In Vitro Effect of Plasmin on Human Platelet Function in Plasma

Inhibition of Aggregation Caused by Fibrinogenolysis

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Background. Plasmin has been reported both to activate platelets and to inhibit platelet functions. The latter effect was thought to be caused by proteolysis of the main membrane glycoproteins.

Methods and Results. We found that incubation of citrated human platelet-rich plasma with streptokinase (SK) (300 IU/ml) does not induce any detectable activation but leads to a time-dependent inhibition of ADP-induced aggregation accompanied by substantial fibrinogenolysis. These effects were abrogated by previous addition of a plasmin inhibitor, aprotinin. Crossover experiments (SK-treated or control platelets mixed with SK-treated or control plasma) demonstrated that the platelets remained functional and that the aggregation defect was caused by fibrinogenolysis. Further experiments (addition of purified fibrinogen to fibrinogen-depleted plasma with either SK or thrombin) suggested that in addition to the low residual level of fibrinogen, fibrinogen degradation products had an inhibitory effect. Under the same conditions, tissue-type plasminogen activator (t-PA) (3,000 ng/ml) had no effect on platelet aggregation, and plasma fibrinogen was not significantly lowered. The effects on glycoproteins IIb-IIIa of incubation with SK, t-PA, or plasmin were assessed with immunoblotting with murine monoclonal antibodies directed against either part of the complex, which is the receptor for fibrinogen. Proteolysis was detected only in the presence of EDTA, a potent chelator of divalent cations.

Conclusions. The incubation of human platelets in citrated plasma with SK concentrations obtained during therapy leads to an aggregation defect that is related to the decrease in fibrinogen, the adhesive protein involved in this function, and to the impeding effect of fibrinogen degradation products on its binding onto platelets but not to an alteration of the corresponding platelet receptor, the heterodimer glycoproteins IIb-IIIa. (Circulation 1992;85:935–941)

Key Words • platelets • glycoproteins • plasmin • thrombolytic agents • plasminogen activators • streptokinase • monoclonal antibodies

There is renewed interest in the effects of thrombolytic agents on platelet functions.1 Indeed, it was suggested that, particularly in the setting of thrombolytic treatment of acute myocardial infarction, plasmin altered platelet function.2,3 Platelet effects of plasmin could be involved both in bleeding complications and in rethrombosis.1 One of the first reports on this topic, which appeared in 1973, indicated that plasmin could be a platelet activator.4 Thereafter, several studies were published that indicated both platelet activation and inhibition by plasmin in vitro.5-8 The reasons for these dual opposite effects remain obscure. Even the mechanism of the impairment of platelet aggregation remains a matter of debate. Furthermore, thrombolytic agents were studied over a wide range of concentrations, some of them apparently irrelevant to therapeutic conditions.2,5,9 Therefore, we decided to reinvestigate the in vitro effect of plasmin on human platelets, either added directly to a washed platelet suspension or generated from plasma plasminogen by the addition of streptokinase (SK) or tissue-type plasminogen activator (t-PA). These two thrombolytic agents are currently the most widely used for the treatment of acute myocardial infarction, and we selected concentrations that may be achieved therapeutically in vivo.10-14

Methods

Prostaglandin (PG) E1, albumin, and HEPES were from Sigma Chemical Co., d-glucose was from Prolabo, and human fibrinogen was from Kabi. The aggregating agents were ADP (Sigma), arachidonic acid (Sigma), collagen (Horm), and thrombin (Leo). In addition, the following platelet-activating monoclonal antibodies were used:15 AL B6, a gift from Claude Boucheix, directed at the CD9 antigen, and PL2-49, directed at the heavy chain of glycoprotein (GP) IIb (classified as CD 41).16 The thrombolytic agents were SK from
Hoechst (Activitase) and recombinant t-PA from Boehringer (Actilyse) conditioned for clinical use. Throughout the experiments described below, the thrombolytic agents SK and t-PA were used at pharmacological concentrations, 300 IU/ml and 3,000 ng/ml, respectively, and plasmin at 2 CU/ml (CU, caseinolytic unit). Aprotinin from Choay (Iniprol) was used according to Fitzgerald et al at a final concentration of 900 KIU/ml (KIU, kallikrein inhibitor unit) unless otherwise stated. We verified that aprotinin had no effect on the platelet aggregation.

