Dependence of Plasmin-Mediated Degradation of Platelet Adhesive Receptors on Temperature and Ca\(^{2+}\)

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The effects of activation of plasminogen by streptokinase and tissue-type-plasminogen activator on platelet activation and the membrane glycoproteins (GPs) that mediate platelet adhesion and aggregation are not yet fully defined. To clarify effects on platelets during activation of plasminogen in vitro, we used monoclonal antibodies (MoAbs), flow cytometry, and platelet surface-labeled with \(^{125}\)I to characterize changes in receptors for fibrinogen (GPIIb-IIIa), von Willebrand factor (GPIb), and collagen (GPIa-IIa). Activation of plasminogen in plasma with pharmacologic concentrations of plasminogen activators did not degrade GPIIb-IIIa or GPIb, and caused only a modest decrease in GPIa. In washed platelets GPIIb-IIIa was extensively degraded by plasmin at 37°C in the absence of exogenous Ca\(^{2+}\), conditions that destabilize the IIb-IIIa complex. Degradation of GPIb in washed platelets displayed a similar although less-marked dependence on temperature and the absence of Ca\(^{2+}\). The binding of activation-specific MoAbs did not increase during activation of plasminogen in plasma. We conclude that during pharmacologic fibrinolysis, reported inhibition of platelet function in plasma is not due to degradation of platelet-adhesive receptors. In addition, platelet activation observed during thrombolytic therapy does not appear to be a direct consequence of plasminogen activation.

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Materials and Methods

Materials. The plasminogen activators studied were SK (Kabi-Vitrum, Stockholm, Sweden) and single-chain recombinant t-PA (Genentech, San Francisco, CA). Human plasminogen was purchased from Enzyme Research Labs (South Bend, IN) and \(^{125}\)I was obtained from Amersham (Arlington Heights, IL). D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK) and lactoperoxidase were purchased from Calbiochem (La Jolla, CA). Reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Biorad Labs (Richmond, CA), and pre-stained molecular weight standards were purchased from Bethesda Research Labs (Gaithersburg, MD). Adenosine diphosphate (ADP) was obtained from Helena Labs (Beaumont, TX). The chromogenic substrate S-2251 (D-Val-Leu-Lys-p-nitroanilide) and...
human plasmin were purchased from KabiVitrum. All other reagents were from Sigma (St Louis, MO).

Preparation of platelets. Platelets were obtained from normal volunteers who gave written informed consent and were not taking any medications known to interfere with platelet function. Antecubital venipuncture was performed with a 19-gauge needle after application of a light tourniquet. Whole blood was anticoagulated with either 1/10 vol of 3.8% (wt/vol) trisodium citrate, pH 7.4, or acid citrate dextrose (ACD, 0.038 mol/L citric acid, 0.075 mol/L trisodium citrate, 0.14 mol/L α-D-glucose), pH 4.5. Heparin sodium (1 U/mL) or ethylenediaminetetraacetic acid (EDTA, 10 mmol/L) was used for anticoagulation in several studies. Neither ACD nor EDTA was used in experiments designed to assess the effects of activation of plasminogen or platelet activation. Platelet-rich plasma (PRP) was isolated after centrifugation of anticoagulated whole blood at 150g for 15 minutes. For some studies autologous platelet-poor plasma was prepared by centrifuging citrated PRP at 1500g for 10 minutes.

To prepare washed platelets for flow cytometry studies, an additional 1/10 vol of ACD and PGE, (10 μmol/L) were added to the PRP, and the platelets were pelleted at 1500g for 10 minutes. Platelets were resuspended in 20 mL of washing buffer (0.14 mol/L NaCl, 2.7 mmol/L KC1, 12 mol/mL NaHCO3, 0.5 mmol/L Na2PO4, 5.5 mmol/L α-D-glucose, 5.0 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid [HEPES], 1/10 vol ACD, 10 μmol/L PGE, pH 6.5, [Tyrode-HEPES wash buffer]) and centrifuged at 1500g for 10 minutes. The initial wash was repeated and the platelets were resuspended in Tyrode-HEPES buffer (no ACD or PGE, no divalent cations), pH 7.4. For specific experiments 2 mmol/L CaCl2 was added as described.

For studies with platelets surface-labeled with 125I, platelets were pelleted from PRP as described above and washed twice by centrifugation with 20 mL of the following buffer: 0.15 mol/L NaCl, 0.05 mol/L Tris, 1/10 vol ACD, 10 μmol/L PGE, and 5.5 mmol/L α-D-glucose, pH 6.5 (TBS wash buffer). Platelets were then resuspended in TBS wash buffer and surface-labeled with 125I by the lactoperoxidase method.29 Surface-labeled platelets were then washed two more times by centrifugation with TBS washing buffer and resuspended in autologous citrated plasma or Tyrode-HEPES buffer (no ACD or PGE, no divalent cations), pH 7.4. Incubations in buffer were performed in the presence or absence of 2 mmol/L CaCl2.

