Platelet-Dependent Thrombin Generation After In Vitro Fibrinolytic Treatment

David L. Aronson, MD; Ping Chang, MD; and Craig M. Kessler, MD

Background. Fibrinolytic therapy is associated with frequent rethrombosis. There is evidence of both increased coagulation and platelet activation.

Methods and Results. Platelet-rich plasma (PRP) or washed platelets were incubated with the fibrinolytic agents urokinase, recombinant tissue-type plasminogen activator (rt-PA), or plasmin at concentrations consistent with those in the plasma of patients treated for myocardial infarction. All of the fibrinolytic agents induced a more rapid generation of thrombin and decreased the clotting times of non-contact-activated PRP than in untreated PRP. This effect was not blocked by the inclusion of thrombin inhibitors during the fibrinolytic treatment. Washed platelets derived from rt-PA-treated PRP induced more rapid thrombin generation when resuspended in untreated plasma or treated plasma. Washed platelets were treated with plasmin, rt-PA, and urokinase and added to platelet-poor plasma. Platelets treated with either plasmin or rt-PA increased the ability of washed platelets to support thrombin generation, but urokinase was without significant effect.

Conclusions. These results indicate not only that plasmin can cause increased platelet support of prothrombin activation but also that rt-PA in the absence of plasminogen can have a direct effect on the platelet, which increases thrombin generation. (Circulation 1992;85:1706–1712)

Key Words • plasminogen activators • urokinase • platelets

The introduction of fibrinolytic agents has had a major impact on morbidity and mortality from acute thrombotic disease. The major concern with fibrinolytic agents has been the occurrence of significant bleeding complications. However, questions concerning increased platelet activation and possible prothrombotic effects have been raised by both in vitro and in vivo experiments.1 There is evidence that fibrinolytic therapy activates platelets and/or the plasma coagulation components both in vitro and in vivo (Table I).1,17 If true, this may prevent reperfusion or be allied with reocclusion.

Proteolytic or pharmacological activation of platelets increases their ability to support coagulation. This is associated with both the binding of coagulant proteins on the platelet surface and the translocation of coagulant active phospholipids to the outer platelet membrane.18–27

The experiments presented below were done to investigate procoagulant effects of fibrinolytic agents using measurements of the beginning of thrombin generation as the end point.

Methods

Materials

Recombinant tissue-type plasminogen activator (rt-PA) was obtained from Genentech Inc. (South San Francisco, Calif.) as a mixture of one-chain and two-chain rt-PA in the clinical formulation. Urokinase (UK) as a mixture of high- and low-molecular-weight two-chain UK was kindly supplied by Drs. Jack Henkin and Arthur Sasahara (Abbott Laboratories, North Chicago, Ill.). The clinical formulation used contains albumin and mannitol. Plasmin was obtained from KabiVitrum (Stockholm, Sweden) and standardized in casein units (CU). Thrombin was prepared by Dr. John Fenton and supplied by Dr. Deborah Beebe (Center for Biologies Review and Research, Bethesda, Md.). Hirulog, a hirudin derivative, was supplied by Dr. John Maraganore (Biogen, Cambridge, Mass.); collagen was obtained from Bio/Data Co. (Hatboro, Pa.); heparin sodium from The Upjohn Co. (Kalamazoo, Mich.); and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) from Calbiochem (San Diego, Calif.). Thrombin substrate S-2238 (H-d-Phe-Pip-Arg-NH-NO₂-2HCl) was purchased from Helena Laboratories (Beaumont, Tex.), and Immulon microtiter plates were obtained from Dynatech (Chantilly, Va.).

Preparation of Platelet-Rich Plasma and Washed Platelets

Venous blood samples were collected from normal volunteers and anticoagulated with 3.8% sodium citrate (9 vol blood:1 vol sodium citrate). Unless otherwise noted, none of the donors had taken any medications known to interfere with coagulation or platelet function for at least 10 days before donation.

Platelet-rich plasma (PRP) was prepared by centrifuging blood at 800g for 3 minutes and removing the top two thirds of the supernatant plasma. The residual
Assay of Thrombin Generation

Typically, 0.5 ml of PRP was placed in a 12×75-mm polypropylene tube, and 20 μl of 1 M CaCl₂ was added to start clotting. At the noted intervals, 10 μl of sample was placed in the wells of a microtitr plate containing 90 μl of 3.8% sodium citrate. At the end of the incubation time, 50 μl of 0.5 mM S-2238 in 1 M Tris, pH 8.1, was added and the plate was read kinetically for 2 minutes at a wavelength of 405 nm on a Vmax microtitr plate reader (Molecular Devices, Palo Alto, Calif.). One unit of thrombin per well yields a value of 10 mOD/min. The time to clot formation was measured from the time of calcium addition until visible clot formation. In the absence of contact with glass or other mechanisms to activate factor XII and platelets, this can be defined as a noncontacted coagulation test.

