Plasmin Effect on Platelet Glycoprotein Ib–von Willebrand Factor Interactions

By Burt Adelman, Alan D. Michelson, Joseph Loscalzo, Joshua Greenberg, and Robert I. Handin

We have studied the effect of streptokinase on platelets in platelet-rich plasma (PRP) and of plasmin on washed platelets. By three and one-half minutes after the addition of 50,000 IU/mL streptokinase to PRP, the maximum rate of ristocetin-induced platelet agglutination declined 40%, and by 60 minutes, it declined 70%. During the same time interval, the thrombin time increased from 20 seconds to over 120 seconds. At a concentration as low as 50 IU/mL, streptokinase reduced the maximum rate of ristocetin-induced platelet agglutination by 50% and prolonged the thrombin time to 1.5 times control value. Streptokinase added to PRP also caused inhibition of platelet aggregation following stimulation by 2.9 µmol/L adenosine diphosphate, 0.25 µ/mL thrombin, and 0.025 mg/mL collagen. Plasmin, 0.05 to 1.0 CU/mL, reduced ristocetin-mediated agglutination of washed platelets in the presence of von Willebrand factor (vWF) from 66% of control to 2% of control, following a one-hour incubation. Autoradiograms produced following sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) of plasmin-treated 125I-surface-labeled platelets demonstrated progressive loss of a protein with a molecular weight (mol wt) of 180,000; simultaneously, a protein with mol wt 135,000 appeared on autoradiograms produced following SDS-PAGE of the surrounding platelet medium. These proteins are similar in molecular weight to glycoprotein (gp) Ib, a platelet surface receptor for vWF, and glycocalcin, a proteolytic fragment of gpIb. By use of an enzyme-linked immunosorbent assay (ELISA) based immunoinhibition assay for glycocalcin, we were able to demonstrate that plasmin treatment of washed platelets released a glycocalcin-related antigen into the surrounding medium and that appearance of this material corresponded to loss of vWF-dependent, ristocetin-induced agglutination.

PLASMIN is a potent serine protease1 that can degrade a number of coagulation proteins in addition to fibrin, which is its primary substrate. Under normal circumstances, alpha-2-antiplasmin should immediately inhibit any plasmin that might diffuse from a localized site of fibrinolysis.5-4 However, during systemic fibrinolytic therapy, the alpha-2-antiplasmin level declines and allows uninhibited, bio logically active plasmin to circulate.5,6 Under these circumstances, plasmin may proteolyze susceptible proteins in plasma or on the surface of circulating cells. Atichartakarn et al demonstrated in vitro that plasmin rapidly destroys the coagulant activity of factor VIII but spares von Willebrand factor (vWF) activity.7 In a similarly designed study, Henriksson and Nilsson7 showed that plasmin destroys the activity of coagulant factors, V, VIII, and IX, and, in contrast to what

Atichartakarn et al found, there was rapid loss of vWF activity.

Platelet adhesion to the subendothelium after vascular injury is a crucial event in primary hemostasis8 and in the initiation of venous and arterial thrombosis. Current evidence suggests that this process is, in part, dependent on the interaction of vWF with the platelet membrane protein, glycoprotein Ib (gpIb).10-14 Studies of patients with the Bernard-Soulier syndrome support this theory. These patients have a hemorrhagic disorder that is characterized by a reduced capacity of platelets to adhere to subendothelium,10,15 and they lack gpIb on their platelets.15-17 In most instances, Bernard-Soulier platelets do not respond to or bind vWF and have a reduced number of thrombin binding sites.18,19

Glycoprotein Ib, which contains receptor sites for vWF, is readily degraded by a number of proteases,20-25 all of which release large fragments of gpIb into the surrounding medium.21,22,25 In general, these fragments are closely related to glycocalcin, which Okumura et al24,25 and Clemetson et al26 have characterized as being a glycoprotein of 150,000 molecular weight (mol wt), derived from gpIb. There is increasing evidence that glycocalcin contains the vWF binding site present on intact gpIb.27 Furthermore, proteolytic cleavage of glycocalcin from the platelet surface correlates with loss of vWF binding to the platelet.21,23-26,28 Of further interest is a report by Coller et al29 that states that glycocalcin is present in plasma and increases in concentration during conditions associated with activation of the coagulation system. The nature of the platelet defect caused by loss of gpIb has led us to
postulate that its potential release from the platelet surface during fibrinolytic therapy would have important therapeutic consequences.