Plasminogen was activated in citrated PRP with pharmacologic and suprapharmacologic concentrations of SK (1,000 or 20,000 U/mL) or t-PA (2.5 or 20 μg/mL). For studies of platelets surface-labeled with 125I, the platelets were resuspended in autologous platelet-poor plasma. Washed platelets were incubated with the same doses of SK or t-PA in Tyrode-HEPES buffer supplemented with 21 μg/mL of human plasminogen in the presence or absence of 2 mmol/L CaCl2. Control incubations included plasminogen and a matched volume of t-PA (0.2 mol/L arginine phosphate, pH 7.2) or SK (0.9% NaCl) buffer. In studies of both washed platelets and PRP, activation of plasminogen was accomplished at 23° or 37°C for up to 60 minutes. The platelet count was 1 to 2 × 105/mL during incubations with plasminogen activators.

In studies of platelets surface-labeled with 125I, incubations were terminated by the addition of 5 μmol/L PPACK and 200 KIU/mL aprotinin. After 10 minutes, 1/10 vol ACD was added and the platelets were pelleted for 2 minutes at 12,000g. The supernatant was removed by aspiration and the platelet pellet was dissolved by boiling for 10 minutes in electrophoresis gel buffer (0.063 mol/L Tris, 2% SDS, 10% glycerol, 0.001% wt/vol bromphenol blue) with or without 1.0% 2-mercaptoethanol. Samples were analyzed on 6% polyacrylamide gels (SDS-PAGE) by the method of Laemmli.30 The gels were dried under vacuum and autoradiography was performed with X-omatic AR-5 film and an image-intensifier screen (Kodak, Rochester, NY). Gels were developed after 4 to 7 days at −70°C. Major platelet GP bands were analyzed by use of a laser densitometer (LKB 2400, Bromma, Sweden).

Results obtained with 125I surface-labeled platelets during pharmacologic activation of plasminogen in citrated plasma were compared to similar autoradiographic studies performed with platelets obtained at hourly intervals from a patient receiving 100 mg of t-PA for AMI.

Flow cytometry of platelets. Platelet-surface GP degradation was quantitated by the use of FITC-labeled MoAbs and analysis of their binding to specific receptors by flow cytometry. MoAb 10E5, a complex specific anti–GP Ib-IIIa antibody, was a gift of B. Coller (State University of New York, Stonybrook).31,32 MoAb 6D1 (B. Coller) binds to the glycolylcin portion of GP IIb.33 MoAb 12F1 binds to the GP IIb subunit of GP IIa (gift of V. Woods, University of California, San Diego).34–36 MoAbs specific for platelet activation were PAC1, which binds to the activated GP IIb-IIIa complex (gift of S. Shattil, University of Pennsylvania, Philadelphia),37–39 and S12, which recognizes the α granule protein (GMP-140 or PAGDEM) expressed on the platelet surface after platelet secretion (gift of R. McEver, University of Oklahoma, Tulsa).40–42 For flow cytometry MoAbs 10E5, 6D1, 12F1, and S12 were used at a final concentration of 5 to 10 μg/mL. MoAb PAC1 was used at 9 to 18 μg/mL.

Before Fluorescein isothiocyanate (FITC) labeling, MoAbs were dialyzed overnight at 4°C in 0.1 mol/L Na2HPO4, pH 7.4. FITC stock was prepared immediately before use at 0.8 mg/mL in 0.5 mol/L Na2CO3, pH 9.5. FITC stock was added to MoAb at 15 wt/wt, the pH was readjusted to 9.5, and the incubation was allowed to proceed at 23°C for up to 2 hours with intermittent vortexing. The peak corresponding to labeled MoAb was separated from free FITC on a 30.0 × 0.5 cm Sephadex G-25-300 (Pharmacia, Uppsala, Sweden) gel filtration column equilibrated with 0.1 mol/L Na2HPO4.

For flow cytometry studies platelets were incubated with t-PA or SK in citrated plasma or buffer in the presence of plasminogen as described above. After 60 minutes, 50-μL aliquots of platelets were removed and added to Tyrode-HEPES buffer, PPACK (5 μmol/L), aprotinin (200 KIU/mL), and FITC-MoAb at 23°C in a final volume of 200 μL. When calcium had been omitted from washed-platelet incubations it was added to a final concentration of at least 100 μmol/L during incubation with MoAbs before flow cytometry. After a 30-minute incubation, flow cytometric analysis was performed by use of a Coulter Epics model 753 flow cytometer (Hialeah, FL) with a 250-mW argon laser emitting at 488 nm. Fluorescence emission was detected at 525 nm with a band pass filter. Platelets were distinguished from cellular fragments by their characteristic forward- and side-light scatter, and a gate was set to analyze the fluorescence from single platelets. The bound fluorescence from 25,000 platelets was analyzed and plotted as a histogram of platelet number versus fluorescence intensity per platelet. The fluorescence scale extends over a 3-decade log scale divided into 256 channels. At the gain settings used, ≥95% of nonspecific binding fell in the first 15 channels. For the activation-specific MoAbs PAC1 and S12, activated platelets were defined as those whose fluorescence fell above channel 15. For quantitation of changes in GPs Ib, IIb-IIIa, and Ia-IIa the mean population fluorescence was calculated with on-line software. Mean fluorescence channel number was converted from log to linear format.

