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Differential effects of low-dose tissue plasminogen activator and streptokinase on platelet aggregation

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Despite increasing success with low-dose intra-arterial thrombolysis, early rethrombosis still occurs. Platelet aggregation is thought to play a major part in this process. We have therefore investigated the effects of recombinant tissue plasminogen activator (rt-PA) and streptokinase on platelet function at doses currently used for peripheral arterial thrombolysis. Platelet-rich plasma was stirred at 37°C, with either streptokinase (100, 300 or 1000 units ml^{-1}) or rt-PA (10 (T10), 30 (T30) and 100 (T100) mg l^{-1}), with immediate addition of an agonist for platelet aggregation (thrombin, collagen, adenosine diphosphate (ADP) or adrenaline) at a predetermined threshold dose. Significant inhibition of collagen-induced and adrenaline-induced platelet aggregation was produced with rt-PA at all doses used ($P<0.05$). With adrenaline as the agonist, T100 produced disaggregation to a mean(s.d.) level of 26 (11) per cent. Thrombin-stimulated platelet aggregation was significantly reduced by T100 ($P<0.001$) and T30 ($P<0.01$) only, disaggregation being dose-dependent and complete with T100. Using ADP as the agonist, T100 produced a significant reduction in maximum platelet aggregation ($P<0.01$), and disaggregation was achieved to a mean(s.d.) level of 48(13) per cent. Streptokinase did not produce any significant changes in any parameter of aggregation.

Keywords: Platelet aggregation, fibrinolytic agents, thrombolysis

Recombinant tissue plasminogen activator (rt-PA) has a high fibrinolytic activity[1,2] associated with a short half-life of <5 min[3,4]. It is relatively fibrin specific, activates fibrin-bound plasminogen to form plasmin, and does not significantly activate circulating plasminogen[5,6]. Experience with this agent for the treatment of peripheral arterial thrombosis is still limited[7–10] compared with that of low-dose streptokinase[11–19]. However, rethrombosis remains a problem with both agents; exposed endothelial collagen at the site of the thrombus causing procoagulant effects on the thrombus surface itself[20], residual thrombus[21–23], and direct activation of platelets and coagulation factors[24–27] may all play an important part. In coronary thrombolysis, rethrombosis rates of up to 45 per cent have been reported[28,29]. Inadequate inflow and/or inadequate outflow cannot always be blamed and platelets are undoubtedly important in the pathogenesis of rethrombosis[30]. In coronary thrombolysis, the combination of streptokinase and aspirin has now been shown to be superior, in terms of the risk of mortality, to the use of either agent alone[31]. As yet there are no such similar studies in peripheral arterial thrombolysis. In peripheral limb arteries the volume and length of the occluding thrombus is many times greater than that in coronary thrombosis. The thrombi tend to be older and, therefore, more mature with greater cross-linking. Consequently thrombolysis takes longer. If there are significant differences in the effects of different thrombolytic agents on platelet function, this may be of considerable importance in determining the duration of lysis, the incidence of rethrombosis and the potential therapeutic benefit of concurrent administration of thrombolytic and antiplatelet agents.

We have therefore determined the effect of rt-PA and streptokinase on platelet aggregation. Doses of rt-PA and streptokinase used in the experiments were based on the equivalent maximal concentration of each agent used in this centre for peripheral arterial thrombolysis.

Method

Using no or minimal tourniquet time, 80 ml of blood were obtained from each of three healthy male volunteers. Blood was collected into 0·109 M trisodium citrate at a ratio of 9:1. All samples were then centrifuged at 500*g* for 10 min. The supernatant platelet-rich plasma was pipetted off and platelet counts performed on a Coulter S Plus IV cell counter (Coulter Electronics Ltd., Luton, UK). The samples were centrifuged again at 2000*g* for 10 min to obtain platelet poor plasma. The platelet-rich plasma was diluted with platelet-poor plasma to a platelet count of 250×10^9 ml^{-1}.

