Increased Thrombin Levels During Thrombolytic Therapy in Acute Myocardial Infarction
Relevance for the Success of Therapy

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Background. It has been suggested that thrombolysis in a feedback reaction may generate pro-coagulant activities.

Methods and Results. Fifty-five patients were treated with urokinase-preactivated protamine-urokinase (n=35) or tissue-type plasminogen activator (n=20) for acute myocardial infarction and underwent coronary angiography at 90 minutes and at 24-36 hours into thrombolysis, and fibrinogen (Ratnoff-Menzie), D-dimer (ELISA) and thrombin-antithrombin III complex levels (ELISA) were measured. Primary patency was achieved in 39 patients (70.9%), 13 of whom (33.3%) suffered early reoclusion. Nonsignificant decreases in fibrinogen levels were observed while D-dimer levels increased +3,008±4,047 μg/l (p<0.01), differences not being significant in respect to the thrombolytic agents or to the clinical course. In contrast, while thrombin-antithrombin III complex levels decreased -4.4±13.0 μg/l in patients with persistent patency, they increased +7.5±13.6 μg/l in case of nonsuccessful thrombolysis (p<0.02) and +11.9±23.8 μg/l in case of early reoclusion (p<0.001). For patients with thrombin-antithrombin III complex levels greater than 6 ng/l 120 minutes into thrombolysis, the unfavorable clinical course was predicted with 96.2% sensitivity and 93.1% specificity.

Conclusion. Generation of thrombin, occurring during thrombolysis, is a major determinant for the success of therapy and thrombin-antithrombin III levels may serve as predictors for the short-term prognosis. (Circulation 1991;83:937-944)

During the past two decades, evidence has accumulated that coronary artery thrombosis plays a pivotal role in the precipitation of acute myocardial infarction.1-3 Today, current concepts include high-dose intravenous thrombolytic therapy to reperfuse the occluded coronary arteries. Recently, large scale, double-blinded multicenter trials unequivocally demonstrated a reduction in mortality due to infarction, especially when thrombolysis was performed during the early hours after onset of symptoms.4-7

After successful thrombolysis, however, the reperfused artery is in jeopardy of thrombotic reocclusion, offsetting the benefit of therapy.8 Reoclusion has been shown to depend on two major determinants: 1) the incidence of residual coronary artery thrombosis,9,10 and 2) the severity of the underlying coronary artery stenosis.10,11 As indicated in recent reports,12-19 rethrombosis may also be related to deteriorations in the hemostatic system, resulting in persistent clotting activation. Early detection of an increased clotting tendency may allow rethrombosis to be predicted early, and thus, specific remedial measures such as aortocoronary bypass operations could be performed immediately.

Clotting activation always leads to the generation of active thrombin. By taking advantage of the rapid, irreversible complexation of free thrombin by antithrombin III,20 a new enzyme-linked immunosorbent assay (ELISA) technique for in vivo thrombin measurements was recently introduced.20,21 Applying this
technique to patients with thromboembolic diseases renders direct insight into the extent of the activation of the clotting cascade.22

This study was designed to elucidate the impact of thrombin on the results of thrombolysis in patients with acute myocardial infarction and to determine whether increased thrombin activities are related to either non-reopening or early reocclusion of the coronary arteries.

**Methods**

**Selection of the Patients**

Patients were studied who had acute myocardial infarctions and who underwent thrombolytic therapy with fibrin-specific plasminogen activators (recombinant tissue-type plasminogen activators [rt-PA] or a combination of low-dose urokinase and pro-urokinase) within 4 hours after onset of symptoms. The criteria for study inclusion were 1) history of typical chest pain of at least 30 minutes in duration not responding to sublingual nitrates or nifedipine, and 2) ST segment elevation of 0.2 mV or more in at least two contiguous electrocardiographic leads. The criteria for study exclusion were 1) contraindications to cardiac catheterization, 2) cardiogenic shock, 3) major illness, especially those predisposing the patient to an increased hemorrhagic risk, 4) age greater than 75 years, and 5) all contraindications to thrombolytic agents. Before study entry, informed consent was obtained from each patient.

**Study Design**

This study was designed as an open-labeled, prospective, nonrandomized trial. In all patients who were enrolled, a central venous line was placed through one cubital vein. All intravenous medications including nitrates (≥75 mg/day), were given through this catheter. All other medications were given as clinically required. An intravenous heparin bolus (5,000 IU) was given immediately preceding thrombolytic therapy, which was immediately followed by a continuous intravenous heparin infusion of 1,250 IU/hr. Intravenous infusion of the thrombolytic agent then was started with a minimum time delay.