Blood samples were also obtained from patients receiving t-PA (100 mg) for AMI. Plasmin was inhibited with PPACK (5 μmol/L) and aprotinin (1,000 KIU/mL) and PRP obtained as described above. Binding of FITC-MoAb 10E5 (anti-GP IIb-IIIa) and 6D1 (anti–GP IIb) to platelets was quantitated by flow cytometry. All patient samples for flow cytometry and autoradiographic studies
were obtained in accordance with the guidelines of the Human Studies Committee at Washington University Medical School.

**Plasmin and α-2-antiplasmin assays.** Activation of plasminogen was performed in plasma or buffer as described. After 60 minutes aliquots were removed and the platelets were pelleted at 12,000g for 2 minutes. The supernatant was collected and immediately frozen at −70°C. Samples were thawed and diluted 1:20 with 0.11 mol/L NaCl, 0.05 mol/L Tris, pH 7.4, and added to the wells of a microtiter plate (Costar, BioRad, Richmond, CA). The chromogenic substrate for plasmin activity, S-2251, was added to a final concentration of 312 µmol/L. Activity of purified human plasmin was used to develop a standard curve from 0.0025 to 0.02 caseinolytic units (CU) per milliliter. The change in absorbance due to hydrolysis of the substrate was measured every 20 seconds for 6 minutes with a programmable automated kinetic plate reader at 23°C (Molecular Devices, Menlo Park, CA). The lower limit of detectibility for samples assayed with a 1:20 dilution was 0.05 CU/mL plasmin activity. The human plasminogen preparation exhibited no plasmin activity in this assay.

α-2-Antiplasmin was measured in PRP with an assay based on the plasmin-specific chromogenic substrate S-2251 as modified from the manufacturer’s instructions for use with microtiter plates (KabiVitrum). Plasmin was added to samples in excess and residual plasmin activity against the substrate S-2251 was measured with the automated kinetic plate reader. α-2-Antiplasmin levels were derived by comparison with a standard curve developed with serial dilutions of pooled human plasma.

**Statistical methods.** Mean channel fluorescence on flow cytometry was converted to relative mean linear fluorescence units as described and presented as the mean ± standard error (SEM). Differences between the means of control and t-PA– or SK-treated platelets were compared by paired analysis through use of the Wilcoxon signed-rank method, and a significant difference was considered to exist when \( P < .05 \).

**RESULTS**

**Effects on GPs after activation of plasminogen in plasma.** To characterize structural changes in membrane GPs, platelets surface-labeled with \(^{125}\)I were suspended in autologous-citratred plasma and incubated with t-PA or SK for up to 60 minutes at 37°C. The migration of platelet GPs Ibα, IIbα, and IIIa as determined by reduced 6% SDS-PAGE was unchanged after incubation with pharmacologic concentrations of t-PA (2.5 µg/mL) or SK (1,000 U/mL, Fig 1). Similar results were obtained with incubations at 23°C (data not shown). With suprapharmacologic concentrations of t-PA (20.0 µg/mL) and SK (20,000 U/mL) there was some proteolysis of minor GPs migrating above GP Ibα, but the migrations of GPs Ibα, IIbα, and IIIa remained unchanged (Fig 1). However, with t-PA (20 µg/mL) densitometry demonstrated a mean decrease of 30% in the GP Ibα band at 60 minutes (n = 2). The degradation of GP Ibα observed with suprapharmacologic concentrations of t-PA did not appear to be a function of plasmin activity alone, as SK generated higher plasmin activity in plasma than t-PA (Table 1). As expected, α-2-antiplasmin was depleted during incubations with t-PA or SK in PRP (data not shown).

Analysis of the binding of specific MoAbs with flow cytometry confirmed the apparent lack of degradation of...
GPs Iba, Ilb, and IIIa after activation of plasminogen in PRP with pharmacologic concentrations of t-PA or SK (Fig 2). Binding of MoAb 10E5 (anti-GPIIb-IIIa) to platelets did not decrease compared with control after incubation with both pharmacologic and suprapharmacologic concentrations of t-PA or SK, as indicated by a lack of change in relative mean fluorescence (Fig 2A). Similar results were obtained with MoAb 6D1 (anti-GPIb), although there was a trend toward decreased MoAb 6D1 binding with 20 pg/mL of t-PA that failed to achieve significance (P = .07, Fig 2B).