Preparation of Human Platelet-Rich Plasma

All the experiments were performed with platelets prepared from blood obtained from healthy volunteers (blood bank of our hospital, Dr. Georges Andreu). Citrated platelet-rich plasma (cPRP) was prepared from venous blood anticoagulated with sodium citrate (11 × 10⁻³ mol/l) and adjusted to 300,000/µl. Platelet response to arachidonic acid was checked to ensure that the cyclo-oxygenase pathway was unaltered. These platelets were used for the initial functional studies and for the biochemical experiments using either SK or t-PA to generate plasmin.

Preparation of Washed Platelets

Platelets were washed essentially according to Mestard’s method, with PGE₁ (10⁻⁷ mol/l) added to the Tyrode’s buffer. Aspirin, a gift of Dr. Cazenave (Strasbourg, France), was used as described in Reference 19 at a concentration able to inhibit ADP-induced aggregation by about 90%. These platelets were resuspended at a concentration of 300,000/µl in Tyrode’s buffer with no PGE₁, a 10-fold lower concentration of aspirin than the one used during washing, calcium (2 × 10⁻³ mol/l), and magnesium (10⁻⁵ mol/l). These platelets were used for some functional tests and for studies on the biochemical effects of plasmin.

Crossover Experiments

Platelets as cPRP were incubated with SK for 15 minutes at 37°C in the presence of PGE₁ (10⁻⁷ mol/l) to prevent any platelet activation. After supplementation with citric acid, citrate, and dextrose (ACD) (6:1 vol:vol) and heparin (20 units/ml), platelets were washed essentially as described above, resuspended at a concentration of 600,000/µl, and kept at 37°C for at least 30 minutes before testing. The autologous citrated plasma was incubated at 37°C for 45 minutes with SK, after 45 minutes, aprotinin (3,000 KIU/ml) was added. Control platelets and control plasma were incubated with saline instead of SK. For the crossover experiments, the platelet suspension supplemented with citrate was diluted with an equal volume of autologous plasma (see Figure 1).

In further experiments, the effect of SK-treated plasma on washed platelets was compared with that of fibrinogen-depleted plasma with thrombin so that fibrinogen degradation products (FDPs) were not formed (see Figure 2).

Platelet Aggregation

Aggregation was monitored with 300-µl samples using a turbidimetric aggregometer connected with a computer able to calculate the area under the curve during the first 4 minutes. Fibrinogen levels were measured by the Clauss method.

Biochemical Methods

The cPRP was incubated with SK or t-PA in the presence or absence of a calcium and magnesium chelator (EDTA). The washed platelets were also incubated with plasmin for 15 minutes in the presence of calcium (2 × 10⁻³ mol/l). Immunobots were performed as described in the legend of Figure 3 with two murine monoclonal antibodies, MB9 and Y2-51 (a kind gift of Dr. Cordell, Oxford, UK). These two antibodies were classified during the course of the fourth workshop on leukocyte differentiation antigens as CD41 and CD61, respectively.

Results

Effects of Streptokinase on Platelet Aggregation

ADP was used at the lowest concentration able to induce an irreversible aggregation with a double-wave aspect. Twenty-one samples were studied, 13 with ADP at a concentration of 2.5 × 10⁻⁶ mol/l and eight with ADP at a concentration of 5 × 10⁻⁴ mol/l. Platelet aggregation in response to ADP was inhibited after preincubation of cPRP with SK (300 IU/ml) for 15 minutes at 37°C (preliminary time course experiments had shown that the maximal inhibition was achieved at this time
point). Both the extent and the pattern of the inhibition induced by the incubation with SK were quite variable among controls, consisting of a loss of the double wave (upper panel, Figure 4) or an overall decrease in every parameter with persistence of a similar aspect of the curve (lower panel, Figure 4). Nevertheless, the velocity was always affected, being reduced to an average of 65% of the control values. The area under the curve during the first 4 minutes after stimulation declined to 40%, on average, of the control values (Table 1); this difference is statistically significant ($p<0.001$, Wilcoxon’s test).