Data already exist to suggest that plasmin affects platelet function. Plasmin treatment of human platelets results in decreased aggregation after stimulation by thrombin, collagen, and adenosine diphosphate (ADP). Ex vivo plasmin treatment of rabbit platelets reduces their half-life when reinfused and changes the electrophoretic pattern of their surface glycoproteins. However, there have been no studies that directly address the effect of plasmin on human platelet gpIb-IIIa interactions.

In this article, we demonstrate that both streptokinase activation of plasmin in platelet-rich plasma (PRP) and the incubation of platelets with purified plasmin can proteolyze gpIb, thereby diminishing vWF-mediated platelet agglutination. Our results suggest that activation of the fibrinolytic system may have an important effect on platelet function.

MATERIALS AND METHODS

The water used was deionized and filtered. All chemicals were reagent grade. NaCl was purchased from New England Nuclear, Boston; ristocetin from Bio-Data Corp, Horsham, Pa; thrombin from Parke-Davis, Detroit; collagen from Hormon-Chemie, Munich; and streptokinase from Pharmacia Laboratories, Piscataway, NJ. Plasmin (KABI through Helena Laboratories, Beaumont, Tex), 1 CU/40 μL, was dissolved in 10 mmol/L Tris-0.15 mol/L NaCl, pH 7.4, and stored in aliquots at –40 °C. Caseinolytic units (CU) were defined according to Sguoiris et al; 1 CU equals 1.14 CTA units (Committee on Thrombolytic Agents). Eight percent SDS-PAGE of this plasmin demonstrated two bands of nearly identical molecular weight. The chromogenic substrate S-2228 was obtained from Sigma Chemical Corp, St Louis. Purified human von Willebrand factor was produced by the method of Kao et al, with the following modifications: the starting material was cryoprecipitate rather than factor VIII concentrate, the final gel filtration step utilized Sephacryl S-1000 (Pharmacia Fine Chemicals, Uppsala, Sweden), and purity of the fractions utilized was determined by both reduced SDS-PAGE and by the ability to support ristocetin-dependent agglutination for formaldehyde-treated platelets. Platelet glycopcalcin was purified by the method of Solum et al; however, the material eluted from the wheat germ lectin column was further chromatographed on a Sephadex 6B-CL column prior to final concentration. Glycopcalcin molarity was calculated from its molecular weight and protein concentration. It was assumed that it contained 60% carbohydrate by weight.

Washed Human Platelets

Venous blood was drawn into 3.8% sodium citrate (wt/wt). Nine parts blood were added to one part anticoagulant. After mixing, the blood was centrifuged at 200 g for ten minutes at room temperature and the upper two thirds of the PRP collected. An equal volume of washing buffer (10 mmol/L Tris, 0.15 mol/L NaCl, 20 mmol/L EDTA, pH 7.4) was added to the PRP, and this mixture was centrifuged at 3,200 g for 15 minutes at room temperature. The platelet pellet was resuspended in 10 mL of washing buffer and washed an additional four times. The platelets were finally suspended in the same buffer at a concentration of 4 × 10^9/μL.

Formaldehyde-Treated Platelets

Formaldehyde-treated platelets were prepared by mixing an equal volume of TBS (10 mmol/L Tris, 0.015 mol/L NaCl, pH 7.4) and 2% paraformaldehyde with PRP and incubating for one hour at 37 °C. The platelets were either immediately washed or stored at 4 °C for up to 16 hours, after which time they were washed twice with washing buffer and twice with TBS. The platelets were resuspended at a concentration of 4 × 10^9/μL and used immediately or stored for up to two weeks at 4 °C.

Effect of Streptokinase on Ristocetin-Induced, von Willebrand Factor-Dependent Agglutination of Platelets in PRP

Streptokinase, which was dissolved in TBS, was added to PRP and the mixture incubated at room temperature. Control PRP was handled in a similar fashion, but streptokinase was not added. At the indicated times, 400-μL samples of PRP were removed from the incubation mixture and placed in aggregometer cuvettes. Agglutination was initiated by adding 50 μL of a 10 mg/mL solution of ristocetin to each PRP sample while the sample was stirred at 37 °C in a Pytson Dual Channel Aggregometer (Buffalo). The slope of the steepest segment of the reaction curve was determined. Aggregation studies were carried out with thrombin, 0.25 U/mL, ADP, 2.9 μmol/L, and collagen, 0.025 mg/mL.