Binding of MoAb 12F1, which binds to the la subunit of GPla-IIa, did not change after incubation with t-PA, although modest decreases in binding of 15% to 20% occurred after SK (P = .03, Fig 2C). Examination of individual histograms of platelet number versus fluorescence per platelet did not show any distinct subpopulations of platelets deficient in the GP receptors characterized with MoAbs 10E5, 6D1, or 12F1. In contrast to the findings at 37°C, results of flow cytometric studies of platelets incubated in plasma with pharmacologic and suprapharmacologic concentrations of t-PA or SK at 23°C demonstrated no decrease in the binding of MoAb 10E5, 6D1, or 12F1 (data not shown).

Effects on GP Iib-IIIa after activation of plasminogen in buffer. To characterize the effects of plasminogen activation in a purified system, platelets surface-labeled with $^{125}$I were incubated at 37°C with 1,000 U/mL of SK in divalent cation-free Tyrode-HEPES buffer supplemented with 21 μg/mL of human plasminogen. Analysis of autoradiograms from control platelets incubated in the absence of SK demonstrated no change in GP IIb-IIIa on either reduced or nonreduced gels (Fig 3). In contrast, activation of plasminogen with SK produced striking degradation of GPs IIb and IIIa (Fig 3). Proteolysis was evident by 10 minutes, with the subsequent generation of three new fragments with apparent mol wts of 78, 71, and 66 Kd on 6% SDS-PAGE under nonreducing conditions. Reduced SDS-PAGE confirmed the proteolysis of GPs IIb and IIIa, with degradation fragments migrating as a broad band with a mol wt of 65,000 to 70,000 (Fig 3).

Previous studies have demonstrated dissociation of GPIIb-IIIa at 37°C in the presence of strong Ca$^{2+}$-chelating agents. To determine whether the presence of Ca$^{2+}$ or the incubation temperature affected the proteolysis of GPIIb or IIIa in a purified system, incubations of surface-labeled platelets with 2.5 μg/mL of t-PA were performed under the following conditions: 23°C without added Ca$^{2+}$, 37°C without added Ca$^{2+}$, and 37°C with 2 mmol/L Ca$^{2+}$ (Fig 4). Control incubations in the absence of plasminogen activators at 23°C and 37°C with or without Ca$^{2+}$ demonstrated no change in the migration of GPs IIb and IIIa. At 23°C in the absence of added Ca$^{2+}$, GPIIb-IIIa was resistant to plasmin-mediated proteolysis (Fig 4). At 37°C in the presence of Ca$^{2+}$, GPIIa was resistant to proteolysis while GPIIb underwent a modest degradation. However, activation of plasminogen with 2.5 μg/mL of t-PA at 37°C in the absence of added Ca$^{2+}$ resulted in almost complete degradation of both GPs IIb and IIIa (Fig 4). Marked proteolysis was evident despite generation of only 0.08 ± 0.01 CU/mL of plasmin activity against the chromogenic substrate S-2251 after activation of plasminogen with t-PA or SK in citrated plasma or buffer. Mean ± SEM (n = 8 to 18).

### Table 1. Plasmin Activity (CU/mL)

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<th>Cirtated PRP (37°C)</th>
<th>Washed Platelets (37°C/−Ca$^{2+}$)</th>
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<tr>
<td>t-PA 2.5 μg/mL</td>
<td>0.19 ± 0.03</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
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<td>t-PA 20.0 μg/mL</td>
<td>0.30 ± 0.02</td>
<td>0.27 ± 0.02</td>
<td>0.24 ± 0.03</td>
<td>0.22 ± 0.06</td>
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<tr>
<td>SK 1,000 U/mL</td>
<td>0.30 ± 0.03</td>
<td>0.24 ± 0.02</td>
<td>0.17 ± 0.01</td>
<td>0.13 ± 0.01</td>
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<tr>
<td>SK 20,000 U/mL</td>
<td>0.87 ± 0.08</td>
<td>0.67 ± 0.04</td>
<td>0.49 ± 0.07</td>
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Plasmin activity against the chromogenic substrate S-2251 after activation of plasminogen with t-PA or SK in citrated plasma or buffer. Mean ± SEM (n = 8 to 18).
plasmin activity with 2.5 μg/mL of t-PA in washed-platelet preparations (Table 1). In addition, activation of plasminogen with t-PA (2.5 μg/mL) or SK (1,000 U/mL) in plasma anticoagulated with 10 mmol/L EDTA, a potent chelating agent, also resulted in marked degradation of GP IIb-IIIa at 37°C (data not shown).