The inhibition of ADP-induced aggregation was accompanied by a decrease in the concentration of clottable fibrinogen, which is an indirect index of the activation of the fibrinolytic system. There was a decrease of about 90% in fibrinogen concentration after incubation of cPRP with SK for 15 minutes (Table 1) ($p<0.001$, Wilcoxon’s test). The mean residual concentration of fibrinogen was 0.27 g/l. This effect of SK was mediated by plasmin generation, because the effects of SK were inhibited by previous addition of aprotinin. We failed to observe any potentiating effect of a short incubation (1 minute) with SK (300 IU/ml) on ADP ($2.5 \times 10^{-6}$ mol/l)–induced aggregation (data not shown).

**Effects of Tissue-Type Plasminogen Activator on Platelet Aggregation**

Under the same conditions (incubation for 15 minutes at 37°C), t-PA (3,000 ng/ml) had no significant effect on platelet aggregation. Plasma fibrinogen was not significantly changed, suggesting poor plasmin generation. Plasmin generation occurred with a high concentration of t-PA (>5,000 ng/ml for 15 minutes), when slight alterations of platelet aggregation could be seen (data not shown), or during a prolonged incubation (60 minutes with 3,000 ng/ml).

**Platelet Aggregation: Crossover Experiments**

The sensitivity to various stimuli of platelets preincubated or not preincubated with SK in citrated plasma
Graphs show two representative examples of the inhibition of ADP-induced aggregation after incubation of citrated platelet-rich plasma (cPRP) with streptokinase (SK). Panels A: Control cPRP. Panels B: cPRP incubated with SK (300 IU/ml) for 15 minutes at 37°C. AUC, area under the curve during the first 4 minutes after addition of ADP (2.5 \times 10^{-6} \text{ mol/l}); Max. Veloc., maximal velocity. Horizontal axis, time scale (minutes); vertical axis, changes in light transmission (arbitrary units, from 0% to 100%, corresponding to unstimulated PRP and plasma, respectively).

and then washed as described above was first tested in the resuspending Tyrode's buffer. There was no difference between the responses of SK-treated and control platelets to thrombin (1 unit/ml), collagen (2.5 and 10 \mu g/ml), arachidonic acid (10^{-4} \text{ mol/l}), and two activating monoclonal antibodies (7.5 \mu g/ml) directed at different proteins (the low-molecular-weight CD9 antigen and GP IIb) and the responses to ADP (from 1.25 to 10 \times 10^{-6} \text{ mol/l}) in the presence of exogenous fibrinogen (from 0.2 to 0.9 g/l) (data not shown). Then these platelet suspensions were mixed with different plasmas. The responses to ADP of SK-treated platelets diluted with control plasma were not affected. By contrast, the addition of SK-treated plasma to control platelets reproduced the defect of the mixture of SK-treated platelets and SK-treated plasma and that of SK-treated cPRP described in the first part of the “Results” as well (Figure 1 and Table 2).

Further experiments were carried out with mixtures of washed platelets and plasmas depleted in fibrinogen incubated with SK or with thrombin and eventually supplemented with various amounts of exogenous fibrinogen. As shown in Figure 2, there was a relation between the concentration of fibrinogen and aggregation as assessed by the area under the curve. It also appears that with SK-treated plasma, the platelet response could not always be restored to control values (fibrinogen increased up to 2 g/l), whereas with fibrinogen-depleted plasma after thrombin treatment, control values were reached with a slight supplementation with fibrinogen (at most 0.5 g/l). On average, 50% of the control response is achieved with 0.25 and 0.10 g/l, respectively.

