Platelet receptor interplay regulates collagen-induced thrombus formation in flowing human blood

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The platelet glycoproteins (GPs) Ib, integrin $\alpha_2\beta_1$, and GPVI are considered central to thrombus formation. Recently, their relative importance has been re-evaluated based on data from murine knockout models. To examine their relationship during human thrombus formation on collagen type I fibers at high shear (1000 s^{-1}), we tested a novel antibody against GPVI, an immunoglobulin single-chain variable fragment, 10B12, together with specific antagonists for GPIbα (12G1 Fab_2) and $\alpha_2\beta_1$ (6F1 mAb or GFOGER-GPP peptide). GPVI was found to be crucial for aggregate formation, Ca^{2+} signaling, and phosphatidylserine (PS) exposure, but not for primary adhesion, even with more than 97% receptor blockade. Inhibiting $\alpha_2\beta_1$ revealed its involvement in regulating Ca^{2+} signaling, PS exposure, and aggregate size. Both GPIbα and $\alpha_2\beta_1$ contributed to primary adhesion, showing overlapping function. The coinhibition of receptors revealed synergism in thrombus formation: the coinhibition of adenosine diphosphate (ADP) receptors with collagen receptors further decreased adhesion and aggregation, and, crucially, the complete eradication of thrombus formation required the coinhibition of GPVI with either GPIbα or $\alpha_2\beta_1$. In summary, human platelet deposition on collagen depends on the concerted interplay of several receptors: GPIb in synergy with $\alpha_2\beta_1$ mediating primary adhesion, reinforced by activation through GPVI, which further regulates the thrombus formation. (Blood. 2004;103:1333-1341)

Introduction

The platelet response to exposed subendothelial matrix is fundamental to thrombosis and hemostasis. Uniquely, collagen, the most abundant vessel wall protein, mediates platelet adhesion and activation, localizing and regulating the hemostatic response at sites of injury. Discovering the molecular mechanisms that control platelet-collagen interaction is crucial for understanding the pathogenesis of arteriothrombotic diseases such as stroke and myocardial infarction. Under high shear rate conditions, the glycoprotein (GP) Ib/V/IX complex allows initial platelet rolling over von Willebrand factor (VWF) bound to subendothelial collagen fibers, and subsequently collagen receptors come into contact with their specific binding sequences in the collagen. For the next step, platelet arrest and activation, firm evidence exists of a role for only 2 receptors, integrin $\alpha_2\beta_1$ and immunoglobulin superfamily member GPVI, despite the apparent redundancy in collagen receptors (for a review, see Siljander PR-M and Farndale RW[1]).

According to the 2-site, 2-step model, high-affinity interaction through $\alpha_2\beta_1$ stops the platelet, allowing low-affinity binding of GPVI, which generates signaling required for the subsequent thrombus formation. Platelet deposition under flow was found to be dependent on GPIb/V/IX and $\alpha_2\beta_1$,[2-4] whereas no platelet deposition occurred on the GPVI-specific substrate collagen-related peptide (CRP), even under low shear rates.[5] The limited number of studies with human platelets deficient in either GPVI or $\alpha_2\beta_1$ support the 2-site, 2-step model. Blood depleted of functional GPVI showed only severe impairment of the collagen-induced "second-wave" aggregation response under flow.[6] Although thrombus volume was reduced, the initial adhesive layer of platelets was fully preserved. The few patients with defective $\alpha_2\beta_1$ displayed bleeding problems, suggesting a central role for the integrin in hemostasis.[7-10] Genetic diversity of $\alpha_2\beta_1$ expression also correlates with predisposition to thrombotic events (for a review, see Kunicki TJ[11]).

