Platelet Activation Results in a Redistribution of Glycoprotein IV (CD36)

Alan D. Michelson, June D. Wencel-Drake, Anita S. Kestin, Marc R. Barnard

Abstract To investigate the possibility that thrombin and/or other platelet activators change the platelet surface expression of glycoprotein IV (GPIV, CD36), we used a panel of five GPIV-specific monoclonal antibodies (OKM5, 5F1, FA6-152, 8A6, and F13) directed against different epitopes. All these antibodies bound to resting platelets in a concentration-dependent and saturable manner, as determined by flow cytometry of washed platelets. Thrombin (1 U/mL) induced an approximately twofold increase in the platelet surface binding of each of these monoclonal antibodies. Immunofluorescence microscopy demonstrated an internal pool of GPIV that, after thrombin stimulation, redistributed to the platelet surface. In a whole-blood flow-cytometric assay, α-thrombin and the thromboxane A2 analogue U46619 each resulted in an approximately twofold increase in the platelet surface binding of OKM5, whereas ADP had a more modest effect, and collagen and epinephrine had little effect. The activation-induced up-regulation of the platelet OKM5 epitope occurred in vivo as demonstrated by flow cytometric analysis of whole blood emerging from a standardized skin puncture site. In summary, both in vitro and in vivo platelet activation results in increased platelet surface expression of GPIV, as a result of a redistribution of GPIV from an internal pool. (Arterioscler Thromb. 1994;14:1193-1201.)

Key Words • platelets • CD36 • glycoprotein IV • thrombin • flow cytometry • monoclonal antibodies

Glycoprotein (GP) IV, also known as GPllb and CD36, is present on the surface of human platelets and other cells, including endothelial cells, monocytes, erythrocytes, and megakaryocytes. GPIV is composed of a single highly glycosylated polypeptide chain with an apparent molecular mass of 88 kD. The structure of GPIV has several homologies with a recently described lysosomal integral membrane protein (LIMP II), suggesting that GPIV and LIMP II belong to a new gene family. There are between 12,000 and 25,000 copies of GPIV on the platelet surface. GPIV is very resistant to protease digestion.

GPIV may play a number of important roles in platelet function and pathophysiology. First, GPIV has been reported to be a collagen receptor involved in the initial phase of platelet-collagen adhesion. Second, GPIV has been reported to be a receptor for thrombospondin and may therefore have a role in thrombospondin-dependent platelet aggregation and thrombospondin-dependent platelet-monocyte adhesion. Third, platelet GPIV is physically associated with the fyn, lyn, and yes protein tyrosine kinases and may be involved in platelet signal transduction. Fourth, GPIV is a receptor for the adhesion of Plasmodium falciparum-infected erythrocytes. Fifth, GPIV may have a role in the pathophysiology of thrombotic thrombocytopenic purpura (TTP), because the plasma of a subgroup of patients with TTP contains a protein (p37) that agglutinates platelets by binding to GPIV. Finally, platelet GPIV is relatively increased in myeloproliferative disorders.

The platelet-specific antigen Nak* is present on GPIV. The Nak*-negative phenotype occurs with a high incidence (≈3%) in Japanese blood donors but occurs in only 0.2% of US blood donors. Because they lack detectable GPIV, the platelets of Nak*-negative individuals have been used to study the role of GPIV in platelet function. Nak*-negative individuals show reduced adhesion to collagen in flowing whole blood. The lack of evidence of a hemostatic defect in Nak*-negative individuals suggests that binding of collagen to other proteins (eg, the GPIa-IIa complex and GPVI) may be sufficient to avoid an overt bleeding diathesis. Thrombospondin binding to the platelets of Nak*-negative individuals appears to be normal, which calls into question the role of GPIV as a thrombospondin receptor. Nak*-negative platelets and monocytes fail to support the adhesion of Plasmodium falciparum–infected erythrocytes, thereby providing evidence for the role of GPIV as the involved receptor. Nak*-negative individuals are at risk for the development of refractoriness to human leukocyte antigen–matched platelet transfusions.