Platelet aggregation was measured as described by Poindexter and Fratantoni28 in microtitr plates. PRP (120 μl) was added to plates containing ADP. After they were shaken for 10 minutes, the optical density was read on the Vmax plate reader. The percent aggregation was calculated linearly between the optical densities of the PRP and PPP.

Statistical analysis was by paired t tests.

Results

rt-PA (final concentration, 1.5 μg/ml) or an equal volume of Tris-buffered saline was added to 0.5 ml of PRP in 12×75-mm polypropylene tubes and incubated for 10 minutes at 22°C. CaCl₂ (1 M) was added to a final concentration of 0.025 M, and timing was started. Serial 10-μl samples were removed and placed into wells containing sodium citrate, and the thrombin was measured as described above. The clotting time of the incubation mixture was also noted, and the clot was removed as it formed. Figure 1A shows the typical pattern of thrombin generation in PRP as assessed by S-2238 hydrolysis. Thrombin generation was more rapid in rt-PA-treated PRP than in control PRP, which in turn was more rapid than in PPP. The difference in the time to thrombin generation as assessed by S-2238 hydrolysis to reach 20 mOD/min averaged 10 minutes (n=9) and was significant at the level of p=0.005. The 95% confidence interval for the difference was 7–14 minutes. The same procoagulant effect was seen in PRP from donors who had taken 250 mg of aspirin 2–24 hours before blood sampling (Figure 1B). The addition of formalinized platelets to PPP had no effect on thrombin generation.

### Table 1. In Vitro Effects of Fibrinolytic Agents

<table>
<thead>
<tr>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregation</td>
<td></td>
</tr>
<tr>
<td>Increased</td>
<td>2–4, 12</td>
</tr>
<tr>
<td>Decreased</td>
<td>7</td>
</tr>
<tr>
<td>No effect</td>
<td>6</td>
</tr>
<tr>
<td>Dense granule release</td>
<td>2, 6</td>
</tr>
<tr>
<td>Protein phosphorylation</td>
<td>3</td>
</tr>
<tr>
<td>Increased cytosolic Ca²⁺</td>
<td>3</td>
</tr>
<tr>
<td>Thromboxane production</td>
<td>5, 14</td>
</tr>
<tr>
<td>PADGEM expression</td>
<td>4</td>
</tr>
<tr>
<td>Decreased glycoprotein Ib</td>
<td>2</td>
</tr>
<tr>
<td>Increased platelet clearance</td>
<td></td>
</tr>
<tr>
<td>Coagulation activation</td>
<td>8–11</td>
</tr>
</tbody>
</table>

PADGEM, α-granule protein.
Similar experiments were done with UK treatment of PRP. UK at a final concentration of 1,000 and 2,000 IU/ml in PRP also decreased the lag time for thrombin generation. The average shortening of the time for thrombin generation was 16 minutes, with a 95% confidence interval of 11–21 minutes (n=6). There is a direct effect of UK on the substrate S-2238, and there has been no correction for this baseline substrate hydrolysis.

Other experiments were done to examine the possibility of a synergistic effect of collagen and UK treatment. Collagen was added to PRP (10 μg/ml) in the absence or presence of 2,000 IU/ml of UK and stirred at approximately 1,000 rpm with a 3-mm Teflon-coated stirring bar. The platelets stirred with collagen shortened the time for thrombin generation compared with control stirred platelets. The addition of UK alone shortened the time substantially more than collagen. The combination had no more effect than UK alone (data not shown).

Both UK and rt-PA are plasminogen activators and have in common the production of plasmin in the PRP. If this is the mechanism of the more rapid thrombin generation, plasmin added to PRP should produce the same effect. The addition of plasmin (0.9 CU/ml) to PRP resulted in the shortening of the time to thrombin generation by an average of 10.4 minutes (n=5) and a 95% confidence interval of 5.6–15.2 minutes.

The impact of UK, rt-PA, and plasmin could also be observed as a dose-dependent shortening of the clotting time of PRP. Figure 2 shows the effect of rt-PA, plasmin, and UK on the shortening of the clotting time of PRP. The test mixtures used were identical to that used for measuring thrombin by S-2238, i.e., 0.5 ml PRP and 20 μl of 1 M CaCl₂ in 12×75-mm polypropylene tubes. The clotting time of the PRP paralleled the time at which thrombin generation reached 20 mOD/min (Figure 3). In all cases, the clotting time and the time to start thrombin generation of the fibrinolytically treated PRP was shorter than its matched control. These differences in clotting time were all significant at the level of p<0.005.

