Effect of Thrombin Inhibition on the Dynamics of Thrombolysis and on Platelet Function During Thrombolytic Therapy

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To evaluate the effect of thrombin on the dynamics of thrombolysis, we infused rabbits with heparin or hirudin alone or in conjunction with tissue-type plasminogen activator (t-PA) and monitored the kinetics of fibrinolysis and changes in ex vivo platelet aggregation responses over time. Both heparin and hirudin enhanced total fibrinolysis in an ex vivo arteriovenous shunt preparation: 82±2% and 79±2%, respectively, compared with 51±8% for t-PA alone (p<0.05) and 50±4% for t-PA plus aspirin (p<0.05). Heparin coadministered with t-PA significantly reduced the half-time for clot lysis compared with t-PA alone (p<0.05), whereas hirudin coadministered with t-PA significantly reduced the half-time for clot lysis compared with that for t-PA alone, t-PA plus aspirin, and t-PA plus heparin (5.5±0.6 versus 12.1±2.0 versus 12.6±2.2 versus 10.0±0.8 minutes, respectively; p<0.05). Both heparin and hirudin prevented the increase in ADP-induced platelet aggregation normally seen with t-PA alone (p<0.01 by t test; p<0.05 by two-way analysis of variance). These data demonstrate that selective, antithrombin III–independent thrombin inhibitors can enhance the efficacy of thrombolysis by modulating the dynamics of the process and preventing platelet activation associated with plasminogen activator therapy.

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KEY WORDS • heparin • hirudin • thrombolysis • platelet aggregation

With the advent of the use of thrombolytic agents, considerable progress has been made over the past several years in the treatment of patients with acute myocardial infarction. Although most patients receiving thrombolytic therapy manifest reperfusion,1–3 a significant number of these individuals experience delays in time to reperfusion, incomplete lysis, or reocclusion.1–3

The mechanism for reocclusion is not entirely clear but is thought to be the result of stimulated thrombotic processes engendered by fibrinolytic therapy.4 We,5 as well as others,6–8 have shown that platelets are activated early during thrombolytic therapy and that adjunctive therapy with antiplatelet agents can prevent the increase in platelet aggregation and enhance clot lysis. Several groups9–11 have found that fibrinopeptide A is elevated in patients receiving thrombolytic therapy, indicating increased thrombin activity in patients with acute myocardial infarction; this increase has been noted in patients treated with both streptokinase and recombinant tissue-type plasminogen activator (t-PA), therefore indicating the specificity of the measurement as a marker for thrombin action (and not simply the direct action of t-PA on fibrinogen). Since the action of thrombin generated during thrombolytic therapy would attenuate lysis both by activating platelets and producing fibrin, we chose to administer antithrombin agents in combination with t-PA and to measure the effects of thrombin inhibition on the kinetics of clot lysis in an ex vivo arterial system using a gamma camera imaging technique. This procedure allows for immediate on-line measurement of the rate of fibrinolysis (kinetics) unlike the standard plasma sampling procedure. To mimic more closely the coronary artery thrombus seen clinically, we used an arterial shunt system rather than a venous shunt system. We also measured changes in platelet function over time. With these data, we hope to gain a better understanding of the relative functional importance and mechanistic action of thrombin during thrombolysis with therapeutic plasminogen activation.

Materials and Methods

Clot Lysis Model

Shunt preparation. The ex vivo shunt was made using a combination of polyethylene (PE-90) tubing and expanded PTFE tubing (1 mm i.d., Gortex). An interposed segment of the PTFE tubing served as the repository for the radiolabeled clot. The polyethylene
tubing was inserted into the native artery and vein, extending the shunt from the rabbit’s body to the gamma camera. A PE-10 cannula arising from the center of the shunt was sealed in place with cyanoacrylate glue and used to inject a mixture of radiolabeled human fibrinogen, rabbit whole blood, and thrombin into the 1-cm segment of PTFE tubing, which was isolated with microaneurysm clamps. A thin cotton thread was passed through the length of the PTFE portion of the shunt to ensure the stability and location of the clot. A fine wire was also passed through the central portion of the shunt but exited through the junction of the PTFE tubing and the venous polyethylene tubing. The wire was removed just before the start of the infusions of the pharmacological agents of interest and was used to produce a small channel in the aged clot that facilitated exposure of drugs and fresh arterial blood to the clot.

Camera setup. A NaI crystal and detector assembly (Bicron Corp., Newbury, Ohio) was used in conjunction with a multichannel analyzer (EG+G Ortec, Oak Ridge, Tenn.) for on-line monitoring of radioactivity. The camera fit into the top portion of a 5-mm-thick lead cylindrical chamber, and the base of the chamber formed a basin that enclosed the ex vivo shunt. The base also contained a 37°C circulating 9.9% saline bath. The entire camera and chamber assembly was stabilized by a fixed support apparatus. The camera’s data output was collected and stored in a Master Data Emulation (MAESTRO II) software program that generates the kinetic decay curves. This removes subjectivity in generating the curves.