Whereas other inherited deficiencies of platelet glycoproteins (Bernard-Soulier syndrome and Glanzmann's thrombasthenia) were first detected in patients who presented with clinical bleeding, Nak*-negative (ie, GPIV-negative) individuals are apparently healthy without evidence of hemostatic abnormalities. However, except for the index case, all Nak*-negative individuals thus far identified have been self-selected as young, healthy blood donors. It remains to be determined whether the Nak*-negative phenotype predisposes individuals to any disease states. In fact, it has been speculated that the reduced rate of response of
Thrombin increases the platelet surface expression of Nak°-negative platelets to collagen 10 might have a pro-
activation of GPIV in vitro and in vivo.

Methods

Murine Monoclonal Antibodies

GPIV-Specific

OKM5 was provided by Dr Patricia Rao, Ortho Diagnostic Systems Inc, Raritan, NJ. FA6-152 was provided by Dr Lena Edelman, Institute Pasteur, Paris, France. 5A6 was provided by Dr John W. Barnwell, New York University Medical Center, New York, NY. 5F1 and F13 were provided by Dr Irwin D. Bernstein, Fred Hutchinson Cancer Research Center, Seattle, Wash. OKM5 has been reported to be di-
rected against a thrombospondin binding site on GPIV. The epitopes of the other four GPIV-specific monoclonal antibodies differ.5,16,40-42

P-Selectin–Specific

KC4 (provided by Drs Barbara C. Furie and Bruce Furie, New England Medical Center, Boston, Mass59,65 and S12 (provided by Dr Rodger P. McEver, University of Oklahoma, Oklahoma City) were used as markers of platelet activation and α-granule secretion. P-selectin* (also known as granule membrane protein 140 [GMP-140]), platelet activation-de-
dependent granule-external membrane (PADGEM) protein,40,43 and CD62P* is a component of the α-granule membrane of resting platelets that is expressed on the platelet plasma membrane only after platelet activation and secretion.37,38

GPIb-Specific

AK3 (provided by Dr Michael C. Berndt, Baker Medical Research Institute, Melbourne, Australia) is directed against the macroglycopeptide portion of GP Ibα.45

GPIIIa-Specific

Y2/51 (DAKO, Carpinteria, Calif) is directed against plate-
let membrane GPIIIa.46

Some antibodies were either biotinylated47 or conjugated with fluorescein isothiocyanate (FITC), except for FITC-conjugated OKM5, which was provided by Dr Rao (Ortho). The biotinylated and FITC-conjugated antibodies functioned normally, in that their binding to platelets was saturable at antibody concentra-
tions similar to that of unlabeled antibodies.