Threshold values for each of four agonists: thrombin (Diagnostic Reagents Ltd., Oxon, UK), collagen (Hormon-Chemie, Munich, GDR), adenosine diphosphate (ADP) and adrenaline (Sigma Chemical Company Ltd., Dorset, UK), were determined for each subject.

Plasma with a platelet count of 250×10^9/ml was stirred at 37°C with either saline or rt-PA (Boehringer Ingelheim, Bracknell, Berkshire, UK) at final concentrations of 10 (T10), 30 (T30) and 100 (T100) mg l^{-1}, with immediate addition of an agonist at the appropriate threshold value. The same protocol was used for streptokinase (Streptase, Hoechst UK Ltd., Hounslow, UK), which was added to achieve final concentrations of 100 (S100), 300 (S300) and 1000 (S1000) units ml^{-1}. A Chronolog 440 dual channel aggrometer (Coulter Electronics Ltd., Harpenden, UK) was used at 37°C with the chart recorders set at 0·5 mm s^{-1}. Aggregation was measured from the change in light absorbance, represented in millimetres on the chart recorder. The rate of aggregation was calculated as half maximal aggregation (in millimetres) divided by the time taken to achieve half maximal aggregation (in seconds). After the lowest optical density had been reached, disaggregation was measured as the maximal increase in optical density and expressed as a percentage of the lowest optical density reached. In the case of curves that were non-reversible, maximal aggregation was taken as the aggregation present at 3 min (ADP, thrombin) or 4 min (collagen, adrenaline) after starting the experiment. The response to threshold doses was measured before and after each set of rt-PA or streptokinase concentrations was tested, and all experiments were performed at least in triplicate on prepared plasma from each of the three volunteers. Results are expressed as mean(s.d.). All statistics were performed using the Mann–Whitney *U* test.

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Table 1 *Effects of recombinant tissue plasminogen activator at final concentrations of 10 (T10), 30 (T30) and 100 (T100) mg l^{-1} on platelet aggregation*

Agonist	Control	Final concentration of rt-PA (mg l^{-1})		
		T10	T30	T100
Collagen				
Rate of aggregation (mm s^{-1})	0·6(0·2)	0·5(0·1)	0·5(0·1)	0·3(0·1)*
Maximal aggregation	107·9(4·9)	100·7(9·3)*	101·1(6·9)*	68·7(17·2)†
Adrenaline				
Rate of aggregation (mm s^{-1})	0·7(0·2)	0·7(0·2)	0·6(0·2)	0·5(0·1)
Maximal aggregation	97·5(4·9)	73·1(21·5)*	58·0(26·7)*	39·2(8·5)†
Thrombin				
Rate of aggregation (mm s^{-1})	0·8(0·4)	1·0(0·2)	0·8(0·3)	0·5(0·2)*
Maximal aggregation	85·2(14·9)	86·4(20·2)	32·7(11·9)†	26·3(10·3)‡
ADP				
Rate of aggregation (mm s^{-1})	1·1(0·2)	1·0(0·3)	1·0(0·2)	0·9(0·2)*
Maximal aggregation	76·6(9·7)	75·4(5·8)	72·4(9·3)	60·4(6·5)†

Results are given as mean(s.d.); * $P<0·05$; † $P<0·01$; ‡ $P<0·001$; rt-PA, recombinant tissue plasminogen activator