Recently, several lines of evidence strongly encouraged revision of the step-wise model, promoting the idea that GPVI is the primary collagen receptor. Integrin $\alpha_2\beta_1$ was found to change its affinity for collagen when stimulated by noncollagenous agonists such as adenosine diphosphate (ADP) or thrombin, or through GPVI,[12] implying that high-affinity interaction through $\alpha_2\beta_1$ requires the involvement of platelet receptors that induce secretion or inside-out signaling. Moreover, mice with Cre/loxP-mediated loss of $\alpha_2\beta_1$ from the platelets displayed undisturbed bleeding times, and their platelets exhibited normal collagen adhesion and signaling.[13] Similarly, $\alpha_2(-/-)$ mice showed no changes in platelet aggregation and adhesion to fibrillar collagen,[14] whereas Fcγ$(-/-)$ mice, generating a deficiency in the coexpressed GPVI, completely lacked a response to collagen under flow, as did wild-type mouse blood incubated with an anti-GPVI monoclonal antibody (mAb), indicating a key role for GPVI.[13]

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Following the deposition of platelets, their procoagulant response is essential for thrombus formation. Platelet aggregates are stabilized through fibrin formation, and newly generated thrombin recruits further platelets and activates other cells. At an advanced stage of activation, collagen-adherent platelets undergo a procoagulant transformation that includes the exposure of phosphatidylserine (PS), the secretion of coagulation factors, and the distinctive morphology of blebbing cells and microvesiculation.[15] We have previously shown that GPVI is essential for triggering this response in collagen-adherent platelets, which is preceded by extensive intracellular Ca^{2+}-signaling.[16] Supporting this, prothrombinase activity was shown to correlate with GPVI density[17] and genotype.[18]

This study was undertaken to clarify the role of the human collagen receptors GPVI and $\alpha_2\beta_1$ in thrombus formation under flow. Studies with GPVI have been hindered by the lack of antagonists of human GPVI. Recently, 10B12, an anti-GPVI, single-chain, variable domain antibody fragment (scFv) was cloned by phage display from a library of human immunoglobulin variable domains, using its capacity to block the recombinant GPVI-CRP interaction.[19] Because it blocks CRP- and fibrillar collagen-induced platelet activation, 10B12 provides a small and efficient tool for human studies. To inhibit $\alpha_2\beta_1$ function, a collagen-derived triple-helical peptide, GFOGER-GPP,[20] was used along with well-defined inhibitory mAbs.[21,22] Simultaneous ADP and collagen receptor antagonism were also studied. Under high shear rate conditions, specific parameters of thrombus formation—surface coverage of platelets, aggregate size distribution, and PS exposure—were monitored. Further, the intracellular Ca^{2+} response, which regulates these platelet processes and secretion and thromboxane A_2 (TxA_2) production, was measured from adherent platelets.

Our results show that GPVI is crucial to collagen-induced Ca^{2+} responses, aggregate formation, and PS expression but that $\alpha_2\beta_1$ also contributes to these processes. Although GPVI is not directly involved in primary platelet adhesion to collagen, the inhibition of GPVI, along with GPIb and $\alpha_2\beta_1$, was mandatory to eradicate thrombus formation.

Materials and methods

Fibrillar type I collagen (Horm) from equine tendon was obtained from Nycomed (Munich, Germany). Apyrase (grade 7), heparin, and MRS2179, a $P2Y_1$ antagonist, were from Sigma (St Louis, MO), H-Phe-Pro-Arg chloromethyl ketone (PPACK) was from Calbiochem (La Jolla, CA), and Fluo-3 and calcein acetoxymethyl esters were from Molecular Probes (Leiden, The Netherlands). Annexin V (Apoptest) labeled with Oregon Green 488 (OG488) was obtained from Nexins Research (Hoeven, The Netherlands). AR-C69931MX, an antagonist of the $P2Y_{12}$ receptor, was from Astra-Zeneca (Charnwood, United Kingdom). Acetylsalicylic acid (ASA) was from Lorex Synthelabo (Maarssen, The Netherlands), and low-molecular–weight heparin (Fragmin) was from Pharmacia N.V. (Puurs, Belgium).

The GFOGER-GPP and CRP peptides were synthesized as described.[20,23] ScFv antibodies 10B12 and 1C3[19] and mAb 12G1 against GPIbα has been previously described[24,25]: 12G1 inhibits platelet deposition to type 1 collagen by 90% at 5 μg/mL, ristocetin-induced platelet aggregation with an IC_{50} of 1.25 μg/mL, ristocetin-induced VWF binding to GPIb by 35%,[24] and 35% of thrombin generation under intermediate shear.[25] Fab_2 fragments were generated by standard methodology. The mAb 6F1 against α_2 was a generous gift from Prof B. Coller (Mount Sinai Hospital, New York, NY).[21] The anti-β_1 mAb 4B4 was from Beckman Coulter (High Wycombe, United Kingdom). Saratin was kindly provided by Dr M. Hoylaerts (KU, Leuven, Belgium).[26]