It has been proposed that plasmin directly activates prothrombin to thrombin, which in turn activates the platelets. PRP was incubated with rt-PA (1.5 μg/ml), UK (1,000 IU/ml), plasmin (0.9 CU/ml), or thrombin (0.06 IU/ml) for 10 minutes in the presence or absence of heparin (0.8 units/ml). Following the incubation, heparin was added to those tubes to which it was not previously added, followed by the addition of protamine (12.5 μg/ml) to minimize the heparin effect on the test system. Despite the presence of heparin during the treatment with the fibrinolytic agents, there was a shortening of the time to thrombin generation and the clotting time (Figures 4A and 4B). When the PRP was treated with 0.06 IU/ml thrombin, a similar effect was seen on the clotting time and the time to thrombin.
TABLE 2. Thrombin Generation With Washed Platelets

<table>
<thead>
<tr>
<th>Platelets</th>
<th>Plasma</th>
<th>Time to 10 mOD/min [average (range)] (min)</th>
<th>Paired t test p vs. normal platelets, normal plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA</td>
<td>Normal</td>
<td>28 (24–32)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Normal</td>
<td>rt-PA</td>
<td>36 (28–46)</td>
<td>p&gt;0.4</td>
</tr>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>37 (30–48)</td>
<td></td>
</tr>
</tbody>
</table>

rt-PA, recombinant tissue-type plasminogen activator.

250,000 and 350,000 platelets/µl were placed in 400 µl of control or rt-PA–treated PPP, and the thrombin generation was measured. More rapid thrombin generation was dependent on the presence of rt-PA–treated platelets, not of the rt-PA–treated plasma (Table 2) (Figure 5). A similar experiment was done with PRP derived from a patient with severe factor XI deficiency. When the rt-PA–treated platelets were readded to normal plasma, there was again significant shortening of the clotting time and the time of thrombin generation, indicating that this effect was independent of the contact phase of coagulation.

Washed platelets derived from UK-treated PRP demonstrated a dose-dependent effect. At 500 and 1,000 units/ml of UK, there was a consistent pattern similar to that seen with rt-PA, whereas after treatment with 2,000 units/ml, the washed platelets had less ability to support the thrombin generation.

To rule out thrombin generated in the PRP and subsequently carried over into the PPP as the platelet activator, a similar experiment was done with the inclusion of thrombin inhibitors. The hirudin analogue, Hirulog, (0.5 µg/ml) was added to PRP, followed by incubation with rt-PA (1.5 µg/ml), and platelets were washed as above with Tyrode’s solution and placed back

FIGURE 5. Graph showing generation of thrombin by washed platelets previously treated with recombinant tissue-type plasminogen activator (rt-PA). Platelet-rich plasma (PRP) was treated with 1.5 µg/ml of rt-PA or left untreated. The rt-PA and control platelets were washed and placed in either the control plasma or the rt-PA–treated plasma, and the thrombin generation was measured: ■, rt-PA–treated platelets and rt-PA–treated plasma; ○, rt-PA–treated platelets and untreated plasma; ●, rt-PA–untreated platelets and treated plasma; △, untreated platelets and untreated plasma.

generation. However, the inclusion of heparin before the addition of thrombin almost completely blocked the more rapid thrombin generation. The differences between Figures 4A and 4B are typical of differences between days and between donors in this test system.

To differentiate whether the effect of the fibrinolytic agents on the PRP is a result of changes in the plasma as opposed to the action on the platelet, washed platelets and PPP were prepared from PRP treated with rt-PA, UK, plasmin, and control PRP. Washed platelets (100 µl) resuspended in Tyrode’s buffer to between
into PPP; or, PPACK (1 μM) was present in Tyrode's solution for the first washing of rt-PA-treated or untreated platelets; they were then washed several times without PPACK. Despite the presence of potent thrombin inhibitors, there was again a shortening of the time for thrombin generation (Figure 6).

If the impact of the fibrinolytic agents is mediated solely by the action of plasmin, platelets treated with plasmin should shorten the clotting time and the time to thrombin generation when washed and added back to control plasma. In contrast, the plasminogen activators would not be expected to have any effect on the activation of washed platelets in the absence of plasminogen. Washed platelets were prepared from PRP and treated with the appropriate fibrinolytic agent. The treated and control platelets were rewashed twice and resuspended in Tyrode's buffer. The washed platelets (100 μl) were then readded to PPP to a platelet count of 50,000/mm³.