Flow Cytometric Analysis of Washed Platelets

The method has been previously described.49-51 Peripheral blood from healthy aspirin-free volunteers was drawn into a 1:8 volume of acid-citrate-dextrose (in mmol/L: trisodium citrate 85, citric acid 71, dextrose 111), pH 4.5, and centrifuged (150g, 15 minutes, 22°C), and the platelet-rich plasma was removed. The platelets were washed twice by centrifugation (2000g, 10 minutes, 22°C) and resuspension in modified Ty-
rode’s buffer (138 mmol/L NaCl, 2.9 mmol/L KCl, 12 mmol/L NaHCO3, 0.4 mmol/L Na2HPO4, 0.1% glucose, 0.35% bovine serum albumin), pH 6.5, with 50 ng/ml prostaglandin E1 (PGE1).48 The final platelet resuspension was at a concentra-
tion of 2.5 x 10^6/μL in modified Tyrode’s buffer, pH 7.3, with 1.5 mmol/L CaCl2 or 2.5 mmol/L EDTA, with or without 50 ng/mL PGE1. The platelets were incubated (10 minutes, 37°C) with various concentrations of purified human α-thrombin (provided by Dr John Fenton II, New York Department of Health, Albany, NY) or control buffer, fixed with 1% formal-
dehyde, and washed as previously described.49 The washed platelets were incubated (20 minutes, 22°C) with a saturating concentration of either an FITC-conjugated goat anti-mouse IgG antibody or an FITC-conjugated monoclonal antibody, washed, and resuspended in phosphate-
buffered saline (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L NaH2PO4, 8.1 mmol/L Na2HPO4, pH 7.4, with 0.35% bovine serum albumin) before flow cytometric analysis. In the assays with a biotinylated antibody, there was an additional incubation (20 minutes, 22°C) with phycoerythrin-streptavidin (Jackson ImmunoResearch) followed by a further wash in phosphate-buffered saline. In other assays, monoclonal anti-
body binding was detected by an indirect method as previously described.49 In these assays, the platelet-specific monoclonal antibodies were unlabeled, and there was an additional incubation (20 minutes, 22°C) with a saturating concentration of an FITC-conjugated goat anti-mouse IgG antibody (Cooper Bio-
medical) followed by two washes. In some assays, platelet samples were activated by thrombin in the presence of 5 mmol/L CaCl2, subsequently incubated (10 minutes, 37°C) with either 5 mmol/L EDTA or buffer only and then fixed and washed. In other assays, platelets were fixed with 1% formal-
dehyde in platelet-rich plasma, as previously described.51 The platelet-rich plasma was prepared from peripheral blood drawn into a 1:6 volume of acid-citrate-dextrose, resulting in a final pH of 6.5.51 The platelets were then washed, incubated with a saturating concentration of a monoclonal antibody, and prepared for flow cytometric analysis as above. Background fluorescence, determined by incubation of the platelets with the appropriate normal mouse IgG or conjugate (Calbio-
chem), was subtracted from all samples. Samples were ana-
lyzed in either an EPICS Profile flow cytometer (Coulter Cytometry) or an FACS 440 (Becton Dickinson FACS Sys-
tems) as previously described.50,52 For each sample, the fluo-
rescence signal from 10 000 individual cells was measured. Any nonplatelet contaminating cells (including monocytes) were excluded from the analysis as previously described.50

Preparation of Peripheral Whole-Blood Samples for Flow Cytometric Analysis

The method has been previously described in detail.52 To analyze individual platelets, experimental conditions were de-
sign to avoid platelet activation and the formation of platelet aggregates. Thus, in the preparation of whole blood for flow cytometric analysis, there were no washing, centrifugation, gel filtration, vortexing, or stirring steps. Blood was drawn by veni-
puncture from healthy adult volunteers who had not ingested aspirin within the previous 10 days. The blood was drawn into a sodium citrate evacuated tube (Vacutainer, Becton Dickinson). To minimize platelet activation during blood drawing, only a light tourniquet and a 19-gauge needle were used, and the first 2 mL of blood was discarded. Within 15 minutes of drawing, the blood was diluted 1:4 in modified A/2-hydroxyethylpiperazine-A/2-
ethane sulfonic acid (HEPES)-Tyrode’s buffer (137 mmol/L 
NaCl, 2.8 mmol/L KCl, 1 mmol/L MgCl2, 12 mmol/L NaHCO3, 0.4 mmol/L Na2HPO4, 0.35% bovine serum albumin, 10 mmol/L HEPES, 5.5 mmol/L glucose, pH 7.4). This method of dilution of blood into buffer before the addition of agonist was based on the method of Shattil et al.49 The blood is diluted into buffer to decrease the platelet concentration, thereby preventing platelet-to-platelet aggregation after the addition of agonist. This dilution is critical because quantitation of the amount of surface antigen per platelet by flow cytometry requires that platelets be analyzed individually. After dilution, the sample was incubated (22°C, 15 minutes) undisturbed (to prevent platelet aggregation) with an
agonist: (1) purified human α-thrombin 0.001 to 10 U/mL (in the presence of 2.5 mmol/L of the peptide glycyl-L-prolyl-L-arginyl-L-proline [GRPRP, Calbiochem] to inhibit fibrin polymerization and platelet aggregation), (2) the thromboxane A₂ analogue U46619 (Cayman Chemical) 0.01 to 100 μmol/L, (3) ADP (Sigma, St Louis) 0.01 to 10 μmol/L, (4) collagen type I (Hormon-Chemie) 0.5 to 10 μg/mL, (5) epinephrine (Sigma) 1 to 100 μmol/L, or (6) buffer only. Fifteen minutes after addition of the agonist, the samples were fixed with formaldehyde (1% final concentration). Samples were diluted 10-fold in modified HEPESTyrode's buffer, pH 7.4, and incubated (22°C, 20 minutes) with a subsaturating concentration of an FITC-conjugated GPIIIa-specific monoclonal antibody (Y2/51) and a saturating concentration of biotinylated monoclonal antibody OKMs. The samples were then incubated (22°C, 15 minutes) with phycoerythrin-streptavidin, diluted 12-fold in modified HEPESTyrode's buffer, pH 7.4, and stored at 4°C before flow cytometric analysis within 24 hours. This method results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of antibody tagging.52

