Thrombin-Induced Increase in Surface Expression of Epitopes on Platelet Membrane Glycoprotein IIb/IIIa Complex and GMP-140 Is a Function of Platelet Age

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Platelets are heterogeneous in the content of membrane glycoprotein (GPIIb/IIIa complex. To determine whether this heterogeneity is related to changes associated with platelet aging in the circulation, newly released platelets, obtained during recovery from nonimmune-mediated acute experimental thrombocytopenia in baboons, were studied. Monoclonal antibody (MoAb) binding to epitopes expressed on GPIIb/IIIa complex (LJ-CP8), GMP-140 (S12), and GPIa/IIa (12F1) was measured on control platelets (comprising platelets with a normal age distribution; mean age 60 to 72 hours) and newly formed platelets (mean age 12 hours), both in the resting state and after thrombin stimulation. Whereas LJ-CP8 binding to resting control platelets increased by 35% upon stimulation by \( \gamma \)-thrombin from 30,885 \( \pm \) 1,171 to 41,458 \( \pm \) 1,311 molecules/platelet at saturating concentrations of antibody, LJ-CP8 binding to resting young platelets did not increase significantly upon thrombin stimulation (31,878 \( \pm \) 3,330 and 33,791 \( \pm \) 3,486 molecules/platelet, respectively). Similarly, binding of antibody S12 in response to maximal thrombin stimulation was reduced by 42% from 10,246 \( \pm \) 834 molecules/platelet at saturating concentrations of S12 for control platelets to 5,971 \( \pm \) 865 molecules/platelet for young platelets (\( P = .001 \)). S12 binding to unstimulated platelets was less than 10% of the binding observed after thrombin stimulation at all concentrations of S12 for both control and young platelets. However, maximal binding of antibody 12F1 to resting control platelets did not differ significantly from that observed with resting young platelets (2,926 \( \pm \) 167 and 2,857 \( \pm \) 208 molecules/platelet, respectively), and 12F1 binding was unchanged after thrombin stimulation for both control and young platelets. We conclude that the thrombin-induced increase in the expression of epitopes on platelet membrane GPIIb/IIIa complex and GMP-140 is a function of platelet age.

MATERIALS AND METHODS

Animals studied. Juvenile male baboons (Papio cynocephalus anubis) weighing 9 to 12 kg were studied. All animals were observed to be disease-free for at least 6 weeks before study. The experimental protocols were approved by the institutional animal care and use committee and were in accordance with the Federal Guide for the Care and Use of Laboratory Animals (1985). All animals had an exteriorized arteriovenous (A-V) access shunt surgically implanted between the femoral artery and vein as described previously.16,19 The

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Membrane glycoproteins (GPs) are critical for maintenance of platelet functional integrity. They serve as cytoadhesins to mediate both platelet interactions with the subendothelium and platelet aggregation. Platelet membrane GPIIb/IIIa complex serves as the receptor for several adhesive proteins that mediate aggregation, including fibrinogen,13 fibronectin,4,6,8 vitronectin9,10 and von Willebrand factor (vWF).4,11-13

Although flow cytometry has established that individual platelets contain widely differing amounts of GPIIb/IIIa complex,14 the origin and significance of this heterogeneity remain unclear, and the extent to which cytoadhesins are modified as platelets age in the circulation is not known. Since the property of increased hemostatic effectiveness has been attributed to young platelets,15-22 and platelet senescence in vivo has been reported to be associated with a process of platelet membrane fragmentation,17,24 it is feasible that platelets may lose fibrinogen receptors by mechanisms involving the detachment of membrane GPs during the process of reversible hemostatic interactions. Alternatively, an enhanced functional capacity of young platelets could be a consequence of the presence of GPIIb/IIIa complexes with increased receptor function which undergo conformational changes or redistribution after reversible interactions with agonists or the vascular endothelium. Such modifications may result in a progressive decrease in receptor affinity for fibrinogen as platelets age in the circulation.