Binding of MoAb 10E5 to the IIb-IIIa complex was examined by flow-cytometric analysis of washed platelets

\[ M_r \times 10^{-3} \]

\[ 0 \quad 15 \quad 30 \quad 60 \]

Fig 3. Autoradiograms of GPs from platelets surface-labeled with \(^{125}\)I and subjected to 6% SDS-PAGE under nonreducing conditions (NR, top row) and after reduction of disulfide bonds (R, bottom row). Platelets were incubated with SK in Tyrode-HEPES buffer containing 21 μg/mL of plasminogen for 60 minutes at 37°C in the absence of added Ca\(^{2+}\). Aliquots were removed at 0, 1, 5, 10, 15, 30, and 60 minutes as indicated. Migration of platelet surface GPs Iβα, IIβα, IIIa, and IV are indicated by the arrows. After activation of plasminogen with SK in the absence of added Ca\(^{2+}\), proteolysis of GPs Iβα, IIβα, and IIIa occurred, indicated by a decrease in the intensity of these bands as well as the appearance of lower mol wt degradation fragments of these GPs. Control platelets incubated with plasminogen alone in the absence of plasminogen activator are shown in the first panel of each row. The migration of mol wt standards is indicated on the left side of each gel.

\[ M_r \times 10^{-3} \]

\[ 0 \quad 15 \quad 30 \quad 60 \]

Fig 4. Autoradiograms of GPs from platelets surface-labeled with \(^{125}\)I and subjected to 6% SDS-PAGE under reducing conditions. Surface-labeled washed platelets were incubated in Tyrode-HEPES buffer with t-PA (2.5 μg/mL) and 21 μg/mL of plasminogen for 0, 15, 30, and 60 minutes under the following conditions: 23°C without added Ca\(^{2+}\), 37°C with 2 mmol/L Ca\(^{2+}\) in the buffer, and 37°C without added Ca\(^{2+}\). A representative incubation of washed platelets with plasminogen alone at 37°C in the absence of added Ca\(^{2+}\) is shown in the first panel (control). Platelet surface GPs Iβα, IIβα, and IIIa are indicated by the arrows. Marked degradation of platelet GPs Iβα and IIIa, and to a lesser degree GPlβα, occurred when platelets were incubated with t-PA and plasminogen at 37°C without added Ca\(^{2+}\). The migration of mol wt standards is indicated on the left side of each gel.
incubated with t-PA or SK in Tyrode-HEPES buffer supplemented with 21 μg/mL of plasminogen at 23°C or 37°C with or without added Ca²⁺. Platelets incubated at 37°C for 60 minutes in buffer containing no added Ca²⁺ demonstrated an 80% loss of MoAb 10E5 binding, as indicated by a marked decrease in relative fluorescence even in the absence of t-PA or SK (Figs 5 and 6C). Loss of MoAb 10E5 binding in the absence of t-PA or SK at 37°C without the presence of added Ca²⁺ was detectable as early as 15 minutes, which corresponded to the time course of plasmin-mediated degradation of GPIIb-IIIa when analyzed by SDS-PAGE and autoradiography (Figs 3 and 4). Because the migration of GPs IIb and IIIa as detected by SDS-PAGE was unchanged in the absence of t-PA or SK at 37°C without added Ca²⁺ (Figs 3 and 4), the decrease in MoAb 10E5 binding with flow cytometry suggested a conformational change in GPIIb-IIIa, leading to loss of the complex specific epitope recognized by this antibody. Indeed, activation of plasminogen by t-PA or SK at 37°C in the absence of exogenous Ca²⁺ produced only an additional 5% to 10% decrease in MoAb 10E5 binding, suggesting that the small number of GP IIb-IIIa complexes that retain native conformation under these conditions are relatively resistant to degradation (Fig 6C). In contrast, activation of plasminogen at 23°C in the absence of Ca²⁺ or at 37°C in the presence of added Ca²⁺, conditions that maintain the integrity of the IIb-IIIa complex, produced only small decreases in MoAb 10E5 binding (Fig 6A and B).

Effects on GPs IIb and IIIa after activation of plasminogen in buffer. To determine whether GPIb displayed a similar temperature- and Ca²⁺-dependent susceptibility to plasmin-mediated proteolysis, washed platelets surface-labeled with [125I] were incubated with t-PA or SK in Tyrode-HEPES buffer in the presence of plasminogen. Densitometric analysis of autoradiograms from platelets incubated with either t-PA (2.5 μg/mL) or SK (1,000 U/mL) at 37°C in the absence of Ca²⁺ demonstrated a 50% decrease in GPIb by 60 minutes (Figs 3 and 4). At 23°C in the absence of Ca²⁺, activation of plasminogen with 2.5 μg/mL of t-PA produced no change in GPIbα (Fig 4), while incubation at 37°C in the presence of Ca²⁺ resulted in a 30% loss of GPIbα (Fig 4). The temperature and Ca²⁺-dependent proteolysis of GPIb was most striking with 20 μg/mL of t-PA (Fig 7). Activation of plasminogen with suprapharmacologic concentrations of t-PA produced a 60% loss of GPIbα at 23°C in the absence of Ca²⁺, a 75% loss at 37°C in the presence of Ca²⁺, and a 90% decrease at 37°C in absence of added Ca²⁺ (Fig 7). A similar though less marked temperature- and Ca²⁺-dependent proteolysis of GPIb occurred with 20,000 U/mL of SK (Fig 7).