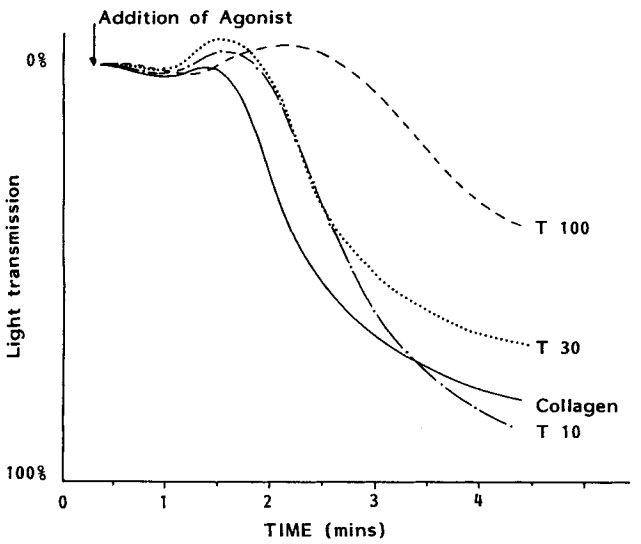

Figure 1 *Example of dose-dependent inhibition of platelet aggregation by recombinant tissue plasminogen activator following addition of collagen (1 mg l^{-1} final concentration)*

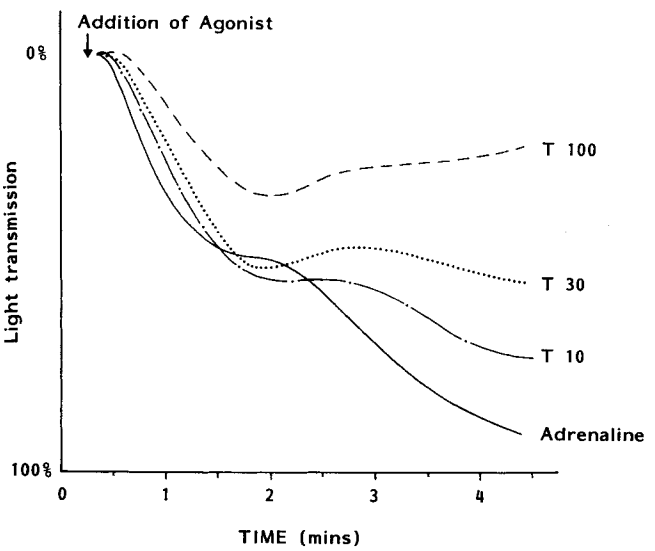

Figure 2 *Example of dose-dependent inhibition of platelet aggregation by recombinant tissue plasminogen activator (rt-PA) following addition of adrenaline (5 μmol l^{-1} final concentration). Marked disaggregation was also seen following addition of 100 mg l^{-1} rt-PA (T100)*

Results

Tissue plasminogen activator

The effects of rt-PA on platelet aggregation are described in *Table 1*.

Collagen. A single experimental example is shown in *Figure 1*. Overall, rt-PA produced significant inhibition of the maximum extent of platelet aggregation induced by collagen at all doses used ($P<0·05$ for T10 and T30, $P<0·01$ for T100). The rate of aggregation was also significantly reduced by T100 ($P<0·05$). No disaggregation occurred in either the presence or the absence of rt-PA.

Adrenaline. An experimental example is shown in *Figure 2*. Overall, adrenaline-induced platelet aggregation was significantly inhibited ($P<0·05$ for T10 and T30, $P<0·01$ for T100). At a final dose of 100 mg l^{-1}, rt-PA produced significant disaggregation to a mean level of 26(11) per cent ($P<0·01$).

Thrombin. An experimental example is shown in *Figure 3*. Overall, thrombin-stimulated platelet aggregation was significantly reduced by T100 ($P<0·01$) and T30 ($P<0·01$) only. The rate of aggregation was also significantly reduced by T100 ($P<0·05$). Disaggregation was dose-dependent with a mean level of 49(20) per cent with T30 ($P<0·001$) and 146(34) per cent with T100 ($P<0·001$).

Adenosine diphosphate. An experimental example is shown in *Figure 4*. Overall, T100 produced a significant reduction in the rate ($P<0·05$) and maximum platelet aggregation ($P<0·01$) following addition of ADP. Disaggregation was consistently achieved only with T100, with a mean level of 48(13) per cent ($P<0·001$).

Streptokinase

Streptokinase did not produce inhibition of either the rate of aggregation or the extent of aggregation, following the use of any of the four agonists in an analogous protocol.