We therefore evaluated epitopes expressed on GPIIb/IIIa complex on the surface membrane of newly formed platelets released into the circulation within 24 hours after onset of nonimmune-mediated acute thrombocytopenia in baboons. We also defined the thrombin-induced changes in the expression of GPIIb/IIIa epitopes on newly formed platelets. Since an inducible pool of GPIIb/IIIa, associated with the platelet \( \alpha \)-granule, has been demonstrated,23 we concurrently studied the surface expression of the \( \alpha \)-granule membrane protein GMP-14024 (granule membrane protein, molecular weight [mol wt] 140,000, previously identified as GPIIa) or platelet activation-dependent granule-external membrane (PAD-GEM)25 protein, which is expressed on the platelet surface only after degranulation. We report that although expression of GPIIb/IIIa epitopes on unstimulated young platelets is similar to that on unstimulated control platelets, the thrombin-induced increase in expression of GPIIb/IIIa and GMP-140 epitopes is diminished on newly formed platelets.

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A-V shunt did not detectably shorten platelet survival or produce measurable platelet activation.30

Acute experimental thrombocytopenia. Baboons were rendered acutely thrombocytopenic by a rapid, nonimmune procedure developed in this laboratory and previously reported in detail.31 Essentially, platelets were irreversibly removed from the circulation by perfusion of heparinized blood (heparin sodium, 100 U/kg, ESI Pharmaceuticals, Cherry Hill, NJ) through a series of columns containing spherical glass microbeads (Ferro, Jackson, MI) and distally placed 20 μm pore size pediatric transfusion filters (Fenwal, Deerfield, IL) interposed between the arterial and venous limbs of the A-V shunt. This procedure efficiently removed >90% of all circulating platelets during a one-hour exposure period. Typically, the platelet count decreased acutely from 509,000 ± 107,000/μL to 36,000 ± 13,000/μL. Platelet counts recovered in a linear manner at the rate of 115,000 ± 11,000 platelets/μL/day.

Autologous baboon platelets were labeled with 51Cr sodium chromate before the procedure to determine the number of "residual" platelets remaining in the circulation at serial times during the recovery phase. At 24 hours after onset of thrombocytopenia, the platelet population consisted of 72 ± 4.5% (n = 11) newly released platelets (by subtraction of the "residual" population from the total platelet population). The young platelet population (defined as a platelet population with a mean age ≤12 hours) was harvested for evaluation of platelet surface membrane GPs as described below.

New platelets released into the circulation under these conditions were subsequently removed from the circulation by predominantly senescent mechanisms after an average lifespan of 6.2 days.32 This procedure enables a narrow age-cohort platelet population to be harvested during recovery from thrombocytopenia, before changes in megakaryocyte maturation rate, size, number and ploidy occur. Thus, platelets released into the circulation during the immediate recovery phase derive from mature megakaryocytes which have not been exposed to the potential adverse effects of drugs,13-33 radiation,17 or antiplatelet sera.23,24 In contrast to previous studies in which antiplatelet antibodies were used to induce acute thrombocytopenia,23-34 platelets produced in response to nonimmune-mediated acute thrombocytopenia in baboons do not have an increased mean platelet volume.35 We reason that the properties of the newly produced platelets in these studies more accurately reflect those of normal young platelets than do platelets produced after use of other methods to induce acute thrombocytopenia.

Monoclonal antibodies (MoAbs). LJ-CP8 (IgG), a murine MoAb directed against GPLIIb/IIIa complex on human platelets, was supplied by Dr Z. Ruggeri (Scripps Clinic and Research Foundation, La Jolla, CA) and purified from ascites fluid by our laboratory using chromatography on Affi-gel Blue48 (Bio Rad, Richmond, CA). This MoAb is complex specific.13 Purity of the IgG preparation was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) (SDS-PAGE).13 MoAb S12 (IgG2a) was a gift from Dr R. McEver (University of Oklahoma, Oklahoma City). This antibody reacts with the α-granule membrane glycoprotein GMP-140,14,29 expressed on human platelets only after degranulation, and was purified from ascites fluid by a previously described method.46 MoAb 12F1 (IgG1), which recognizes the GPla/IIa heterodimer on human platelets was prepared as previously described.46 All purified MoAbs were stored at −70°C before use.