Blood collection

Blood from 9 healthy volunteers was collected in 40 μM PPACK in 0.1 vol saline, supplemented hourly with 10 μM PPACK. Donors had not taken medication for 2 weeks. Platelet counts were determined with a Coulter counter (Coulter Electronics, Hialeah, FL). Donors were genotyped for receptor polymorphisms: GPVI (a/b), $\alpha_2\beta_1$ (α2 807 C/T), and GPIb (5-C/T) using TaqMan at the National Blood Service (Cambridge, United Kingdom). All donors expressed high GPVI levels (7 aa, 2 ab).[18] Five donors were CT for C807T α_2 polymorphism, implying an average density of $\alpha_2\beta_1$ on the platelet surface. Three donors were low expressors of $\alpha_2\beta_1$ (CC), and one was a high expressor (TT). In this small population, C807T α_2 polymorphism had no apparent effect on any of the platelet responses. Two donors were CT for GPIb C5T.

Static platelet adhesion

Static platelet adhesion to CRP and fibrillar collagen was performed exactly as previously described,[27] using cation-free conditions to examine the adhesive role of GPVI in the absence of $\alpha_2\beta_1$.

Intracellular Ca^{2+} and thrombus volume measurements

Acid citrate dextrose–platelet-rich plasma (ACD-PRP) was incubated with 7 μM Fluo-3 acetoxymethyl ester or calcein for 30 minutes at 37°C. Platelets were centrifuged with 0.1 vol ACD, washed once with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer containing 10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 0.1% glucose, 0.1% bovine serum albumin (BSA), pH 6.6, centrifuged again with 0.1 vol ACD, and suspended in the original volume of PRP with HEPES buffer, pH 7.45. PPACK-anticoagulated blood was spiked with Fluo-3–labeled platelets at 10% of the original platelet count and with 30 μM PPACK.

In control perfusions over collagen, 30% of 7 μM calcein-prelabeled autologous platelets were added. In real-time, *Z*-stacks of *x*-*y* scans were taken during the perfusion (box volume of 171.8 × 171.8 × 50 μm) at a 2-photon excitation wavelength of 800 nm and an emission of 485 to 515 nm (8% of full power) using a Radiance 2000 multiphoton laser scanning fluorescence microscope system (BioRad, Hemel Hempstead, United Kingdom), equipped with a pulsed sub-picosecond Tsunami Ti:sapphire laser (Spectra-Physics, Mountain View, CA). Two-photon images were analyzed with Laserpix software (Bio-Rad).

Flow experiments and image recording

Whole blood perfusion experiments were performed essentially as described for mouse blood.[28] Briefly, glass coverslips were coated with collagen fibers (12.5 μg/cm^2) and blocked with HEPES buffer, pH 7.45, containing 1% BSA and 1% glucose. The blood was placed in a syringe and perfused over the coverslip through a transparent 50 μm deep chamber using a pulse-free pump,[29] at a shear rate of 1000 s^{-1} for 4 minutes. Blood was incubated for 15 minutes before perfusion with various concentrations of scFv 10B12 or 1C3, 10 to 20 μg/mL 6F1, 500 μg/mL GFOGER-GPP, 10 μg/mL 4B4, 20 μg/mL Fab_2 fragment 12G1, ASA (100 μM), MRS2179 (40 μM), and AR-C69931MX (20 μM) or 1 U/mL apyrase, as described. Where indicated, autologous Fluo-3–labeled platelets were added to the blood before antagonists.

Microscopic phase-contrast and fluorescent images from Fluo-3–labeled platelets were recorded in real-time using a Visitech digital imaging system (Sunderland, United Kingdom) equipped with 2 intensified, charge-coupled device (CCD) cameras.[30] After perfusion, flow chambers were rinsed at the same flow rate for 4 minutes with HEPES buffer, pH 7.45, supplemented with 1 U/mL heparin and 2 mM $CaCl_2$. Phase-contrast and fluorescent images were collected with a 40× UV-transparent objective and 15× to 20× image magnification. Exposure of PS was detected after incubation of the slide with 100 μL HEPES/$CaCl_2$ buffer, pH 7.45, containing OG488-labeled annexin V (1 μg/mL) for 5 minutes. Antibodies and antagonists were also added to the rinsing buffer and the annexin V incubation.