Binding of MoAb 6D1 (anti-GPⅠb) as analyzed by flow cytometry remained stable when platelets were incubated for up to 60 minutes in Tyrode-HEPES buffer with plasminogen (21 μg/mL) in the absence of t-PA or SK, regardless of temperature or Ca²⁺ conditions (Fig 8). Activation of plasminogen with pharmacologic concentrations of t-PA produced no change in the binding of MoAb 6D1, regardless of temperature or Ca²⁺ conditions (Fig 8). Incubations with SK (1,000 U/mL) at 37°C in the absence of Ca²⁺ produced a 30% decrease in MoAb 6D1 binding, but this change was not significant by paired analysis (P = .07, Fig 8C). Incubations of washed platelets with 20 μg/mL of t-PA resulted in a 50% decrease in MoAb 6D1 binding at 37°C in the absence of added Ca²⁺ (P = .04). The results of autoradiography and flow cytometry suggest that GPⅠb may undergo partial proteolysis in washed platelets while binding of MoAb 6D1, an antibody that binds close to the ligand-binding domain of GPⅠb, is preserved. Another explanation for the discrepancy between the magnitude of GPIb degradation on autoradiography and the amount of MoAb 6D1 binding on flow cytometry may be the replacement of degraded receptors from the internal platelet pool of GPⅠb.  However, both autoradiographic and flow-cytometric studies demonstrate the temperature- and Ca²⁺-dependence of GPIb degradation by plasmin (Figs 4, 7, and 8).

In contrast to the findings with GPs IIb-IIIa and Ib, there was no evidence of a decrease in MoAb 12F1 (anti-GPⅠa) binding to washed platelets during activation of plasminogen with t-PA or SK at 23°C or 37°C in the presence or absence of Ca²⁺ (data not shown).

Patient studies. Although our observations in citrated plasma demonstrate that platelet-adhesive GP degradation does not occur following activation of plasminogen with pharmacologic concentrations of t-PA or SK (Figs 1 and 2), it is conceivable that prolonged platelet exposure to activation of plasminogen in vivo may result in proteolysis of platelet receptors. To examine this possibility, platelets were obtained from patients receiving a 3-hour infusion of t-PA...
MoAbs 10E5 (anti-GP1b-IIIa) and 6D1 (anti-GP1b) following t-PA therapy for AMI.

**Activation-specific MoAbs during activation of plasminogen in plasma.** Platelets were incubated in citrated plasma at 37°C with pharmacologic and suprapharmacologic concentrations of t-PA or SK for periods of up to 1 hour. The binding of PAC1, an MoAb that binds to the GP1b-IIIa fibrinogen receptor on platelets after activation, did not increase when platelets were incubated with either t-PA or SK, consistent with a lack of platelet activation in response to activation of plasminogen (Fig 10). Even after incubation with 20,000 U/mL of SK there was no evidence of platelet activation or aggregation ($P > .3$, $n = 5$), despite the generation of substantial plasmin activity of $0.87 \pm 0.08$ CU/mL, a level comparable with that reported to induce platelet activation by others. Platelets incubated with t-PA or SK in plasma could still be activated with $10 \mu$mol/L ADP, as evidenced by an ADP-induced increase in PAC1 binding comparable to that observed with control platelets (Fig 10). The absence of platelet activation in citrated plasma during activation of plasminogen was confirmed by the lack of increased binding of MoAb S12 documented with flow cytometry. This antibody binds to the α granule protein (GMP-140), which is expressed on the platelet surface after platelet secretion.

**DISCUSSION**

Our results indicate that activation of plasminogen in citrated plasma by pharmacologic concentrations of t-PA or SK does not result in degradation of platelet adhesive receptors for fibrinogen and WF. Although some degradation of the collagen receptor occurs in plasma with SK, it is modest. However, plasmin-mediated proteolysis of GPIb-IIIa in washed platelets is marked at physiologic temperatures in the absence of added Ca$^{2+}$. Similarly, plasmin-mediated degradation of GPIb in washed platelets is increased at physiologic temperatures, in the absence of added Ca$^{2+}$, in the presence of increased plasmin activity, and with suprapharmacologic concentrations of t-PA. Furthermore, we could not find any evidence that activation of plasminogen in citrated plasma by t-PA or SK directly activates platelets. Consequently, the platelet activation observed during pharmacologic activation of plasminogen does not appear to result from the direct action of plasmin on platelets. In addition, the resistance of platelet-adhesive receptors to plasmin-mediated degradation in plasma suggests that the inhibition of platelet aggregation in plasma observed by others is most likely due to factors other than the proteolysis of GPs, Ib, IIb, and IIIa.

