Proteolysis of von Willebrand Factor After Thrombolytic Therapy in Patients With Acute Myocardial Infarction

By Augusto B. Federici, Scott D. Berkowitz, Theodore S. Zimmerman, and Pier Mannuccio Mannucci

In 20 patients with acute myocardial infarction (AMI) treated with streptokinase (SK, n = 7), recombinant single-chain tissue plasminogen activator (rt-PA, n = 7) or urokinase (UK, n = 6), the behavior of plasma von Willebrand factor (vWF) was studied before and 1.5, 3, 24, 48, and 72 hours after beginning thrombolytic therapy. vWF antigen (vWF:Ag) was high in plasma, especially after SK. The ristocetin cofactor (Ricof) activity of vWF, high before therapy, tended to decrease soon after therapy. This pattern of vWF changes was paralleled by the early loss of higher molecular weight multimers. By immunoblotting of immunopurified and reduced vWF and monoclonal antibody epitope mapping, we found that vWF was degraded after thrombolysis, especially after SK, as indicated by the higher values of two plasmin-generated fragments of 176 and 145 Kd. There were more plasmin-generated fragments in the five patients who had bleeding complications than in the remaining 15 who did not. In conclusion, quantitative and qualitative changes of vWF compatible with proteolytic degradation of the protein occur during thrombolytic therapy. Such degradation, roughly proportional to the degree of the general lytic state induced by each agent, might be a cofactor of the bleeding complications occurring in treated patients.

© 1992 by The American Society of Hematology.

Materials and Methods

Reagents. Sepharose CL-4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Electrophoresis-pure reagents, including acrylamide, bis-acrylamide, dithiothreitol (DTT), ammonium persulfate, nitrocellulose paper, sodium dodecyl sulfate (SDS), and TEMED, were purchased from Bio-Rad (Richmond, CA), and I2I from Amersham (Arlington Heights, IL). All other reagents were obtained from Sigma (St Louis, MO). Rabbit anti-mouse IgG was obtained from Zymed (South San Francisco, CA), and 12I from Amersham (Arlington Heights, IL). All other reagents were obtained from Sigma or Mallinkrodt (Paris, KY) and were of the best reagent grade.

Thrombolytic agents. Single-chain rt-PA was supplied by Boehringer Ingelheim during the Plasminogen Activator Italian Multicenter Study (PAIMS).12 SK and UK were purchased from Hoechst and Serono (Milano, Italy). For in vitro experiments, human urinary UK was purchased from Behring Diagnostics, La Jolla, CA.

Monoclonal antibodies to vWF. The anti-vWF monoclonal antibodies M7 and M31 used in these experiments were prepared as previously described.11 M7 recognizes an epitope on reduced vWF between Leu31 and Met31. M31 recognizes an epitope on reduced vWF between Leu44 and Met45. Both antibodies react with the intact vWF subunit of 225 Kd. M7 specifically reacts with the native fragment of 140 Kd and with the plasmin-generated fragment of 176 Kd. M31 reacts with the two native fragments of 189 and 176 Kd and with the plasmin-generated fragment of 145 Kd.

Patients. Twenty consecutive patients with AMI were admitted to the Cardiology Division of the IRCCS Maggiore Hospital of Milan. The majority (n = 14) were participating in the PAIMS, which compared the coronary patency found after rt-PA with that
after SK.12 The remaining six patients were treated under an open protocol designed to study the effects of relatively low doses of UK in AMI. All patients were treated with thrombolytic agents within 8 hours of chest pain. They were informed that blood samples were obtained for experimental purposes and gave informed consent.

**Treatment protocols and blood sampling.** SK and UK were each administered intravenously (IV) as constant infusions over 60 minutes at a dosage of 1,500,000 IU. A total dose of 100 mg rt-PA was administered over 3 hours (an initial bolus of 10 mg followed by 50, 20, and 20 at each successive hour).13 After either thrombolytic agent, heparin was administered by a continuous IV infusion (1,000 U/h for 72 hours). None of the patients was treated with aspirin before and during the 72-hour period of the study. A heparin-bonded catheter placed in the pulmonary artery or the right atrium was used to draw blood after flushing with saline. Samples were obtained immediately before starting the infusion and at 1.5, 3, 24, 48, and 72 hours. The 1.5-hour time point corresponded to 30 minutes after the end of the 60-minute SK and UK infusions and to the mid-time interval during the 3-hour rt-PA infusion; the 3-hour time point corresponded to the end of the rt-PA infusion.