Effect of Plasmin on Ristocetin-Induced, von Willebrand Factor-Dependent Agglutination of Washed Platelets

Washed platelets, 4 × 10^9/μL in washing buffer, were incubated with various concentrations of plasmin at room temperature. At various time intervals, 200-μL aliquots of the incubation mixture were placed in aggregometer cuvettes containing 200 μL washing buffer and combined with aprotinin and vWF. Agglutination was initiated by the addition of ristocetin. 1.25 mg/mL. In this and all other studies, the amount of vWF added was sufficient to support agglutination at a rate equivalent to that produced by 100 μL of pooled human plasma. The amount of aprotinin added was always in excess of the amount needed to totally inhibit plasmin activity in the S-2228-based assay (see below).

Plasmin Digestion of 125I-Labeled Platelets

The surface proteins of intact platelets were labeled with 125I using the lactoperoxidase technique developed by Phillips. The labeled platelets, 9 × 10^7/μL, were suspended in TBS containing 4 mmol/L EDTA, pH 7.4. Before the platelets were digested with plasmin, an aliquot was added to buffer containing vWF; agglutination was then measured in the aggregometer after 50 μL of 10 mg/mL ristocetin was added. The labeled platelets were divided into control and experimental aliquots, and plasmin, 2.5 CU/μL, was added to the experimental aliquot. At one minute, 15 minutes, and 30 minutes, samples from the control and experimental incubations were removed, aprotinin added, and the platelets pelleted by centrifugation at 8,700 g in a Beckman Microfuge B (Beckman, Palo Alto, Calif). The platelets and supernatants were separated, and the platelets washed three times in TBS containing 4 mmol/L EDTA. For each time interval, samples of platelets and supernatant from the control and plasmin-treated platelets were subjected to SDS-PAGE according to the method of Laemmli. Six percent polyacrylamide slab gels were used. To ensure comparability, the samples applied to the gels
contained identical counts per minute. Autoradiograms of the dried gels were made by exposing x-ray film (Kodak, Rochester, NY) to the gels at −20 °C. A Dupont Cronex intensifying screen was used (Du Pont, Wilmington, Del) to enhance development of the autoradiograms. To make sure that the labeled platelets had been affected by plasmin, 200-μL samples of treated and control platelets were suspended in 200 μL of buffer that contained vWF and were subjected to ristocetin-induced agglutination as described above. The maximum rate of agglutination of the plasmin-treated platelets declined by 13% at one minute, 57% at 15 minutes, and 60% at 30 minutes of value.

**Detection of Glycocalcin-Related Antigen in the Supernatant of Plasmin-Treated Washed Platelets**

Washed platelets, 1.2 × 10^7/μL, in TBS containing 20 mmol/L EDTA, pH 7.4, were subjected to plasmin digestion as described above. At ten minutes, 30 minutes, and 90 minutes, individual aliquots of plasmin-treated and control platelets were removed and combined with aprotinin. As described above, the platelets were centrifuged at 8,000 g and the supernatants removed; the platelets were resuspended and tested for agglutination in the presence of vWF and ristocetin.

The supernatants from the plasmin and control treatment groups were analyzed for the presence of glycocalcin-related antigen using an enzyme-linked immunosorbent assay (ELISA) based inhibition assay. The concentration of added glycocalcin was determined by its ability to inhibit the binding to platelets of a mouse monoclonal antibody, 6D1, which is directed against platelet gplB. This antibody was generously provided by Dr Barry Coller, SUNY, Stony Brook, NY. In this assay, the platelets were fixed to microwell plates. One hundred microliters of washed platelets, 1 × 10^5/μL in TBS containing 4 mmol/L EDTA, pH 7.4, were added to individual microwells of Immulon 1 flat-bottomed polystyrene plates (Dynatech, Alexandria, Va) that had been previously coated with poly-L-lysine (1.0 μg/mL). The plates were then centrifuged at 800 g for five minutes. Without removing the buffer, 50 μL of 0.5 formaldehyde (wt/vol) in TBS with 4 mmol/L EDTA, pH 7.4, was added to each microwell; the plates were then incubated at room temperature for 15 minutes and washed twice with TBS. Each microwell was covered by a confluent monolayer of platelets that showed negligible leukocyte or red cell contamination by phase-contrast microscopy. Experiments with 125I-labeled platelets demonstrated that platelet fixation to the microwells was reproducible and that the platelets remained adherent throughout the assay.