Image analysis

Surface coverage from phase-contrast images was analyzed using Image-Pro (Silver Spring, MD) software version 4.1, and it was analyzed from platelets stained with OG488-annexin V using Quanticell software (Visitech). Distribution of aggregate sizes in phase-contrast images was

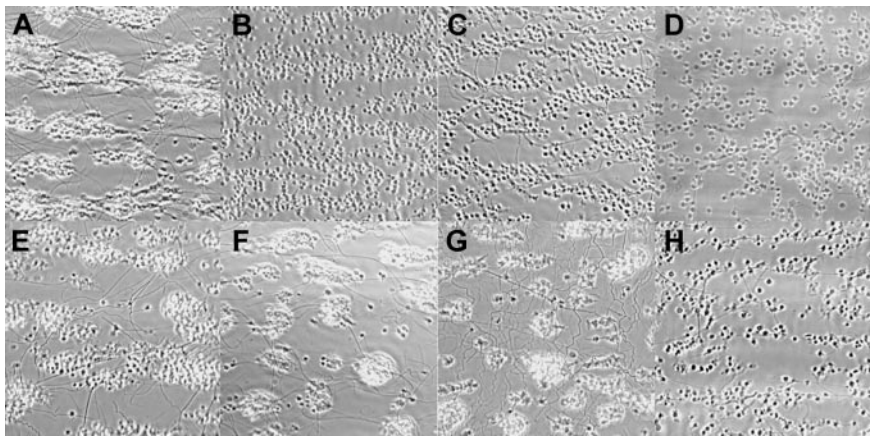

Figure 1. Effects of inhibitory antibodies and receptor antagonists on thrombus formation. Images (120 × 120 μm) were recorded using a phase-contrast microscope after the perfusion of PPACK-anticoagulated whole blood over a collagen-coated surface for 4 minutes at a shear rate of 1000 s^{-1} Antibodies or inhibitors were added 15 minutes before perfusion. (A) Control. (B-D) 10B12 at 50, 100, or 300 μg/mL, respectively. (E) scFv 1C3 at 100 μg/mL. (F) 6F1 at 20 μg/mL. (G) GFOGER-GPP at 500 μg/mL. (H) ADP-receptor and TxA_2 blockade by 40 μM MRS2179, 20 μM AR-C69931MX, 1 U/mL apyrase, and 100 μM ASA. Images shown are representative of 3 to 9 independent experiments. Collagen fibers are visible in some of the panels. Original magnification, × 60.

measured using Leica QWin image analysis software (Leica Imaging Systems, Cambridge, United Kingdom). Changes in Fluo-3 fluorescence from individual platelets were converted to $[Ca^{2+}]_i$ as described.[31] To provide a measure of the proportion of procoagulant cells independent of platelet deposition, the ratio of annexin V–binding surface coverage to phase-contrast surface coverage was calculated and was termed procoagulant index (Pi). Although the procoagulant area was overestimated through fluorescent glare in the optics, Pi provided a means of distinguishing the effects of treatments on procoagulant expression from those on platelet deposition.

Experimental design and statistics

Each experimental condition was tested on at least 3 occasions using blood from different donors, and each donor provided blood for at least 10 perfusion experiments. Control conditions were included for each blood sample, together with other permutations of conditions. For each perfusion surface, images from 9 random microscopic fields were collected, and the average percentage area covered by adherent platelets was measured either by phase-contrast or by OG-labeled annexin V fluorescence. Phase-contrast, fluorescent surface coverage (mean ± SE) and Pi were compared among all experimental groups using analysis of variance (ANOVA), with Newman-Keuls or Dunnett posttesting. Data derived from the same donors were compared by paired sample *t* tests or ANOVA. The effect of ADP blockade under different conditions was tested by 2-way ANOVA.

Image analysis provided estimates (area and roundness of separate features, such as platelets and aggregates) of thrombus size and shape. Features were segmented digitally and measured with minimal operator intervention. From an average of 9 images, using 3 different donors, at least 200 different features were measured for each treatment, the effects of which were determined by χ^2 analysis. Data are plotted as frequency distributions with mean values per field (± SE). Prism (GraphPad Software, San Diego, CA) was used throughout for multiple comparisons and linear regression.