Fibrogen and fibrinogen-fibrin degradation products (FDP) were measured by adding 9 mL venous blood to 1 mL anticoagulant to achieve a final concentration of 0.125 mmol/L citrate and 100 kallikrein inhibitory units (KIU) per mL of aprotinin. For vWF measurements, another 9 mL blood was added to 1 mL anticoagulant to achieve final concentrations of 0.125 mmol/L citrate, 5 mmol/L EDTA, 6 mmol/L N-ethylmaleimide, and 600 KIU/mL aprotinin. The latter broad-spectrum protease inhibitor was added to inhibit plasmin proteolysis after blood sampling,14 whereas EDTA and N-ethylmaleimide were added to inhibit calcium-dependent proteases.15 Each sample was centrifuged at 10,000 x g for 20 minutes at 4°C and then the plasma was transferred to another tube and centrifuged at 40,000 x g for 20 minutes to remove platelets more completely. Plasma was frozen in an ethanol/dry ice mixture and stored at −70°C until tested within 1 month.

**Definition of bleeding complications.** The following bleeding episodes were defined as major complications before starting the study: any hemorrhage severe enough to require transfusion, gastrointestinal bleeding causing melena or hematemesis, macroscopic hematuria, computed tomography scan-confirmed intracranial hemorrhages, and unexplained decreases in hemoglobin to a level requiring transfusion. For the purpose of this study, we considered only those bleeding complications that developed within 24 hours of the start of thrombolytic therapy. We surmised that during this period complications were due to the hemostatic disturbances induced by thrombolytic agents and heparin, whereas after 24 hours most antithrombotic effects of thrombolytic agents had decreased or subsided and hence most hemostatic disturbances should be due to heparin only.

**Laboratory measurements.** Fibrogen was measured as fibrin polymerization time with a commercial kit (Biochemia Stago, Milan, Italy). FDP were measured with a commercial kit (Biochemia Stago). vWF:Ag was measured by electroimmunoassay, using a commercial anti-human vWF rabbit antisera (Istituto Behring, Scoppito, Italy).13 Ristocetin cofactor (RCof) activity was measured by the aggregometric method of MacFarlane et al., using formalin-fixed platelets.16 The prothrombin time and activated partial thromboplastin time (APTT) were obtained with standardized commercial reagents (Organan Teknika, Milan, Italy). Multimeric analysis of plasma vWF was performed according to Ruggeri and Zimmerman,19 adding 1% SDS directly to the sample and electrophoresing it through 1.4% LGT agarose gel. The proportion of vWF present as high molecular weight (HMW) multimers was measured by densitometrically scanning the autoradiographs with a computerized system (Cliniscan 2, Helena Laboratories, Milano, Italy).20,21 In brief, the area under the densitometric curves corresponding to the larger multimers (arbitrarily defined as the area comprising 30% of the length of each curve starting from the cathodal origin of each gel) was computed and expressed as percentage of the total area. The between-assay reproducibility of the densitometric method is expressed by a coefficient of variation of 5.9% for a normal plasma and of 6.2% for a post-desmopressin (DDAVP) plasma with high vWF levels and supranormal multimeric structure, both run in seven different electrophoretic gels.

**Immunopurification of plasma vWF.** vWF was immunopurified from plasma obtained before and after thrombolytic agents with the anti-vWF monoclonal antibody 5.5.72 coupled to cyanogen bromide-activated Sepharose CL 4B at a density of 4 mg IgG per mL beads, as previously described.19 vWF was also immunosolated from normal plasma incubated in vitro with UK for 16 hours at 37°C (384 Plough U/mL dissolved in phosphate-buffered saline). This sample was used as a positive control for the effect of thrombolytic agents on vWF. Attempts to set up positive controls using SK and rt-PA were not reproducible. Therefore, UK controls were used throughout. Negative controls (normal plasma with no added UK) were prepared in parallel.