The assay was performed in the following fashion: a previously prepared microwell plate was thawed and washed three times with 10 mmol/L Tris, Tween-20, pH 8.0 (washing buffer supplied by New England Nuclear). Each microwell was filled with bovine gamma-globulin solution (5 mg/100 mL in TBS) and incubated for 30 minutes at 37 °C. After three washes with washing buffer, 25 μL of varying known concentrations of glycocalcin in TBS or an unknown sample and 25 μL of a 1:5,000 or 1:10,000 dilution of anti-gplB antibody (6D1) was added to each well. After incubation for 15 minutes, 30 minutes and three washes with washing buffer, 50 μL of a 1:250 dilution of a sheep anti-mouse F(ab')2 fragment conjugated with horseradish peroxidase (New England Nuclear) was added to each microwell. After incubation for 60 minutes at 37 °C and six washes with washing buffer, 50 μL of a 1:1:18 (vol/vol/vol) mixture of 4% o-phenylenediamine, 0.3% H2O2, and 17 mmol/L citric acid in 65 mmol/L phosphate, pH 6.3, was added to each well. After incubation for 30 minutes at 37 °C, the color development was stopped by the addition of 50 μL of 4.5 mol/L H2SO4. The optical density (OD) was read at 492 nm (Bio-Tek EIA reader, Bio-Tek Instruments, Burlington, Vt) and expressed as a percentage of the OD in assays with 6D1 without glycocalcin (100% binding). The OD of assays with neither 6D1 or glycocalcin defined 0% binding. The concentration of glycocalcin-related antigen in unknown samples was determined by comparison with a standard curve prepared by assaying known amounts of glycocalcin at concentrations of 0 to 100 nmol/L. The standard curve was analyzed by log-logit technique.

**Thrombin Time**

Thrombin time determinations were performed on citrated platelet-poor plasma (PPP) kept at 37 °C. Thrombin was diluted in TBS, pH 7.4, to a concentration of approximately 5 U/mL and warmed to 37 °C. At zero time, 200 μL of thrombin solution was added to 200 μL of plasma and gently rocked until a clot was formed. The length of time for this to occur was recorded. The concentration of thrombin in the buffer solution was adjusted so that the control thrombin time was between 18 and 24 seconds.

**Plasmin Activity**

Plasmin activity was determined using the chromogenic substrate S-2288, following the manufacturer's method. The assay was performed at 37 °C in a spectrophotometer equipped with a recording chart (Giford 2400, Giford, Oberlin, Ohio). Kinetic data supplied by the manufacturer indicate that for a substrate concentration of 10^-3 mol/L and a plasmin concentration of 4 × 10^-4 mol/L, change in absorbence per minute (ΔA/min) at 405 nm is 0.042. One caseinolytic unit plasmin equals approximately 0.45 nmol plasmin.

**Protein Determination**

All protein determinations were by the method of Lowry.

**RESULTS**

The Effect of Streptokinase on Platelets in Platelet-Rich Plasma

To study the effects that activation of fibrinolysis may have on the platelet, we added streptokinase to PRP and measured platelet function. The addition of 50,000 IU/mL streptokinase to PRP resulted in rapid loss of ristocetin-induced agglutination (Fig 1). By three and one-half minutes, the maximum rate of agglutination declined to 60% of the control value, and by 60 minutes, it declined to 30% of the control value.

![Fig 1. Effect of streptokinase on ristocetin-induced platelet agglutination in PRP. Streptokinase, 50,000 IU/mL, was added to PRP: at various time points during incubation at room temperature, aliquots of PRP were mixed with ristocetin and the maximum rate of agglutination determined. Each point represents the mean of two determinations.](image-url)
The same dose of streptokinase also inhibited ADP-, thrombin-, and collagen-induced platelet aggregation. ADP-induced platelet aggregation declined to 24% of the control value at ten minutes, 20% at 30 minutes, and 16% at 60 minutes. The effect on thrombin- and collagen-induced aggregation was less marked: at 60 minutes, the maximum rate of aggregation declined to 64% of control with thrombin and to 69% of control value with collagen.

