Recognition of Platelet-Associated Fibrinogen by Polyclonal Antibodies: Correlation With Platelet Aggregation

By E.I.B. Peerschke

Progressive decreases in platelet-bound fibrinogen accessibility to antibody and enzymes were recently reported to occur after adenosine diphosphate (ADP)-induced fibrinogen binding. Because previous studies also indicated that platelets that are activated but not aggregated by ADP in the presence of fibrinogen lose their ability to aggregate in a time-dependent manner despite negligible changes in fibrinogen binding, the present study examined the relationship between platelet aggregation and accessibility of platelet-bound fibrinogen to specific polyclonal antibody F(ab′)$_2$ fragments over a 60-minute time course. Although ^{125}I-fibrinogen binding remained virtually unchanged, comparison of antifibrinogen antibody F(ab′)$_2$ binding and platelet aggregation 5 minutes and 60 minutes after platelet stimulation with ADP or thrombin showed decreases in F(ab′)$_2$ binding of 62% ± 13% and 73% ± 7% (mean ± SD, n = 5), respectively, and decreases of 65% ± 16% and 60% ± 10% in platelet aggregation. In contrast, platelets stimulated with A23187 or chymotrypsin retained 87% ± 16% and 76% ± 12% of their ability to aggregate over the same time course, and lost only 39% ± 14% and 36% ± 12% of their ability to bind antifibrinogen antibody F(ab′)$_2$ fragments, respectively. Pretreatment of ADP-stimulated platelets with chymotrypsin largely prevented the progressive loss of platelet aggregability and the accompanying decreased recognition of bound fibrinogen by antifibrinogen F(ab′)$_2$ fragments. Preincubation of platelets with cytochalasin D (30 μg/mL) also inhibited the decrease in platelet aggregation after exposure of ADP-treated platelets to fibrinogen over a 60-minute time course. This was accompanied by only a 25% ± 18% decrease in antifibrinogen antibody F(ab′)$_2$ binding. Present data support the hypothesis that qualitative changes in platelet-bound fibrinogen correlate with loss of the ability of platelets to aggregate, and implicate both the platelet cytoskeleton and chymotrypsin-sensitive surface membrane structures in modulating qualitative changes in bound fibrinogen on the platelet surface.

INDUCTION OF FIBRINOGEN receptors is a consequence of platelet stimulation by a wide variety of agonists.[1] These include small molecules such as adenosine diphosphate (ADP) and epinephrine, proteases such as thrombin and chymotrypsin, large molecules such as collagen, and various calcium ionophores. Whereas it is certain that platelet stimulation is a prerequisite for fibrinogen binding, the mechanism of fibrinogen receptor induction is incompletely understood. A variety of intracellular mediators have been implicated including G-proteins, protein kinase C activation, IP_3, and calcium mobilization.[2] Although the number of fibrinogen receptors exposed after platelet stimulation varies with agonist concentration and type,[1] fibrinogen binding characteristics are similar. Binding occurs predominantly to the platelet membrane glycoprotein (GP) IIb-IIIa complex,[1] is divalent cation dependent,[1] and can be inhibited by synthetic peptides mimicking the α-chain Arg-Gly-Asp (RGD) sequence or the γ-chain pentadecapeptide sequence of fibrinogen.[3,4]

Fibrinogen binding is a prerequisite for platelet aggregation.[1] Although fibrinogen binding is necessary, several studies suggest that it may not be sufficient for aggregation.[5-7] For example, platelets that are activated but not aggregated by ADP in the presence of fibrinogen lose their ability to aggregate in a time-dependent manner, despite negligible changes in fibrinogen binding.[5] This dissociation between fibrinogen binding and platelet aggregation, and recent observations of progressive decreases in platelet-associated fibrinogen accessibility to antibody with time after equilibrium fibrinogen binding, suggest the hypothesis that qualitative changes in surface membrane-bound fibrinogen after platelet stimulation may correlate with the ability of activated platelets to aggregate. This hypothesis is consistent with reports by Lindon et al,[8] who showed a direct relationship between platelet adhesion to fibrinogen-coated surfaces and antifibrinogen antibody binding to deposited fibrinogen. Thus, the present study was designed to evaluate the potential correlation between the ability of ADP-, thrombin-, A23187-, or chymotrypsin-treated platelets to aggregate and changes in bound fibrinogen recognition by antifibrinogen F(ab′)$_2$ fragments at various times after platelet exposure to fibrinogen.

