Evaluation of the Inhibition by Heparin and Hirudin of Coagulation Activation During r-tPA–Induced Thrombolysis

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During the last decade a number of thrombotic agents have become available for the treatment of thrombotic disease. These drugs were originally used for the therapy of deep-vein thrombosis and pulmonary embolism and are now also used for treating acute peripheral arterial thrombosis and acute coronary thrombosis. The major problems associated with thrombolytic therapy are bleeding and rethrombosis.

Comprehensive comparative studies of thrombolytic agents are available only for coronary artery thrombosis. Streptokinase (SK), the most widely used, effectively accomplishes coronary thrombolysis and restores coronary patency either by intravenous (IV) infusion or intracoronary administration. More recently several trials have been performed using recombinant tissue-plasminogen activator (r-tPA). In these studies the degree of systemic fibrinogenolysis (evaluated by the decrease in plasma fibrinogen levels) was lower with r-tPA compared with SK. However, analysis of the patients from the first thrombolysis-in-myocardial-infarction (TIMI) study showed a very high reocclusion rate after short-term r-tPA administration despite early heparinization.

Rethrombosis after thrombolytic therapy may be related, as reported by Marder and Sherry, to the same factors that are responsible for the initial occlusive events: residual high-grade coronary lesion and re-exposure of blood to the ruptured atherosomatic plaque. It may also be related to the activation of platelets and clotting factors by thrombolytic agents. Oxygen-free radicals have also been implicated in myocardial ischemia secondary to reperfusion.

Furthermore, thrombin released from the clot during thrombolysis may lead to coagulation, since it has been shown that thrombin is incorporated into the clot in an active form. Thrombin is released as thrombin-FDP complex and is inactivated by heparin-AT III complex. During fibrinolysis, thrombin is released as thrombin-FDP complex and is inactivated by heparin-AT III. However, as successive fibrin layers are removed, inaccessible molecules of thrombin are exposed at the surface of the residual clot, possibly contributing to the occurrence during thrombolytic therapy of coagulation that is poorly controlled by heparin. We have investigated the accessibility of fibrin-bound thrombin to hirudin. The results clearly show that two recombinant hirudin variants neutralize thrombin both in solution and fibrin bound. Furthermore, we have found that in vitro models, hirudin present in the surrounding medium of a clot under lysis is more efficient than heparin in preventing the activation of coagulation. This observation suggests that hirudin may be effective in the prevention of the rethrombotic process frequently encountered during thrombolytic therapy.

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Asserachrom fpa from Diagnostica Stago. Specific fibrin degradation products (FbDP) determination was performed by enzyme-linked immunosorbent assay (ELISA) using a D neo monoclonal antibody (MoAb) as previously described.27

Protocols Used

Fibrin adsorption to polystyrene beads and quantitation of thrombin available on immobilized fibrin surface. This was performed by the technique described by Wilner et al.26 to 1 g of cleaned polystyrene beads (Biobeads SX2, 200 to 400 mesh from Bio-Rad) was added 2.5 mL of fibrinogen at 2.5 mg/mL in phosphate-buffered saline (PBS) or 2.5 mL of ovalbumin at the same concentration as control beads. The mixtures were incubated overnight at 4°C under agitation. The unbound material was then removed by five washings in 50 mL PBS, and the fibrinogen-coated beads were then resuspended in 10 mL PBS. The amount of fibrinogen adsorbed to the beads was determined by a competitive ELISA26 and showed that 600 µg fibrinogen was bound to each gram of beads.

To determine thrombin adsorption to fibrin, aliquots of 1 mL of fibrinogen-coated polystyrene bead suspension were centrifuged, the supernatants discarded, and 0.2 mL of thrombin (at serial dilutions from 50 to 6 U/mL) were added to the pellets with continuous mixing in a vortex agitator. After ten minutes incubation at room temperature with continuous agitation, the beads were extensively washed five times in 10 mL PBS and then suspended in 1 mL buffer. The suspensions were incubated for a further two hours at room temperature, centrifuged, and thrombin quantified both in the supernatants and in the pellets by amidolytic activity using chromogenic thrombin-sensitive substrate. For the following experiments a suspension of fibrin-coated bead on which 0.3 U/mL of thrombin was available on its surface was used. To determine thrombin release during thrombolysis. One hundred milligrams of fibrin-coated beads were incubated at 37°C with 200 µL of plasmin (1 CU/mL) with periodic agitation. At intervals, 20,000 U aprotinin were added to block plasmin, and plasmin (1 CU/mL) with periodic agitation. At intervals, 20,000 U aprotinin were added in the incubation mixture. For the following experiments a suspension of fibrin-coated bead on which 0.3 U/mL of thrombin was available on its surface was used. To determine thrombin release during thrombolysis. One hundred milligrams of fibrin-coated beads were incubated at 37°C with 200 µL of plasmin (1 CU/mL) with periodic agitation. At intervals, 20,000 U aprotinin were added to block plasmin, and released thrombin was measured in the supernatants by its amidolytic activity. It was previously checked that addition of aprotinin totally blocked plasmin activity.