**Immunoblotting methods.** SDS-polycrylamide gel electrophoresis and immunoblotting of vWF. Purified vWF was reduced with 65 mmol/L DTT and 2% SDS for 15 minutes at 60°C and then electrophoresed in SDS-5% polyacrylamide gels (PAGE) as described by Laemmli.22 vWF was transferred from the gels onto nitrocellulose membrane with 25 mmol/L Tris-HCl, 192 mmol/L glycine, 20% (vol/vol) methanol buffer, pH 8.3, at 0.25 mA, 37°C for 16 hours. The nitrocellulose membranes were then reacted with a pool of anti-vWF monoclonal antibodies diluted 1:200. This procedure was followed by labeling with 125I-rabbit anti-mouse IgG.23 The membranes were then processed according to the method of Johnson et al.24 Bands were visualized by autoradiography with Kodak XRP-1 film (Eastman Kodak, Rochester, NY) using a Cronex Quanta III intensifying screen (EL, Du Pont de Nemours, Wiimington, DE). The proportion of total vWF present in each band revealed by autoradiography was measured by densitometric scanning of the autoradiographs with the computerized system Cliniscan 2. The autoradiographs used for densitometric scanning were those that had less background radioactivity and exposures used for this purpose were usually different from those used to make the figures.

**Statistical analysis.** Data were evaluated by analysis of variance (ANOVA) for repeated measures (time intervals after starting thrombolysis) and for one factor (thrombolytic agent or bleeding tendency). When ANOVA showed statistically significant differences, values obtained at different times after each thrombolytic agent were compared with baseline values with the Newman-Keuls test. P values less than .05 were accepted as statistically significant.

**RESULTS**

**Changes in plasma fibrinogen and FDP.** Table 1 shows that fibrinogen levels were very low for 24 hours after beginning SK infusion and had recovered only partially at 48 hours. After rt-PA, fibrinogen decreased much less than after SK, and at 72 hours rebounded over baseline values. A modest decrease of fibrinogen was seen 1.5 hours after UK, with mean values returning close to baseline values at 3 to 24 hours. FDP were markedly and persistently increased after SK and rt-PA infusion, with smaller and less lasting increases after UK (Table 1).
Changes in vWF measurements. Table 1 shows that plasma vWF antigen (vWF:Ag), already high before treatment with any of the thrombolytic agents (upper normal limit, 157 U/dL), increased further after SK and remained higher than baseline during the postthrombolytic period. After rt-PA and UK, the early postinfusion increase in vWF:Ag was less marked. Plasma Ricof, also high before thrombolysis (upper normal limit, 157 U/dL), tended to decrease in the early postinfusion period. As a result of these changes in Ricof and vWF:Ag, the Ricof to vWF:Ag ratio became significantly lower than baseline for up to 24 hours after starting SK and rt-PA, with no change after UK (Table 1).

Changes in the relative proportion of HMW multimers are summarized in Table 1. After SK and rt-PA, there was a loss of HMW multimers (reflected by a decrease in their percentage), that was statistically significant for up to 24 hours after starting either thrombolytic agent. These changes coincided with the decrease in the Ricof to vWF:Ag ratio. At subsequent postinfusion times (48 to 72 hours), not only were HMW multimers restored, but larger than normal multimers had also appeared in some cases, although the increase was not statistically significant. No change of the multimeric pattern of vWF was observed after UK.

Proteolysis of vWF. Fig 1 shows the electrophoretic patterns of the native subunit and proteolytic fragments of vWF immunopurified from plasmas and reduced, before and after each thrombolytic agent. Figure 1A shows a representative pattern for SK, Fig 1B for rt-PA, and Fig 1C for UK. In each electrophoretic gel, vWF immunopurified from normal plasma, with or without in vitro addition of UK, was run in parallel with vWF immunopurified from plasmas of patients treated with thrombolytic agents. Normal plasmas with and without UK were used as positive and negative in vitro controls of the action of plasmin on vWF. In all the negative controls (no added UK), the monoclonal antibody M7 identified two bands at the aminoterminal portion of vWF (the 225-Kd subunit and the native 140-Kd fragment), but no plasmin-generated fragment; the monoclonal antibody M31 identified three bands at the carboxyterminal of vWF (the 225-Kd subunit, the 189-, and 176-Kd native fragments), but no plasmin-generated fragment. In all the positive controls (UK added), M7 identified, in addition to native fragments, a new plasmin-generated fragment of 176 Kd and M31 a new plasmin-generated fragment of 145 Kd.
apparent molecular masses between 225 and 176 Kd were detected by M7 in two of the seven cases after rt-PA infusion (Fig 1B). After UK, the 176-Kd plasmin-generated fragment was barely detectable by M7, whereas the 145-Kd fragment was seen throughout the postinfusion period (Fig 1C).