Patients then were taken to the catheterization laboratory, and after a second 5,000-IU heparin bolus, the first coronary angiogram was recorded not later than 90 minutes after onset of thrombolytic therapy. In case of coronary patency, the dose of heparin was adjusted in increments of 250 IU/hr to result in an activated partial thrombin time of 60–80 seconds. Within 6 hours after onset of therapy, all patients were given oral aspirin (250 mg/day). In case of nonpatency, thrombolytic therapy was continued. Then, a second angiogram was recorded 90 minutes into the thrombolytic regimen. In case the coronary artery was still occluded, the investigators were free to continue with intracoronary thrombolytic therapy.

In patients in whom primary patency was achieved, an additional angiogram was recorded 24–36 hours later. Patients receiving intracoronary thrombolytic therapy, however, were classified as “nonsuccessful thrombolysis.” In these patients, no follow-up angiogram was obtained.

**Thrombolytic Therapy**

Two different fibrin-specific plasminogen activators (rt-PA and pro-urokinase) were used for thrombolytic therapy. Patients received either 6.5×10^6 units pro-urokinase infused within 40 minutes, pre-activated by a 250,000-IU urokinase bolus (n=35),23 or they were given 70 mg rt-PA infused within 90 minutes (n=20).24 No maintenance infusion of either of the two plasminogen activators was given.

**Assessment of Angiograms**

Cineangiograms were assessed qualitatively by two experienced, independent investigators to judge the patency of the infarct-related coronary artery. Patency was defined as complete perfusion with prompt filling or as partial perfusion with delayed opaquification of the distal vessel. Coronary occlusion was defined as minimal penetration or absence of contrast beyond the point of coronary obstruction. In case of disagreement between the investigators, a consensus between them was obtained by discussion.

**Blood Collection**

A 2.7F polyethylene cannula was placed in one suitable vein of the arm, contralateral to the arm with the central venous line, through which citrated blood samples containing 200 KIU/ml aprotinin were collected at baseline and at 15, 60, and 120 minutes into the thrombolytic regimen. Aprotinin was added to the citrate to preclude any plasmin activity in the samples. Samples were centrifuged at 2,000 g within 2 hours of collection, were deep frozen, and were kept at −70°C until analyzed.

**Coagulation and Fibrinolysis Assays**

For the determination of fibrinogen levels, the modification by Bang et al25 of Ratnoff–Menzie’s method was applied. D-Dimer26 and thrombin–antithrombin III (TAT) complex measurements21 were obtained with ELISA techniques.

**Assay of Thrombin–Antithrombin III Complexes**

The assay principle applied for measuring TAT complexes was first described by Pelzer et al.20,21 The method is now available commercially (Enzygnost TAT kit, Behringwerke AG, Marburg, FRG). The kit contains tubes coated with a polyclonal rabbit antibody to human thrombin preabsorbed with prothrombin and, thus, made specific. In a 30-minute incubation with the plasma samples at 37°C, the TAT complexes bind to the tubes by the thrombin component. The plasmas then are removed by washing twice with 2 ml phosphate buffer solution containing Tween. In a second 30-minute incubation with peroxidase-conjugated rabbit antibody to human antithrombin III at 37°C, the enzyme is bound to the
TAT complexes coating the tube surfaces. Unbound material is removed by washing three times with phosphate buffer solution containing Tween. Then o-phenylenediamine dihydrochlorid that is dissolved in citrate-phosphate buffer containing 0.3 g/l hydrogen peroxide is added, and while being protected from light, the tubes are incubated at 25°C. After 30 minutes, the reaction is stopped with dilute sulfuric acid. The absorbance is read against a blank with distilled water at 492 nm. Linearity of the standard curve is warranted between 2 and 60 µg/l. In case the samples are of higher TAT content, they are diluted with standard plasma.

Evaluation of the Assay of Thrombin–Antithrombin III Complexes

By 20-fold measurements performed in standard plasmas of different TAT contents (TAT content, 2–60 µg/l) intra-assay reproducibility was tested. The coefficient of variation ranged from 9.4% to 4.1%. The day-to-day variability was tested in three standard plasmas stored at –70°C (TAT content, 6–60 µg/l) by six-fold measurements at different dates during a 2-month period. The coefficient of variation ranged from 6.3% to 5.2%. Dilution parallelism that was evaluated in two standard plasmas (TAT content, 20 and 60 µg/l) was warranted until at least a 10-fold dilution.