Radiiodination of MoAbs. Purified LJ-CP8 and S12 were radiolabeled with carrier-free Na 125I (Amersham, Arlington Heights, IL) by iodogen (Pierce Chemical, Rockford, IL) and the method described by Fraker and Speck.29 Specific activity ranged from 0.3 to 0.9 μCi/μg for LJ-CP8 and from 0.7 to 0.9 μCi/μg for S12. 12F1 was radiolabeled with carrier-free Na 125I using Chloramine T (Eastman Kodak, Rochester, NY).46 Specific activity ranged from 4.0 to 5.2 μCi/μg. Radioactivity was >95% precipitable in 10% trichloroacetic acid (TCA) for all 125I-labeled MoAbs.

Binding of MoAbs to baboon platelets. Although the binding of LJ-CP8, S12, and 12F1 to human platelets had been extensively characterized,39,44-46 their binding to baboon platelets has not been previously evaluated directly. LJ-CP8 inhibited binding of baboon fibrogenin to thrombin-stimulated baboon platelets in vitro and has potent antithrombotic and antithrombogenic effects in vivo.29 In the present study, binding of LJ-CP8, S12, and 12F1 to resting and thrombin-stimulated baboon platelets was evaluated on a quantitative basis.

Binding of LJ-CP8 to unstimulated baboon platelets. Blood was drawn into acid-citrate dextrose (ACD) anticoagulant (1:9 vol/vol) containing 5 mmol/L EDTA and 1 μmol/L prostaglandin E1 (PGE1, Sigma Chemical, St Louis) and platelet-rich plasma (PRP) was prepared by differential centrifugation.31 Platelets were separated from plasma by filtration through Bio-Gel A-50m agarose (Bio Rad) using a modified Tyrode’s buffer, pH 7.47 containing EDTA (5 mmol/L) and PGE1 (1 μmol/L). Gel-filtered platelets were incubated with varying amounts of 125I-LJ-CP8, in a final volume of 200 μL, for 20 to 30 minutes at 22°C. The final platelet concentration in the incubation mixture was 5 × 107/mL, and 125I-LJ-CP8 concentrations ranged from 1.4 μg/mL to 1.4 mg/mL.

Platelets were separated from the reaction mixture by layering triplicate aliquots (50 μL) onto 20% sucrose (300 μL) in modified Tyrode’s buffer containing 2% bovine serum albumin (BSA) in a microcentrifuge tube (Sarstedt, Numbrecht, FRG) and centrifuged at 13,000 g (Savant Instruments, Farmingdale, NY) for four minutes at 22°C. The tip of the tube containing the pelleted platelets was amputated, and the platelet-associated radioactivity was counted in a γ-scintillation counter. The presence of 2% BSA in the sucrose was essential for optimum recovery of labeled platelets.

Nonspecific binding was evaluated by incubating 20- to 100-fold excess unlabeled LJ-CP8 for 30 minutes before adding varying amounts of 125I-LJ-CP8. Binding isotherms were analyzed by the Ligand program,47 assuming one class of noninteracting binding sites, for determination of the number of molecules bound per platelet, with the dissociation constant (kd) defined as the free antibody concentrations required to give 50% maximum binding. Pilot studies showed that maximum binding of antibody occurred after 20-minute incubation under the conditions used. Binding assays performed with PRP instead of gel-filtered platelets yielded essentially identical results. Use of whole blood in the incubation mixture was avoided since sedimentation of erythrocytes through the sucrose resulted in passage of unbound 125I-LJ-CP8 to the tip of the microcentrifuge tube. To evaluate the effect of Ca2+ on binding of LJ-CP8 to unstimulated platelets, we replaced EDTA with CaCl2 (2 mmol/L).

LJ-CP8 binding to thrombin-stimulated baboon platelets. Blood was sampled into ACD anticoagulant (1:9 vol/vol) containing EDTA (5 mmol/L), and gel-filtered platelets were prepared as described previously except that PGE1 was omitted. Gel-filtered platelets (1.0 × 107 to 4.0 × 107 platelets/μL) were incubated with γ-thrombin (a gift from Dr J.W. Fenton II, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany) at 22°C for five minutes before incubation with 125I-LJ-CP8 under the conditions described previously. The amount of γ-thrombin used induced >65% release of 3H-serotonin and exceeded the amount required for maximally increased LJ-CP8 binding.