Since there were between-patient and between-treatment variations in the relative proportions of native and plasmin-generated vWF fragments, we attempted to express such variations semiquantitatively by scanning densitometrically the corresponding autoradiograms obtained after electrophoresis and immunoblotting. The mean percentage of total vWF corresponding to each native and plasmin-generated fragment before and after thrombolytic agents is shown in Fig 2A-C. When results were tested with ANOVA for repeated measures, only the plasmin-cleaved fragments of 176 and 145 Kd were shown to be different between treatments and over time. After SK, there were marked increases of both the plasmin-generated fragments of 145 and 176 Kd at 1.5 hours, with progressive decreases in their percentages at later time intervals. A similar pattern was seen after rt-PA, although the percentages of
both plasmin-generated fragments were lower than after SK. After UK, the changes were even less marked than after rt-PA.

**Bleeding episodes and their relationship to vWF proteolysis.** Major bleeding episodes occurred in three patients treated with SK (gastrointestinal bleeding, bleeding from the point of insertion of a catheter that required two blood transfusions, and a large subcutaneous hematoma that also required blood transfusions), and in two patients treated with rt-PA (hematuria and gastrointestinal bleeding). Platelet count, APTT, prothrombin time, fibrinogen, FDP, and vWF measurements were not significantly different in the patients who bled compared with those who did not (data not shown). However, there were differences in the degrees of vWF fragmentation in the two groups. Figure 3 shows that at 3 and 24 hours, the patients who bled had significantly larger amounts of both the plasmin-generated fragments of 176 and 145 Kd, whereas the native fragments did not differ in these groups of patients.

**DISCUSSION**

We have demonstrated that plasma vWF undergoes degradation during thrombolytic therapy of patients with AMI, that the degree of degradation depends on the type and dose of thrombolytic agent (being greater for SK than for rt-PA and UK at the doses used), and that fragments specifically cleaved by plasmin are present in the circulation after thrombolytic therapy. Degradation of plasma vWF usually occurred in parallel with a postinfusion increase in

---

**Fig 2. Changes of native and plasmin-generated vWF fragments (expressed as percentage of total vWF) before and after thrombolytic agents (A, SK; B, rt-PA; C, UK).** Bars indicate mean values (1 SD is indicated by vertical lines) of the fragments at the aminoterminal site (M7) (Pla 176, plasmin-cleaved fragment of 176 Kd; Nat 140, native fragment of 140 Kd) and at the carboxyterminal site (M 31) (Nat 189 and 176, native fragments of 189 and 176 Kd; Pla 145 Kd) before and at various time intervals after beginning thrombolytic treatment. Significant \( P < .05 \) differences from basal levels are indicated by asterisks. Probability values were calculated only when ANOVA gave significant between-treatment differences. ( ● ), Basal; ( ● ), 1.5 hours; ( ● ), 3 hours; ( ● ), 24 hours; ( ● ), 48 hours.

**Fig 3. Changes of native and plasmin-generated vWF fragments in (A) 15 patients without bleeding complications and (B) five patients with bleeding complication before and 3 and 24 hours after the start of the infusion. Symbols as in Fig 2.*
the plasma concentrations of the protein as measured immunologically (vWF:Ag), in contrast with a relative decrease in RiCof activity (resulting in a lower RiCof to vWF:Ag ratio) and HMW multimers of vWF.

