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Thrombolytic Therapy and Proteolysis of Factor V

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Objectives. **We sought to determine the extent of Factor V proteolysis during thrombolytic therapy.**

Background. **Thrombin- or Factor Xa–activated Factor V is an essential cofactor in the prothrombinase complex. In purified systems, plasmin, the major product of thrombolytic therapy, is known to first activate then inactivate Factor V.**

Methods. **We used quantitative gel electrophoresis and Western blotting to analyze the cleavages in plasma Factor V after thrombolytic therapy.**

Results. **The addition of streptokinase to plasma resulted in the activation then inactivation of Factor V, confirming previous results using purified reagents. We also identified the Factor V fragments resulting from the action of thrombin and plasmin. After thrombolytic therapy, there was considerable Factor V cleavage. The cleavage patterns were consistent with the action of plasmin, with little evidence for the action of thrombin. In the Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries trial (n = 17), we observed an average 58% loss of intact Factor V at 6 h (range 1% to 91%). Samples from the Thrombolysis in Myocardial Infarction trial, Phase II (n = 12), collected on a shorter time scale, showed a loss of up to 99% at 50 min, with the loss of intact Factor V associated with the plasma concentration of plasminogen activator. Samples from patients with bleeding (n = 12) showed extensive Factor V cleavage.**

Conclusions. **Factor V cleavage in thrombolytic therapy is primarily plasmin mediated, rapid and often extensive. It is likely that transient increases, as well as longer term losses, of Factor V cofactor activity play a role in both ischemic and hemorrhagic events subsequent to thrombolytic therapy. The extensive loss of Factor V in some patients may affect the estimation of heparinization.**

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Understanding the interaction of the coagulation and fibrinolytic systems is of key importance in many clinical settings, including thrombolytic therapy after acute myocardial infarction. In this setting, there may be thrombin as well as extensive plasmin production, with the potential for complex and profound alterations in both the coagulation and fibrinolytic systems. Some of these changes are mediated through activation or degradation of Factor V, or both.

Factor Va is the protein cofactor required for the efficient conversion of prothrombin to thrombin. As such, it is a key regulatory component of the coagulant response (for review, see references 1 and 2). Figure 1 illustrates the reactions in which Factor V participates. Activation of the plasma procofactor Factor V to the active cofactor Factor Va is thought to proceed predominantly through its initial activation by Factor Xa, and subsequent feedback activation by thrombin (3). Recently, is has been shown that two monocyte enzymes, elastase and cathepsin G, are also capable of activating Factor V (4). Thrombin is also capable of contributing to the inactivation of Factor Va through interaction with thrombomodulin and the generation of activated protein C (5). Although the proteolytic effects of plasmin on Factors V and Va have been suspected for some time, it has only recently been elucidated at the molecular level (6,7). Plasmin is not only capable of the initial activation of Factor V, but also the subsequent rapid inactivation of Factor Va (7).

To date, little is known about the changes in Factor V associated with either endogenous or pharmacologic fibrinolysis. After tissue-type plasminogen activator (t-PA) therapy, one group reported no change in the clotting activity of Factor V (8), whereas another group reported a decrease of ~50% (9). However, clotting assays are influenced by other alterations occurring in this setting (e.g., fibrinogenolysis, heparin administration). There have been no studies on the molecular alteration of Factor V in any clinical setting. Factor V activation and depletion may affect both the coagulation status of the patient and the activated partial thromboplastin time (aPTT) commonly used to estimate adjunctive heparin therapy. Therefore, we have developed a sensitive Western blotting technique for Factor V analysis, which we used to examine plasma from patients undergoing thrombolytic therapy for acute myocardial infarction.

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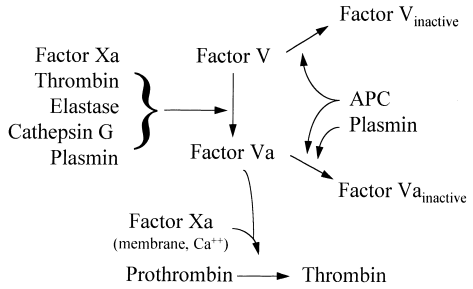
Abbreviations and Acronyms

aPTT	=	activated partial thromboplastin time
FDP	=	fibrin(ogen) degradation product
GUSTO	=	Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries (trial)
IgG	=	immunoglobulin G
PPACK	=	D-Phe-Pro-Arg-chloromethyl ketone
rt-PA	=	recombinant tissue-type plasminogen activator
SDS-PAGE	=	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
t-PA	=	tissue-type plasminogen activator
TIMI II	=	Thrombolysis in Myocardial Infarction trial, Phase II

Methods

Materials. Human plasma Factor V was isolated by immunoaffinity chromatography as described by Katzmann et al. (10), and Factor Va was prepared using thrombin (11). Radioiodinated Factor V was prepared using Bolton-Hunter reagent (ICN Biomedics) to a specific activity of 300 to 800 cpm/ng (12). Prothrombin was prepared as described by Bajaj et al. (13). Human a-thrombin was prepared by activation of prothrombin using *Oxyuranus scutellatus scutellatus* (Taipan snake) venom as described by Owen and Jackson (14). A burro polyclonal a-human Factor V antibody was used in the Western blotting analyses as previously described (15). Biotinylated goat antihorse immunoglobulin G (IgG) (which cross reacts with the burro immunoglobulin), avidin and biotinylated horseradish peroxidase were obtained from Vector Laboratories, Inc. Horseradish peroxidase-conjugated antihorse IgG was obtained from Southern Biotechnology Associates. Nitrocellulose (0.45-μm pore size) was purchased from Schleicher and Schuell. Reagents for the chemiluminescent detection of antigens on Western blots (ECL Reagents) were obtained from Amersham Life Science. All other materials used were of reagent grade, or the highest grade commercially available.