**Biochemical Studies**

Figure 3 shows the results of immunoblotting of GP IIb and IIIa after incubation of cPRP for 60 minutes with t-PA (3,000 ng/ml) in the presence or absence of EDTA. With control platelets, a major peptide of apparent molecular weight of 98 kd under nonreducing conditions corresponding to GP IIIa and a major peptide of apparent molecular weight of 125 kd under reducing conditions corresponding to GP IIb\alpha were detected with the corresponding specific monoclonal antibodies. There was no change after incubation of
platelets with t-PA in the presence of calcium. After incubation with t-PA in the presence of EDTA, fragments resulting from the proteolysis of GP IIbα and IIIa were found. With the antibody against GP IIIa, the band of apparent molecular weight of 98 kd decreased in intensity, and several bands of lower apparent molecular weights (90–75 kd) appeared. Furthermore, two bands of apparent molecular weights greater than 98 kd were revealed (108 and 116 kd). With the antibody against GP IIbα, the band of apparent molecular weight of 125 kd decreased in intensity, and several bands of lower molecular weights appeared (105–49 kd). These effects of t-PA on platelet surface GPs were abolished by a previous addition of aprotinin (not shown).

Similar results were obtained with SK-treated cPRP and with plasmin-treated washed platelets in the presence of 2×10⁻³ mol/l calcium (not shown).

**Discussion**

The outcome of the interaction between plasmin and platelets depends on several complex phenomena: 1) the kinetics of the generation of plasmin when thrombolytic agents are studied, the concentrations that are achieved, and the effects of the physiological inhibitors of plasmin (mainly α₂-antiplasmin); 2) the effects of plasmin on platelets: both activation (leading to aggregation) and proteolysis of the membrane GPs, which are the receptors for adhesive proteins (GP IIb and GP IIb-IIIa), have been reported; and 3) the effects of plasmin on the adhesive proteins present in plasma (von Willebrand factor and fibrinogen) and/or bound to their specific platelet receptors. In this study, we attempted to dissect some of these reactions under well-defined conditions.

To study the effects of thrombolytic agents on platelet functions, we chose the highest concentrations reportedly reached in vivo. Martin showed that the infusion of 100,000 IU of SK per hour gave a plasminatic concentration of 10 IU/ml. Therefore, we assumed that in the setting of acute myocardial infarction, the infusion of 1.5 million IU of SK over 30–90 minutes would yield concentrations of 300 IU/ml at most. Very recently, Martin and Fiebach have infused, in different patients with chronic arterial thrombosis, the same dose of SK as in acute myocardial infarction but over 3–6 hours. After 1 hour of infusion, the plasma SK concentration was found to be about 300 IU/ml, which is in very good agreement with our assumption.

After a 15-minute incubation with SK (300 IU/ml), we observed both inhibition of ADP-induced aggregation and substantial fibrinogenolysis. These effects were inhibited by previous addition of aprotinin and thus were related to plasmin generation. Approximately 0.5–1.0 CU/ml of plasmin was anticipated to be generated, and the recently published results of Winters et al. who measured plasmin with an amidolytic assay under similar experimental conditions, are in agreement with these calculations. Adelman and coworkers also observed an inhibition of ADP-induced aggregation, but they used an extremely high concentration of SK (50,000 IU/ml).

The next step was to localize the responsible biochemical lesion. The binding of fibrinogen to its platelet receptor (GP IIb-IIIa) is the basic mechanism of aggregation; therefore, the aggregation defect could result from proteolysis of either the platelet GPs or the ligand present in plasma. Thus, we performed crossover experiments in which platelets exposed to plasmin (generated in plasma incubated with SK) were resuspended with control autologous plasma and vice versa. Our results suggest that the changes in the plasma composition were the predominant cause of impaired platelet function: aggregation of control platelets mixed with SK-treated plasma was identical to that of SK-treated platelets mixed with SK-treated plasma; by contrast, aggregation of SK-treated platelets mixed with control plasma was virtually unaffected.