There is little previous information about the behavior of vWF in patients treated with thrombolytic agents. Lombardi et al.20 studied patients with deep vein thrombosis after treatment with UK and found high plasma levels of vWF:Ag and qualitative changes of vWF compatible with proteolytic degradation. Hamilton et al.21 studied patients with deep vein thrombosis or AMI, and also healthy volunteers, given a variety of doses of SK over various infusion periods. A general pattern of results emerged: high levels of plasma vWF:Ag and RiCof, and an increased proportion of HMW multimers at early postinfusion times, followed by subsequent decrease of RiCof activity, loss of HMW multimers, and sustained high levels of vWF:Ag. However, a minority of patients had different patterns: an early decrease in HMW multimers and RiCof, followed by subsequent increases in these measurements. Our findings are more similar to the latter, but it is difficult to compare them with those of Hamilton et al.,21 because the infusion rates and doses of SK administered and the timing of blood sampling for vWF measurements were different in the two studies. While the dosage of UK used in our study was experimental and is not widely adopted, the dosages of SK and rt-PA were those currently used in clinical practice for patients with AMI. vWF and RiCof were already high before infusion, as observed by others,20 because vWF is an acute-phase reactant to the tissue injury and necrosis occurring during AMI. The decrease in RiCof after thrombolysis can be explained by the action of plasmin, which reduces this activity of vWF.23 Plasmin is probably also involved in the explanation for the relative loss of HMW multimers, because the enzyme induces a selective loss of HMW multimers when added to purified vWF in vitro.24 Our data are consistent with those obtained in rabbits by Johnstone et al.,25 who showed a significant loss of HMW multimers in response to SK infusion. It is less clear why the decrease in vWF measured immunologically as vWF:Ag increased, particularly after SK, in contrast with the decreased activity of the protein measured as RiCof. Perhaps high vWF:Ag levels might be related to the method used to assay vWF:Ag. Atichartakarn et al.26 and Guisasola et al.27 have shown that the action of plasmin on vWF in vitro determines a heightened reactivity of vWF with the heterologous antibodies used in the electroimmunoassay, an increased height of the precipitation rockets, and hence the measurement of spuriously high levels of the protein.28 Although it is likely that plasmin formation causes most of the vWF changes found after thrombolytic therapy, we cannot exclude a contributory effect of the direct action of other fibrinolytic enzymes (SK-plasminogen, rt-PA, or UK).

Characterization of the vWF fragments that develop from the degradation of the protein by plasmin generated was rendered possible by the use of two monoclonal antibodies that, in addition to reacting with the native subunit (of 225 Kd) and three fragments (of 140, 176, and 189 Kd) of vWF immunopurified from normal and reduced plasma, also react with two vWF fragments cleaved specifically by plasmin (of 145 and 176 Kd). As expected, there were no plasmin-generated fragments in samples taken before thrombolytic therapy or in negative controls (no added UK). Plasmin-generated fragments of 176 and 145 Kd appeared during and after thrombolytic therapy, in larger amounts after SK than after rt-PA, whereas with the low dosage of UK used in this study there was a lesser formation of plasmin-generated fragments. These different patterns are probably related to the different intensity of the lytic states induced by each thrombolytic agent and/or to the different doses given (see the different values of fibrinogen and FDP in Table 1). Interestingly, the appearance of plasmin-cleaved fragments seemed to be associated with the occurrence of bleeding complications after thrombolysis, because a larger proportion of plasmin-generated fragments was actually observed in the five patients who had bleeding complications within 24 hours from starting thrombolysis than in the remaining 15 patients who did not bleed (Fig 3). No other hemostatic measurements (including vWF measurements) were associated with bleeding complications. Although these results can only be considered preliminary because of the small number of patients studied, and the correspondingly high risk of type I error, it is biologically plausible that the degree of degradation by plasmin of a protein with such an important role in hemostasis might be associated with bleeding complications.

In conclusion, our data demonstrate that vWF is degraded after therapy with thrombolytic agents. vWF degradation is produced by the circulating plasmin, and is greater for the thrombolytic agent SK, which gave the largest degree of plasma proteolysis. Plasmin-induced proteolysis of vWF might be a cofactor, with other hemostatic abnormalities induced by thrombolytic agents, of the bleeding complications.

ACKNOWLEDGMENT

We thank Professor A. Lotto, Drs La Marchesina, Mauri, and Barbonaglia from the Cardiology Department, IRCCS Maggiore Hospital of Milan, and Drs Visani and Gali from Boehringer Ingelheim, Firenze, for providing us with samples from patients with AMI enrolled on the PAIMS. We thank also A. Lattuada for scanning the autoradiographs and M.L. Colibretti for her excellent technical assistance. We acknowledge the help of Dr A. Gringeri for the statistical analysis and Dr Z.M. Ruggeri for his helpful criticism. The manuscript was prepared by Elena Zumbo.

REFERENCES


Proteolysis of von Willebrand factor after thrombolytic therapy in patients with acute myocardial infarction
AB Federici, SD Berkowitz, TS Zimmerman and PM Mannucci