Preparation of Whole-Blood Samples From Puncture Sites for Flow Cytometric Analysis

The method has been previously described.52-53 The study was approved by the Human Subjects Committee of The Medical Center of Central Massachusetts. A standardized bleeding time test was performed on healthy adult volunteers who had not ingested aspirin within the previous 10 days. The bleeding time test was performed on healthy adult volunteers who had not ingested aspirin within the previous 10 days. The samples were obtained from healthy aspirin-free volunteers. Washed platelets were subsequently prepared by centrifugation and gel filtration on Sepharose 2B (PharmaCia). In activation studies, platelets were stimulated with 1 U/mL α-thrombin at 22°C for 2 minutes, at which time the cells were fixed with paraformaldehyde, 1% final concentration (Polysciences), on ice. Resting (non-thrombin-stimulated) platelets were fixed in the same way. Unreacted aldehyde was blocked with 20 mmol/L N-acetyl-l-cysteine (Sigma) and poly-l-lysine (Sigma). In some studies, the cells were treated with 200 μmol/L L-proline [GPRP, Calbiochem] to inhibit fibrin polymerization of antibody OKMs (2.0 μg/mL) was used. The ratio of OKMs to KC4 (P-selectin-specific) binding was 1.00 to 0.04 in control samples, biotinylated mouse IgG was used. The samples were then incubated (15 minutes, 22°C) with phycoerythrin-streptavidin, further diluted approximately six-fold in modified Tyrode's buffer, and stored at 4°C before flow cytometric analysis. Immediately before puncture, control samples were run with peripheral blood drawn from the antecubital vein of the opposite arm of the same subject through a 21-gauge butterfly needle and tubing as previously described.52-54 After discarding the first 2 mL, one drop of nonanticoagulated blood from the butterfly tubing was placed on a piece of paraffin, and aliquots were processed for flow cytometry exactly as described for the puncture samples. The platelets in these control samples were in a resting state as demonstrated by lack of binding of the P-selectin-specific monoclonal antibody S12. The binding of S12 to these control samples was less than 5% of parallel samples activated with 10 U/mL thrombin.

Flow Cytometric Analysis of Platelets in Whole Blood

The method has been previously described.52,53,55 Samples prepared as described above were analyzed in an EPICS Profile flow cytometer. The instrument was equipped with a 500-mW argon laser and operated at 15 mW at a wavelength of 488 nm. The fluorescence of FITC and phycoerythrin were determined using 525- and 575-nm band-pass filters, respectively. After identification of platelets by characteristic log forward and orthogonal light-scatter and by either FITC-AK3 positivity (ie, GPIb positivity) or Y2/51 positivity (ie, GPIIIa positivity), binding of biotinylated antibody was determined by analyzing 10,000 individual platelets for phycoerythrin fluorescence. Because GPIb is not present on any circulating blood cell except platelets,96-97 the thrombin-induced decrease in the platelet binding of AK3 or Y2/51 result in a fluorescence below the threshold used to distinguish platelets from other cells. Background fluorescence, obtained from parallel samples with purified biotinylated mouse IgG (Calbiochem), was subtracted from each test sample.