The decrease in fibrinogen concentration and the increase in plasma levels of FDPs in SK-treated plasma were the two potential causes of the functional impairment of the platelets. The next issue was to evaluate the respective roles of these two parameters under our experimental conditions. For this purpose, we added exogenous fibrinogen after blockade of plasmin, and we obtained only a partial restoration of aggregation (Figure 2). We also prepared plasma depleted in fibrinogen by clotting, thus without generation of FDPs. By contrast, defective aggregation of washed platelets resuspended in such a plasma could be easily corrected with minimal amounts of fibrinogen. These latter results are in agreement with data obtained with PRP prepared from patients with constitutional fibrinogenemia. There are two possible explanations for the inhibiting effect of FDPs on aggregation: competition for the fibrinogen receptor by fragments unable to support aggregation or binding of fragments to fibrinogen, leading to impairment of its binding to platelet surface.

There was a striking variability among donors concerning the aggregation defect induced by SK that does not appear to be related to the extent of fibrinogenolysis. This variability may be caused by various complex patterns of FDPs comprising some fragments that bind to activated platelets and still support aggregation, some others that bind to platelets but do not support aggregation, and others that have lost their ability to bind to platelets. This is the subject of ongoing research.

In our experiments, t-PA, even at high concentrations, had no effect either on fibrinogen concentration or on platelet function unless incubation is continued for a sufficiently long time (more than 45 minutes). This is not surprising, because fibrin is crucial for effective activation of plasminogen by t-PA, but the platelet surface was also suggested to provide favorable conditions for plasmin generation by t-PA.

These data concerning the effect of thrombolytic agents on platelet aggregation are consistent with our biochemical results. No changes in GP IIbα and IIIa after the action of SK, t-PA, or plasmin were discernible by means of immunoblots, provided that calcium was present. In contrast, under conditions of near total calcium depletion (in the presence of EDTA), fragments of apparent lower molecular weights resulting from the proteolysis of GP IIbα and IIIa were found. Two additional peptides of apparent molecular weights higher than that of GP IIIa were also revealed with an anti–GP IIIa antibody. As suggested by Beer and Colier, the upward shift in mobility of a GP IIIa–like immunoreactive peptide compared with the parent compound may correspond to the first stages of prote-
olysis without loss of large peptidic fragments and to a conformational change of GP IIa (cleavage within a disulfide-bonded loop). It is well known that incubation of cPRP or a platelet suspension at 37°C in the presence of EDTA leads to dissociation of the GP IIb-IIIa complex.24-26 This dissociation could expose the sites vulnerable to proteolysis by plasmin. During the preparation of this article and after the preliminary release of our data,25 we became aware that another team reached a similar conclusion using two methods: binding of monoclonal antibodies to whole platelets as assessed with flow cytometry and surface labeling of platelet GPs.24 These results rule out the possibility that plasmin in the presence of calcium generates proteolytic fragments that have lost the epitopes for the monoclonal antibodies and thus become no longer detectable.

In summary, we have shown that 1) in the presence of plasma, the main effect of plasmin (generated in the presence of SK) is impairment of aggregation; 2) this effect results from both the degradation of the plasmatic pool of fibrinogen, the main adhesive protein involved in this process, and the inhibiting effects of FDPs; and 3) by contrast, the platelet receptor for fibrinogen (the GP IIb-IIIa complex) is unaltered by plasmin if the experiments are carried out in the presence of calcium.

In contrast to the briefly mentioned data of Fitzgerald et al25 but in agreement with the recently published results of Winters et al,24 we failed to show any activating effect of plasmin on platelets in the presence of citrated plasma. On the other hand, SK (5,000 IU/ml) may induce platelet aggregation when high concentrations of SK-neutralizing antibodies are present.36-37 A possibility we did not encounter with a lower concentration of SK and that was not specifically addressed in our present study.

As recently pointed out,38-40 impairment of primary hemostasis may be one important mechanism of bleeding under thrombolytic therapy. Our data suggest that in contrast to the suggestion of Sane et al,39 the replacement of fibrinogen after blockade of plasmin activity is a more logical theory than platelet infusions.

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