Discussion

Our results show that rt-PA can cause significant inhibition of platelet aggregation *in vitro*. The doses used in this study are based on the theoretical concentration of rt-PA or streptokinase delivered to an occluding thrombus using our standard protocol for local low-dose intra-arterial thrombolysis (33 mg l^{-1} rt-PA; 333 units ml^{-1} streptokinase).

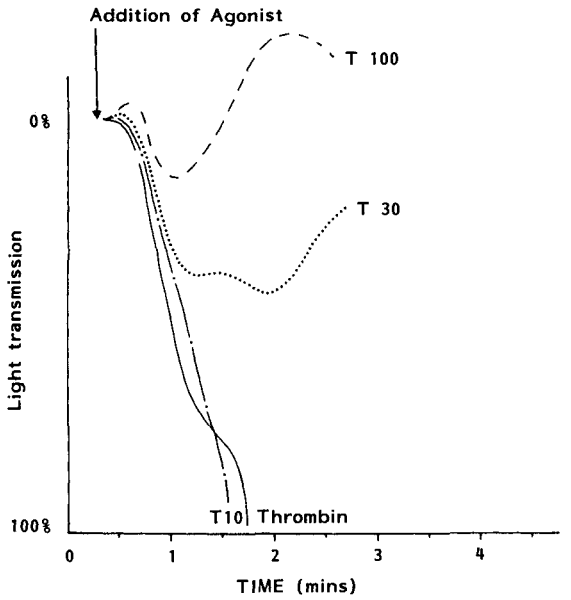

Figure 3 *Example of significant inhibition of platelet aggregation by 30 (T30) and 100 (T100) mg l^{-1} recombinant tissue plasminogen activator (rt-PA) following addition of thrombin (0·3 units final concentration). Disaggregation was seen following addition of 30 and 100 mg l^{-1} rt-PA*

A dose-dependent inhibition of collagen-induced platelet aggregation was produced by rt-PA, which was highly significant at the T100 concentration ($P<0·01$). The rate of aggregation was also significantly reduced with rt-PA at this concentration ($P<0·05$). No disaggregation was seen using the doses of rt-PA studied. In contrast, Loscalzo and Vaughan[32] found that collagen-induced platelet aggregation was resistant to rt-PA. This is because they were primarily studying the effects of rt-PA on disaggregation rather than inhibition of aggregation. In their protocol, rt-PA was added 1 min after the addition of agonist at a point where marked platelet aggregation had already occurred. Although our results demonstrate that rt-PA can significantly inhibit platelet aggregation induced by collagen, we agree that it cannot achieve disaggregation. The explanation of this phenomenon may be that platelets aggregate in response to collagen via fibrinogen-independent, as well as fibrinogen-dependent, mechanisms[33]. Platelets adhering to collagen release ADP, which promotes irreversible platelet aggregation.

Adrenaline-induced platelet aggregation was also significantly inhibited by all doses of rt-PA used. At the highest dose, disaggregation was seen to a mean level of 26(11) per cent.

Dose-dependent inhibition of platelet aggregation induced by thrombin was seen. rt-PA also produced dose-dependent disaggregation to a mean level of 146(34) per cent at the T100 concentration, suggesting that the final light transmission was actually lower than in the original sample. This may be due to disaggregation of previously formed platelet microaggregates, but this is only an hypothesis. These results verify the findings in earlier work[32]. The method of adding thrombin to platelet-rich plasma is not often used because of the difficulty in interpretation of the consequent increase in light transmission. This may be due to a combination of platelet aggregation and coagulation. However, using the same protocol with streptokinase, we did not see any significant effect on the rate or extent of aggregation using clinically equivalent doses, and so the effects seen can be attributed to rt-PA alone.