MATERIALS AND METHODS

Platelet preparation. Blood was obtained from volunteers after receiving informed consent. Samples were collected into 0.1 vol 3.2% sodium citrate and 0.05 vol 1 mmol/L aspirin. Platelet-rich plasma (PRP) was prepared by centrifugation (280*g* for 15 minutes). Platelets were separated from plasma by gel-filtration over Sepharose 2B (Pharmacia, Piscataway, NJ) equilibrated with 0.01 mol/L HEPES-buffered, modified Tyrode's solution (HBMT), pH 7.5, containing no added calcium, 2 mmol/L magnesium, 2 mg/mL bovine serum albumin (fatty acid free; Sigma Chemical Co, St Louis, MO), and 1 mg/mL dextrose, as previously described.[9] Platelet suspensions contained 250,000 to 300,000 platelets/μL.

Prostaglandin E_1 (PGE_1) treated and formalin-fixed platelets were prepared as follows. Unstimulated, gel-filtered platelets were incubated with 0.1 μmol/L PGE_1 (Sigma; 30 minutes at 22°C) or an equal volume of 2% formalin (60 minutes at 22°C). Samples were washed twice by centrifugation (1,000*g* for 20 minutes) and resuspension in 0.15 mol/L NaCl containing 5 mmol/L EDTA. Washed, PGE_1-treated or formalin-fixed platelets were finally resuspended in HBMT.

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Fibrinogen preparation. Band I fibrinogen was purified from fresh-frozen plasma as previously described.[9] It was radiolabeled with ^{125}iodine and an immobilized oxidant (N-chlorobenzenesulfonamide:iodobeads; Pierce Chemical Co, Rockford, IL) according to the instructions provided by the manufacturer. Protein-bound and free iodine were separated by gel-filtration over Sephadex G-25 (Pharmacia) equilibrated with 0.15 mol/L NaCl.

Antifibrinogen antibodies. Affinity-purified, antifibrinogen IgG from rabbits (Accurate Chemical and Scientific Co, Westbury NY) was digested with pepsin to produce $F(ab')_2$ fragments.[10] Fc fragments were absorbed on protein A using a 10% cell suspension of *Staphylococcus aureus* (Sigma). $F(ab')_2$ fragments were labeled with ^{125}iodine using iodobeads (Pierce) as described for fibrinogen above.

Platelet stimulation. Platelets were stimulated with a variety of agonists. ADP (10 μmol/L) or A23187 (5 μmol/L) were added to platelet suspensions in the presence of either ^{125}I-labeled (10 μg/mL) or unlabeled fibrinogen (10 μg/mL, 100 μg/mL). Additional platelet samples were stimulated with 150 mU/mL human thrombin (a kind gift from Dr J Fenton, NY State Department of Health, Albany, NY). Thrombin was neutralized with a 10-fold excess of hirudin 5 minutes after platelet stimulation, and before addition of fibrinogen. Other platelet samples were exposed to chymotrypsin (200 μg/mL; Worthington, Freehold, NJ) for 15 minutes at 37°C. Chymotrypsin was neutralized with a 10-fold molar excess of phenylmethylsulfonyl fluoride (PMSF). EDTA (5 mmol/L) was added to the platelet suspension, and the preparation was centrifuged (15 minutes at 1,000*g* at 22°C). The resulting platelet pellet was resuspended in HBMT, followed immediately by the addition of fibrinogen. In some experiments, chymotrypsin-treated platelets were also stimulated with 10 μmol/L ADP before adding fibrinogen. Stimulated platelets were incubated for up to 60 minutes (22°C) in the presence of fibrinogen without agitation to prevent aggregation. No macroscopic aggregates were noted over this time course.

Chymotrypsin-treated platelets were also evaluated for GPIb degradation and GPIIIa digestion. GPIb degradation was assessed using the 6D1 monoclonal antibody (MoAb) (a gift from Dr B. Coller, SUNY, at Stony Brook).[11] GPIIIa digestion was assessed using a monoclonal anti-GPIIIa antibody (SZ 21; AMAC, Inc, Westbrook, ME) on Western blots of lysed chymotrypsin-treated platelets.[12]

In other experiments, platelets were preincubated (30 minutes at 22°C) with 30 μg/mL cytochalasin D (Sigma) before stimulation. Platelets incubated with dimethyl sulfoxide (DMSO) served as controls. Activation-induced cytoskeleton formation was assessed based on the ability of platelets to retract reptilase-induced (Bothrops atrox serine proteinase; Abbot Laboratories, N. Chicago, IL) fibrin clots at 37°C, as described previously.[13] The extent of clot retraction (percentage) was calculated based on the amount of fluid expressed from the clot relative to the starting volume of the platelet/fibrinogen suspension.