Coagulation activation due to lysis of a whole clot suspended in plasma. Standard clots from 300 µL of blood were obtained after clotting citrated blood with thrombin in the presence of CaCl2 (final concentration of thrombin was 0.5 U/mL of blood and that of CaCl2 was 0.025 mol/L). Clots were stabilized for two hours at room temperature and were then thoroughly washed ten times with a large volume of 0.15 mol/L NaCl (300 mL for 20 clots for ten minutes for each washing) to eliminate thrombin trapped in the fibrin clot. To determine the efficiency of the washing, one clot was kept after each washing and immersed in 1 mL PBS for 15 minutes under gentle agitation. The released thrombin was determined by its amidolytic activity. It was found that 91% of the thrombin was eliminated after the first two washes, and in the sixth wash no thrombin was detectable (less than 0.003 U/mL). The clots were then suspended in the presence of various amounts of t-PA in 1.5 mL of citrated platelet poor plasma (PPP) or in 1.5 mL of plasma depleted in vitamin K-dependent factors to avoid overestimation of thrombin due to prothrombin activation. After two hours incubation at room temperature with gentle rocking, plasmin was blocked by adding 10,000 U aprotinin/mL (final concentration). Thrombin released from the clot was evaluated by its action on surrounding plasma fibrinogen. This was done by semiquantitative determination of soluble fibrin complexes and also by the measurement of fpa released from plasma fibrinogen.

Neutralization by heparin of thrombin released from the clot during thrombolysis in a plasma milieu. Ten milliliters of fibrinogen at 5 mg/mL in the presence of factor XIII (used at the same final concentration as in normal plasma) was clotted by 800 units of thrombin in CaCl2 solution. The final concentration of CaCl2 in the mixture was 0.025 mol/L. After three hours incubation at 37°C, the clots were extensively washed in NaCl 0.15 mol/L and then incubated with plasmin at 1 mg/mL until clots were completely lysed. Then plasmin was inhibited by adding 200,000 units aprotinin; complete inactivation was verified by monitoring amidolytic activity using S 2251 as substrate. Thrombin (present as thrombin-FDP complexes28) in the resulting solution was determined using CBS 3447 and then adjusted by dilution to 10 U/mL. Neutralization of thrombin-FDP by plasma AT III in the presence of heparin was compared with that of free thrombin at the same activity using Stachrom AT III kit (Diagnostica Stago).

Comparison of the inhibitory effects of heparin and hirudin on thrombin bound to the fibrin surface. (1) Experiment using fibrin immobilized on polystyrene beads: To avoid fibrin formation during the test, an afibrinogenemic patient’s plasma was used as a source of AT III. To 100 mg of fibrin-coated beads presenting 0.3 U of thrombin available on its surface were added 300 µL of an afibrinogenemic patient’s plasma containing either saline (control), heparin, or hirudin at several concentrations. After 120 seconds under vortex agitation, the reaction was stopped by adding 5 mL of iced PBS and centrifuged at 10,000 g for 30 seconds. The beads were then immediately washed three times in 5 mL iced PBS, and thrombin amidolytic activity associated with the beads was then measured. (2) Experiment using a standard, whole blood clot immersed in normal plasma: Standard whole blood clots, prepared as above, were suspended in 1.5 mL of normal citrated plasma containing either 0.5 IU/mL heparin or 1 µg/mL hirudin (Hoechst, final concentration). After two hours incubation at room temperature with gentle rocking, fpa was determined in the surrounding plasma. Inhibition by heparin and hirudin of coagulation activation during thrombolysis. Standard whole blood clots prepared as above were suspended in 1.5 mL normal citrated plasma containing 600 ng/mL t-PA in the presence of heparin (heparin Kabi at 0.1, 0.2, 0.5, 1, 2, 5, and 10 IU/mL) or recombinant hirudin used at concentrations that induced the same prolongation of thrombin time as standard heparin, or buffer (for control reaction). After three hours incubation with gentle rocking, plasmin generated was blocked by adding 10,000 U/mL aprotinin (final concentration). Activation of coagulation in plasma was then estimated by two different methods: determination of fpa released from plasma fibrinogen and semiquantitative determination of soluble fibrin.