Results

Blocking of GPVI abolishes aggregate formation, Ca^{2+} signaling, and PS exposure without eradicating primary platelet adhesion

PPACK-anticoagulated whole blood was perfused at high shear rate (1000 s^{-1}) over a surface covered with native collagen type I fibrils. Phase-contrast and fluorescence microscopic images were captured to monitor 2 independent parameters of thrombus formation measured as the surface coverage of deposited platelets and of procoagulant, PS-exposing platelets that stained with OG488-conjugated annexin V. In control perfusions, platelets rapidly adhered to the collagen fibers, forming aggregates that later coalesced (Figure 1A). The deposition of platelets measured by surface coverage was linear in time, indicating regular and continuous scavenging of platelets (Figure 2A). To confirm that the measuring surface area represents a linear progression of thrombus build-up, the thrombus volume was measured by using calcein-labeled platelets from the same donor and real-time scanning of the 2-photon fluorescence signal, which even at more than 50 μm penetration was typically not distorted by the flowing blood. Again, thrombus volume increased linearly in time (Figure 2B). No differences in platelet deposition were observed within the donor population, relating to genetic polymorphisms of GPVI, $\alpha_2\beta_1$, or GPIbα. By the 4-minute end point, 17.9% ± 0.8% of the surface was covered by platelets. Most platelets were deposited as interconnected islets originating from individual aggregates. The mean feature size was 176 ± 16 μm^2, an area corresponding to approximately 40 platelets. Single platelets made up 22% of features, whereas 10% were large aggregates corresponding to 130 platelets or more (Figure 3A). Large numbers of blebbing platelets were observed in direct contact with collagen fibers, and staining with OG488-annexin V revealed 8.3% ± 0.7% coverage of PS-exposing platelets. To provide a measure of procoagulant platelets independent of the total number of adherent cells, the ratio of the 2 surface parameters was expressed as Pi, giving a value of 0.43 ± 0.03. Thrombin generation was absent during and after perfusion because no fibrin or D-dimer was observed. Including Fragmin (0.5 U/mL) as an additional anticoagulant did not change platelet deposition or annexin V binding from PPACK-only controls (data not shown).

Blocking GPVI by 10B12 inhibited CRP- and collagen-induced platelet aggregation in vitro.[19] Static platelet adhesion to CRP was

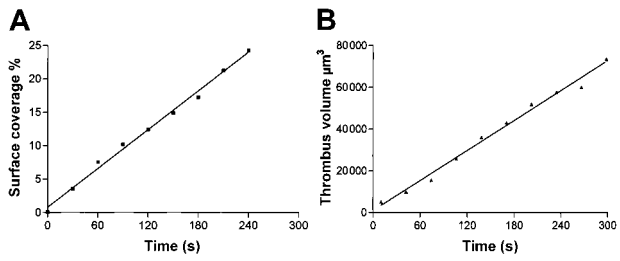

Figure 2. Thrombus formation measured by surface area coverage or thrombus volume is linear in time. (A) Phase-contrast video images captured during the flow of PPACK-anticoagulated whole blood over collagen at 1000 s^{-1} were analyzed for platelet surface area coverage, which was plotted against perfusion time. Linear regression showed R^2 = 0.97-0.99 (n = 3). (B) Thrombus volume was measured using blood supplemented with 30% of calcein-labeled autologous platelets. *Z*-stacks of *x-y* scans were measured in real-time by 2-photon laser scanning microscopy. Fluorescence image stacks were reconstructed 3 dimensionally and analyzed. Plots of thrombus volume compared with time were linear up to 300 s (R^2 = 0.99). Representative plots are shown in panels A and B.