Fibrinogen and antifibrinogen $F(ab')_2$ binding. Stimulated platelets were incubated with ^{125}I-fibrinogen (10 μg/mL) at 22°C without stirring. ^{125}I-fibrinogen binding was assessed after 5 minutes and 60 minutes by centrifugation of duplicate sample aliquots through silicone oil. Platelet-associated radioactivity was quantified in a gamma counter (MicroMedic Systems 4/600; Rohm and Haas Co, Horsham, PA). Nonspecific ^{125}I-fibrinogen binding was assessed in the presence of 10 mmol/L EDTA or 5 mg/mL unlabeled fibrinogen.[9]

In parallel experiments, stimulated platelets were incubated (22°C) in the presence of 10 μg/mL unlabeled fibrinogen. After 5 minutes and 60 minutes, ^{125}I-antifibrinogen $F(ab')_2$ fragments (50 μg/mL) were added. $F(ab')_2$ binding was assessed after a subsequent 30 minutes of incubation (22°C), when equilibrium $F(ab')_2$ binding had occurred.[14] Nonspecific $F(ab')_2$ binding was quantified using stimulated platelets that were incubated with fibrinogen in the presence of 10 mmol/L EDTA to prevent specific platelet-fibrinogen interactions.[9]

Platelet aggregation. The ability of platelets to aggregate was evaluated in parallel with their ability to bind antifibrinogen antibody $F(ab')_2$ fragments. Separate suspensions of stimulated platelets were incubated (22°C) with 0.1 mg/mL unlabeled fibrinogen. Sample agitation was minimal to prevent aggregation during the incubation period. After 5 minutes and 60 minutes, 0.5 mL aliquots were removed and aggregated in a dual channel aggregometer with the temperature set to 37°C (Chronolog Corp, Haverton, PA). Instrument baselines were adjusted to 90% light transmission using a buffer blank, and 10% light transmission using unstimulated platelets. The extent of platelet aggregation was quantified and used as an indicator of platelet function.

RESULTS

A comparison of fibrinogen and antifibrinogen antibody $F(ab')_2$ binding to platelets after exposure to 10 μg/mL exogenous fibrinogen for 5 minutes and 60 minutes is presented in Fig 1. Platelets were stimulated with ADP, thrombin, chymotrypsin, or A23187. As reported previously,[14] antifibrinogen $F(ab')_2$ fragment binding to ADP-treated platelets declined markedly during the 60 minutes of incubation, despite little change in overall fibrinogen binding. Similar observations were made with thrombin-stimulated platelets. In contrast, platelets stimulated with A23187 or chymotrypsin retained significantly more of their ability to bind antifibrinogen $F(ab')_2$ fragments over the same time course ($P < .05$).

Parallel studies showed that decreases in antifibrinogen antibody $F(ab')_2$ fragment binding to ADP- and thrombin-treated platelets correlated with decreases in platelet aggregation (Fig 1). Moreover, platelets stimulated with A23187 or chymotrypsin retained most of their ability to aggregate 60 minutes after exposure to 0.1 mg/mL fibrinogen (Figs 1 and 2A), consistent with the smaller, observed decreases in antifibrinogen antibody $F(ab')_2$ binding. When equal parts of fresh, chymotrypsin-treated platelets were combined with ADP-treated platelets that had been incubated with fibrinogen for 60 minutes, the platelet mixture aggregated

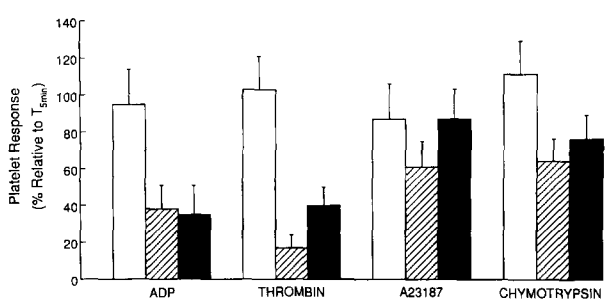

Fig 1. Modulation of fibrinogen binding (□), antifibrinogen antibody $F(ab')_2$ binding (▨), and platelet aggregation (■) 60 minutes after exposure of platelets stimulated with 10 μmol/L ADP, 150 mU/mL thrombin, 5 μmol/L A23187, or 200 μg/mL chymotrypsin to fibrinogen. Platelet responses are presented as a percentage (mean ± SD, n = 5) relative to responses observed 5 minutes after exposure to fibrinogen.