Previous studies of platelet function have indicated that activation of plasminogen in plasma can induce both antiaggregatory and proaggregatory responses. Antiaggregatory effects may be due to generation of fibrinogen-degradation products that compete with fibrinogen for binding to the platelet GPIb-IIIa fibrinogen receptor but do not support platelet aggregation. In addition, proteolysis of WF by plasmin may explain the inhibition of ristocetin-induced platelet agglutination. Although antiaggregatory effects...
Degradation of platelet adhesive receptors

Fig 7. Autoradiograms of GPs from platelets surface-labeled with 125I and subjected to 6% SDS-PAGE under reducing conditions. Surface-labeled washed platelets were incubated in Tyrode-HEPES buffer with 21 μg/mL of plasminogen and either t-PA (top) or SK (bottom) under the following conditions: 23°C without added Ca2+, 37°C with 2 mmol/L Ca2+ added to the buffer, and 37°C without added Ca2+. Aliquots were removed at 0, 15, 30, and 60 minutes. Representative incubations of platelets with plasminogen in the absence of t-PA or SK at 37°C without added Ca2+ are shown in the first panels. Platelet surface GPs IIb, IIIa, and IIia are indicated by the arrows. Marked degradation of platelet GPs IIb and IIia occurred after activation of plasminogen with either t-PA or SK, but only at 37°C in the absence of added Ca2+. Degradation of GPIb was greater after activation of plasminogen by t-PA than after that by SK. Proteolysis of GPIb with activation of plasminogen by t-PA was evident under all three conditions, but was most striking at 37°C without added Ca2+. Migration of mol wt standards is indicated on the left side of each gel.

could also be due to proteolysis of platelet-adhesive GPs by plasmin, resulting in decreased binding of cohesive ligands, our results suggest that significant functional impairment of platelet receptors is unlikely. MoAbs 10E5 (anti–GPIIb-IIIa) and 6D1 (anti–GPIb) both bind close to the ligand-binding domains of their respective receptors.31,33 Preserved binding of these MoAbs (as characterized with flow cytometry in plasma) and the absence of structural change of these GP receptors (as shown by SDS-PAGE during activation of plasminogen in plasma) suggest that the fibrinogen and vWF receptors are likely to retain function. The observation of Loscalzo and Vaughan that platelets disaggregated by suprapharmacologic concentrations of t-PA in plasma could be reaggregated by ADP if resuspended with intact fibrinogen is consistent with functional competence of the GPIIb-IIIa receptor after activation of plasminogen in plasma.32

Degradation of platelet surface GPIb-IIIa has been reported in washed platelets during activation of plasminogen with t-PA.19 Our finding that GPIb-IIIa is not degraded in association with activation of plasminogen in plasma suggests that the results obtained in washed-platelet systems may not be extrapolated to explain antiaggregatory effects in plasma. In addition, degradation of GPIIb-IIIa in washed platelets in the absence of Ca2+ may explain discordant observations in studies of washed platelets in plasma. Irreversible dissociation of GPIIb-IIIa can be induced by incubation of washed platelets at 37°C with strong Ca2+-chelating agents such as EDTA.44-49 Chelation of Ca2+ at 23°C does not result in dissociation of GPIIb-IIIa at physiologic pH,45-49 suggesting that both physiologic temperature and the absence of Ca2+ are required to induce instability of the IIb-IIIa complex. We have shown that as few as two washes in ACD-containing buffer are sufficient to destabilize GPIIb-IIIa when platelets are subsequently incubated at 37°C in the absence of added Ca2+. Under these conditions, even in the absence of plasmin, the results of SDS-PAGE and autoradiography demonstrate that GPIIb and IIIa polypeptides are still present on the platelet surface but that MoAb 10E5 will no longer bind to the IIb-IIIa complex, even following restoration of Ca2+ before incubation with the antibody. In addition, the dissociated GPIIb-IIIa receptors on platelets in buffer at 37°C in the absence of Ca2+ are exquisitely sensitive to plasmin-mediated degradation. Marked degradation occurred with plasmin activity less than 0.1 CU/mL. However, at 37°C in the presence of exogenous Ca2+, binding of the complex specific MoAb 10E5 to the IIb-IIIa complex is maintained even when platelets are incubated with suprapharmacologic doses of t-PA or SK in the presence of plasminogen. Recently Beer and Coller have reported susceptibility of GPIIb to proteolysis by plasmin and other proteases on washed platelets in the presence of EDTA, although temperature conditions were not specified.53 Our observations are in agreement with theirs and suggest that plasmin can degrade both GPIIb and IIIa in the absence of Ca2+ at 37°C. However, our results also demonstrate that the GPIIb-IIIa does not undergo significant plasmin-mediated proteolysis in the presence of Ca2+.