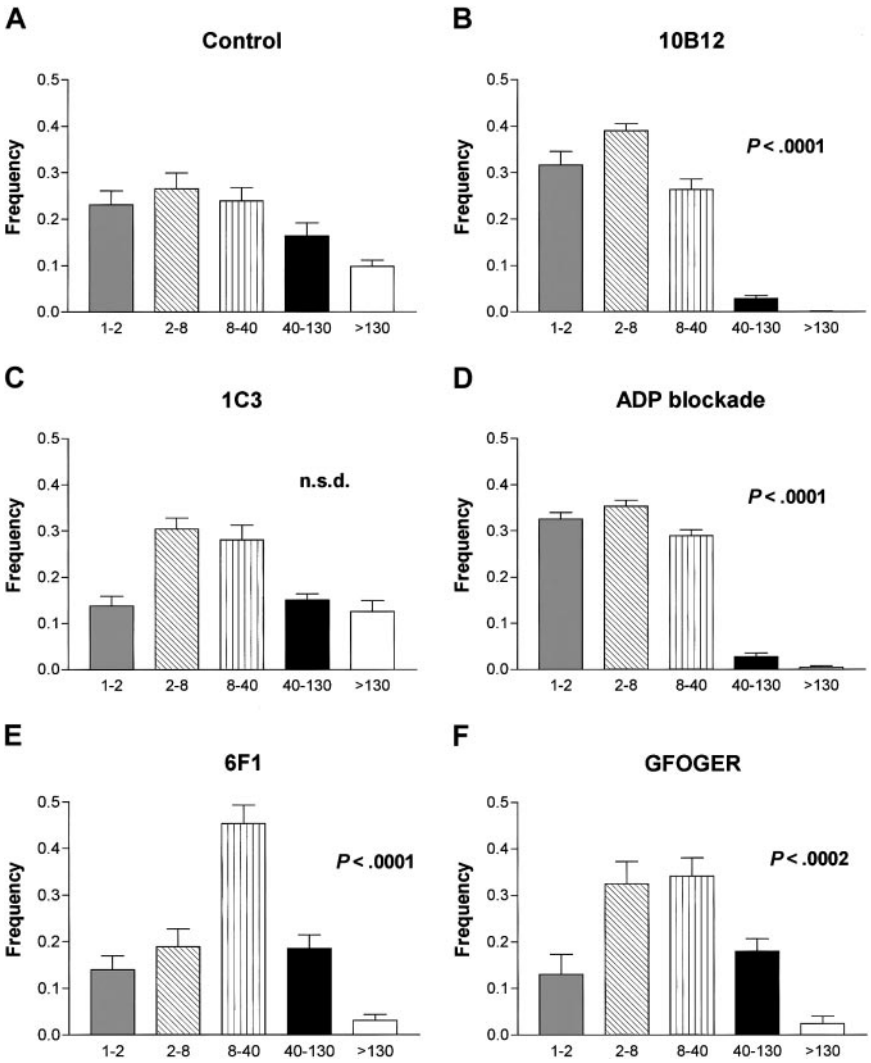

Figure 3. Effects of inhibitory antibodies and receptor antagonists on size distribution of platelet aggregates during thrombus formation. Histograms were obtained by image analysis of phase-contrast micrographs obtained from perfused collagen surfaces. Estimated numbers of platelets per feature were 1 to 2 (▤), 2 to 8 (▧), 8 to 40 (▥), 40 to 130 (■), and more than 130 platelets (□). (A) Control. (B) 10B12 at 50 μg/mL. (C) 1C3 at 100 μg/mL. (D) ADP receptor and TxA_2 blockade. (E) 6F1 at 20 μg/mL. (F) GFOGER-GPP at 500 μg/mL. Data (A-F) are mean ± SE from 3 donors. Statistical significance compared with the control was established from contingency tables (χ^2 analysis).

abolished at 5 μg/mL and decreased by 80% ± 4% (n = 4) to collagen fibers at 25 μg/mL. However, under flow, a concentration curve for 10B12 (Figure 4) showed that, up to 300 μg/mL, surface coverage with platelets did not significantly decrease ($r^2 = 0.22$; $P = .42$), though, dramatically, platelet aggregates were absent (Figure 1B-D). Biosensor data indicated that at 300 μg/mL 10B12, occupancy of recombinant GPVI had reached more than 97% (data not shown). The predominance of single platelets with increasing 10B12 concentrations, obvious to the eye, was quantified by image analysis as almost complete elimination of larger aggregates ($P < .0001$) at 50 μg/mL 10B12, and the mean area was reduced to 37 ± 2 μm^2 from 176 ± 16 μm^2 ($P < .001$) (Figure 3B). In contrast to platelet adhesion, surface coverage of PS-exposing platelets decreased progressively with the 10B12 concentration with an IC_{50} value of 23 μg/mL ($r^2 = 0.94$; $P < .01$) (Figure 4), and blebbing platelets were no longer visible. At the highest concentration of 10B12 tested, the Pi was reduced to 0.02 ± 0.005 ($P < .001$), indicating strong blockade of GPVI function.