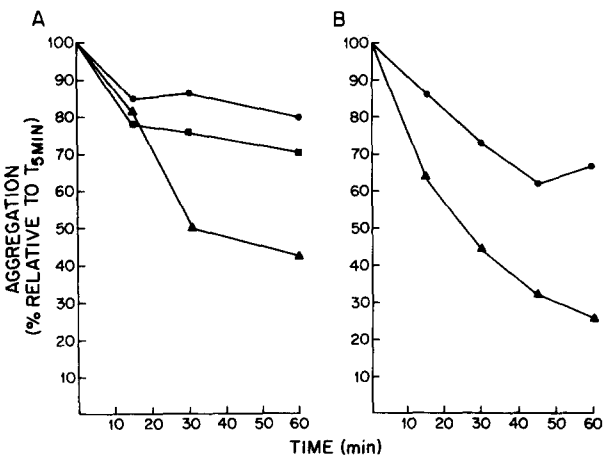

Fig 2. Results of a typical experiment depicting changes in the extent of platelet aggregation as a function of time after platelet exposure to fibrinogen (0.1 mg/mL) at 22°C. (A) Gel-filtered platelets stimulated with 10 μmol/L ADP (▲), 5 μmol/L A23187 (■), or 0.2 mg/mL chymotrypsin (●). (B) Gel-filtered platelets preincubated with 30 μg/mL cytochalasin D (●) or DMSO (▲), and stimulated with 10 μmol/L ADP. At the indicated times, platelet aliquots were stirred at 37°C in an aggregometer and their responses recorded. Values are relative to the extent of aggregation measured immediately after stimulated platelet exposure to fibrinogen.

less well than chymotrypsin-treated platelets alone or chymotrypsin-treated platelets combined with ADP-treated platelets that had been incubated with fibrinogen for 5 minutes (Table 1). Indeed, ADP-treated platelets incubated with fibrinogen for 60 minutes behaved similarly to PGE_1-treated platelets or unstimulated, formalin-fixed platelets in aggregation studies with chymotrypsin-treated platelets (Table 1).

Table 1. Aggregation of Combinations of ADP-Treated Platelets Incubated With Fibrinogen for 5 Minutes or 60 Minutes and Fresh, Chymotrypsin-Treated Platelets

Platelets	Incubation Time (min)*	Aggregation Extent (%)†
Chymo-treated‡	—	100
ADP-treated§	5	69 ± 11
	60	13 ± 4
Chymo-treated + ADP-treated¶	5	83 ± 17
Chymo-treated + ADP-treated¶	60	37 ± 21
Chymo-treated + PGE_1-treated¶	—	15 ± 16
Chymo-treated + formalin-fixed¶	—	24 ± 18

*Incubation time reflects the length of time ADP-treated platelets were exposed to fibrinogen (0.1 mg/mL) before aggregation was initiated.

†Aggregation was induced by stirring samples at 37°C in an aggregometer. The extent of platelet aggregation is expressed relative to that of fresh, chymotrypsin-treated platelets. Values represent mean ± SD, n = 3.

‡Chymotrypsin-treated platelets were aggregated at 37°C in the presence of 0.1 mg/mL fibrinogen by stirring in an aggregometer.

§ADP-treated platelets were aggregated by stirring in an aggregometer either 5 minutes or 60 minutes after exposure to fibrinogen (0.1 mg/mL).

¶Platelet mixtures consisted of equal parts of fresh, chymotrypsin-treated platelets combined with ADP- or PGE_1-treated platelets, or unstimulated, formalin-fixed platelets.