Only the highest concentration of rt-PA produced significant inhibition of platelet aggregation induced by ADP ($P<0·01$). Loscalzo and Vaughan[32] found that rt-PA produced disaggregation when added to a suspension of ADP-aggregated platelets. This effect was dependent on dose and time. The earlier the rt-PA was added, the faster the rate of disaggregation and the greater its extent. The effect was inhibited by incubation of the platelet-rich plasma with α_2-antiplasmin before adding ADP. They concluded that disaggregation was the result of the proteolytic effect of plasmin and not the result of the amidolytic effect of rt-PA itself. Aspirin potentiated the effect on the rate of disaggregation over a wide range of rt-PA concentrations. Some disaggregation was seen[28] at concentrations of rt-PA as low as 5 mg l^{-1}. Whether local concentrations of rt-PA secreted by the vascular endothelium reach these levels is not known. It is unclear why rt-PA does not have such marked effects in our own study. In contrast, using New Zealand White rabbits, Ohlstein *et al.*[34] demonstrated that rt-PA at doses of 10 and 30 $\mu g\ kg^{-1}\ min^{-1}$ produced *ex vivo* platelet hyperaggregability when collagen, arachidonic acid or ADP was used as the aggregating agent. These effects were apparently more pronounced at lower and subthreshold doses of collagen. Platelet counts were significantly decreased (by 16–22 per cent compared with control values). Streptokinase was also used in the same system: after a bolus dose of 40 000 units, a maintenance dose of 4000 units min^{-1} for 15 min was used. A similar platelet hyperaggregability was observed. The authors concluded that, following addition of rt-PA, platelet hyperaggregability was more common but of a shorter duration than following addition of streptokinase. The platelet hyperaggregation and thrombocytopenia observed in this study after systemic administration of rt-PA or streptokinase may be due to enhanced plasmin activity. Plasmin has a biphasic response, causing inhibition of platelet aggregation[35] and thromboxane B_2 production at low doses, but acting as a platelet agonist[36,37] at high doses. The doses used, 10 and 30 $\mu g^{-1}\ kg^{-1}\ min^{-1}$, are respectively equivalent to 42 and 126 mg h^{-1} for a 70 kg man. These are many orders of magnitude higher than those used in our own clinical study in humans and so direct comparison is not possible.

Although we have shown significant inhibition of platelet aggregation by rt-PA, we have shown neither inhibition nor enhancement of aggregation or of disaggregation by streptokinase. However, Fitzgerald *et al.*[25,38] have recently reported marked platelet activation following coronary thrombolysis with streptokinase. With additional *in vitro* tests they showed that the proaggregating effect was dose-dependent and seen over a range of 300–3000 units ml^{-1} with the maximum effect at 1883(440) units ml^{-1}. The mechanism of action is thought to involve plasminogen because, by passing citrated platelet-

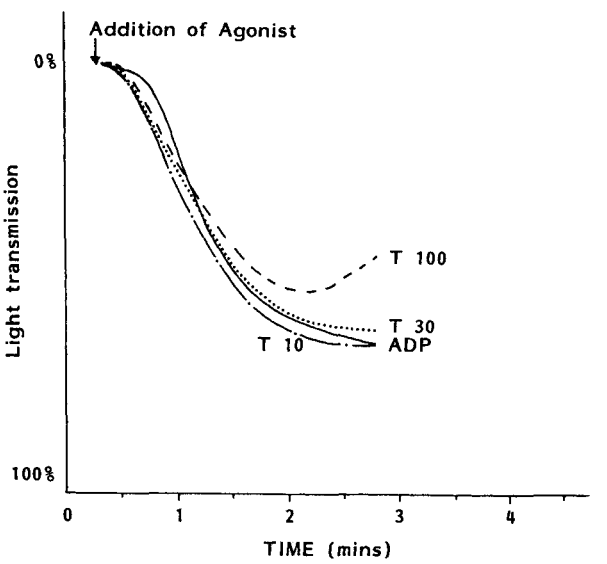

Figure 4 *Example showing that only 100 mg l^{-1} recombinant tissue plasminogen activator (T100) produced significant inhibition of maximal platelet aggregation and of the rate of aggregation following addition of adenosine diphosphate (2 μmol l^{-1} final concentration)*