Inhibition of agglutination by ristocetin was accompanied by a parallel increase in the thrombin time, which is an index of fibrinogen proteolysis. Thrombin times were performed on PPP samples after 50,000 IU/mL streptokinase was added. The thrombin time increased by two times control at three minutes, 2.75 times at 12 minutes, and by greater than eight times at 60 minutes.

We added 50,000 IU/mL streptokinase to PRP in our initial studies to ensure total and rapid plasmin generation. In subsequent studies, we examined the effect of lower doses of streptokinase on ristocetin-induced platelet agglutination and thrombin time (Table 1). Following a 60-minute incubation period, 50 IU/mL streptokinase caused a 50% reduction in the maximum rate of ristocetin-induced platelet agglutination and prolonged the thrombin time to 1.5 times the control (Table 1). These data indicate that low concentrations of streptokinase could activate enough plasmin to affect platelet function.

**Plasmin Effect on Washed Platelets and Purified von Willebrand Factor**

Because plasmin activation is the primary effect of streptokinase in human plasma, we next sought to demonstrate that plasmin treatment of washed platelets and/or vWF could directly affect vWF-ristocetin-dependent platelet agglutination. Plasmin at a concentration of 0.5 CU/mL rapidly reduced the ability of washed platelets to agglutinate in the presence of purified vWF and 1.25 mg/mL ristocetin. By ten minutes, the maximum rate of agglutination had declined to 20% of control rate, and by 60 minutes, was less than 10% of control rate (Fig 2).

The sensitivity of vWF to plasmin digestion was also determined. Von Willebrand factor, 0.1 mg/mL, was incubated with 2.5 CU/mL plasmin. Von Willebrand factor activity was assayed by adding aliquots of plasmin-treated vWF to washed platelets and stimulating agglutination with 1.25 mg/mL ristocetin. By one minute, the rate of vWF-dependent platelet agglutination declined by 36% and then remained at that level for an hour.

**Plasmin Digestion of Formaldehyde-Treated Platelets**

Formaldehyde-treated platelets will agglutinate in the presence of vWF and ristocetin. Cooper and colleagues have shown that a *Serratia marcescens* metalloprotease will digest glycopocalcin from the surface of formaldehyde-treated human platelets. We subjected treated platelets to plasmin digestion and then assayed ristocetin-mediated agglutination. Plasmin, 0.5 CU/mL, progressively inhibited platelet agglutination: by ten minutes, the maximum rate of agglutination had declined to 18% of control rate, and by 60 minutes, was only 15% of the control rate.

**Plasmin Digestion of *125*I-Labeled Platelets**

To determine the effect of plasmin on platelet surface proteins, we exposed *125*I-lactoperoxidase-labeled platelets to plasmin. Figure 3 shows autoradiograms obtained from gels of both the pellets and supernatants at 1, 15, and 30 minutes of incubation.

The control pellet contains six major bands, ranging in estimated mol wt from 89,000 to 180,000. In autoradiograms from the plasmin-treated platelets, there is progressive loss with time of a radiolabeled band having an estimated mol wt of 180,000. At the same time, a radiolabeled band with an estimated mol

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**Table 1. Effect of Streptokinase Concentration on Ristocetin-Induced Platelet Agglutination and Thrombin Time at 60 Minutes**

<table>
<thead>
<tr>
<th>Streptokinase (IU/mL)</th>
<th>Percentage of Maximum Agglutination</th>
<th>Thrombin Time (s)</th>
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<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>20 ± 1.2</td>
</tr>
<tr>
<td>50</td>
<td>51 ± 6.2</td>
<td>39 ± 1.9</td>
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<tr>
<td>100</td>
<td>69 ± 4.8</td>
<td>107 ± 20</td>
</tr>
<tr>
<td>500</td>
<td>60 ± 2.9</td>
<td>&gt; 120</td>
</tr>
<tr>
<td>50,000</td>
<td>38 ± 5.2</td>
<td>&gt; 120</td>
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</table>

