Activation of Plasminogen by Tissue Plasminogen Activator on Normal and Thrombasthenic Platelets: Effects on Surface Proteins and Platelet Aggregation

By Raphael B. Stricker, Dan Wong, Donny Tak Shiu, Patrick T. Reyes, and Marc A. Shuman

Tissue plasminogen activator (TPA) converts plasminogen to plasmin within the fibrin clot, thus localizing activation of fibrinolysis. To determine the extent to which platelets promote activation of plasminogen by TPA, we studied the interaction of TPA and plasminogen with unstimulated platelets. Normal washed platelets incubated in the presence of physiologic concentrations of plasminogen (180 µg/mL) and TPA (20 ng/mL) failed to generate plasmin activity. In contrast, incubation of platelets with TPA concentrations achieved during thrombolytic therapy (40 to 800 ng/mL) produced a tenfold to 50-fold increase in plasmin activity. After exposure to plasminogen and 200 ng/mL of TPA for one hour, platelets failed to agglutinate in the presence of ristocetin. Incubation of platelets suspended in autologous plasma with 400 ng/mL of TPA for one hour also inhibited ristocetin-induced agglutination. Exposure of platelets to plasminogen and increasing concentrations of TPA correlated with a decrease in glycoprotein Ib (GPIb) and an increase in glycoplasticin, as shown by immunoblotting. The glycoprotein GPIb/IIa (GPIIb/IIa) complex and a 250,000-dalton protein also disappeared from washed platelets after incubation with plasminogen and 200 ng/mL of TPA for one hour. These platelets failed to aggregate in the presence of adenosine diphosphate (ADP) or gamma thrombin, although aggregation in response to calcium ionophore A23187 and arachidonic acid remained intact. However, aggregation in response to all four agonists was normal when platelets were incubated with TPA in the presence of autologous plasma. Platelets from a patient with Glanzmann’s thrombasthenia also generated plasmin in the presence of TPA. Hydrolysis of GPIb and inhibition of ristocetin-induced agglutination occurred to a lesser extent with these platelets than with control platelets. We conclude that platelets provide a surface for activation of plasminogen by pharmacologic amounts of TPA. Plasmin generation leads to degradation of GPIb and decreased ristocetin-induced agglutination in normal and thrombasthenic platelets, as well as degradation of GPIIb/IIa in normal washed platelets and inhibition of ADP and gamma thrombin-induced aggregation. These findings suggest that pharmacologic concentrations of TPA may cause platelet dysfunction due to plasmin generation on the platelet surface.

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Tyrode's buffer at 200,000/μL and incubated with plasminogen and TPA for one hour at 37 °C as shown in Table 1.

After incubation, the platelets were centrifuged and the supernatants removed for assay of plasmin activity using substrate S-2251. A standard curve was constructed using substrate and known amounts of plasmin. Platelets from (F) in Table 1 were resuspended with TPA and plasminogen and 276 STRICKER ET AL.

Aggregation was then performed using the same agonist concentrations. PRP incubated with TPA and plasminogen from (F) in Table 1 were resuspended in Tyrode's buffer at 200,000/L and incubated with plasminogen and TPA as shown in Table 1. 

Results

Fig 1. Plasminogen activation by TPA using control and thrombasthenic platelets. Washed platelets were incubated with plasminogen and increasing concentrations of TPA for one hour at 37 °C. The platelets were centrifuged and the supernatant was assayed for plasmin activity as described in Materials and Methods. Incubation of platelets with TPA alone, plasminogen alone, or plasminogen plus a physiologic TPA concentration (20 ng/mL) failed to generate plasmin activity. Platelet exposure to plasminogen and TPA concentrations of 40, 200, 400, and 800 ng/mL yielded plasmin activities of 0.20 to 0.55 CU/mL for control platelets and 0.10 to 0.45 CU/mL for thrombasthenic platelets.