Influence of the Sampling Procedure

In a series of 12 healthy volunteers, the stability of the TAT levels during serial blood collection was tested. A 2.7F polyethylene cannula was placed in one cubital vein, and a citrated blood sample was collected for baseline TAT analysis. A 7,500-IU i.v. bolus of high molecular weight heparin was given thereafter through one contralateral cubital vein. At seven predefined collection times (ranging from baseline to 2 hours), repeated blood samples were drawn through the polyethylene cannula and were analyzed for their TAT content. TAT levels remained stable throughout the observation time and measured 4.1±3.2 µg/l at baseline and 5.5±4.4 µg/l at 120 minutes (NS).

Influence of Concomitant Heparin Administration

In a series of 13 patients undergoing thrombolysis with urokinase plus pro-urokinase infused within 40 minutes, the influence of concomitant administration of heparin was studied. In each odd-numbered patient (n=7), heparin was administered according to the outlined protocol, whereas in each even-numbered patient (n=6) heparin was not given until catheterization for angiography. Apart from the heparin therapy, the outlined study protocol was meticulously followed, and TAT measurements were obtained at baseline and 15, 60, and 120 minutes into the thrombolytic regimen.

Materials

High molecular weight heparin was purchased as Liquemin from Hoffmann LaRoche AG, FRG. High molecular weight urokinase was supplied by Deutsche KabiVitrum GmbH, FRG. Natural pro-urokinase produced in a malignant kidney cell line (TCL 598) was a kind gift from Sandoz AG, FRG. rt-PA was purchased as Actilyse from Dr. Karl Thomae GmbH, FRG. Enzygnost-TAT ELISA test kits were purchased from Behringwerke AG, Marburg, FRG, and ELISA D-dimer test kits were supplied by Boehringer Mannheim GmbH, Mannheim, FRG.

Statistics

Unless otherwise stated, data are given as mean±SD. For statistical analysis, paired and unpaired t tests and one-way analysis of variance were applied. All variables were tested for normal distribution. In case of non-normally distributed variables, logarithmic transformation was performed before they were entered into the test. All reported p values are two sided, and only p values of 0.05 or less were regarded as significant.

Results

Characteristics of Patients

During an 18-month study period, 55 patients (46 men and nine women; mean age, 57.0±9.3 years) were enrolled. Patients were 173.5±7.0 cm tall and weighed 78.1±10.8 kg. Twenty-six patients had an anterior wall, 25 had an inferior wall, and four had a posterolateral wall myocardial infarction. The first 35 patients were given the combined urokinase and pro-urokinase regimen, whereas the second 20 patients were treated with the rt-PA regimen. No significant differences were observed between the two treatment groups.

Angiographic Results

With the outlined criteria, primary patency was achieved in 39 patients (70.9%), whereas the infarct artery remained occluded in 16 patients (29.1%) (11 pro-urokinase, five rt-PA). In all 39 patients in whom primary patency was achieved, a second coronary angiogram was recorded 24–36 hours later that revealed coronary recurrences in 13 (33.3%) (nine pro-urokinase, four rt-PA), six of whom had new symptoms or electrocardiographic changes suggestive of coronary reocclusion. In the remaining 26 patients, persistent patency was diagnosed from the second angiogram. Additional angiograms were not recorded in the 16 patients with nonsuccessful thrombolysis.

Hemostatic Variables

During thrombolysis, the activated partial thrombin time was prolonged to more than 150 seconds in all patients. Fibrinogen levels decreased −0.3±1.0 g/l (NS) from baseline values (Figure 1), with no significant differences between the effects of the two treatment regimens or among the clinical courses of
the three groups of patients (Figure 1). Simultaneously, D-dimer levels increased $+3.008 \pm 4.047 \, \mu g/l$ ($p<0.01$) (Figure 1), indicating marked lysis of cross-linked fibrin. Again, no significant differences were detected among the clinical courses of the three groups of patients or between the effects of the two thrombolytic regimens.

During the observation time, TAT levels in the total group of patients increased slightly but not significantly (Figure 2A), averaging $+2.8 \pm 17.3 \, \mu g/l$. When TAT levels were evaluated with regard to the early clinical course of the patients, however, marked differences were observed. In patients with persistent patency, an average decrease of $-4.4 \pm 13.0 \, \mu g/l$ was observed (Figure 2B), whereas patients with early reocclusion (Figure 2C) and patients with nonsuccessful thrombolysis (Figure 2D) demonstrated an increase in TAT levels averaging $+11.9 \pm 23.8$ and $+7.5 \pm 13.6 \, \mu g/l$, respectively. When the TAT levels of the latter two patient groups were compared with those from the patient group with persistent patency, the differences were highly significant ($p<0.02$ and $p<0.001$, respectively, by one-way analysis of variance). Again, no significant differences were observed with respect to the effects of the two thrombolytic regimens used.