Each value represents the mean ± SD of three or more separate determinations.
Fig 3. (A) Effect of plasmin on platelet surface proteins studied by autoradiography. Washed platelets were labeled by the lactoperoxidase method and incubated with plasmin at room temperature for 1, 15, and 30 minutes. Following incubation, the platelets were then centrifuged and prepared for electrophoresis in 6% nonreduced acrylamide gels. The gels were exposed to x-ray film for 165 hours at -20 °C. This autoradiogram demonstrates the progressive loss of a 180,000 mol wt protein (++) from the surface of the plasmin-treated platelets. In addition, three new proteins, varying in mol wt from 86,000 to 80,000, appear in the plasmin-treated platelets (C, control; P, plasmin-treated). (B) Supernatants removed from the platelets described in A were similarly used to produce autoradiograms. This autoradiogram demonstrates the progressive appearance of a 135,000 mol wt protein (+) in the supernatants removed from plasmin-treated platelets. The autoradiogram was developed after exposure for 240 hours at -20 °C.

wt of 135,000 appears in the autoradiograms of the supernatants from the plasmin-treated platelets. Specific identification of the 180,000 mol wt band seen in the pellets and the 135,000 mol wt band seen in the supernatants is not possible by the methods used here. However, the relative positions and estimated molecular weight of these two bands suggest that they correspond to gpIIb and glycocalicin, which is a water-soluble fragment of gpIIb/IIIa.13,16,24-26,43

There was very little change in the electrophoretic pattern of the other major platelet membrane proteins. The densities of gpIIb and gpIIa did not appreciably change during the course of this experiment. We did note the appearance of three new radiolabeled bands after 15 minutes of digestion. The estimated molecular weight of these bands is 70,000, 75,000, and 83,000. Interestingly, the new band with mol wt of 83,000 disappeared by 30 minutes.

Detection of Glycocalicin-Related Antigen in the Supernatant From Plasmin-Treated, Washed Platelets

To confirm that plasmin digestion of platelet membranes affects gpIIb, we used an ELISA to determine the concentration of glycocalicin-related antigen in supernatants from plasmin-treated platelets. The glycocalicin concentration in the supernatants increased as the period of digestion grew longer; this increase corresponded to a progressive decline in the rate of platelet agglutination with ristocetin (Table 2). Control samples contained 10 nmol/L immunoassayable glycocalicin antigen at the beginning of incubation. After the addition of plasmin, the glycocalicin antigen concentration rose markedly, increasing to 290 nmol/L by 30 minutes and 410 nmol/L at 90 minutes. There was a 70% decrease in ristocetin-induced platelet agglutination by ten minutes and almost total loss of reactivity by 90 minutes.

We next studied the effect that varying doses of plasmin had on gpIIb. Washed platelets were exposed to 0.05, 0.10, 0.50, and 1.00 CU/mL plasmin. The supernatants were analyzed for glycocalicin-related antigen by our ELISA. The data from this study are summarized in Fig 4. A dose-dependent decrease in the rate of

<table>
<thead>
<tr>
<th>Time</th>
<th>Glycocalicin Concentration in Surrounding Medium</th>
<th>Percentage of Decline in Agglutination</th>
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<tbody>
<tr>
<td>10 min</td>
<td>210 nmol/L</td>
<td>4 nmol/L</td>
</tr>
<tr>
<td>30 min</td>
<td>290 nmol/L</td>
<td>13 nmol/L</td>
</tr>
<tr>
<td>90 min</td>
<td>410 nmol/L</td>
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Each value represents the mean of two determinations.
By use of an ELISA, we were able to demonstrate that plasmin treatment of washed platelets caused progressive release of a glycocalcin-related antigen into the surrounding medium. Corresponding to the release of this material was a dramatic decline in vWF-dependent platelet agglutination. Because the monoclonal antibody used in this assay cross-reacts with glycocalcin and gp1b, these data suggest that plasmin is able to proteolyze gp1b, the platelet receptor for vWF.

To further examine the effect of plasmin on the platelet surface, we used SDS-PAGE and autoradiography to analyze the $^{125}$I-labeled surface proteins from platelets incubated with plasmin. These studies demonstrated a progressive disappearance of a labeled protein with an estimated mol wt of 180,000. Marked loss of this protein occurred by 30 minutes and corresponded to a 60% decline in platelet agglutination. Although we cannot specifically identify this protein, its apparent molecular weight in the unreduced state and its position relative to other protein bands on the autoradiogram suggest that it is gp1b. Because gp1b is heavily glycosylated, exact determination of its molecular weight by electrophoretic techniques is difficult; however, using an electrophoretic system similar to ours, Phillips and Poh Agin estimated its molecular weight to be 170,000.