Plasmin (0.45 CU/ml) consistently shortened the time to thrombin generation and the clotting time compared with the control platelets. This effect was dose dependent, with 0.225 and 0.9 CU/ml having less impact. The increased support of thrombin generation was completely inhibited by the presence of aprotinin (10 μg/ml). As predicted, UK-treated (1,000 IU/ml) washed platelets affected neither the thrombin clotting time nor the time to achieve thrombin generation (Table 3).

When washed platelets were incubated with 1.5 μg/ml of rt-PA, washed twice, and added to PPP, there was shortening of the clotting time and the time to thrombin generation (n=4). Analysis by paired t test gave a 95% confidence interval of between 4- and 14-minute shortening of the clotting time (Table 3). The inclusion of aprotinin (10 μg/ml) did not prevent the increased prothrombin conversion and the shortening of the clotting time after treatment with rt-PA, indicating that this was not mediated by the presence of residual plasminogen.

Platelet aggregation was done on the control PRP and the rt-PA-treated PRP. There was no increase or decrease in platelet aggregation in the absence of ADP after treatment of the PRP with rt-PA (Figure 7).

**Discussion**

The impact of fibrinolytic therapy on the hemostatic system has been and is of much concern. The initial view, confirmed in the clinic, was that such therapy would result in abnormal bleeding. Studies on both platelets and plasma components of the hemostatic mechanism reveal ample cause for a hemostatic defect. Recently, there has been concern that such treatment not only leads to increased blood loss but may, paradoxically, lead to a prothrombotic state and limit the effectiveness of thrombolytic therapy.

The prothrombotic effects may be associated with changes in the platelet function or with activation of the plasma coagulation system. Increased fibrinopeptide A, a marker of fibrin formation, is seen even in the presence of heparin during treatment with fibrinolytic agents. Platelets have increased aggregation re-

**Table 3. Effect of Fibrinolytic Agents on Washed Platelets**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>Change in clot time</th>
<th>95% Confidence interval</th>
<th>Change in time to 20 mOD</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>rt-PA</td>
<td>1.5 μg/ml</td>
<td>−12.5</td>
<td>−3, −21</td>
<td>−6</td>
<td>−15.5, +3.5</td>
</tr>
<tr>
<td>Plasmin</td>
<td>0.45 CU/ml</td>
<td>−15.5</td>
<td>−9, −22</td>
<td>−11</td>
<td>−20.4, −1.5</td>
</tr>
<tr>
<td>Urokinase</td>
<td>1,000 IU/ml</td>
<td>−2.5</td>
<td>7.5, −7.8</td>
<td>−1</td>
<td>−10, +8</td>
</tr>
</tbody>
</table>

rt-PA, recombinant tissue-type plasminogen activator; CU, casein units.
Thrombin Generation After Fibrinolytic Treatment

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Response to agonists and demonstrate increased production of thromboxane derivatives after treatment both in vitro and in vivo with fibrinolytic agents. Inhibitors of platelet receptors or of platelet activation minimize reocclusion in animal models.15–17

The test system used here is a sensitive indicator of changes in the interaction of platelets and the plasma coagulation system. The “noncontacted” test system minimizes both the activation of the plasma intrinsic system and the activation of platelets. The slower clotting times allow us to detect consistent differences between the control and fibrinolytically treated samples. The analysis of “time to thrombin generation” is a marker of a decreased lag time and might have more significance than a maximal rate of thrombin generation. The level of thrombin content used above represents conversion of about 5% of the plasma thrombin and approximately the amount needed for rapid clot formation.

These experiments show an increased ability of fibrinolytically treated platelets to support the generation of thrombin. The concentration of the fibrinolytic agents used is consistent with the plasma levels during therapy (1.5 μg/ml for rt-PA and 500–2,000 IU/ml for UK).

Noncontacted PRP treated with either rt-PA, plasmin, or UK demonstrates a marked and consistent decrease in the time for thrombin generation and a decrease in the clotting time (Figures 1 and 3). These phenomena are independent of the activation of the cyclooxygenase pathway; aspirinized and normal platelets react identically. The lack of inhibition of these phenomena by heparin, Hirulog, or PPACK (Figures 4 and 6) argues against the proposal that plasmin generates thrombin, which in turn activates the platelets or factor V,8,9,11

The interrelation between collagen treatment under conditions known to partially stimulate the release reaction and collagen–UK treatment are not inconsistent with published data.2,26,29 The addition of collagen to whole blood decreases the time for the generation of prothrombin fragment F1,2, the first step in thrombin activation.29 Rosing et al26 report some effect of collagen-treated platelets alone but much more striking support of prothrombin generation after the combination of thrombin and collagen. These results are consistent with a partial activation by collagen and stirring and complete activation with UK with stirring, because the addition of collagen to the stirred UK platelets did not have an additive effect.