Table 1. Incubation Concentrations of Platelets, TPA, and Plasminogen

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<thead>
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<th>A</th>
<th>B</th>
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<tr>
<td>Platelets/μL</td>
<td>200,000</td>
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<td>200,000</td>
<td>200,000</td>
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<tr>
<td>TPA ng/mL</td>
<td>—</td>
<td>200</td>
<td>200</td>
<td>20</td>
<td>40</td>
<td>200</td>
<td>400</td>
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<tr>
<td>PLG μg/mL</td>
<td>180</td>
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PLG, plasminogen.
GPIb and a higher mol wt protein with consequent generation of the proteolytic cleavage product glycoprotein IIb. Glycoprotein IIb is also formed when thrombasthenic platelets are exposed to TPA and plasminogen, but the GPIb band is incompletely degraded (Fig 2B). Less GPIb is cleaved in the thrombasthenic platelets exposed to a plasmin concentration of 0.45 CU/mL (Fig 2B, lanes D through F) than in normal platelets exposed to a plasmin concentration of 0.20 CU/mL (Fig 2A, lane C). Thus, thrombasthenic platelets appear to be more resistant to the effect of plasmin than do control platelets. In addition, the 250,000-dalton protein is not seen in the thrombasthenic platelets. Thus, these platelets appear to be deficient in a higher-mol-wt protein that is degraded by plasmin.

Figure 3 shows the results of immunoblotting using rabbit anti-GPIIb/IIIa. In control platelets exposed to TPA alone (lane A), four bands are detected by the GPIIb/IIIa antibody: the 250,000-dalton protein, two proteins of mol wt 140,000 and 130,000 corresponding to GPIIb, and a 90,000-dalton protein corresponding to GPIIIa. After exposure of platelets to plasminogen and increasing concentrations of TPA, the four bands are no longer detectable (lane F). Thrombasthenic platelets do not contain these bands even without exposure to plasminogen and TPA (data not shown). Thus, exposure of platelets to TPA and plasminogen results in degradation of GPIIb/IIIa. In addition, the immunoblot findings with anti-GPIIb/IIIa antibody confirm the absence of the 250,000-dalton protein in thrombasthenic platelets, as suggested by the findings with anti-GPIb antibody.

Figure 4 shows the results of ristocetin-induced aggregation of washed platelets after exposure to plasminogen and 200 ng/mL of TPA for one hour. Ristocetin-induced platelet aggregation was inhibited by 80% compared with control platelets. In contrast, thrombasthenic platelets showed only a 30% inhibition of ristocetin-induced aggregation even after exposure to plasminogen and 400 ng/mL of TPA. However, the aggregation of untreated thrombasthenic platelets by ristocetin was 20% less than that of normal platelets in the washed platelet system. This difference is unexplained.

Figure 5 shows the effect of TPA on ristocetin-induced aggregation of normal platelets suspended in autologous plasma. Although 200 ng/mL of TPA had no effect on platelet aggregation, incubation of PRP with 400 ng/mL of TPA completely inhibited ristocetin-induced aggregation. These TPA concentrations generated plasmin activities of 0.04 and 0.20 CU/mL, respectively, in PRP. Thus, TPA exerts its effect both in a washed platelet system and in PRP, although in PRP, a higher concentration of TPA is needed to achieve the same effect as with washed platelets.

Figure 6 shows the aggregation curves of normal washed platelets in response to ADP, gamma thrombin, arachidonic acid, or calcium ionophore A23187. Exposure of platelets to
Normal Platelets

I. Control (Plg)

C0 ITPA + Plg

Glanzmann Platelets

Time

Time

PAP + TPA 400 ng/ml

Ristocetin-induced agglutination of PRP. Platelets were suspended in autologous plasma at 200,000/μL and incubated with TPA 200 or 400 ng/mL for one hour at 37 °C. Ristocetin 1.5 mg/mL was then added, and aggregometry was performed. PRP incubated with TPA buffer alone was used as a control.

plasminogen and 200 ng/mL of TPA for one hour at 37 °C inhibited the aggregation response to ADP and gamma thrombin by 80%. In contrast, platelet aggregation in response to arachidonic acid and calcium ionophore remained intact after TPA exposure. However, when PRP was substituted for washed platelets, aggregation in response

to all four agonists remained intact, even after platelet exposure to 800 ng/mL of TPA (data not shown).

DISCUSSION

In this study, we have shown that washed platelets incubated in the presence of plasminogen and pharmacologic concentrations of TPA produced a tenfold to 50-fold increase in plasmin activity. Exposure of platelets to TPA and plasminogen resulted in a decrease in GPIb and an increase in glycoprotein, as shown by immunoblotting. The platelets failed to agglutinate in the presence of ristocetin after exposure to pharmacologic concentrations of TPA. In addition, GPIIb/IIIa was no longer immunologically detectable in these platelets, and the aggregation response to ADP and gamma thrombin was inhibited. Thrombasthenic platelets generated slightly reduced amounts of plasmin in the presence of TPA, and ristocetin-induced agglutination was not inhibited to the same extent as in normal platelets. By immunoblotting, the thrombasthenic platelets also appeared to be less susceptible to degradation of GPIb.