S12 binding to thrombin-stimulated baboon platelets. S12 binding was evaluated by incubating γ-thrombin-stimulated platelets with varying concentrations of 125I-S12 ranging in concentration from 0.01 to 10.0 μCi/mL in a final volume of 200 μL under conditions described for LJ-CP8. Nonspecific binding of S12 and
binding of S12 to unstimulated platelets was negligible when PGE1 (1 μmol/L) was used.

12F1 binding to unstimulated baboon platelets. 12F1 binding to baboon platelets was evaluated by incubating platelets with varying amounts of [125I-12F1 ranging from 5.0 ng/mL to 2.0 μg/mL using conditions described for binding of LJ-CP8 to unstimulated platelets, except that an incubation time of 60 minutes was required for maximum 12F1 binding. Binding of 12F1 to baboon platelets did not change on stimulation by γ-thrombin, and nonspecific binding was <2% over the entire antibody concentration range studied.

Serotonin release assay. To assess the degree of γ-thrombin stimulation required for the maximum increase in LJ-CP8 and S12 binding, we evaluated the effect of γ-thrombin dose on the extent of serotonin release and on the increased binding of LJ-CP8 and S12 as compared with unstimulated platelets. In these studies, gel-filtered platelets (100,000 to 300,000/μL) were incubated with [3H]-serotonin (1.0 nCi/mL) for 15 minutes at 37°C. Imipramine (1 μmol/L final concentration) was then added to inhibit uptake of released serotonin. Labeled platelets were incubated with varying amounts of γ-thrombin for five minutes at 22°C, and the amount of [3H]-serotonin released was measured in the supernatant after platelets were separated by centrifugation at 13,000 g for five minutes. Total uptake of [3H]-serotonin was typically >75% under these conditions. Release of [3H]-serotonin ranged from 2% to 80% depending on the dose of γ-thrombin used. Binding of S12 to stimulated baboon platelets was maximum when platelets were stimulated to a degree sufficient to produce >65% serotonin release.

RESULTS

LJ-CP8 binding to baboon platelets. Binding of LJ-CP8 to unstimulated and thrombin-stimulated baboon platelets is shown in Fig 1. Binding to resting control platelets (mean age 60 to 72 hours) increased by 34.2 ± 4.3% on stimulation by γ-thrombin from 30,885 ± 1,171 to 41,458 ± 1,311 molecules/platelet (mean ± SEM, n = 11) (Table 1). This difference was statistically significant (P = .005, t test for comparison of two independent means with unequal variance). Nonspecific binding was <1% for both resting and thrombin-stimulated platelets. The dissociation constant (kd) of LJ-CP8 was not significantly different for thrombin-stimulated platelets as compared with unstimulated platelets (1.74 ± 0.31 x 10^-7 and 1.31 ± 0.25 x 10^-7 mol/L respectively, P = .278). The extent of antibody binding to platelets from different animals varied greatly. Binding of LJ-CP8 varied from 21,886 to 48,013 molecules/platelets for unstimulated platelets and from 26,851 to 48,494 molecules/platelets for thrombin-stimulated platelets. For this reason, paired difference analyses were computed to assess the significance between resting and stimulated platelets. All data displayed Gaussian distributions (Wilk-Shapiro test for normality). Similar results were obtained when Fab' fragments were used instead of IgG (results not shown). In previous studies with human platelets, maximum LJ-CP8 binding ranged from 27,500 to 67,100 molecules/platelet for unstimulated platelets and from 45,200 to 131,900 for thrombin-stimulated platelets. Comparative results were obtained in our laboratory with [125I]LJ-CP8 binding to human platelets (range 52,000 to 97,000 and 78,000 to 130,000 molecules/platelet for unstimulated and thrombin stimulated platelets respectively).