Figure 1. Reactions involving Factor V. Factor V must be activated to Factor Va for thrombin to be generated and coagulation to proceed. Factor Va is the required cofactor for Factor Xa because the prothrombinase complex assembles on a membrane surface in the presence of Ca^{2+}. Factor V activation may be catalyzed by several enzymes: Factor Xa, thrombin, monocyte elastase, cathepsin G and the fibrinolytic enzyme plasmin. Factor V and Factor Va may be rendered inactive by activated protein C (APC), and plasmin is able to inactivate Factor Va as well.



Plasma samples. For in vitro experiments, venous blood samples were collected from healthy volunteers after an approved protocol reviewed by the University of Vermont Committee on Human Research Institution Review Board. We used a two-syringe technique with 1/10 volume 3.8% trisodium citrate as anticoagulant agent. Plasma was rendered platelet-poor by centrifugation at 8,000 × *g* for 15 min at 4°C. The plasma was frozen and stored at −70°C.

Our primary experimental plasma samples were from two large studies of thrombolytic therapy: the Thrombolysis In Myocardial Infarction (TIMI) trial, phase II (16) and the Global Utilization of Streptokinase and t-PA for Occluded arteries (GUSTO) trial (17). The protocols for both studies were approved by all appropriate Institutional Review Boards. In the TIMI II trial, patients received either 100- or 150-mg doses of t-PA in the presence of heparin (18). In the GUSTO trial, patients received one of four possible treatment strategies, utilizing accelerated-dose t-PA or streptokinase, or both, all in the presence of heparin (19). Fifteen samples from GUSTO were chosen randomly from the noninvasive subgroup. Blood was collected for analysis in GUSTO at six time points: before administration of therapy and at 6, 12, 24, 36 and 48 h after therapy. Samples from TIMI II were chosen in two ways. Initially, 12 samples were chosen to represent a range of fibrinogen loss (as an approximation of plasmin generation). Seven patients exhibited only slight fibrinogen loss (low loss group); five were chosen for more extensive fibrinogenolysis (high loss group). Patients from both the 100- and 150-mg treatment groups were selected. An additional 12 samples were chosen randomly from those patients who received 100 mg of t-PA and had experienced major bleeding events during their hospital stay, as described previously (20). Bleeding was classified as major if it did not involve surgical treatment but involved either: 1) intracranial hemorrhage or cardiac tamponade; 2) a decrease in hemoglobin >50 g/l whether or not a bleeding site had been identified; or 3) death from hemorrhage on clinical review by the Mortality and Morbidity Classification Committee of TIMI II (20). Testing, in addition to Western blot analyses, included assays for fibrinogen, fibrin(ogen) degradation products (FDP) and t-PA antigen. There were four blood collection times in the TIMI II trial: before infusion of recombinant tissue-type plasminogen activator (rt-PA), at the peak of infusion (50 min), toward the end of infusion (300 min) and 2 h after the end of infusion (480 min).

Blood from the patients in the GUSTO and TIMI II trials was collected into special collection tubes we prepared containing 20 mmol/liter D-Phe-Pro-Arg-chloromethyl ketone (PPACK; Calbiochem-Novabiochem Corp.), a potent inhibitor of t-PA, thrombin and plasmin; 150 kallikrein inhibiting U/ml of aprotinin (American Diagnostica, Inc.), a serine protease inhibitor; and 4.5 mmol/liter EDTA. These samples were used for Western blotting analyses and t-PA assays. Plasma from a patient totally deficient in plasma and platelet Factor V antigen was used as a negative control in all experiments. Samples in TIMI II were also collected into PPACK plus

sodium citrate and used for FDP and fibrinogen analyses once the excess PPACK had been allowed to hydrolyze by incubation of plasma at 37°C for 40 min (21). All samples were snap-frozen and stored at −70°C until analysis.

Coagulation testing. Fibrinogen was measured on either a BBL fibrometer (Becton Dickinson) or the Stago ST4 instrument (American Bioproducts) using a modification of the Clauss method (21,22) and standardized using standard plasma from Dade (Baxter Healthcare Corp.). Fibrin degradation product samples from patients in TIMI II were assayed by tanned red cell hemagglutination inhibition immunoassay (23). Tissue-type plasminogen activator antigen was measured using a sandwich immunoassay (24) with reagents generously supplied by Dr. Desire Collen from Leuven, Belgium. Because we were interested in the possible activation of Factor V, activity was assessed with a one-stage factor assay using immunodepleted Factor V–deficient plasma and the Stago ST4 instrument.

To estimate the effect of thrombin on Factor V, we added 0.1 ml of 5 nmol/liter $CaCl_2$ in veronal buffer and 0.1 ml of 10-U/ml bovine thrombin (Dade) to 1.8 ml of fresh citrated plasma and incubated this sample at room temperature. Under these conditions, we observed initial clot formation in ~1 min. Samples were withdrawn over time and prepared for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To determine the effect of plasmin on citrated plasma using Western blotting, we added 200 U/ml of streptokinase to a second sample of the identical plasma, incubated this sample at room temperature and removed aliquots over time for analysis. We used 100 U/ml of streptokinase in experiments to estimate the effect of plasmin on Factor V activity.

Western blotting analysis of Factor V. Plasma samples (3.5 μl) were diluted 1:9 in a buffer containing 1% SDS, 2% 2-mercaptoethanol, 0.325 mol/liter Tris base (pH 6.5), 10% glycerol and 0.001% bromphenol blue, and subjected to electrophoresis in either full-size or mini-gel polyacrylamide gradient slab gels (5% to 15%) according to Laemmli (25). After electrophoresis, the separated proteins were transferred to nitrocellulose as previously described (26). After incubation with a burro antihuman Factor V polyclonal antibody as the primary probe, Factor V and its derived peptides were visualized in one of two ways. Initially blots were developed using biotinylated goat antihorse IgG, followed by avidin, biotinylated horseradish peroxidase and the precipitating chromogenic peroxidase substrate 4-chloro-1-naphthol plus hydrogen peroxide. In later experiments, a chemiluminescent detection system was used (Amersham ECL), in which peroxidase-conjugated secondary antibody was detected according to the manufacturer's protocol.