rich plasma over a lysine–sepharose column to remove plasminogen, the proaggregatory effect of streptokinase (but not ADP) was prevented. The effect was subsequently restored by the addition of exogenous plasminogen. In contrast, after rt-PA therapy for myocardial infarction, platelet activity remains relatively unchanged[39]. Although our own study failed to demonstrate an enhancement of ADP-induced aggregation by streptokinase, we used a threshold dose of ADP, which was defined as the lowest concentration of ADP required to achieve progressive aggregation throughout the study period. In order to demonstrate similar enhancement of aggregation by streptokinase, we would need to repeat these experiments with lower doses of ADP to achieve minimal, reversible aggregation like Fitzgerald *et al.*[38]. It is also known that streptokinase can initiate specific antibody-mediated platelet aggregation *in vitro* and this may be associated with clot propagation and thromboembolism *in vivo*[40,41]. The extent of platelet aggregation induced by streptokinase correlates with the titre of antistreptococcal antibodies[42], which are present in up to 10 per cent of patients[43]. Antistreptococcal antibodies were not measured in our subjects; however, any potential augmentation of aggregation was not apparent at the doses of agonist used.

Various animal studies have also demonstrated that platelet activation does modify the response to streptokinase and other fibrinolytic agents[44,45]. Platelet aggregation is accompanied by platelet release reactions, which may have adverse physiological effects. The increase in biosynthesis of thromboxane A_2, a potent platelet agonist and vasoconstrictor[46], in Fitzgerald's study far exceeded the level in patients with unstable angina[47], in whom there is a beneficial effect of aspirin[48,49].

Various theories have been proposed to explain the platelet effects. Fibrinogenolysis will impair ADP-induced platelet aggregation as fibrinogen is a necessary cofactor[50]. Further impairment of aggregation and adhesion[51] is seen as a consequence of cleavage of glycoproteins Ib and IIb/IIIa (receptors for von Willebrand factor and fibrinogen respectively[52,53]), following activation of platelet-bound plasminogen[54]. Plasmin-induced cleavage of thrombospondin, fibronectin and fibrin can disrupt the matrix and result in platelet disaggregation[32]. This mechanism is enhanced by fibrinolytic agents, resulting in lysis of the fibrin framework that holds the platelets together.

The clinical importance of these enhanced platelet effects is uncertain. Groups of patients with thrombosis or at risk of thrombosis appear to have increased platelet adhesiveness[55], increased spontaneous aggregation[56], a reduced threshold to a range of agonists[57], and increased platelet release reactions[58]. However, although the mean values of all these parameters achieve significance for the groups as a whole, there is sufficient overlap with control populations to make their use in individual cases clinically unreliable. Nevertheless, an agent with platelet inhibitory rather than proaggregatory characteristics would appear highly desirable.

The concurrent use of an antiplatelet agent and a fibrinolytic agent may cause concern because of the potential bleeding complications. However, in the ISIS-2 study[31], although the combination of aspirin and streptokinase was associated with a 0·7 per cent increase in frequency of minor bleeds, no increase in major bleeds was noted and there was a 0·5 per cent reduction in the risk of stroke.

In our study rt-PA has been shown to have important dose-dependent inhibitory effects on all four platelet aggregation agonists assessed. Furthermore, using the same protocol with streptokinase, no significant inhibition of any parameter for any agonist is seen. These effects may help to explain the more rapid lysis seen with rt-PA in peripheral arterial thrombolysis compared with streptokinase[10], and may also help to reduce early rethrombosis. Concurrent therapy with antiplatelet agents may confer a greater effect with streptokinase than with rt-PA. Further *ex vivo* assessment of platelet behaviour using patients receiving peripheral arterial thrombolysis is required to substantiate these findings.

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