Immunofluorescence Microscopy

Immunofluorescent staining of platelets was performed as previously described.58 Briefly, platelet-rich plasma was prepared from acid-citrate-dextrose-anticoagulated whole blood obtained from healthy aspirin-free volunteers. Washed platelets were subsequently prepared by centrifugation and gel filtration on Sepharose 2B (Pharmacia). In activation studies, platelets were stimulated with 1 U/mL α-thrombin for 2 minutes, at which time the cells were fixed with paraformaldehyde, 1% final concentration (Polysciences), on ice. Resting (non-thrombin-stimulated) platelets were fixed in the same way. Unreacted aldehyde was blocked with 20 mmol/L N-acetyl-l-cysteine (Sigma; essentially globulin free once recrystallized) and incubated at 22°C for 20 minutes with either a GPIV-specific murine monoclonal antibody (OKMs or 5F1) or irrelevant ascites. After being rinsed, permeable or intact cells then were rinsed with Tris-buffered saline containing 0.1% bovine serum albumin (Sigma; essentially globulin free once recrystallized) and incubated at 22°C for 20 minutes with either a GPIV-specific murine monoclonal antibody (OKMs or 5F1) or irrelevant ascites. After being rinsed, permeable or intact cells then were stained for 5 minutes at 22°C with Tris-buffered saline containing 0.1% bovine serum albumin (Sigma). In all studies, the cells were treated with 200 μmol/L L-proline [GPRP, Calbiochem] to inhibit fibrin polymerization of antibody OKMs. The samples were run with peripheral blood drawn from the antecubital vein of the opposite arm of the same subject through a 21-gauge butterfly needle and tubing as previously described.52-54 After discarding the first 2 mL, one drop of nonanticoagulated blood from the butterfly tubing was placed on a piece of paraffin, and aliquots were processed for flow cytometry exactly as described for the puncture samples. The platelets in these control samples were in a resting state as demonstrated by lack of binding of the P-selectin-specific monoclonal antibody S12. The binding of S12 to these control samples was less than 5% of parallel samples activated with 10 U/mL thrombin.

Results

Binding of Monoclonal Antibody OKMs to Resting and Activated Platelets

To assess the binding to resting platelets of a GPIV-specific monoclonal antibody (OKMs), platelets fixed in platelet-rich plasma were analyzed by flow cytometry. OKMs bound to the platelets in a concentration-dependent and saturable manner (data not shown). In all subsequent experiments, a saturating concentration of OKMs (2.0 μg/mL) was used. The ratio of OKMs to KC4 (P-selectin-specific) binding was 1.00 to 0.04 in experiments using saturating concentrations of these antibodies and FITC-conjugated goat anti-mouse secondary antibody (mean of the ratios of eight experiments). This lack of binding of the P-selectin-specific monoclonal antibody confirmed that the platelets were in a resting state.