Table 2. Pretreatment of ADP-Stimulated Platelets With Chymotrypsin: Effect on Platelet Aggregation and Antifibrinogen Antibody $F(ab')_2$ Fragment Binding 60 Minutes Compared With 5 Minutes After Platelet Exposure to Fibrinogen

Agonist	Antifibrinogen $F(ab')_2$ Binding (%)*	Aggregation Extent (%)†
ADP	34 ± 23	30 ± 15
Chymotrypsin	89 ± 36‡	80 ± 25§
Chymotrypsin + ADP	69 ± 14‡	60 ± 10§

Platelets were stimulated with 10 μmol/L ADP, 0.2 mg/mL chymotrypsin, or chymotrypsin followed by ADP, as described in Materials and Methods.

*Antifibrinogen antibody $F(ab')_2$ binding, 60 minutes after exposure of stimulated platelets to 0.01 mg/mL fibrinogen at 22°C, is expressed relative to binding noted at 5 minutes. Values represent mean ± SD, n = 5.

†Platelet aggregation 60 minutes after exposure of stimulated platelets to 0.1 mg/mL fibrinogen is expressed relative to platelet aggregation at 5 minutes. Values represent mean ± SD, n = 5.

‡§Differences between paired samples were not statistically significant ($P > .2$), as determined using the Student's t-test.

Decreases in antifibrinogen $F(ab')_2$ binding and platelet aggregation accompanying the exposure of ADP-stimulated platelets to fibrinogen for 60 minutes could be prevented by pretreating platelets with chymotrypsin (Table 2). As GPIb and GPIIIa degradation result from chymotrypsin treatment,[15,16] studies were performed to examine the relationship between chymotrypsin-induced GPIb and GPIIIa digestion and modulation of antifibrinogen antibody $F(ab')_2$ binding. As summarized in Table 3,

Table 3. Effect of Chymotrypsin-Induced GPIb and GPIIIa Digestion and Modulation of Antifibrinogen Antibody $F(ab')_2$ Binding to Platelets After Subsequent Stimulation with ADP

Chymotrypsin Dose	GPIb (%)*	GPIIIa 66-Kd Fragment (%)†	Antifibrinogen $F(ab')_2$ Binding (%)‡
0	100	0	24 ± 17
10	97 ± 5	5 ± 3	64 ± 20
25	85 ± 8	10 ± 5	56 ± 16
50	54 ± 17	15 ± 12	67 ± 13
100	26 ± 14	17 ± 9	67 ± 16
200	12 ± 6	33 ± 13	61 ± 11

Platelets were incubated with increasing doses of chymotrypsin for 15 minutes at 37°C. Chymotrypsin was neutralized with PMSF and platelets washed, resuspended in HBMT, and stimulated with 10 μmol/L ADP. All values represent the mean ± SD of three separate experiments.

*Degradation of GPIb was quantified using the 6D1 MoAb.[11] 6D1 binding to chymotrypsin-treated platelets is expressed as a percentage relative to 6D1 binding to untreated, control platelets.

†Degradation of GPIIIa to its 66-Kd fragment was assessed on Western blots of lysed chymotrypsin-treated platelets using a commercial anti-GPIIIa MoAb.[12] The data represent the amount of the 66-Kd fragment seen as a percentage relative to total platelet GPIIIa.

‡Antifibrinogen antibody $F(ab')_2$ binding reflects binding to stimulated platelets 60 minutes compared with 5 minutes after exposure to 10 μg/mL fibrinogen. Binding at 5 minutes was taken as 100%.

platelets exposed to increasing concentrations of chymotrypsin showed an increased loss of surface GPIb, inferred from decreased binding of the 6D1 monoclonal anti-GPIb antibody.[11] In addition, chymotrypsin treatment was accompanied by the appearance of a 66-Kd GPIIIa fragment, as assessed by immunoblotting of lysed chymotrypsin-treated platelets. No correlation between GPIb degradation or the appearance of the 66-Kd GPIIIa fragment and changes in antifibrinogen antibody $F(ab')_2$ binding emerged, however.