The loss of gp1b from the surface of $^{125}$I-labeled platelets during plasmin treatment was accompanied by the appearance of a labeled protein in the supernatant medium. Following SDS-PAGE of supernatant samples, this protein was identified on autoradiograms and its molecular weight estimated to be 135,000. It was the only band seen and probably is the material detected as glycocalcin-related antigen in the ELISA.

Our demonstration that progressive loss of gp1b from plasmin-treated platelets is associated with declining vWF-dependent agglutination is consistent with findings from similar studies and supports current theory regarding gp1b function. Cooper et al. used a Serratia marscescens-derived metalloprotease to cleave gp1b from platelets and thereby reduce agglutination in the presence of bovine vWF. Okumura and Jamieson demonstrated that trypsin treatment of platelets, known to degrade gp1b, would also inhibit platelet agglutination.

In addition to its effect on ristocetin-induced platelet agglutination, plasmin generated in PRP causes a marked reduction in ADP-induced aggregation. Thrombin- and collagen-induced aggregation are also affected, but not to the same extent. These changes occur rapidly and parallel prolongation of the thrombin time. Although each of these effects deserves investigation, this report focuses on plasmin-mediated inhibition of vWF-dependent platelet agglutination.

platelet agglutination was clearly associated with a corresponding rise in the amount of glycocalcin-related antigen in the supernatant. At the lowest plasmin concentration used, 0.05 CU/mL, gp1b was proteolyzed and platelet agglutination reduced. This amount of plasmin is undoubtedly available in vivo during fibrinolytic therapy.

To ensure the accuracy of the ELISA assay, a known quantity of purified glycocalcin was subjected to plasmin digestion. Glycocalcin levels at ten minutes, 30 minutes, and 60 minutes were all within 93% of the control level. These results demonstrate that plasmin digestion of glycocalcin does not interfere with the epitope recognized by the monoclonal antibody used in the ELISA.

**DISCUSSION**

We have presented evidence that plasmin added to washed platelets or generated in PRP by addition of streptokinase can inhibit platelet function. Our results indicate that (1) plasmin directly reduces the ability of platelets to agglutinate in the presence of vWF and ristocetin, (2) this effect is associated with proteolysis of gp1b, (3) the extent of plasmin action can be determined by measuring released glycocalcin-related antigen, and (4) the concentration of plasmin necessary to induce this effect is no greater than that which occurs during fibrinolytic therapy.

In addition to its effect on ristocetin-induced platelet agglutination, plasmin generated in PRP causes a marked reduction in ADP-induced aggregation. Thrombin- and collagen-induced aggregation are also affected, but not to the same extent. These changes occur rapidly and parallel prolongation of the thrombin time. Although each of these effects deserves investigation, this report focuses on plasmin-mediated inhibition of vWF-dependent platelet agglutination.

**Fig 4.** Effect of plasmin on washed platelets. Washed platelets in TBS containing 20 mmol/L EDTA were incubated at room temperature with varying doses of plasmin. After one hour, the reaction was stopped by the addition of aprotinin and the platelet pellet centrifuged. The supernatants were assayed via ELISA for glycocalcin-related antigen (O--O). The platelets were resuspended in buffer containing vWF and the maximum rate of agglutination determined following the addition of ristocetin (O--O). Each point represents the mean of two determinations.

According to Phillips and Poh Agin, an electrophoretic system similar to ours, allowed the detection of a labeled protein in the supernatant with an estimated mol wt of 180,000. Marked loss of this protein occurred by 30 minutes and corresponded to a 60% decline in platelet agglutination. Although we cannot specifically identify this protein, its apparent molecular weight in the unreduced state and its position relative to other protein bands on the autoradiogram suggest that it is gp1b. Because gp1b is heavily glycosylated, exact determination of its molecular weight by electrophoretic techniques is difficult; however, using an electrophoretic system similar to ours, Phillips and Poh Agin estimated its molecular weight to be 170,000. The loss of gp1b from the surface of $^{125}$I-labeled platelets during plasmin treatment was accompanied by the appearance of a labeled protein in the supernatant medium. Following SDS-PAGE of supernatant samples, this protein was identified on autoradiograms and its molecular weight estimated to be 135,000. It was the only band seen and probably is the material detected as glycocalcin-related antigen in the ELISA.