RESULTS

Thrombin Adsorption to Fibrin

There was a dose-dependent relationship between the thrombin added to the fibrinogen-coated beads and that adsorbed to the fibrin beads. Under the conditions used, 5% to 6% of the thrombin was found to be associated with the fibrin surface as determined by its amidolytic activity on a thrombin-sensitive substrate.

Release of Thrombin Into the Surrounding Medium Induced by Fibrin Lysis

Thrombin associated with fibrin beads was progressively released during incubation of the beads with plasmin (results not shown). In our experimental conditions, all the thrombin
was released after one hour incubation, since after that time the beads did not induce any amidolytic activity on the thrombin-sensitive chromogenic substrate, and the amidolytic activity of the supernatant reached plateau levels.

**Activation of Coagulation Induced by Lysis of a Whole Blood Clot**

When the clot was suspended in PPP containing r-tPA (150, 300, and 600 ng/mL), a linear dose response was found between the extent of fibrin degradation (expressed as the concentration of FbDP in the supernatant) and the activation of coagulation in the surrounding plasma (expressed as the quantity of fPA released from plasma fibrinogen; Table 1).

It should also be noted that when clots were suspended in plasma in the absence of r-tPA, there was some evidence of coagulation activation but at a lower rate than that occurring in the presence of r-tPA (Table 1). This coagulation is dependent on thrombin bound to the clot, since it was also observed using a plasma depleted in vitamin K-dependent factors (Table 1).

**Neutralization of Thrombin Released From the Clot, Under Lysis, by Heparin—AT III Complex**

Thrombin release from the clot (thrombin-FDP complexes) and free thrombin used at the same amidolytic activity are similarly inhibited by addition of normal plasma 1/20 (as a source of AT III) in the presence of heparin: after one minute, residual thrombin activities were identical in the two mixtures (results not shown).

**Inhibition of Thrombin Bound to the Fibrin Surface by Heparin and Hirudin**

As shown in Fig 1, thrombin bound to fibrin polystyrene beads was poorly neutralized by incubation for 120 seconds with heparin in an afibrinogenemic patient’s plasma. In contrast, hirudin, used in the same conditions and at the same antithrombin activity as heparin, was much more effective in inhibiting the thrombin adsorbed to fibrin beads.

However, the difference between heparin and hirudin was less pronounced when the fibrin beads were incubated for several minutes with the afibrinogenemic patient’s plasma containing heparin or hirudin (results not shown).

Furthermore, when standard whole blood clots were immersed for two hours in plasma, thrombin bound to fibrin clot induced a release of 680 ng/mL of fPA from plasma fibrinogen. The amount of fPA generated was reduced to 160 ng/mL when 0.5 IU heparin was added to 1 mL of plasma and to 24 ng/mL when 1 μg of hirudin/mL of plasma was used (1 μg of hirudin has the same antithrombin activity as 0.5 IU heparin).

**Inhibitory Effect of Heparin and Hirudin on Thrombolysis-Induced Coagulation Activation**

Since rethrombosis occurs in some patients under thrombolytic therapy despite simultaneous treatment with heparin, the influence of hirudin on the activation of coagulation was tested in comparison with that of heparin. The following observations were made: (1) The degradation of the fibrin clot (expressed as the concentration of FbDP in the supernatant) was not affected by the presence of heparin or hirudin in the plasma, since the levels of FbDP generated were identical whether heparin or hirudin were used (results not shown). (2) The action of hirudin in the prevention of thrombolysis-induced coagulation activation is stronger than that of heparin. The levels of fPA released from plasma fibrinogen during thrombolysis of the suspended clots were much lower when plasma contained hirudin than when it contained equivalent doses of heparin. The use of very high heparin concentrations, in excess of pharmacologic doses, failed to inhibit completely the activation of coagulation (Fig 2).

Similar results were obtained with the determination of soluble complexes: these complexes were still present in the plasma containing 0.1 or 0.2 U/mL heparin in which clots were suspended. Soluble complexes were not found with equivalent concentrations of hirudin.

**DISCUSSION**

The activation of coagulation during thrombolytic therapy has already been reported. Heparin at pharmacologic concentrations is not sufficient to prevent the risk of rethrombosis, as already observed in clinical studies. In this work we propose that in addition to the ischemic reperfusion mechanism, this activation is due, at least in part, to thrombin bound to the fibrin network and progressively exposed at the fibrin surface during thrombolysis and to thrombin released during fibrinolysis as complexes with fibrin degradation products.