Binding of LJ-CP8 to human platelets, whether stimulated or not, was decreased by >80% when platelets were preincubated with EDTA (5 mmol/L) for 30 minutes at 37°C as compared with binding with Ca2+ added (2 mmol/L).44 Pilot studies of the effect of EDTA and divalent cations showed that binding of LJ-CP8 to baboon platelets was unchanged at 22°C with Ca2+ added (2 mmol/L), Mg2+ (2 mmol/L), or EDTA (5 mmol/L), for both resting and thrombin-stimulated platelets.

Unstimulated young platelets bound 31,878 ± 3,330 molecules/platelet at saturation, a value that was not significantly different from control platelets (comprising platelets with a normal age distribution, P = .932, Table 1). Maximum antibody binding to thrombin-stimulated young platelets at saturating concentrations of LJ-CP8 was 33,791 ± 3,486 and was not significantly different from that observed for unstimulated platelets (P = .695, Table 1). Binding to thrombin-stimulated platelets was maximum under the conditions used; higher doses of γ-thrombin did not result in further increases in LJ-CP8 binding. Thus, the thrombin-induced increase in binding of LJ-CP8 to control platelets was abolished with newly released platelets. The kd of LJ-CP8 binding to thrombin-stimulated young platelets was not significantly different from the value obtained for unstimulated young platelets (1.1 x 10^-7 and 8.4 x 10^-6 mol/L, respectively, P = .359) or for control platelets (P = .099, Table 1).

The thrombin-induced increase in the number of molecules of [125I]LJ-CP8 bound/platelet at different concentrations of [125I]LJ-CP8 is shown in Fig 2. Binding to control platelets at saturation increased by 10,068 ± 1,511 mole-
does not significantly increase after thrombin stimulation. Young platelets at saturating concentrations of $^{125}$I-LJ-CP8 bound 2,857 ± 208 molecules/platelet, a value that was not significantly different from that obtained with young platelets (2,857 ± 208 molecules/platelet, 48 hours after onset of thrombocytopenia, S12 binding at saturation after maximum stimulation by γ-thrombin was intermediate between newly released platelets and control platelets (results not shown), indicating a progressive shift toward the normal GMP-140 response profile as platelets age in vivo.

**S12 binding to baboon platelets.** Binding of S12 to thrombin-stimulated and unstimulated platelets is shown in Fig 3. Binding to control platelets was 10,246 ± 317 molecules/platelet (kd = 9.7 ± 0.4 x 10$^{-10}$ mol/L) after maximal thrombin stimulation at saturating concentrations of S12. The number of molecules bound was the same in the presence of EDTA (5 mmol/L) or Ca$^{2+}$ (2 mmol/L). In contrast, maximum binding to young platelets was decreased by 42% (P < 0.001) to 5,971 ± 665 molecules/platelet (kd = 1.1 ± 0.3 x 10$^{-9}$ mol/L). It has been shown for human platelets that the thrombin-induced increase in S12 binding occurs concurrently with $^{14}$C-serotonin secretion, in a dose-dependent manner.9 In the present study, $^{3}$H-serotonin secretion from young platelets was essentially identical to that measured for control platelets (66.5% ± 2.6% and 68.2% ± 4.0%, respectively) in response to the same dose of γ-thrombin. Higher doses of γ-thrombin did not result in further increases in S12 binding for either control or young platelets. Previous studies have shown that young platelets harvested in this manner are not deficient in the α-granule proteins platelet factor-4 and β-thromboglobulin.11 Thus, the decreased expression of epitopes associated with GMP-140 on young platelets is unlikely to be due to a reduction in α-granule number. S12 has been suggested to serve as a useful indicator of in vivo platelet activation.39,48 In the present study, S12 did not bind to unstimulated young platelets (Fig 3), indicating the absence of circulating platelets that had undergone degranulation. At 48 hours after onset of thrombocytopenia, S12 binding at saturation after maximum stimulation by γ-thrombin was intermediate between newly released platelets and control platelets (results not shown), indicating a progressive shift toward the normal GMP-140 response profile as platelets age in vivo.