Proteolysis of platelet GPIb has also been reported in washed platelets incubated with plasmin and with t-PA in the presence of plasminogen.17,19 Although the function of GP Ib does not require the presence of divalent cations,44 our observations in washed platelets suggest that it is more susceptible to plasmin-mediated degradation at 37°C in the absence of Ca2+. In incubations without plasminogen activators the binding of MoAb 6D1 is maintained regardless of temperature or Ca2+ conditions. This argues against a major change in the conformation of GPIb at 37°C in the absence of Ca2+. However, the increased proteolysis of GPIb at 37°C in
the absence of Ca\(^{2+}\) suggests that subtle changes in the conformation of GPIIb or changes in its microenvironment under these conditions render GPIIb more susceptible to proteolysis by plasmin.

We also observed a discrepancy between the degree of GPIIb degradation in washed platelets determined by SDS-PAGE and autoradiography and MoAb 6D1 binding with flow cytometry. For example, activation of plasminogen with t-PA (2.5 \(\mu g/mL\)) at 37°C in the absence of added Ca\(^{2+}\) resulted in a 50% decrease in GPIIb on autoradiography (Fig 4), while similar incubations caused no change in MoAb 6D1 binding on flow cytometry (Fig 8). This suggests that GPIIb receptors degraded by plasmin may be replaced by intact receptors from the internal platelet pool of GPIIb described by Michelson et al and others.\(^{51}\) However, we could detect no decrease in \(^{125}\)I surface-labeled GPIIb during activation of plasminogen in plasma with pharmacologic concentrations of t-PA and SK (Fig 1). Preliminary patient studies during fibrinolytic therapy for AMI have yielded discordant results.\(^{50,55}\) Administration of t-PA resulted in a small increase in platelet surface GPIIb, with a concomitant 19% decrease in total platelet GPIIb, compatible with replacement of degraded GPIIb receptors from the internal platelet pool.\(^{50}\) In contrast, SK therapy for AMI produced a 19% decrease in surface GPIIb but no change in GPIIIa.\(^{55}\) Our autoradiographic- and flow cytometric patient studies suggest that the amount of platelet surface GPIIb, GPIIb, and IIla does not change during infusion of t-PA, although the possibility of continuous replacement of degraded GPIIb receptors in vivo cannot be excluded by this method.

These discordant results will require further clinical study, but presently the following conclusions appear warranted from our observations and previous studies. First, GPIIb is readily hydrolyzed on washed platelets by high doses of plasmin.\(^{17,18}\) Second, proteolysis of GPIIb on washed platelets can be enhanced by physiologic temperatures and chelation of Ca\(^{2+}\). Third, degradation of GPIIb is modest in plasma
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during activation of plasminogen by pharmacologic concentrations of plasminogen activators and may be offset by replacement of degraded receptors from internal platelet stores.36,21 Finally, in view of the resistance of GPIb to plasmin-mediated proteolysis in plasma, the marked inhibition of ristocetin-induced platelet agglutination described by others following activation of plasminogen in plasma is likely due to factors other than the degradation of GPIb.17,19 Although small decreases in MoAb 12F1 binding to GPIa were seen with SK in plasma, generally GPIa was resistant to degradation in both plasma and washed-platelet studies. Because MoAb 12F1 does not block collagen binding to GPIa-IIa, it is possible that plasmin-mediated degradation of the collagen binding site of GPIa-IIa may occur. Because GPIa is difficult to locate consistently on one-dimensional SDS-PAGE studies, definitive conclusions concerning the effects of plasmin on Mg^{2+}-dependent platelet adhesion to collagen will require functional assays and perhaps the use of MoAbs that inhibit the function of GPIa-IIa.

Fitzgerald et al have demonstrated elevated levels of thromboxane metabolites indicative of platelet activation during plasminogen activation with SK and t-PA.4,13,14 However, activation of platelets in vivo may result from other factors, such as release of thrombin from clot,21 re-exposure of collagen in the ruptured atherosclerotic plaque,56,57 a procoagulant surface on the residual clot,21 or direct procoagulant effects attributable to plasmin interactions with the clotting system.29-31 We found no increase in the binding of MoAb PAC1, which specifically recognizes the GPIIb-IIIa complex on activated platelets, or MoAb S12, which binds to the α granule protein (GMP-140) expressed on the platelet surface after platelet secretion, demonstrating the absence of direct activation of platelets during activation of plasminogen in citrated plasma. In conclusion, while GPIIb-IIIa, and to a lesser extent GPIb, can be degraded by plasmin in purified systems in the absence of Ca^{2+}, these receptors are preserved during pharmacologic activation of plasminogen in plasma. Our observations suggest that functional impairment of the major platelet-adhesive receptors is unlikely to occur during activation of plasminogen in plasma. Thus, platelets in patients treated with fibrinolytic agents appear to retain the necessary complement of surface receptors required to mediate coronary reocclusion after successful fibrinolytic therapy.27 The mechanisms for platelet activation and impaired platelet function during pharmacologic thrombolysis remain undefined, but they appear to result from effects of activation of plasminogen on plasma components rather than direct effects on platelets.

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Dependence of plasmin-mediated degradation of platelet adhesive receptors on temperature and Ca2+

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