Additional studies were performed to assess the role of actin polymerization in modulating fibrinogen expression on the platelet surface. Compared with control platelets treated with DMSO, platelets preincubated with cytochalasin D retained more of their ability to aggregate (Fig 2B) and showed significantly ($P < .02$) smaller decreases in antifibrinogen antibody $F(ab')_2$ binding 60 minutes as compared with 5 minutes after stimulation with ADP (Fig 3). Time-dependent changes in the ability of cytochalasin-treated platelets to aggregate after stimulation with ADP and exposure to fibrinogen were modest compared with those demonstrated by DMSO-treated samples. Indeed, the largest decrease in cytochalasin-treated platelet aggregation over a 60-minute time course is shown in Fig 2B. In comparison, DMSO-treated, control platelets lost twice as much of their ability to aggregate in parallel studies. As reported previously,[17] cytochalasin D-treated platelets bound less fibrinogen and aggregated less well than their DMSO-treated counterparts. The data presented in Fig 3, therefore, reflect changes in fibrinogen binding, antifibrinogen antibody $F(ab')_2$ binding, and platelet aggregation relative to control values (T = 5 minutes) for each treatment group.

DISCUSSION

Platelet aggregation is the result of a sequence of reactions that occur largely at the surface membrane and include agonist binding, signal transduction, modification of GPIIb-IIIa, and fibrinogen binding.[2] Although a strong correlation exists between platelet aggregation and fibrinogen binding,[1] discrepancies between platelet-fibrinogen interactions and platelet aggregation have been described.[5-7] For example, it is well known that platelets lose their ability to aggregate if they are exposed to agonist in the presence of fibrinogen before platelet-platelet interactions are initiated by stirring or otherwise agitating the sample.[18] Previous characterization of such platelets showed no decreases in fibrinogen binding.[5] Results from the present study suggest that qualitative, not quantitative, changes in bound fibrinogen are likely to contribute to the observed decrease in platelet aggregation.

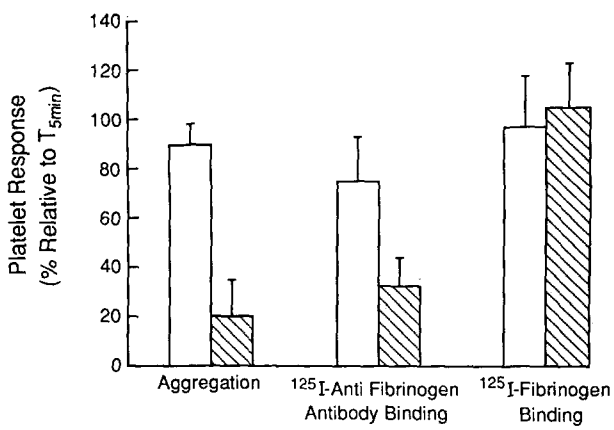

Fig 3. Effect of cytochalasin D (□) on the modulation of platelet aggregation, antifibrinogen antibody $F(ab')_2$ binding, and fibrinogen binding 60 minutes after exposure of ADP (10 μmol/L)-treated platelets to fibrinogen. Platelet responses are presented as a percentage (mean ± SD, n = 4) relative to responses observed 5 minutes after platelet exposure to fibrinogen. DMSO-treated platelets (▨) served as controls.

Indeed, the present study provides evidence for a strong correlation between time-dependent decreases in fibrinogen accessibility to antibody and platelet aggregability. Platelets stimulated with ADP or thrombin gradually lost their ability to aggregate, and exhibited substantial decreases in antifibrinogen antibody $F(ab')_2$ binding within 60 minutes after exposure to fibrinogen. In contrast, platelets stimulated with A23187 or chymotrypsin showed a significantly smaller decrease in antifibrinogen antibody $F(ab')_2$ binding over the same time course, and retained more of their ability to aggregate.

The data further show that ADP-treated platelets fail not only to interact with each other after 60 minutes of incubation with fibrinogen at 22°C, but also with freshly stimulated, chymotrypsin-treated platelets. Aggregation responses recorded for mixtures of chymotrypsin-treated platelets and ADP-treated platelets exposed to fibrinogen for 60 minutes resembled those obtained with combinations of chymotrypsin-treated platelets and PGE_1-treated platelets or unstimulated, formalin-fixed platelets. These findings suggest that ADP-treated platelets, incubated with fibrinogen for 60 minutes, are as inert in terms of aggregating with fresh chymotrypsin-treated platelets as unstimulated platelets. If platelet aggregation is the result of fibrinogen binding to receptors on adjacent platelets, these observations may further suggest that fibrinogen bound to stimulated platelets for 60 minutes has lost its ability to combine with available receptors on neighboring platelets.