Our findings have three significant implications. First, we have shown that unstimulated platelets incubated with a physiologic concentration of TPA do not generate plasmin. Therefore, under physiologic conditions, platelets do not provide a mechanism for plasminogen activation by TPA. Second, it appears that unstimulated platelets can interact with pharmacologic doses of TPA with consequent plasmin generation. Third, the amount of plasmin generated by low therapeutic levels of TPA is capable of degrading GPIb, the von Willebrand factor binding site on platelets, thereby inhibiting platelet adhesion. GPIIb/IIIa also appears to be degraded by plasmin, although this does not occur in PRP.
Thus, TPA has the potential to cause platelet dysfunction at concentrations well within the therapeutic range by means of plasmin generation and degradation of GPIb.

Our study suggests that platelet-dependent activation of plasminogen by TPA does not require GPIIb/IIIa. However, thrombasthenic platelets appear to be less susceptible to the action of plasmin on GPIb, since there was less inhibition of ristocetin-induced agglutination and less degradation of GPIb in these platelets at a moderate plasmin concentration (0.45 CU/mL). These findings imply that GPIIb/IIIa may be involved in the interaction of plasmin with platelets. Alternatively, thrombasthenic platelets may contain more GPIb than do control platelets, so that cleavage of this membrane glycoprotein by plasmin is incomplete. The residual GPIb would therefore be sufficient to allow platelet agglutination by ristocetin even in the presence of moderate levels of plasmin activity.

An additional finding in normal platelets is that a mol wt = 250,000 protein disappears in the presence of plasmin. In contrast, the 250,000-dalton band is not seen in thrombasthenic platelets even before exposure to plasmin. Furthermore, the 250,000-dalton protein was not detected by immunoblotting in platelets from four other thrombasthenic patients (data not shown). The nature of this protein and its role in platelet function are unknown. It represents either an additional protein that is decreased or missing in thrombasthenic platelets or a dimer of GP I Ib-IIIa.

Previous studies using rabbit platelets or human platelets have shown that plasmin levels of 0.9 CU/mL or more can directly cause platelet activation and aggregation. Our results demonstrate that TPA does not generate enough plasmin to cause direct platelet aggregation, in accord with the observation of Schafer and Adelman that plasmin concentrations of 0.5 CU/mL do not aggregate washed human platelets. However, at this plasmin concentration, partial platelet activation does seem to occur, as evidenced by release of 3H-serotonin. Our findings differ from those of Deguchi et al., who described the interaction of a platelet extract with Lys-plasminogen in the presence of trace amounts of TPA. In contrast, our study used intact platelets with physiologic amounts of Glu-plasminogen and pharmacologic concentrations of TPA. Thus, plasmin generation in moderate quantities appears to occur on the platelet surface in the presence of TPA.

A major question concerns the applicability of our findings to in vivo platelet function in the presence of TPA. We know of no previous study in which platelet function was examined during TPA administration. Adelman et al. showed that when platelets are incubated with streptokinase at concentrations producing as little as 0.05 CU/mL of plasmin, ristocetin-induced platelet agglutination is inhibited. Little data exists on in vivo correlates of plasmin activity, however. Our results using PRP suggest that plasmin generated by TPA levels achieved during thrombolytic therapy may interfere with platelet adhesion despite the presence of circulating plasmin inhibitors. However, we could show no effect on platelet aggregation in response to ADP, gamma thrombin, arachidonic acid, or calcium ionophore when PRP was substituted for washed platelets. Further studies in patients receiving TPA therapy are necessary to determine whether TPA interferes with platelet function by means of plasmin generation in vivo as well as in vitro.

In summary, we have shown that TPA interaction with plasminogen and washed platelets leads to plasmin generation, inhibition of platelet aggregation, and degradation of glycoproteins Ib and IIb/IIIa. Plasmin is generated on control and thrombasthenic platelets, but the effect of plasmin on thrombasthenic platelets appears to be decreased. The mechanism of TPA interaction with platelets and the risk of bleeding due to this interaction remain to be determined.

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