Bβ15-42 and Bβ1-14 do not react with the antibody (26), while larger fibrinogen fragments are removed by the ethanol precipitation step.

Stabilization of blood samples containing pharmacologic levels of t-PA in difficult because the enzyme is not easily inhibited (33). Thus there is ongoing conversion of plasminogen to plasmin. The effectiveness of this plasmin in inducing ex vivo fibrinogenolysis can be minimized by collecting blood into an anticoagulant containing a large excess of the plasmin inhibitor trasylol. When 1 μg/ml of rt-PA was added to plasma containing 200 U/ml of trasylol, we found proteolysis of 0.65% of the initial fibrinogen per hour, which is insignificant compared with the 30% proteolysis observed in patients who received rt-PA.

Because the plasma level of fibrinopeptide Bβ1-42 reflects the rate at which fibrinogen is being converted to fragment X, the integral of the Bβ1-42 concentration over time reflects the cumulated amount of fibrinogen converted to fragment X. We observed rapid and extensive degradation of fibrinogen in response to SK, whereas there was slower and less extensive degradation in patients who received rt-PA. Continuing plasmin action on fragment X leads to its further degradation to fragments Y and D. Thus the amount of fragment X formed does not equal the amount of fragment X in the circulation. It is to be expected that conditions that lead to the generation of large amounts of fragment X would also favor its further degradation. Thus while more fragment X was formed in response to treatment with SK, we would expect that more fragment X would be converted to fragments Y and D. It is clear from the immunoblots that the patients who received SK had extensive degradation of fragment X, with high levels of fragments Y and D. These blots also show that fragment X persists in the circulation for 24 h after thrombolytic therapy.

In summary, the data clearly demonstrate that at the dose used in this study rt-PA induced significant fibrinogenolysis. Fibrinogen degradation to fragment X occurs in response to SK and rt-PA, and some fragment X persists in the circulation for as long as 24 h after cessation of therapy. Due to the different degree to which fragment X is further degraded in the two groups, the concentrations of fragment X in the circulation in the two groups is similar despite the twofold difference in the extent of fibrinogenolysis. Because fragment X containing clots are weak and highly susceptible to further plasmin action, the bleeding diathesis may well be exacerbated by the incorporation of fragment X into the hemostatic plug. The demonstration of a lag before fibrinogen proteolysis becomes evident in patients treated with rt-PA, suggesting the possibility that manipulation of the regimen might minimize fibrinogen proteolysis. This in turn may minimize bleeding complications.

Acknowledgments

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