The quantity of intact Factor V (i.e., the 330-kDa band) was estimated using scanning densitometry (Microscan 1000, TRI). Subject plasmas, as well as a normal standard plasma (at four dilutions), were run on each gel so that gel-specific standard curves could be constructed. Standard curves were linear from 25 to 200 ng/lane of Factor V. For blots using the precipitating peroxidase chromogenic substrate, photographs were taken and the negatives used to expose sheet film, making a "positive" transparency for densitometry. For chemiluminescent blots, the directly exposed sheet film was used for densitometry. In both cases, the standard curve on each gel acted as an internal standard. Factor V concentrations are expressed either as the percentage of Factor V in the standard plasma or as mass units (ug/ml) based on the known Factor V concentration in the standard plasma.

Statistical analyses. Data are expressed as mean values ± SEM. All statistical analyses were done using SPSS for Windows version 6.0.1. We tested for differences between mean values using either analysis of variance or the Student *t* test (two-sided). Associations were estimated using the Pearson correlation coefficient. Significance was set at $p \leq 0.05$.

Results

In vitro studies of Factor V cleavage by thrombin and plasmin. We used Western blotting techniques to examine the cleavage patterns of plasma Factor V when thrombin and plasmin were used to effect proteolysis (Fig. 2). Consistent, time-dependent changes in the Factor V Western blotting patterns were observed. Purified Factor V, seen predominantly as a band of 330,000 apparent molecular weight (330 kDa), was the major band observed in whole human plasma at the "0" time point. However, even with our best efforts at blood collection and sample preparation, there were always at least small amounts of immunoreactive material at bands of 280, 220 and 150 kDa. The 330-kDa band was not observed in human serum (data not shown), and no immunoreactive bands were observed in plasma from a Factor V–deficient individual (Fig. 2, lane C). Both plasmin and thrombin generated bands at 280 kDa, although thrombin did so to a considerably greater extent than did plasmin. Both enzymes also generated bands at 220 and 150 kDa and a doublet at 74/72 kDa. Thrombin generated a specific band at 105 kDa, whereas plasmin generated specific bands at 130, 110 and 92 kDa. These Western blot patterns of whole plasma agree well with the known thrombin- and plasmin-mediated electrophoretic cleavage patterns of Factor V in purified systems (1,2,11,27–29). The action of Factor Xa as a Factor V cleavage enzyme cannot be ruled out, because Factor Xa–mediated cleavage produces peptides of 105 and 220 kDa (29). The analysis of bands at lower apparent molecular weights was hindered by nonspecific adsorption of the immune reagents and the chromogenic and chemiluminescent visualization materials.

Because plasmin is known to both activate Factor V and inactivate Factor Va, we analyzed Factor V activity over time in plasma treated with streptokinase. Figure 3 shows that Factor V activity initially increases and then decreases over time, confirming previous results obtained using purified reagents (7). As discussed previously (7), it remains unclear which particular bands generated under these conditions are associated with cofactor activity.

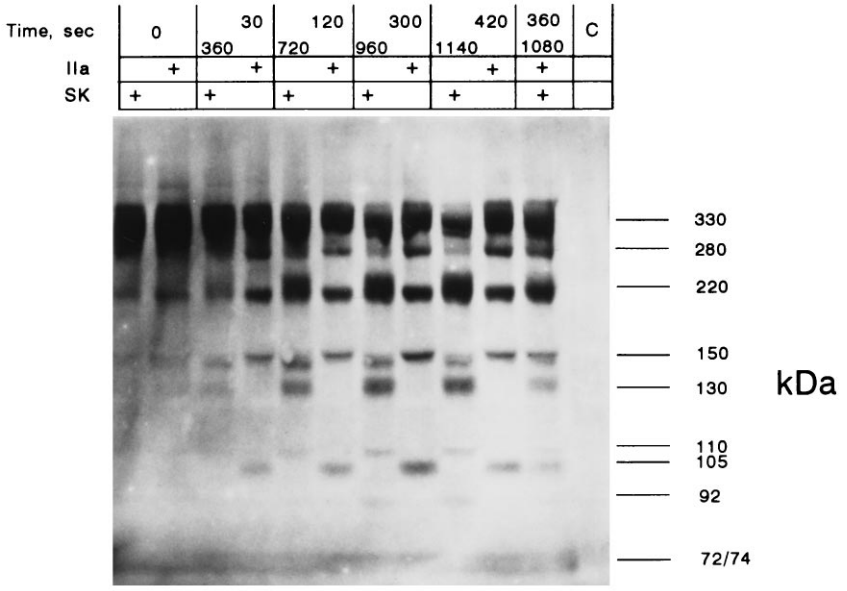
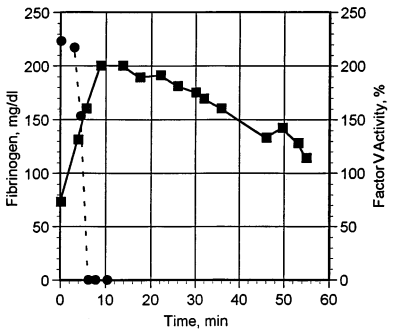


Figure 2. Western blotting analysis of normal plasma treated with thrombin and streptokinase. Fresh citrated plasma was treated with either thrombin or streptokinase as described in the text. C = control plasma from a subject with a congenital lack of Factor V; kDa = kilodaltons; Time = time after start of incubation at which samples were removed and prepared for electrophoresis; + = reagent—thrombin (IIa) or streptokinase (SK)—that was added to sample; **Numbers at right** = apparent molecular weight of selected bands based on SDS-PAGE migration, as estimated from the migration of protein standards.