To assess the effect of platelet activation on the binding of antibody OKMs, washed platelets were incubated with...
various concentrations of purified human α-thrombin. Increasing concentrations of thrombin resulted in increasing binding of OKM5 (Figs 1 and 2, solid bars). The ability of the flow cytometric technique to analyze individual platelets enabled us to demonstrate that the thrombin-induced increase in OKM5 binding was not restricted to a distinct subpopulation of platelets. This is shown in Fig 1 for both partially and maximally thrombin-activated platelets by the gradual shift to the right of a single major peak. The maximal increase in OKM5 binding to platelets occurred with approximately 0.5 U/mL thrombin (Fig 2, solid bars). In experiments with biotinylated OKM5, the maximal thrombin-induced change in OKM5 binding to platelets was a 112.5±26.0% (mean±SEM, n=4) increase over resting platelets. In experiments with FITC-conjugated OKM5, the maximal thrombin-dependent change in OKM5 binding to platelets was a 90.5±12.8% (mean±SEM, n=3) increase over resting platelets (data not shown). These thrombin-induced increases in OKM5 binding were not due to platelet aggregation because there was no associated change in forward light-scatter (an index of particle size): mean peak forward light-scatter channel was 58.7±2.9 for resting platelets and 59.0±1.7 for thrombin-activated platelets (mean±SEM, n=3) (data not shown). With the indirect method (FITC-conjugated goat anti-mouse antibody), the maximal thrombin-dependent change in OKM5 binding to platelets was a 119.3±6.3% (mean±SEM, n=7) increase over resting platelets (data not shown). Addition of antibody OKM5 before thrombin incubation and fixation resulted in a similar thrombin-induced increase in OKM5 binding to platelets (117%; mean of two experiments). Irrespective of whether platelets were fixed before or after the OKM5 incubation, OKM5 bound to resting platelets to a similar extent, and thrombin induced a similar increase in OKM5 binding (Fig 3). In matched experiments, thrombin in the absence of PGE1 resulted in a 123.6±10.0% (mean±SEM, n=3) maximal increase in OKM5 binding to platelets, whereas thrombin in the presence of 50 ng/mL PGE1 resulted in a 67.1±8.2% increase. Thus, the response to thrombin in the absence of PGE1 was 84.2% greater than that observed with thrombin in the presence of PGE1.

Origin of the Thrombin-Induced Increase in Platelet Surface Binding of OKM5

In matched experiments, we assessed the effect of thrombin on the platelet surface binding of five different GPIV-specific monoclonal antibodies (OKM5, 5F1, 8A6, FA6-152, and F13) directed against different epitopes and a P-selectin–specific monoclonal antibody (S12). All five GPIV-specific monoclonal antibodies bound to resting platelets. Increasing concentrations of thrombin resulted in approximately parallel rises in the platelet surface binding of each of the five GPIV-specific antibodies (Fig 2). In the absence of thrombin, there was minimal binding of S12 (1.4±0.5% of the binding with 1 U/mL thrombin), thereby confirming the resting state of the platelets. As expected, increasing concentrations of thrombin resulted in a marked rise in
the platelet surface binding of the P-selectin-specific monoclonal antibody (data not shown).

The parallel thrombin-induced increases in the platelet surface binding of the five different GPIV-specific monoclonal antibodies (Fig 2) suggested that these increases were not the result of a change in the conformation and/or microenvironment of platelet surface GPIV but were the result of a redistribution of GPIV from an internal pool. To directly test this hypothesis, experiments were performed with immunofluorescence microscopy (Figs 4 and 5). Intact resting platelets stained with monoclonal anti-GPIV antibodies (OKM5 and 5F1) revealed a fluorescent rim pattern consistent with surface localization (Fig 4, a and d). Permeabilization of the platelets with lysophosphatidylcholine revealed extensive intracellular staining for GPIV, which generally codistributed with fibrinogen (Fig 4, compare b to c and e to f). In parallel experiments, platelets were stimulated with 1 U/mL thrombin for 2 minutes. These cells were subsequently stained in the permeable state and revealed a centralized clearing of the GPIV antigen (Fig 5, compare d to a and e to b). In contrast, resting or thrombin-stimulated platelets stained with an irrelevant ascites control failed to demonstrate either surface or intracellular staining (Fig 5, c and f).

Taken together, the flow cytometric data with the panel of five different GPIV-specific monoclonal antibodies (Fig 2) and the data obtained by immunofluorescence microscopy (Figs 4 and 5) strongly suggest that: (1) in addition to the platelet surface pool of GPIV, there is a significant internal pool of GPIV; and (2) thrombin stimulation results in a redistribution of the internal pool of GPIV to the platelet surface.