Our demonstration that progressive loss of gp1b from plasmin-treated platelets is associated with declining vWF-dependent agglutination is consistent with findings from similar studies and supports current theory regarding gp1b function. Cooper et al. used a Serratia marscescens-derived metalloprotease to cleave gp1b from platelets and thereby reduce agglutination in the presence of bovine vWF. Okumura and Jamieson demonstrated that trypsin treatment of platelets, known to degrade gp1b, would also inhibit platelet agglutination. In addition, immunochemical experiments utilizing antibodies directed against gp1b have provided complementary evidence in support of gp1b function as a platelet receptor for vWF.

That plasmin can degrade gp1b and liberate a glycocalcin-related fragment is not surprising. As mentioned above, various proteases have been shown to affect gp1b. Trypsin cleaves a 120,000 mol wt glyco-
dependent protease cleaves a 145,000 mol wt fragment from gpIb46,47; a platelet-derived, calcium-dependent protease cleaves a 145,000 mol wt fragment from gpIb48; and a Serratia metalloprotease releases a 156,000 mol wt glycopeptide.31 In each instance, these fragments have been shown to be closely related to glycocalcin.

We observed that thrombin-induced platelet aggregation was also reduced when streptokinase was added to PRP. Others have demonstrated that plasmin may bind thrombin. In a study by Jenkins et al,45 platelets were treated with an antiligycocalcin antibody and stimulated by thrombin; the platelets retained their ability to secrete 14C-serotonin, but aggregated at a diminished rate. Cooper et al21 noted a similar response to thrombin stimulation after gpIb was enzymatically removed from platelets by a metalloprotease. Both groups concluded that gpIb may modulate the response of platelets to thrombin. Our data appear to support this view; however, we cannot be sure that the only effect of plasmin on the platelet membrane is the proteolysis of gpIb.

There is also a reduction in ADP-induced platelet aggregation after the addition of streptokinase to PRP. Our studies did not, however, explore the cause of this effect. ADP-induced platelet aggregation is dependent on the presence of an intact platelet surface receptor, release of stored ADP, and calcium and fibrinogen in the surrounding medium.49 In addition, high levels of fibrinogen degradation products in plasma may interfere with ADP-induced platelet aggregation.50,51 Niewiarowski and colleagues demonstrated that plasmin treatment of platelets causes release of stored adenine nucleotides in association with decreased response to ADP.30 Plasmin generation in PRP reduces the fibrinogen concentration, generates fibrin(ogen) degradation products, and possibly alters the platelet surface receptor for ADP. Thus, further study is necessary to explain the observed effect of streptokinase in PRP on ADP-induced platelet aggregation.

This study demonstrates that plasmin digestion of vWF results in a small decline in its ability to support ristocetin-induced platelet agglutination. These results agree with those of Henriksson and Nilsson,8 but differ from those of Atichartakarn et al.7 Further study is needed to resolve this question.

Plasmin generation can occur during a number of pathologic conditions, including disseminated intravascular coagulation, carcinomatosis, and liver disease,52,53 and as a result of fibrinolytic activator therapy.8 In each of these instances, affected patients may suffer from excessive bleeding. The etiology of this bleeding is usually ascribed to the combination of a decreased plasma fibrinogen concentration, a large amount of circulating fibrin(ogen) split products, and to the action of circulating plasmin on clots necessary for hemostasis.31 Interestingly, Castillo et al34 noted decreased ristocetin-induced platelet agglutination in a group of patients with advanced liver disease and evidence of systemic fibrinolysis. Additional studies by Ordinas et al55 suggest that platelets from similarly ill patients were deficient in surface glycoproteins from the group I complex.

The amount of uninhibited plasmin present in plasma during fibrinolytic therapy probably varies among individuals. The average concentration of plasminogen in human plasma is 2.5 μmol/L and of alpha-2-antiplasmin is 1.0 μmol/L.33 After streptokinase is added to human plasma, approximately 0.60 CU/mL of free plasmin is generated.32 In our studies, less than 10% of this amount of plasmin, 0.05 CU/mL, caused washed platelets to become less responsive to vWF and ristocetin and released glycocalcin-related antigen.

Our data suggest that systemic activation of the fibrinolytic system can cause significant platelet dysfunction. This effect may contribute importantly to the bleeding complications of primary fibrinolysis and fibrinolytic activator therapy.36-38 However, platelet inhibition may also be an important antithrombotic effect of such therapy. Further in vivo investigation of these platelet effects seems warranted in light of current interest in fibrinolytic therapy.

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