In vivo cleavage of Factor V after thrombolytic therapy. To explore the extent of change in Factor V concentration associated with pharmacologic fibrinolysis, using Western blots we analyzed samples from 17 patients undergoing thrombolytic therapy as part of the GUSTO trial. The results for four representative patients are shown in Figure 4. The time points represented are "0" time (before drug administration) and 6, 12, 24, 36 and in one case 48 h after the start of infusion. Several alterations in Factor V banding patterns are evident. First, at the "0" time point all samples we examined had evidence of various amounts of Factor V cleavage, consistent with endogenous plasmin activity. In only one case (Subject 1) did we observe a band specific for thrombin activity (105 kDa). After pharmacologic plasminogen activation, we observed decreased immunoreactivity of the 330-kDa intact Factor V band, as well as increased immunoreactivity at bands more consistent with plasmin-mediated cleavage than with thrombin: 220, 150, 130, 110 and 92 kDa and the 74/72-kDa doublet. We also detected the presence of a band at ~180 kDa, which we did not observe in the in vitro experiment.

Figure 3. Streptokinase-mediated change in plasma fibrinogen and Factor V levels. Streptokinase (100 U/ml) was added to fresh citrated plasma and incubated at room temperature. The samples were withdrawn over time and assayed for fibrinogen (clottable fibrinogen [22]) and Factor V activity (one-stage clot-rate assay using Factor V–deficient plasma). Factor V activity is expressed as percentage of a standard plasma.



To quantitate the change in intact Factor V associated with thrombolysis, standard curves were constructed on each gel, using a control plasma followed by densitometric analyses of the Western blots (Fig. 5). Densitometry of the 330-kDa band in all 17 GUSTO samples was done to assess the extent and time course of Factor V cleavage. Figure 6 illustrates the mean values (± SEM) of the density associated with each subject's 330-kDa band expressed as a percentage of the standard plasma. The average intact Factor V level in this group before infusion was 205%. Factor V cleavage was maximum at the time point closest to drug infusion, with levels slowly recovering over the next 2 days. The range, at 6 h after infusion, for the percentage of starting intact Factor V, was 11% to 99%, indicating a variable response to pharmacologic thrombolysis. At 24 h, the range was 14% to 111% of starting intact Factor V.

To examine changes in Factor V over a shorter time frame (0 to 8 h), we analyzed samples from 12 patients in TIMI II, who were chosen based on either relatively large or relatively small thrombolysis-related losses in fibrinogen. Factor V antigen along with fibrinogen, FDP and t-PA were compared between the two groups (Fig. 7). Low fibrinogen loss, compared with extensive fibrinogenolysis, was associated with a lower mean t-PA antigen value at 50 min, higher mean FDP values at 50, 300 and 480 min and relatively small amounts of Factor V cleavage. In the low fibrinogenolysis group, intact Factor V levels at 50 min averaged ~70% of the preinfusion mean level, compared with 14% in the high fibrinogenolysis group. The Western blot patterns were consistent with the action of plasmin (data not shown). The time course of Factor

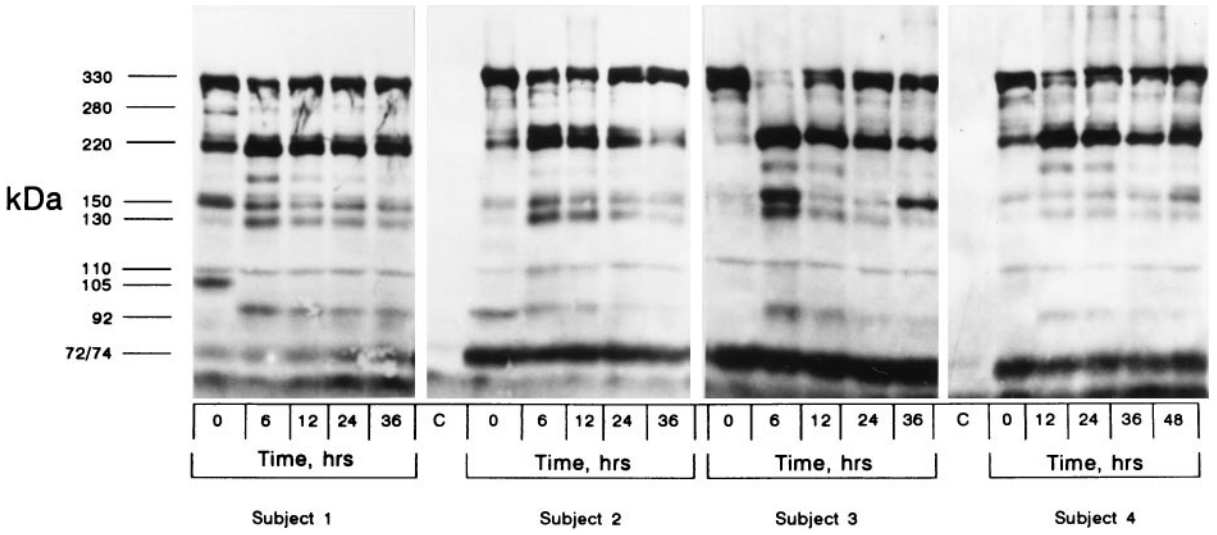
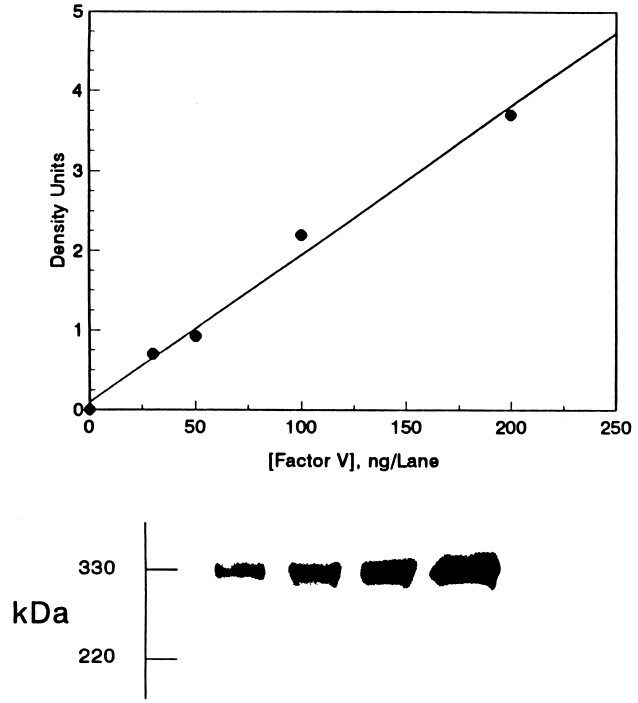


Figure 4. Western blotting analyses of Factor V in plasma samples from subjects receiving thrombolytic therapy in the GUSTO trial. Plasma samples were collected and processed for Western blotting as described. C = control plasma from a subject with a congenital lack of Factor V; kDa = kilodaltons; **numbers at left** = apparent molecular weight of selected bands based on SDS-PAGE migration, as estimated from the migrations of protein standards.