Role of Calcium in the Platelet Binding of OKM5

To determine whether the binding of antibody OKM5 to platelets was Ca²⁺ dependent or independent, experiments were performed in the presence of either CaCl₂...
Effect of Ca\(^{2+}\) on the Platelet Surface Binding of Glycoprotein IV-Specific and P-Selectin-Specific Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Resting or Activated Platelets</th>
<th>Fluorescence Intensity (mean±SEM, n=4)</th>
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<td>Ca(^{2+})</td>
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<tr>
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<td>Activated</td>
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<tr>
<td>KC4</td>
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<td>KC4</td>
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Washed platelets in modified Tyrode's buffer containing 1.5 mmol/L Ca\(_{Cl_2}\) were incubated (10 minutes, 37°C) with or without 1 U/mL thrombin and then incubated (10 minutes, 37°C) in the presence of either 1.5 mmol/L Ca\(_{Cl_2}\) or 5 mmol/L EDTA as indicated. Platelets were then fixed in 1% formaldehyde and analyzed by flow cytometry using either monoclonal antibody OKM5 (glycoprotein IV-specific) or monoclonal antibody KC4 (P-selectin-specific), followed by a fluorescein isothiocyanate-conjugated goat anti-mouse antibody. The fluorescence intensity of OKM5 binding to resting platelets in the presence of 1.5 mmol/L Ca\(_{Cl_2}\) was assigned 100 arbitrary linear units.

or EDTA. When washed platelets were activated by thrombin in the presence of 1.5 mmol/L Ca\(_{Cl_2}\) and then incubated (10 minutes, 37°C) with or without 1 U/mL thrombin and then incubated (10 minutes, 37°C) in the presence of either 1.5 mmol/L Ca\(_{Cl_2}\) or 5 mmol/L EDTA as indicated. Platelets were then fixed in 1% formaldehyde and analyzed by flow cytometry using either monoclonal antibody OKM5 (glycoprotein IV-specific) or monoclonal antibody KC4 (P-selectin-specific), followed by a fluorescein isothiocyanate-conjugated goat anti-mouse antibody. The fluorescence intensity of OKM5 binding to resting platelets in the presence of 1.5 mmol/L CaCl\(_2\) was assigned 100 arbitrary linear units.

ccrease in OKM5 binding (1.5±5.2%, n=3) compared with non-thrombin-treated platelets in the presence of 1.5 mmol/L CaCl\(_2\). These findings demonstrate that: (1) the thrombin-induced upregulation of the OKM5 epitope and thrombin-induced platelet secretion were both partially Ca\(^{2+}\) dependent (as previously reported for platelet secretion\(^{48}\)); and (2) the identification of epitopes on GPIV and P-selectin by monoclonal antibodies OKM5 and KC4, respectively, was Ca\(^{2+}\) independent on both resting and activated platelets (as previously reported for P-selectin\(^{43}\)).

**Effect of Physiological Agonists on Platelet Membrane GPIV in Whole Blood**

All of the experiments described above were performed in washed platelet systems. To further determine the physiological relevance of the activation-induced increase in the platelet surface expression of GPIV, experiments were performed in a whole blood system\(^{53}\) with a number of physiological platelet agonists (Fig 6). Both thrombin and U46619 (a thromboxane A\(_2\) analogue) resulted in marked concentration-dependent increases in the platelet surface binding of OKM5 in whole blood (Fig 6A and B), whereas ADP resulted in a more modest increase (Fig 6C). A modest increase in the platelet surface binding of OKM5 occurred only with a maximal concentration of collagen (Fig 6D). There was only a minimal increase with epinephrine (Fig 6E). Compared with resting platelets, the maximal increases in OKM5 binding induced by these five agonists were: 101±11% (mean±SEM, n=3) with 10 U/mL thrombin, 81±5% with 10 μmol/L U46619, 47±2% with 10 μmol/L ADP, 32±9% with 10 μg/mL collagen, and 17±6% with 100 μg/mL epinephrine. In these whole blood experiments with human α-thrombin (but not the other agonists), 2.5 mmol/L GPRP was included to inhibit fibrin clot formation.\(^{53}\)