Progressive, qualitative changes in bound fibrinogen and platelet aggregation associated with exposure of ADP-treated platelets to fibrinogen over a 60-minute time course could be prevented by the pretreatment of platelets with chymotrypsin. These data support the hypothesis that chymotrypsin-sensitive surface membrane structures participate in the observed modulation of fibrinogen expression on ADP-treated platelets. No correlation between chymotrypsin-induced degradation of GPIb[15] or digestion of GPIIIa to its 66-Kd fragment[16] and loss of platelet aggregability or changes in platelet-associated fibrinogen accessibility to antibody could be demonstrated, however. Proteolytic events preceeding the appearance of the 66-Kd GPIIIa fragment,[19] degradation of GPIIb, and/or proteolysis of the GPIIb-IIIa microenvironment may play potential roles in the modulation of fibrinogen expression on stimulated platelets. The observation that pretreatment of platelets with as little as 10 μg/mL chymotrypsin was sufficient to prevent ADP-induced qualitative changes in fibrinogen binding suggests

that the membrane protein(s) apparently contributing to this process is/are highly susceptible to proteolysis.

Further studies implicate actin polymerization in the modulation of fibrinogen expression on ADP-treated platelets and platelet aggregation. Platelets pretreated with cytochalasin D retained much of their ability to aggregate after 60 minutes of exposure to fibrinogen. Moreover, platelet-associated fibrinogen remained largely accessible to antifibrinogen $F(ab')_2$ fragments. This apparent correlation between cytoskeleton activation and observed, qualitative changes in bound fibrinogen on the surface of ADP-treated platelets may also explain the relative lack of such changes after platelet stimulation with chymotrypsin or A23187. Chymotrypsin has been shown to expose fibrinogen receptors via proteolysis rather than intracellular signal transduction, as evidenced by the lack of effect of metabolic inhibitors and PGE_1 treatment on fibrinogen binding and platelet aggregation.[20] The lack of cytoskeleton activation by these platelets has been inferred from studies demonstrating an inability of chymotrypsin-treated platelets to retract fibrin clots.[13] In comparison, platelet stimulation with A23187 is accompanied by activation of intracellular calpains and degradation of cytoskeletal proteins, including actin-binding protein, which plays a role in actin filament organization.[21] Indeed, degradation of actin-binding protein was a consistent finding after platelet stimulation with A23187 in the present studies (data not shown).

The association between time-dependent, qualitative changes in bound fibrinogen on the platelet surface and platelet cytoskeleton activation may suggest bound fibrinogen redistribution, or internalization. Cytoskeleton-dependent events such as receptor clustering,[22] sequestration of bound fibrinogen in channels of the surface canalicular system,[23] and fibrinogen internalization have been described.[24,25] Internalization is unlikely under present experimental conditions, as bound fibrinogen remains accessible to proteolysis by plasmin or chymotrypsin.[14] Fibrinogen sequestration in areas of the open canalicular system, which may not be readily accessible to antibody and partially accessible to proteases, however, may relate to present observations.

Recently, Gralnick et al[26] also reported qualitative changes in bound fibrinogen on thrombin-stimulated platelets using an MoAb recognizing an epitope in the fibrinogen D domain. These investigators showed decreases in whole antibody and $F(ab')_2$ binding to platelets over a 60-minute time course, but no significant changes in Fab fragment binding, consistent with stearic constraints limiting antibody access to platelet-associated fibrinogen. Interestingly, studies with polyclonal antibodies[14] showed similar time-dependent decreases for both antifibrinogen IgG and Fab fragment binding to platelets, thus raising the possibility that certain epitopes on fibrinogen may remain relatively more accessible to specific MoAb Fab fragments after fibrinogen binding to stimulated platelets.

Additional studies are required to elucidate the mechanism(s) responsible for the apparent qualitative changes in bound fibrinogen on stimulated platelets. The present study demonstrates an agonist specific modulation that is dependent on cytoskeleton activation and is sensitive to proteolysis by chymotrypsin. The data further provide a correlation between qualitative changes in bound fibrinogen on the surface of stimulated platelets and the ability of platelets to aggregate. Interestingly, other adhesive proteins, fibronectin and von Willebrand factor,[4] when bound to thrombin-treated platelets, remain largely unchanged as assessed recently using polyclonal antibodies.[27] These findings may have implications for the regulation of hemostasis and thrombosis. For example, qualitative changes in bound fibrinogen on ADP- or thrombin-treated platelets could provide a protective mechanism whereby the exposure of platelet-associated fibrinogen that does not participate in platelet-platelet contacts is modified to limit thrombus formation.

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