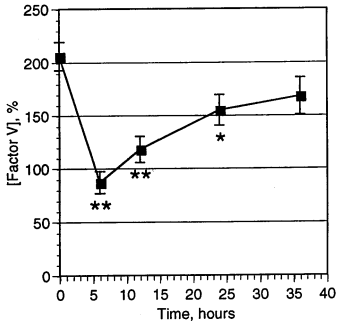
Figure 5. Standard curve for the quantitative Western blotting analyses of intact Factor V in the plasma samples. The plasma samples were analyzed by Western blotting as described in the text. Densitometric data were collected on the 330-kDa band. Each individual gel contained a standard curve. The figure illustrates a representative standard curve made from densitometric data of a single gel. The quantity of Factor V present in the standardized plasma was determined by radioimmunoassay (12). kDa = kilodaltons.



V loss was different from that of fibrinogen in the high fibrinogen loss group. The nadir for Factor V was at the 50-min time point, whereas fibrinogen was lowest at the 480-min point.

Assessment of Factor V cleavage in patients with bleeding. An additional 12 patients in TIMI II were studied because of serious bleeding events during their stay in the hospital. There were significant differences between the 0- and 50-min time points for fibrinogen, FDP, t-PA and Factor V (Fig. 8). At 50 min, the average loss in fibrinogen and generation of FDPs were similar to those seen in the low fibrinogen loss group. The t-PA antigen value was intermediate between the low and high fibrinogen loss groups. In contrast, the decrease in intact Factor V at 50 min (average 77% loss, range 21% to 100%) closely resembled that of the high fibrinogen loss group. The correlation of t-PA antigen values and loss of intact Factor V, using data from all patients in TIMI II for whom we had both t-PA antigen values and Factor V Western blot data (n = 21), was significant ($r = 0.4688$, $p \leq 0.05$).

Figure 6. Changes in the amount of intact Factor V in the plasma samples of subjects from the GUSTO trial. The original chemiluminescent blots corresponding to Figure 4, and others, were analyzed densitometrically at the 330-kDa band (intact Factor V), with values expressed as a percentage of the standard plasma used on each gel. Data are presented as mean value ± SEM. *$p \leq 0.05$ and **$p \leq 0.01$ versus the 0 time point. Results from analysis of variance using all five time points: $p \leq 0.001$.



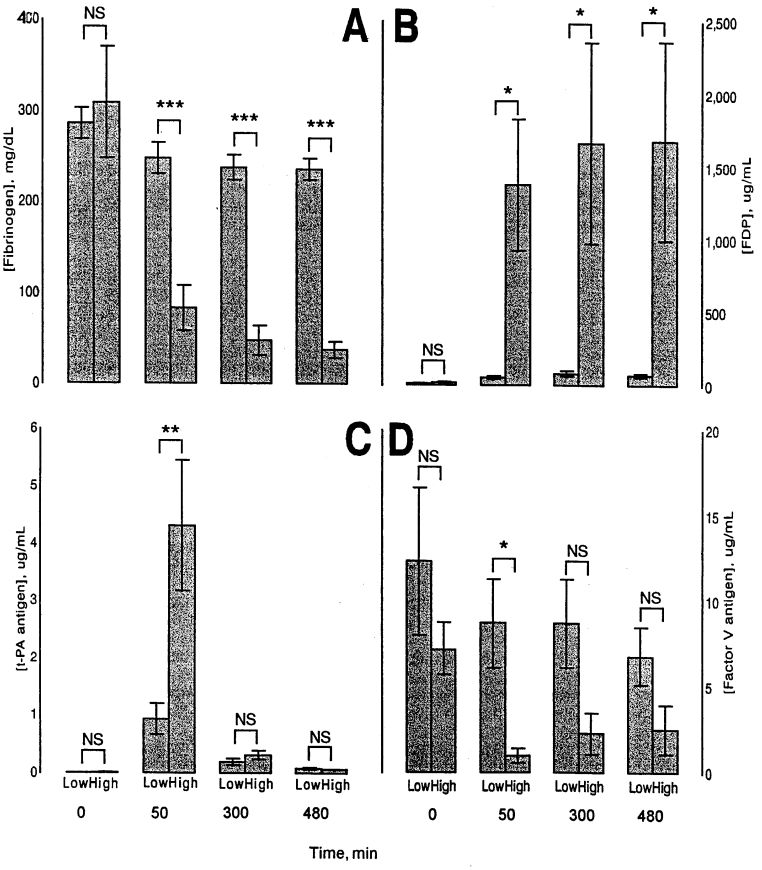


Figure 7. Changes in plasma fibrinogen, FDP, t-PA antigen and intact Factor V after thrombolytic therapy in samples from patients in TIMI II with either minimal or extensive fibrinogenolysis. Fibrinogen (**A**), FDP (**B**), t-PA antigen (**C**) and 330-kDa Factor V antigen (**D**) were measured as described in the text in two sets of patients in TIMI II—those with low fibrinogenolysis (**left-hand bars** in panels A to D; n = 7) and those with high fibrinogenolysis (**right-hand bars**, n = 5). Data from samples taken before infusion (0 min) and from 50, 300 and 480 min after the start of infusion are expressed as mean value ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, for between-group comparisons of mean values.