Since thrombin may favor both fibrin formation and

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**Table 1. Relation Between the Intensity of Fibrin Degradation (Expressed in FbDP) and Activation of Coagulation in the Surrounding Plasma (Expressed in fPA Released From Plasma Fibrinogen)**

<table>
<thead>
<tr>
<th>Concentration of t-PA (ng/mL)</th>
<th>Extent of thrombolysis (in FbDP ng/mL) in Plasma in Which a Clot Was Suspended</th>
<th>Concentration of t-PA in Plasma (ng/mL) - Control in the Absence of Clot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 150 300 600 600</td>
<td></td>
</tr>
<tr>
<td>FPA released from normal plasma (ng/mL)</td>
<td>500 45,000 72,000 120,000 135</td>
<td></td>
</tr>
<tr>
<td>FPA released from vitamin K-depleted plasma (ng/mL)</td>
<td>680 835 1,124 1,540 135 135 135</td>
<td></td>
</tr>
<tr>
<td>FPA released from plasma containing 0.1 or 0.2 U/mL heparin in which clots were suspended</td>
<td>648 880 1,300 1,610 135 135 135</td>
<td></td>
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platelet adhesion to collagen, we have tested in our in vitro model strategies to neutralize thrombin, both associated with the clot and released during thrombolysis, to diminish the risk of rethrombosis during thrombolysis.

We have found, as have others, that thrombin bound to either fibrin polystyrene beads or whole blood clots remains active and therefore may promote in situ the extension of the thrombus: the thrombin bound to fibrin-polystyrene beads hydrolyzed a thrombin-sensitive substrate. Furthermore, using a more physiologic system, standardized whole blood clots, extensively washed to eliminate thrombin trapped in the fibrin network, induced fibrin formation when suspended in either normal plasma or vitamin K-dependent factor-free plasma. This activation of coagulation was demonstrated by the substantial generation of both fibroplasmin (fP) and soluble-fibrin monomer complexes.

Therefore, we have compared the efficiency of two antithrombin drugs on fibrin-bound thrombin: heparin and hirudin. Hirudin is an antithrombotic agent extracted from leeches and that is now available in large amounts, produced by genetic engineering. From our results it appears that heparin-AT III complex, which is a good inhibitor of free thrombin, was a poor inhibitor of thrombin bound to fibrin, as already described by Linardic and Greenberg. In contrast, we have found that hirudin at an equivalent dose neutralized both free and fibrin-bound thrombin.

The difference between the effect of heparin and hirudin on fibrin-bound thrombin neutralization was less pronounced with prolonged incubation time, as observed both with fibrin beads and standard clots suspended in plasma. When the fibrin clot was degraded by thrombolytic agents, there was a release of thrombin activity into the surrounding medium, as demonstrated in a purified system. Using the whole blood system, despite the addition of heparin to the plasma in which the clots were suspended, both fP and soluble fibrin are generated, and this generation is higher during in vitro thrombolysis. This fibrin formation is evident even at very high doses of added heparin (up to 10 U/mL). Since thrombin released from the clot during thrombolysis (as thrombin-FDP complexes) may be inactivated by hepa-
rin-AT III complex, we postulate that the thrombin bound to fibrin that is poorly accessible to heparin-AT III is responsible for the activation of coagulation.

As it has been reported that degradation of a thrombus by exogenous plasminogen activators occurs at its surface, we assume that the successive removal of fibrin layers occurring during fibrin clot lysis induces a further exposure of thrombin at the surface of residual clot. This thrombin is poorly accessible to heparin-AT III and is thus responsible for both coagulation and platelet activation. Thrombin released into the circulation would have little or no procoagulant effect. Our hypothesis is supported by the fact that at pharmacologic doses or even at very high doses, heparin does not totally prevent fP A release from plasma fibrinogen.

In contrast to heparin, hirudin, which neutralizes both free thrombin and fibrin-bound thrombin, even at low doses, prevents more efficiently the activation of coagulation induced by clots suspended in plasma both before and during thrombolysis. fP A released from fibrinogen was much lower than that released in the presence of heparin used at the same antithrombin doses, and soluble fibrin was never detected in the plasma in which hirudin was added. Consequently, adequate anticoagulation by hirudin should be effective in preventing rethrombosis occurring after thrombolytic therapy. A further potential advantage of hirudin in this context is suggested by the reported inhibition of thrombin-dependent platelet adhesion to collagen.

From these results, we propose that hirudin should be tested in clinical trials for the prevention of rethrombosis associated with thrombolytic fibrinogen.

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