By secondary analysis, we tested whether the use of TAT levels could distinguish patients with persistent patency from those with an unfavorable clinical course (nonsuccessful thrombolysis and early reocclusion). A TAT level of $6 \, \mu g/l$ (twice the upper limit in healthy volunteers) was selected as the borderline value in distinguishing the groups. We tested the hypothesis that levels greater than $6 \, \mu g/l$ would identify patients with an unfavorable clinical course (Figure 3A), whereas levels of $6 \, \mu g/l$ or less would indicate patients at low risk (Figure 3B and 3C). When the group of patients with an unfavorable early clinical course was subdivided into those with nonsuccessful thrombolysis and early reocclusion, distinction between the groups with persistent patency and early reocclusion was more marked (sensitivity, 100%; specificity, 92.9%; Figure 3B) than when patients with persistent patency were compared with patients in whom thrombolysis had not been successful (sensitivity, 92.5%; specificity, 93.3%; Figure 3C).

**Influence of Concomitant Heparin Administration**

Thirteen additional patients undergoing thrombolysis with a combination of urokinase and pro-urokinase were studied. The odd-numbered patients ($n=7$) received heparin according to the outlined protocol, and the even-numbered patients ($n=6$) received heparin only at catheterization for angiography. In the latter patients, TAT levels increased continuously from $+9.5 \pm 1.2$ to $27.5 \pm 9.6 \, \mu g/l$ (mean±SEM), whereas in the former patients, TAT levels first decreased from $13.4 \pm 7.8$ to $7.6 \pm 2.2 \, \mu g/l$ (mean±SEM) and then increased to $16.8 \pm 7.6 \, \mu g/l$ (mean±SEM) (NS). Thus, the increase in TAT levels was not completely suppressed by heparin given at clinical and routine doses.
Discussion

Confirming previous reports,12–18,27 we found that TAT levels at baseline indicate marked circulating thrombin levels. Also, thrombin generation is further enhanced by thrombolytic drugs. When differentially analyzed for clinical course (persistent patency, non-successful thrombolysis, or early reocclusion), marked differences among the three groups, however, were found. Rapid normalization occurred in patients with successful thrombolysis, and steady increases in TAT levels occurred in patients who did not undergo successful thrombolysis or who suffered reocclusion of the infarcted artery within 24–36 hours. To interfere with the activation process of thrombin, intravenous heparin was given to all patients enrolled in the study. Despite massive retardation of blood clotting, as reflected by the prolongation of the activated partial thrombin time, however, TAT levels were normalized in only half of the patients, whereas in the other patients, a further increase in TAT levels was observed. Thus, the short-term success of thrombolytic therapy seems highly dependent on whether the individual patient can cope with the thrombin generation.

To exclude any interference with fibrinogen split products, this study was performed with fibrin-specific plasminogen activators only. However, the increase in TAT levels has been reported to be even more marked with the use of streptokinase.12,13 Furthermore, with streptokinase, increased thrombin levels may be associated with early rethrombosis.28 Thus, thrombin generation most likely is a general effect observed during thrombolysis.

Fibrinogen levels throughout the observation time decreased only slightly, and the decrease was similar.
to all three groups of patients. Thus, unlike the suggestions from previous reports of patients treated with streptokinase,\textsuperscript{29,30} in patients treated with fibrin-selective plasminogen activators, massive degradation of circulating fibrinogen does not play a major role in determining the outcome of therapy. Furthermore, the increases in D-dimers, a surrogate of the thrombolytic activity directed toward cross-linked fibrin, were least marked in patients with persistent patency. Hence, factors other than the thrombolytic power induced by the thrombolytic drugs seem to govern the short-term success of thrombolysis.