Discussion

The study of Factor V in the setting of thrombolytic therapy is important for several reasons. 1) The activation of Factor V in this setting may be a key factor in recurrent ischemia and thrombosis (7). Eisenberg et al. (30,31) and other investigators (32) have suggested that thrombolytic therapy is accompanied by activation of the coagulation system. This activation may result from reexposure of the procoagulant surface that presumably triggered the initial thrombotic response, in addition to activation of key regulatory cofactors such as Factor V. 2) Degradation of Factor V may play a role in bleeding complications associated with systemic fibrin(ogen)olysis, which can range from mild to severe (20,33). 3) The degrada-

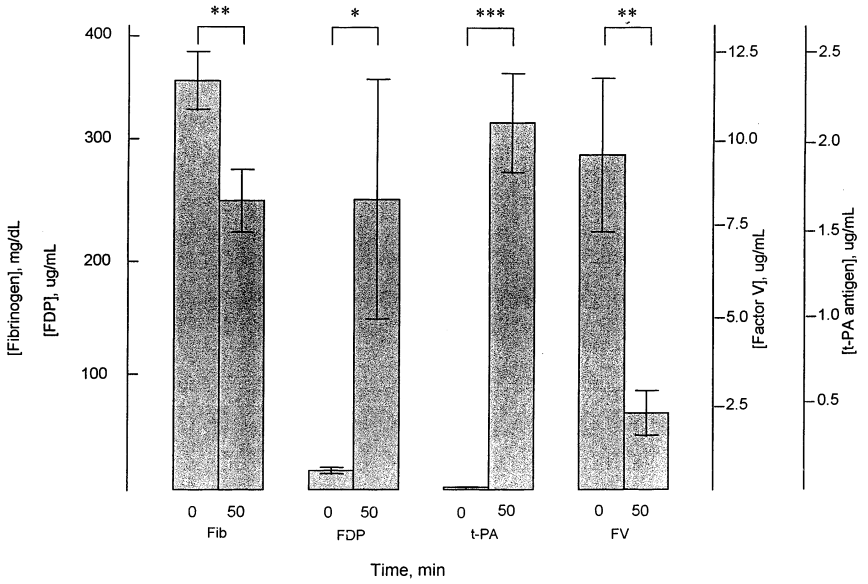


Figure 8. Changes in plasma fibrinogen (Fib), FDP, t-PA antigen and intact Factor V (FV) after thrombolytic therapy in samples from patients in TIMI II with hemorrhagic events. Fibrinogen, FDP, t-PA antigen and Factor V were measured as described in the text in 12 patients in TIMI II who experienced hemorrhagic events during their hospital stay and received thrombolytic therapy (t-PA, 100 mg) for acute myocardial infarction. Data from samples taken before infusion (0 min) and from 50 min after the start of infusion are expressed as mean values ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, for comparison of mean values between 0 and 50 minutes.

tion of Factor V, if extensive, will prolong the aPTT value, which is used to estimate the heparin concentration and to adjust the dosage. Adjunctive heparin therapy has been shown to be of critical importance in the optimal utilization of t-PA (34–37), and a lack of understanding the factors that influence aPTT will affect optimal heparin adjustment.

Description of cleavage patterns of Factor V due to thrombolytic therapy. To our knowledge, this report is the first to describe the molecular events associated with the in situ proteolysis of human Factor V during thrombolytic therapy. Although there have been numerous references to the loss of Factor V activity during fibrinolytic activation—plasmin has been reported to be the enzyme responsible (38)—it has only been shown recently that plasmin, in vitro, is capable of proteolytic cleavage of Factor V (6,7). Interestingly, in vitro results suggest that plasmin is capable of first the activation and then the inactivation of this key coagulation pro-cofactor.

We have confirmed that, in whole plasma, the thrombin and plasmin cleavage patterns of Factor V are different. Because thrombin and Factor Xa activities result in bands of identical apparent molecular weights, with the exception that Factor Xa does not produce bands at 280 and 74 kDa, we could not differentiate thrombin and Factor Xa activities in our system.

Significance of plasmin-mediated Factor V cleavage during thrombolytic therapy. Concerning the plasmin-mediated cleavages we observed, it is unclear which cleavage products are associated with Factor Va cofactor activity and which result in inactive degradation products. Therefore, we are unable to determine from the electrophoretic analysis presented here whether specific cleavages are accompanied by activation or inactivation of Factor V. Preliminary results from our laboratory indicate that similar plasmin cleavages are seen in the setting of disseminated intravascular coagulation and are most frequently associated with decreased Factor V activity in Factor V clotting assays (data not shown). In the current study, we report that hemorrhagic events are commonly associated with relatively large losses in intact Factor V (Fig. 7). Because the time course of Factor V activation/inactivation is relatively rapid (7), it seems likely that sustained plasmin generation, such as is seen in thrombolytic therapy, will result in an *overall* decrease in functional Factor V activity. However, transient localized increases also seem likely given the in vitro results shown in Figure 3. The degradation phase is particularly difficult to predict because it is membrane dependent (7). The lack of evidence for thrombin-mediated Factor V cleavage after thrombolytic therapy may indicate that this is a relatively rare event, and that plasmin-mediated cleavages predominate. This, in turn, suggests that plasmin may play a significant role in any posttherapy recurrent thrombotic events. More studies are needed to definitively address these points. Collecting blood samples for accurate Factor V activity assays in this setting will continue to be difficult because of the need to prevent in vitro artifacts. Therefore, it would be helpful to be able to associate Factor Va cofactor activity with specific cleavage products, as observed on Western blots.

We have found only three recent studies that have reported Factor V plasma concentrations after thrombolytic therapy (see ref. 39 for review). Kasper et al. (40) reported on 22 patients who received intracoronary anistreplase (acylated streptokinase–plasminogen complex). Using a one-stage assay sensitive to Factor Va levels, they showed that Factor V values dropped from a preinfusion mean (± SD) value of 96 ± 11% to a nadir mean value of 53 ± 26%. Factor V values were lowest at 6 h after infusion and had normalized by ~10 h. Of the 22 patients, 18 showed a >75% decrease in Factor V. Blood specimens for this study, unfortunately, were collected in sodium citrate without protease inhibitors, so these results must be considered cautiously.