Animal preparation and protocol. New Zealand White female rabbits (3–5 kg) were anesthetized with ketamine (50 mg/kg i.m.) and subsequently given sodium pentobarbital (5–10 mg/kg i.v.) to maintain the level of anesthesia. All animals were surgically prepared with right femoral vein and artery cannulation for drug infusion and blood sampling, respectively. The left common femoral vessels were carefully dissected to expose the bifurcation of the common femoral artery. A 3-cm exposure of clean vessel proximal and a 2-cm exposure distal to the arterial bifurcation were required for insertion of the shunt. All unused branches of the artery and vein were ligated. The ascending branch of the common femoral artery served as the insertion site of the arterial end of the shunt. The venous end of the shunt was inserted into the common femoral vein. A small (PE-10) cannula was placed in an arterial branch just proximal to the tip of the arterial end of the shunt for a bolus injection of 10% of the total t-PA dose immediately before the start of the infusion. Before insertion, the saline-filled shunt was clamped at both ends of the polyethylene tubing to isolate the shunt from the animal’s circulation. The clamps remained in place throughout the injection and incubation of the clot and were removed immediately before the start of the infusions. Once the shunt was in place, autologous whole blood from the rabbit was drawn and promptly mixed with [125I]fibrinogen and thrombin in vitro, and the mixture was immediately injected into the PTFE tubing by way of the small branch of PE-10 tubing arising from the center of the PTFE tubing. The blood was allowed to clot and stabilize for 1 hour before releasing the clamps and starting the drug infusions.

Control animals received either saline (2.5 ml/hr), aspirin (7.5 mg/kg bolus), heparin (71 units/kg bolus and 15 units/kg/hr for 1 hour), or hirudin (0.3 mg/kg/hr for 1 hour). These doses of heparin and hirudin were chosen based on studies of Agnelli et al,12 who showed that comparable doses of heparin and hirudin provided equivalent increases in activated partial prothrombin time. The treatment groups included t-PA alone (0.1 mg/kg bolus immediately proximal to the shunt plus 0.9 mg/kg/hr for 1 hour), t-PA plus aspirin, t-PA plus heparin, and t-PA plus hirudin, all given at the same dose as in the controls. Infusions were started after the release of clamps diverting blood flow through the arteriovenous shunt containing the ex vivo blood clot. All infusions were administered for 1 hour, and the clot was monitored with the gamma camera during this time. We continued to monitor the clot for an additional 30 minutes after the infusions were stopped.

Platelet Aggregation Time Course

The rabbits were anesthetized as described above. The right femoral artery and vein were cannulated for blood sampling and infusion, respectively. After surgery, the animals were allowed a recovery period of ~1 hour. An infusion of t-PA plus heparin, t-PA plus hirudin, heparin alone, or hirudin alone was started. In the 3-hour experiments, t-PA was given in a dose of 0.9 mg/kg/hr for 1 hour, followed by 0.3 mg/kg/hr for the second and third hours. A bolus injection of heparin (71 units/kg) was given, followed by a continuous infusion of 15 units/kg/hr for 3 hours. Hirudin was infused at a dose of 0.3 mg/kg/hr. Blood samples (4 ml) were collected in sodium citrate (13 mM trisodium citrate) and aprotinin (216 kallikrein inhibiting units/ml) or D-phenyl-l-prolyl-t-arginyl chloromethylketone (1 μM final concentration). Blood samples were obtained at 0, 1, 5, 10, 30, 60, 120, 180, and 240 minutes after the start of the infusion of drug.

Platelet-rich plasma was prepared by centrifuging whole blood at 138g for 10 minutes at 22°C. The platelet-rich plasma (upper two thirds of plasma) was removed for determination of platelet counts using a Coulter counter (model ZM, Coulter Corp., Hialeah, Fla.). Platelet-poor plasma was obtained by centrifuging the remaining blood after platelet-rich plasma removal at 1,240g for 10 minutes. Platelet-poor plasma was added to platelet-rich plasma to give a final platelet count of 1×10^6 platelets/μl.

Platelet aggregation was induced with 20 μM ADP. Aggregation was monitored at 37°C while stirring at 900 rpm in a dual-channel aggregometer (Payton Scientific Inc., Buffalo, N.Y.). Aggregation responses were quantified using maximal rates of change in light transmittance. Values were normalized to pretreatment rates in all cases.

Fibrinogen Iodination

Fibrinogen was iodinated using the Iodobead method as previously described.13 The average specific activity of the radiolabeled fibrinogen was 5,500 cpm/ng.