**12Fl binding to baboon platelets.** Binding of 12Fl to unstimulated platelets is shown in Fig 4. Control platelets at saturating concentrations of 12Fl bound 2,926 ± 167 molecules/platelet (kd = 3.1 ± 0.3 x 10$^{-10}$ mol/L), a value that was not significantly different from that obtained with young platelets (2,857 ± 208 molecules/platelet,
Evidence shows that young platelets are hemostatically more effective in vivo than old platelets. Blajchman et al. examined differences in the hemostatic function, survival, and membrane GPs in young and old platelets from rabbits during marrow suppression and recovery and suggested that platelets become smaller and hemostatically less effective as they age in the circulation. Studies in rabbits by George et al., demonstrating that platelets lose surface GPs as they age, appear to support that hypothesis. Haver and Gear showed that young platelets are functionally more active than old platelets and suggested that these differences are related to their larger size. However, more recent studies by Thompson et al. have shown that in the baboon, size and age are both determinants of platelet function, but through independent mechanisms. Furthermore, data from our laboratory have shown that whereas platelets produced in response to nonimmune-mediated acute thrombocytopenia in baboons, theoretically, several abnormalities of the GPIIb/IIIa complex could lead to decreased platelet function. These include (a) a deficiency of these GPs, possibly by fragmentation of platelets and shedding of membranes in response to platelet activation; (b) a conformational instability of the GPIIb/IIIa complex; and (c) a defect in either fibrinogen receptor exposure or fibrinogen binding.

The present data show that the number of GPIIb/IIIa antigenic sites for LJ-CP8 on unstimulated young platelets (mean age < 12 hours) does not differ significantly from that measured on normal control platelets (mean age 60 to 72 hours). Therefore, an actual loss of GPIIb/IIIa complex probably does not account for the putative decrease in hemostatic function associated with platelet aging in vivo. This conclusion is consistent with the observation that heterozygote carriers for Glanzmann’s thrombasthenia show reduced platelet membrane GPIIb/IIIa complexes but have normal platelet function without excessive bleeding. However, this may reflect, in part, the ability of platelets to adhere to subendothelial surfaces by interactions with vWF through GPIb and collagen through GPIa. A wide range in the number of GPIIb/IIIa epitopes expressed on normal individual human platelets has been reported, from 27,500 to 67,100 sites/platelet for unstimulated platelets, and from 45,200 to 131,900 sites/platelet for thrombin-stimulated platelets. Thus, the number of GPIIb/IIIa complexes on normal platelets probably exceeds the minimum number required for optimum hemostatic effectiveness. In the present study, no significant differences were noted in the kd of LJ-CP8 binding to unstimulated young platelets and unstimulated control platelets. However, these data do not exclude possible differences in the affinity of the GPIIb/IIIa complex for fibrinogen.

Thrombin stimulation of control baboon platelets resulted in an increased number of surface-oriented epitopes associated with GPIIb/IIIa. The magnitude of this increase (35%) was lower than that reported for human platelets, in which a twofold increase in LJ-CP8 binding was observed. The increased antibody binding to GPIIb/IIIa epitopes on normal platelets in vivo may lead to expression of functional receptors, new membrane GPs, such as GMP-140 and thrombospondin, or an increased expression of preexisting epitopes, as with GPIIb/IIIa. Binding of MoAb 12F1 to another major membrane GP, GPIa (VLA-2), a receptor for collagen, was not increased after thrombin stimulation of control baboon platelets. Thus, the increased binding of LJ-CP8 appears to be specific for GPIIb/IIIa rather than a nonspecific consequence of changes associated with platelet activation.

Although abnormalities in platelet membrane GPs associated with congenital bleeding have been well documented, changes in intrinsic plasma membrane GPs associated with platelet aging in the circulation have not yet been defined. Since changes in platelet surface GPs during the life span of platelets may contribute to platelet senescence and be associated with an increased risk for bleeding or thrombosis, we evaluated the surface expression of GPIIb/IIIa epitopes on newly formed platelets released into the circulation within 24 hours after onset of nonimmune-mediated acute thrombocytopenia in baboons.
platelets contains the GPIIb/IIIa complex as part of its structure, which, unlike the receptor on unstimulated platelets, does not require a cofactor. Although the GPIIb/IIIa complex is primarily located on the surface plasma membrane, thrombin stimulation of platelets may result in translocation of GPIIb/IIIa molecules from intracellular pools. Wencel-Drake et al demonstrated the presence of GPIIb/IIIa in the α-granule membrane which becomes fused with the surface membrane on granule secretion. Other researchers have suggested that the increased number of GPIIb/IIIa epitopes expressed on stimulated human platelets is related to the presence of GPIIb/IIIa complexes within the superficial canalicular membrane system which become accessible upon stimulation.