Experiments with washed platelets derived from fibrinolytically treated PRP indicate that this increased clotting activity resides in the platelets. In all experiments, the treated platelets generated thrombin more rapidly than the untreated platelets when added back to normal plasma, with the exception of the platelets derived from PRP treated with 2,000 IU/ml UK. It may be that the UK-treated platelets are unstable during the washing procedure.

Treatment of washed platelets indicates that these effects can be mediated directly by plasmin. When washed platelets are treated with plasmin, there is a dose-dependent effect, with the greatest activation seen at 0.45 CU/ml and less activation at 0.9 CU/ml. UK treatment of washed platelets does not change their ability to support thrombin generation, indicating an indirect effect presumably mediated by UK activation of plasminogen. However, the experiments with washed platelets treated with rt-PA and returned to PPP after more washing also shortened the time to thrombin generation and the clotting time of the plasma. This indicates a direct effect of rt-PA on the platelet. The washed platelets might contain residual plasminogen. There was no inhibition of the rt-PA effect in the presence of 10 μg/ml of aprotinin; a quantity sufficient to inhibit 0.9 CU of plasmin. UK, another plasminogen activator, did not have this effect. Another possibility is binding of rt-PA to the platelets; when these platelets are placed in plasma, this would activate the plasminogen. However, the binding of rt-PA and plasminogen is dependent on divalent cations such as calcium.30–32 There are no reports of macromolecular substrates for rt-PA other than plasminogen or fibrinogen.33

Several mechanisms have been proposed for the role of platelets in prothrombin activation to thrombin. The platelet content of phospholipid, often referred to as platelet factor 3, is an established mechanism to promote prothrombin activation. There are also platelet membrane protein–specific mechanisms. Walsh and Griffin27 have studied the interactions of platelets and factor IX and factor XII and have proposed that the platelet serves as a specific binding region for these components. However, platelets derived from rt-PA–treated PRF from a patient with a severe deficiency of factor XI significantly shortened the clotting time and the thrombin generation when added back to normal plasma.

Exquisitely small quantities of thrombin will activate platelets and allow them to bind activated factor X, with a concomitant increase in the rate of thrombin generation.23–25 One mechanism appears to be the binding of factor Xa to factor V released from the α-granule. Plasmin releases the α-granule protein PADGEM (GMP-140)4 and may release the platelet factor V at the same time.

Others report that the fibrinolytic agents are capable of generating small amounts of thrombin, possibly independently of the platelets.8,9 It is proposed that thrombin would activate prothrombin, and the thrombin thus formed could then activate the platelets and increase their ability to support coagulation. Streptokinase produced more thrombin than other fibrinolytic agents. In these experiments, rt-PA produced only small changes in the quantity of fibrinopeptide A when the plasminogen activator was added to citrated plasma as was done above. Further, since the same qualitative effect is seen in the presence of heparin, the shortened clotting time and platelet support of thrombin activation would appear to be dependent on a different mechanism. Another plausible mechanism is that plasmin activates factor V,24 which binds to the platelets.

Previous studies of the effect of fibrinolytic agents on platelet aggregation indicate more or less the same aggregation properties. Our studies did not reveal any effect of fibrinolytic treatment on platelet aggregation (Figure 7). This diversity of result would indicate that under different conditions, one may see differing effects.

These experiments demonstrate a novel phenomenon associated with fibrinolytic agents. The data indicate that the “platelet activation” seen is not a result of thrombin activation, because thrombin activation is
completely blocked by the presence of heparin, whereas the fibrinolytic effect is independent of the inclusion of heparin.

The above data indicate that not only plasmin but also rt-PA can directly mediate prothrombin changes in the platelet. The increase in the platelet support of prothrombin activation is similar to that seen with thrombin. This event could result from either receptor binding or proteolysis, and the mechanism could be through changes in either membrane glycoproteins or phospholipids.

The term “platelet activation” is operationally defined by the test systems of each laboratory. Fibrinolytic agents have been demonstrated to activate platelets by all of the criteria seen in Table 1. We have extended these measurements of platelet activation by fibrinolytic agents to include increased support of prothrombin activation. This has already been shown for other platelet agonists.18,26,29 This may be the mechanism of increased thrombin generation in patients treated with fibrinolytic agents as assessed by increase in the level of the thrombin–antithrombin III complex.35,36 Further studies are under way to confirm whether this phenomenon will be seen in patients treated with fibrinolytic agents.

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