As previously described in detail,\(^{53}\) this whole blood flow cytometric assay results in analysis of individual platelets in whole blood. Therefore, as in the washed...
Evidence by the shift of a single peak to the right (Fig 7B). The increase in platelet surface GPIV expression induced by the puncture site was not a nonspecific increase in surface glycoprotein expression because in matched samples there was a reciprocal decrease in the platelet surface expression of the GPIb-IX complex.

Discussion

In this study, we have demonstrated that platelet activation results in an increased platelet surface expression of GPIV (CD36). Thrombin, one of the most physiologically important platelet activators, induced an approximately twofold increase in the platelet surface binding of the GPIV-specific monoclonal antibody OKM5, both in a washed platelet system and in whole blood. We were able to study the effect of human α-thrombin in the physiological milieu of whole blood by using the peptide GPRP, an inhibitor of fibrin polymerization. ADP and the stable thromboxane A₂ analogue U46619 also increased the platelet surface expression of the OKM5 epitope in whole blood. Furthermore, analysis of whole blood emerging from a standardized puncture site established that the activation-induced increase in the platelet surface GPIV could occur in vivo. The use of a whole blood assay made it very unlikely that the upregulation of the platelet surface expression of the OKM5 epitope observed during the bleeding time was an in vitro artifact. The ability of the flow cytometric method to analyze individual platelets both in a washed platelet system and in whole blood enabled us to demonstrate that neither the in vitro nor the in vivo activation-induced increase in platelet surface GPIV was restricted to a distinct subpopulation of platelets.

To determine the origin of the activation-induced increase in the platelet surface expression of the OKM5 epitope, we used a panel of four other GPIV-specific monoclonal antibodies (FA6-152, 8A6, 5F1, and F13). Whereas OKM5 is directed at a thrombospondin binding site on GPIV, 5F1 is directed against different epitopes of GPIV. In the present study, increasing concentrations of thrombin resulted in approximately parallel rises in the platelet surface binding of OKM5, FA6-152, 8A6, 5F1, and F13. These data suggest that the thrombin-induced increase in the platelet surface expression of the OKM5 epitope was the result of a change in the conformation and/or microenvironment of platelet surface GPIV, but was the result of a redistribution of GPIV from an internal pool. Direct evidence for this hypothesis was obtained by immunofluorescence microscopy. These experiments demonstrated that there is an internal pool of GPIV that after thrombin stimulation is redistributed to the platelet surface. The observed general codistribution of GPIV with fibrinogen suggests that at least a portion of the intracellular pool of GPIV is associated with platelet α-granules. In a study published while the present work was under review, Berger et al performed immunocytochemical studies of GPIV at the ultrastructural level. These authors confirmed our hypothesis that GPIV is present in platelet α-granules, as well as in membranes of the open canalicul system, and that thrombin stimulation results in a redistribution...
of GPIV to the platelet plasma membrane. In this study, consistent with an alpha-granule location for a portion of the internal pool of GPIV, agonists that are strong degranulators (thrombin, U46619) markedly increased platelet surface GPIV, whereas agonists that are weak degranulators (collagen, epinephrine) had little effect on platelet surface GPIV.

Although the exact functions of GPIV remain controversial,1 the presently described activation-induced increase in the platelet surface expression of GPIV may amplify the role of GPIV as a thrombospondin receptor,3,4 a collagen receptor,9,10 and a signal transduction molecule,1,15 and/or a cytoadherence ligand on Plasmodium falciparum-infected erythrocytes.16,17 Furthermore, we have recently reported that the activation-induced increase in the platelet surface expression of GPIV may be a more sensitive clinical marker of platelet activation than the activation-induced increases in the platelet surface expression of P-selectin and the GPIIb-IIIa complex.55

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