By reducing the procoagulant powers indicated from the elevated TAT levels, the potentiative effect of heparin on thrombolytic drugs that was observed in some studies\textsuperscript{31,33} may be derived. On the other hand, incomplete inhibition of thrombin activation may also offset the benefit of thrombolytic therapy.\textsuperscript{33,34} Therefore, we tried to determine how and to what extent concomitant heparin treatment may have interfered with the effects observed. After an initial decrease on heparin injection, TAT levels then increased after 60 minutes, and within 120 minutes into the thrombolytic regimen, levels increased to more than 200% of the minimum levels. Thus, as previously reported,\textsuperscript{33–35} heparin, in standard doses, does not cope with the thrombin liberation observed during thrombolysis. Therefore, to improve the results of thrombolysis, more effective regimens for thrombin inhibition must be developed and used. New and promising drugs that meet the desired profiles, such as the specific thrombin inhibitor hirudin\textsuperscript{36} and the GP IIb/IIIa platelet receptor inhibitors,\textsuperscript{37} are currently being tested.

With fibrinopeptid A as a marker for thrombin activities,\textsuperscript{38} similar results were recently reported by Rapold et al.\textsuperscript{33} They concluded that fibrinopeptide A levels of less than 5 ng/ml measured within half an hour after heparin administration identified patients with a particularly low reocclusion risk. For the limited number of patients enrolled in their study, however, these results could not be confirmed statistically. Furthermore, coronary angiography was performed only at 12.5\pm6.1 days after thrombolysis. Thus, both patency and reocclusion had only been diagnosed from clinical data. Furthermore, Weitz et al\textsuperscript{39} demonstrated that t-PA in high concentrations (5 \(\mu\)g/ml) may also liberate fibrinopeptide A from purified fibrinogen by a non-plasmin-mediated reaction. One might, therefore, object to the findings of Rapold et al\textsuperscript{33} that elevated fibrinopeptide A levels during thrombolysis with rt-PA may not necessarily reflect free thrombin activities.

In contrast, the present study used TAT complex levels as thrombin markers. In comparison to fibrinopeptide A levels, TAT levels offer the advantage of a higher sensitivity,\textsuperscript{13,22} greater ease in acquiring samples, and less sensitivity to in vitro artifacts. Furthermore, TAT levels may serve as direct measures for thrombin quantities in plasma samples.\textsuperscript{20} With a half-life of less than 5 minutes (data not presented here), they do not accumulate, thus directly reflecting the actual thrombin concentration in circulating blood. By studying TAT complex levels, we have minimized possible bias.

This question arises: From which source do the increasing thrombin quantities derive? Fibrin is known to incorporate active thrombin, which on plasmin digestion may reversibly be liberated.\textsuperscript{40}
Hence, the clot itself may be a source for active thrombin. The small mass of a coronary artery thrombus, however, does not make this hypothesis very likely to be the only thrombin source. On the other hand, thrombolysis reexposes the site of plaque rupture to the blood. Thus, the pathomechanism of clot formation may get reinduced. The close relation between the TAT levels and the short-term clinical outcome, however, supports a third concept: a balance between the coagulation and fibrinolytic cascades, which when disturbed in favor of thrombolysis counteracts with increased clotting activation.

By the use of fibrinopeptide A measurements, previous studies have demonstrated that thrombin is also released when streptokinase, urokinase, or t-PA is added to citrated or EDTA-containing plasma as well as to nonanticoagulated whole blood. This effect was markedly attenuated on the addition of heparin. Supporting these findings, Barthel et al.43 recently reported from in vitro experiments, which they performed in plasma samples collected at baseline from patients, who demonstrated a continuous increase in TAT complex levels during thrombolysis. When they added pro-urokinase and urokinase to these nonheparinized plasma samples, the increases in TAT levels observed in vivo were reproduced. Thus, the generation of thrombin seems to be due to a direct stimulation of the clotting system.

This feedback mechanism may be platelet related. The experiments of Barthel et al.,43 however, were performed in platelet-poor plasma. Therefore, it may as well be the other way around, and platelet activation may only occur as a secondary phenomenon. The later hypothesis is supported by the fact that thrombin itself is one of the strongest platelet activators known. Furthermore, in comparison to the other plasminogen activators, streptokinase has the strongest procoagulant effect. One may speculate, therefore, that plasmin itself acts as clotting activator. This hypothesis is further supported by the fact that factor V in a direct reaction is activated by plasmin.

In conclusion, our data support a transient, but significant, increase of thrombin as a response to thrombolysis. The increased thrombin levels are strongly related to the short-term outcome of thrombolysis, and the short-term outcome of thrombolysis can be predicted from TAT measurements. Our data, however, do not allow us to draw any definite conclusion on the mechanism of thrombin generation. From the results of in vitro studies, however, it is tempting to speculate that the increased thrombin levels may be due to direct stimulation of the clotting cascade by the plasminogen activators or by plasmin.

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