Since significant amounts of GPIIb/IIIa complex are believed to be present in the α-granule membrane, the process of granule secretion could result in direct expression of receptor complexes for adhesive proteins at the cell surface. Such a mechanism would differ considerably from one in which latent receptors are confined to the surface membrane, undergo conformational changes on platelet stimulation, and subsequently bind to ligands that are either secreted from platelets or are present in the plasma. Adhesive proteins may already be bound to α-granule membrane receptors in unstimulated young platelets and direct surface expression of the receptor–adhesive protein complex may occur after platelet stimulation. Such a model could explain the increased functional efficiency of young platelets by allowing rapid expression of adhesive proteins on the cell surface during platelet stimulation. This model also permits a decrease in surface expression of latent epitopes for GPIIb/IIIa.

To investigate further the expression of α-granule membrane GPs in young platelets, we concurrently studied surface expression of the α-granule protein GMP-140 with MoAb S12. Maximum antibody binding to GMP-140 after thrombin-induced secretion was markedly reduced in young platelets to 58% of the value obtained with thrombin-stimulated control platelets. Although this reduction may reflect an actual decrease in the platelet content of GMP-140, previous studies from this laboratory demonstrated that newly released platelets are not deficient in α-granule proteins, platelet factor 4, or β-thromboglobulin. Our data support the following concepts: (a) α-granule membrane GPs, including GMP-140 and GPIIb/IIIa, may undergo a change in structure or conformation or be influenced by a change in the surrounding microenvironment during platelet maturation in the circulation; (b) fusion of α-granule membrane components with the external membrane after granule secretion may be incomplete or impaired in young platelets; (c) α-granule membrane receptors in unstimulated young platelets may already have ligands bound which become directly exposed on the platelet surface after granule secretion. Thereby inhibiting antibody binding to antigenic sites on these receptors. Although the functional role of GMP-140 is currently unknown and the consequences of its deficiency are undefined, it mays serve as an internal receptor for α-granule coalescence, movement, or release or may mediate the receptor and procoagulant activities of the stimulated platelet membrane. Since our studies and those of other investigators indicate that on the average 10,500 molecules exist on the plasma membrane of stimulated platelets, GMP-140 appears to constitute a major component of platelet α-granule membranes and could therefore play an important role in hemostasis.

The decrease in immunoreactive GMP-140 on stimulated young platelets may be selectively severe in a small subpopulation of platelets or may reflect a partial decrease in epitope expression on all platelets. Platelet analysis by flow cytometry should help clarify this by permitting evaluation of platelets at the single-cell level. Johnston et al measured the number of molecules of fluorescein-labeled S12 antibody bound to individual platelets by flow cytometry and demonstrated that normal human platelets display marked heterogeneity in expression of GMP-140 epitopes in response to thrombin stimulation. Thus, in some platelets, expression of GMP-140 epitopes was complete in response to minimal thrombin concentrations, whereas other platelets failed to express any GMP-140 epitopes for S12 even at the highest thrombin concentrations. Based on our studies, we propose that heterogeneity in GMP-140 epitope expression is related to changes associated with platelet aging in the circulation in addition to factors such as the heterogeneity of platelet α-granule content or differences in sensitivity of individual platelets to thrombin stimulation. In the present study, S12 binding to thrombin-stimulated young platelets was strikingly reduced as compared with control platelets despite the same degree of stimulation (>. 50% 3H-serotonin release). Since higher concentrations of thrombin did not elicit further S12 binding to either young platelets or control platelets, we conclude that maximum binding of S12 had occurred and that differences in sensitivity to thrombin stimulation could not account for the observed decrease in S12 binding to young platelets.

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Thrombin-induced increase in surface expression of epitopes on platelet membrane glycoprotein IIb/IIIa complex and GMP-140 is a function of platelet age

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