Drugs and Reagents

Recombinant desulfatohirudin was kindly provided by CIBA-GEIGY, Basel, Switzerland. Predominantly single-chain recombinant t-PA was obtained from Ge-
Figure 1. Decay curve for clot lysis in an arteriovenous shunt model. The open squares represent data for the control group (saline, n=4), and the open circles represent data for the group treated with tissue-type plasminogen activator (0.1 mg/kg bolus followed by infusion of 0.9 mg/kg for 1 hour, n=4). For experimental details, see “Materials and Methods.” The initial abrupt decrease in counts noted in both the control and treatment groups was due to a mechanical shift in the location of the clot beneath the camera.

Statistical Analysis

All data within groups were analyzed by one-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparison or Dunnett’s test. Data among groups were analyzed by multifactorial analysis using Duncan’s multiple range test after ANOVA (Human Systems Dynamics, Northridge, Calif.). Values are given as mean±SEM. Values of p<0.05 were considered statistically significant.

Results

Figure 1 shows the radioactive decay curves for the saline control group and the group receiving t-PA alone. Radioactivity was normalized to the baseline pretreatment value for a given experiment. There was an immediate modest reduction in detected radioactivity owing to slight movement of the clot on restoration of perfusion pressure with the release of the shunt clamps. This adjustment occurred within the first 10 minutes of the infusion. However, no further significant decrease was noted during the subsequent 80 minutes of observation in the saline control group. The other control groups—aspirin, heparin, or hirudin alone (data not shown)—did not differ significantly from the saline control group.

The treatment group receiving the t-PA infusion showed a continued significant reduction of radioactive counts within the clot beyond that seen in the saline control group (p<0.02 by two-way ANOVA). To analyze closely the effect of the different drug regimens on the kinetics of clot lysis, the extent of lysis was analyzed by normalizing the data to the 10-minute time point, the time at which no further mechanical shifting of the clot was evident. The adjusted extent of lysis for the saline control group was 9±1% at 60 minutes, whereas the t-PA group manifested a significantly greater loss of labeled clot during the same 10–60-minute time interval (51±8%, p<0.005).

The adjusted extent of clot lysis at 60 minutes for the saline control and all treatment groups is shown in Figure 2A. All treatment groups manifested significantly greater lysis than the saline control group. Aspirin confused with t-PA did not result in a different extent of lysis from that seen with t-PA alone (50±4% versus 51±8%, respectively). However, adjunctive treatment with heparin (82±2%) or hirudin (79±2%) resulted in significantly greater lysis than either t-PA alone or t-PA plus aspirin (p<0.01).

Comparison of the time at which 50% of the original clot lysed (t_{1/2}) showed (Figure 2B) that adjunctive

FIGURE 2. Bar graphs of the effects of drug regimens on the kinetics of clot lysis. Panel A: The effect on extent of lysis for the saline control group and all treatment groups. All treatment groups received tissue-type plasminogen activator (t-PA), and the adjunctive treatment groups received aspirin, heparin, or hirudin as detailed in “Materials and Methods.” *p<0.005 compared with saline control; †p<0.05 compared with t-PA treatment alone. Panel B: The effect on the rate of lysis for all treatment groups. The time at which 50% of the original clot lysed (t_{1/2}) is shown for the three groups receiving adjunctive therapies versus t-PA treatment alone. *p<0.01 compared with t-PA or t-PA plus aspirin; †p<0.05 compared with t-PA, t-PA plus aspirin, or t-PA plus heparin.
aggregation measured ex vivo (Figure 3A). Heparin prevented the increase in platelet aggregation during the first hour of t-PA infusion (Figure 3B). Hirudin similarly prevented the early enhanced aggregation response normally seen with t-PA alone (Figure 3C). Heparin, however, also appeared to attenuate the later decrease in platelet aggregatory response at 180 minutes, whereas hirudin did not. With hirudin, the platelet aggregation response was reduced by ~30% (p<0.01) at 180 minutes, similar to that observed with t-PA alone. Heparin given with t-PA, however, failed to attenuate significantly the platelet aggregation response at 180 minutes.

Discussion

We examined the effect of antithrombin treatment on fibrinolysis using an ex vivo arterial shunt preparation. Our arterial shunt system was designed to resemble an arterial thrombus seen in the clinical setting more closely than the model we have used in the past, which mimics a venous thrombus. The ex vivo assembly allowed for on-line monitoring via a gamma camera with very little background interference. This addition provides for immediate surveillance of the clot after treatment and thus affords one a better indication of the kinetics of fibrinolysis.

Our results show that coinfusion of t-PA with either heparin or hirudin leads to an enhanced extent of fibrinolysis in an ex vivo arteriovenous shunt preparation. Heparin and hirudin, when given adjunctly with t-PA, also prevented the early increase in platelet aggregation normally seen with t-PA alone. The results also suggest that adjunctive hirudin, a specific inhibitor of thrombin, was more effective than heparin in enhancing the rate of fibrinolysis but was similar to heparin in blocking early platelet activation after t-PA administration.