Topol et al. (8) studied 30 patients who received intravenous t-PA. The average dose was 55 mg of two-chain t-PA (estimated to be approximately equivalent to 90 mg of one-chain t-PA [39]), and the average peak antigen value was ~850 ng/ml. Blood was collected with sodium citrate as an anticoagulant agent, with added aprotinin as a protease inhibitor. These investigators reported no significant effect on plasma Factor V. However, Collen et al. (9), using similar collection and assay methods, reported different results. They studied 86 patients who received t-PA (0.75 mg/kg body weight per 90 min; mean peak value of 1.2 μg/ml)—53 who received streptokinase (1.5×10^6 IU/60 min) and 33 who received placebo (9). The t-PA group had an average drop in Factor V of 15% at 60 min and 40% at 90 min; the streptokinase group had average drops of 58% and 68%, respectively. Interestingly, the placebo group showed an average drop of 19% by 90 min. Although individual-specific data were not presented, the large distributions of Factor V values at each time point suggest that, in contrast to the report of Topol et al. (8), some individuals achieved quite low Factor V concentrations during infusion of either t-PA or streptokinase.

Subject-to-subject variability in cleavage of Factor V. Our results are consistent with the position that some Factor V processing occurs in all patients receiving t-PA, but that the extent is highly variable, ranging from relatively mild to virtually complete. The degree of loss of intact Factor V was associated with the t-PA antigen level, which is consistent with the activity of plasmin. Because the patients in TIMI II with major bleeding abnormalities showed an average 77% loss in intact Factor V at 50 min, but only an average 29% fibrinogen loss, Factor V may be a preferred substrate under some conditions. If the majority of this processing results in degradation (even if there is transient activation), this may give rise to a plasmin-mediated coagulation defect that is not reflected in a drop in the fibrinogen value. This is consistent with the poor predictive ability of fibrinogenolysis for hemorrhagic events in the TIMI II trial (20), and suggests that further studies of Factor V proteolysis may be instructive in understanding the pathophysiology of hemorrhage after thrombolytic therapy. The loss of fibrinogen and the loss of intact Factor V followed different time courses in the five patients exhibiting high fibrinogenolysis (Fig. 7). This observation suggests that one protein may be a superior substrate to the other under certain conditions. Alternatively, this may reflect the

different half-lives of these two proteins: ~3 days for fibrinogen and 12 h for Factor V (41). The importance of Factor V proteolysis may be concentrated in the early time course of infusion, especially with the newer "front-loaded" rt-PA dosage regimens (17,19).

Potential effect of Factor V cleavage on estimation of heparinization. The extent of intact Factor V loss we observed is, at least for some patients, clearly of a magnitude that would have an effect on the aPTT, if it were associated with degradation. There is much current interest in aPTT and its use in defining optimal heparin administration. Also, the manner in which anticoagulation adjuvant therapy is terminated in any given patient has come under study, because a possible "rebound" effect has been identified (i.e., increased tendency toward thrombosis after the discontinuation of anticoagulation) (42). We have reported that under some conditions, aPTT prolongation may be associated with recurrent thrombosis (Tracy RP, Kleiman NS, Thompson B, et al., unpublished data, 1991). A possible hypothesis is that unrecognized Factor V degradation might contribute to aPTT prolongation, causing an inappropriate downward adjustment of the heparin infusion rate in some patients and putting the patient at risk for thrombosis as the Factor V level starts to increase.

Finally, concerning Factor V cleavage, we have little information on any role played by the fibrin specificity of the plasminogen activator used as thrombolytic therapy. The GUSTO study examined streptokinase, t-PA and the combination of both. Although the number of samples we report on here is too small to address this issue, a larger study should prove informative. If fibrin specificity results in decreased Factor V proteolysis, this would provide increased rationale to consider newer agents with even greater degrees of fibrin specificity, such as TNK–t-PA (43) and staphlokinase (44).