During thrombolysis, both thrombolytic and thrombotic events occur concurrently. Lysis results when thrombolytic events outweigh thrombotic processes. Reclosure and delays in time to reperfusion may be the consequences of thrombotic processes predominating in a given individual. Previous studies have shown that platelets are intimately involved in thrombosis and reclosure during thrombolysis. Thrombolytic treatment is associated with platelet activation and this activation occurs during a time in treatment when plasmin activity is greatest. The mechanism of this activation is not completely understood. Coinfusion of antiplatelet agents, prostaglandins I2 and E1, prevents the activation normally seen with plasminogen activator infusions. Platelet inhibition also reduces the time to reperfusion and the frequency of reclosure after thrombolytic therapy. Using a canine model of coronary thrombosis, Golino et al demonstrated that adjunctive therapy with thromboxane A2 and serotonin receptor antagonists can also reduce the delay to reflow while significantly reducing the number of animals that rethrombose during continued thrombolytic therapy. Specific blockade of the glycoprotein IIb/IIIa platelet fibrinogen receptor also leads to a reduction in time to reperfusion and rate of reclosure.

These studies initially suggested that plasmin itself may contribute to enhanced platelet aggregation responses during thrombolysis or that plasmin may lead to the release or production of a platelet agonist(s) that...
contributes to ongoing thrombosis during thrombolysis. It has recently been suggested that plasmin generation increases thrombin elaboration.\textsuperscript{9–11} Given the potency of thrombin as a platelet agonist, we hypothesized that its generation alone may account directly for enhanced platelet activity during thrombolytic therapy. We have shown in this study that thrombin inhibition with either heparin or hirudin prevents the hyperaggregable phase of platelet aggregation usually seen during t-PA infusion, suggesting that this hypothesis is correct. Antiplatelet agents such as prostaglandin \( I_2 \) and \( E_2 \), which increase cAMP levels, may prevent platelet activation by reducing the susceptibility of the platelet to agonists such as thrombin.\textsuperscript{18} Moreover, thrombin activation of platelets will, of course, stimulate the release of thromboxane \( A_2 \) and serotonin,\textsuperscript{19} and this action may account for the results of Golino et al\textsuperscript{2} using thromboxane \( A_2 \) and serotonin receptor blockade.

This study shows that the efficacy of clot lysis is enhanced considerably and perhaps in proportion to the degree of specificity of thrombin inhibition. Others have shown that thrombus growth can be inhibited by specific thrombin inhibitors.\textsuperscript{12,20,21} In this study, heparin, a catalyst for antithrombin III inhibition of thrombin, was not as efficacious as hirudin, a specific inhibitor of thrombin, in increasing the overall kinetics of clot lysis by t-PA. These results are similar to those of other investigators who have compared heparin with more specific thrombin inhibitors.\textsuperscript{2,22} This difference in efficacy could be due to the ability of hirudin to inhibit clot-bound thrombin more effectively than heparin.\textsuperscript{22} Additionally, heparin has been shown to have stimulatory effects on platelets,\textsuperscript{23} which may counteract its antithrombotic actions. Plasmin generation normally produces a biphasic platelet response, with enhanced then reduced platelet aggregation. Both antithrombin agents prevented the enhanced aggregatory phase, but heparin also prevented the reduced aggregatory phase, perhaps as a result of its ability to promote platelet activation, negating any inhibitory action of plasmin.

We also compared the action of aspirin with that of antithrombin agents on fibrinolysis. Aspirin in our model did not improve on the action of t-PA alone. Although aspirin is an effective platelet inhibitor, it does not have an effect on fibrin. As stated earlier, both platelet and fibrin deposition is important in the dynamics of thrombus formation. Antithrombin agents may be more efficacious than aspirin because both platelet and fibrin deposition are affected by inhibiting thrombin, whereas aspirin exerts its effect principally by inhibiting platelets and does so relatively weakly.

In summary, these data show that adjunctive treatment with antithrombin agents, heparin and hirudin, enhances clot lysis by t-PA. Heparin and hirudin completely prevent the early phase of enhanced platelet aggregation observed during lytic therapy, suggesting but not unequivocally proving that thrombolytic agents for this adverse effect of treatment with plasminogen activators. Hirudin appeared more effective that heparin in enhancing the rate of lysis in this model, perhaps owing to the ability of this leech product to inhibit clot-bound thrombin more effectively than heparin. Thus, these data suggest that the prothrombotic responses engendered by lytic therapy may be attenuated with the use of antithrombin agents. Thrombin inhibitors may, then, exert their beneficial effect by inhibiting both fibrin production and platelet activation, thereby facilitating dissolution of thrombi and extending patience.

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