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References

1. Jenny RJ, Mann KG. Factor V: a prototype co-factor for vitamin K–dependent enzyme complexes in blood clotting. Baillieres Clin Haematol 1989;2:919–44.
2. Mann KG, Jenny RJ, Krishnaswamy S. Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. Ann Rev Biochem 1988;57: 915–56.
3. Mann KG. Normal Hemostasis. In: Kelley W, editor. Textbook of Internal Medicine. New York: J.B. Lippincott, 1992:1240–5.
4. Allen DH, Tracy PB. Human coagulation factor V is activated to the functional cofactor by elastase and cathepsin G expressed at the monocyte surface. J Biol Chem 1995;270:1408–15.
5. Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. J Biol Chem 1987;264:4743–53.
6. Omar MN, Mann KG. Inactivation of factor Va by plasmin. J Biol Chem 1987;262:9750–5.
7. Lee CD, Mann KG. Activation/inactivation of human factor V by plasmin. Blood 1989;73:185–90.
8. Topol EJ, Bell WR, Weisfeldt ML. Coronary thrombolysis with recombinant tissue-type plasminogen activator: a hematologic and pharmacologic study. Ann Intern Med 1985;103:837–43.
9. Collen D, Bounameaux H, DeCock F, Lunen HR, Verstraete M. Analysis of coagulation and fibrinolysis during intravenous infusion of recombinant human tissue-type plasminogen activator in patients with acute myocardial infarction. Circulation 1986;73:511–7.
10. Katzmann JA, Nesheim ME, Hibbard LS, Mann KG. Isolation of functional human coagulation factor V by using a hybridoma antibody. Proc Natl Acad Sci USA 1981;78:1622–7.
11. Nesheim ME, Mann KG. Thrombin-catalyzed activation of single-chain bovine factor V. J Biol Chem 1979;254:1326–38.
12. Tracy PB, Eide LL, Bowie EJW, Mann KG. Radioimmunoassay of factor V in plasma and platelets. Blood 1982;60:59–67.
13. Bajaj SP, Rapaport SL, Prodanos C. A simplified procedure for purification of human prothrombin, factor IX and factor X. Prep Biochem 1981;11:397–405.
14. Owen WG, Jackson CM. Activation of prothrombin with *Oxyuranus scutellatus scutellatus* (Taipan snake) venom. Thromb Res 1973;3:705–10.
15. Viskup RW, Tracy PB, Mann KG. The isolation of human platelet factor V. Blood 1987;69:1468–75.
16. The TIMI Study Group. Comparison of invasive and conservative strategies after treatment with intravenous tissue plasminogen activator in acute myocardial infarction. N Engl J Med 1989;320:618–27.
17. The GUSTO Investigators. An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. N Engl J Med 1993;329:673–82.
18. Braunwald E, Knatterud GL, Passamani E. Announcement of protocol change in thrombolysis in myocardial infarction trial [letter]. J Am Coll Cardiol 1987;9:467.
19. The GUSTO Investigators. The effects of tissue plasminogen activator, streptokinase, or both on coronary-artery patency, ventricular function, and survival after acute myocardial infarction. N Engl J Med 1993;329:1615–22.
20. Bovill EG, Terrin ML, Stump DC, et al. Hemorrhagic events during therapy with recombinant tissue-type plasminogen activator, heparin, and aspirin for acute myocardial infarction: results of the Thrombolysis in Myocardial Infarction (TIMI), phase II trial. Ann Intern Med 1991;115:256–65.
21. Geffken DF, Keating FG, Kennedy MH, Cornell ES, Bovill EG, Tracy RP. The measurement of fibrinogen in population-based research: studies on instrumentation and methodology. Arch Pathol Lab Med 1994;118:1106–9.
22. Clauss A. Gerinnungs-physiologische schnell-methode zur bestimmung des fibrinogens. Acta Haematol 1957;17:237–46.
23. Merskey C, Lalezari P, Johnson AJ. A rapid, sensitive method for measuring fibrinolytic split products in human serum. Proc Soc Exp Biol Med 1969; 131:871–5.
24. Holvoet P, Cleemput H, Collen D. Assay of human tissue-type plasminogen activator (t-PA) with an enzyme-linked immunosorbent assay (ELISA) based on three murine monoclonal antibodies to t-PA. Thromb Haemost 1985;54:684–97.
25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
26. Tracy RP, Monkovic D, Andrianorivo A, Calore JD, Mackay AM. The effect of pre-staining prior to immunoreaction during electrophoretic blotting. Electrophoresis 1987;8:350–7.
27. Suzuki K, Dahlback B, Stenflo J. Thrombin-catalyzed activation of human coagulation factor V. J Biol Chem 1982;237:6556–65.
28. Esmon CT. The subunit structure of thrombin-activated factor V. J Biol Chem 1979;254:964–74.
29. Monkovic DD, Tracy PB. Activation of human factor V by factor Xa and thrombin. Biochemistry 1990;29:1118–28.
30. Eisenberg PR, Sobel BE, Jaffe AS. Activation of prothrombin accompanying thrombolysis with recombinant tissue-type plasminogen activator. J Am Coll Cardiol 1992;19:1065–9.
31. Eisenberg PR, Sherman L, Rich M, et al. Importance of continued activation of thrombin reflected by fibrinopeptide A to the efficacy of thrombolysis. J Am Coll Cardiol 1986;7:1253–62.
32. Gulba PC, Barthels M, Westhoff-Bleck M, et al. Increased thrombin levels

during thrombolytic therapy in acute myocardial infarction: relevance for the success of therapy. Circulation 1991;83:937–44.

33. Rao AK, Pratt C, Berke A, et al. Thrombolysis in Myocardial Infarction (TIMI) trial—phase I: hemorrhagic manifestations and changes in plasma fibrinogen and the fibrinolytic system in patients treated with recombinant tissue plasminogen activator and streptokinase. J Am Coll Cardiol 1988;11: 1–11.
34. Turpie AGG, Robinson JG, Doyle DJ, et al. Comparison of high-dose with low-dose subcutaneous heparin to prevent left ventricular mural thrombosis in patients with acute transmural anterior myocardial infarction. N Engl J Med 1989;320:352–7.
35. Arnout J, Simoons M, de Bono D, Rapold HJ, Collen D, Verstraete M. Correlation between level of heparinization and patency of the infarct-related coronary artery after treatment of acute myocardial infarction with alteplase (rt-PA). J Am Coll Cardiol 1992;20:513–9.
36. Prins MH, Hirsh J. Heparin as an adjunctive treatment after thrombolytic therapy for acute myocardial infarction. Am J Cardiol 1991;67:3–11A.
37. Eisenberg PR. Role of heparin in coronary thrombolysis. Chest 1992;101: 131–9S.
38. Francis CW, Marder VJ. Mechanisms of fibrinolysis. In: Williams W, Beutler E, Erslev A, Lichtman M, editors. Hematology. 3rd ed. New York: McGraw-Hill, 1983:1266–76.
39. Sane D, Califf R, Topol E, Stump D, Mark D, Greenberg C. Bleeding during thrombolytic therapy for acute myocardial infarction: mechanisms and management. Ann Intern Med 1989;111:1010–22.
40. Kasper W, Erbel R, Meinertz T, et al. Intracoronary thrombolysis with an acylated streptokinase-plasminogen activator (BRL 26921) in patients with acute myocardial infarction. J Am Coll Cardiol 1984;4:357–63.
41. Williams WJ. Life span of plasma coagulation factors. In: Williams W, Beutler E, Erslev A, Lichtman M, editors. Hematology. 3rd ed. New York: McGraw-Hill, 1983:1230–8.
42. Gold HK, Torres FW, Garabedian HD, et al. Evidence for a rebound coagulation phenomenon after cessation of a 4-hour infusion of a specific thrombin inhibitor in patients with unstable angina pectoris. J Am Coll Cardiol 1993;21:1039–47.
43. Keyt BA, Paoni NF, Refino CJ, et al. A faster-acting and more potent form of tissue plasminogen activator. Proc Natl Acad Sci USA 1994;91:3670–4.
44. Collen DC, Lijnen HR. Staphlokinase, a fibrin-specific plasminogen activator with therapeutic potential? Blood 1994;84:680–6.