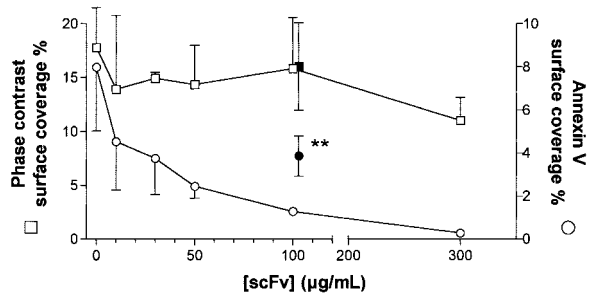

Figure 4. Inhibition of GPVI causes dose-dependent reduction in platelet PS exposure but not in total surface coverage. Blood was treated with 10B12 at 10-300 μg/mL (white symbols) or 1C3 at 100 μg/mL (black symbols). Total surface coverage of platelets (squares) and surface coverage of OG488-labeled annexin V binding platelets (circles) were measured after 4 minutes of perfusion. Data represent mean ± SE from 3 to 17 experiments performed on the blood of 3 donors. **Significant difference from 10B12 treatment ($P < .001$) and from control ($P < .05$).

ScFvs 1C3 and 10B12 recognizes distinct epitopes, and 1C3 is incapable of blocking static platelet adhesion to CRP or collagen-induced platelet aggregation.[19] As expected, 1C3 (100 μg/mL) did not alter either the surface coverage (Figure 1E) or the size and morphology (Figure 3C) of the formed thrombi. However, 1C3 halved the PS expressing surface coverage (Figure 4), suggesting that the 1C3-binding site is relevant for GPVI function, possibly by influencing receptor clustering or dimerization. The Pi obtained with 1C3 was reduced to 0.24 ± 0.07 ($P < .05$, relative to control), a significantly higher Pi than with 100 μg/mL 10B12 ($P < .001$). Coinhibition with 10B12 and 1C3 did not change surface coverage from that of 10B12 alone (data not shown).

To study the dynamics of platelet activation, Ca^{2+} signal generation was measured in real-time under flow from single, Fluo-3-labeled platelets coming in contact with collagen. Control

platelets exhibited a strong Ca^{2+} response, sometimes preceded by an initial Ca^{2+} spike (Figure 5A). A submaximal dose (50 μg/mL) of 10B12 decreased the Ca^{2+} signal amplitude (Figure 5B). Although blocking GPVI did not abolish primary adhesion, the increased translocation of platelets across the collagen surface was observed during Ca^{2+} measurements. Increasing the 10B12 concentration to 300 μg/mL reduced the Ca^{2+} signal slightly further (Figure 5C). No difference from controls was observed in the averaged Ca^{2+} response with 1C3 (100 μg/mL), though Ca^{2+}-spiking occurred in individual platelets (Figure 5D). Together, these data indicate that blocking of GPVI with 10B12 potently suppressed collagen-dependent platelet activation pathways without eradicating primary adhesion.

Blocking of GPIb or $\alpha_2\beta_1$ reduces primary adhesion and aggregate formation, but blocking only $\alpha_2\beta_1$ inhibits PS exposure

High levels of VWF and high $\alpha_2\beta_1$ density have been correlated with increased platelet deposition on collagen,[32,33] whereas patients with VWF concomitant with low $\alpha_2\beta_1$ expression bleed more than patients with either condition alone.[34] The essential nature of GPIb and $\alpha_2\beta_1$ in human platelet deposition on collagen under flow has been emphasized,[2,3,32,35] though other studies failed to detect consistent involvement of $\alpha_2\beta_1$.[4] This prompted us to revisit the topic in the context of molecular interplay with GPVI using the following antagonists: 12G1 Fab_2 fragment, recognizing amino acids 1 to 59 of GPIbα, hinders platelet adhesion to collagen by inhibiting VWF-GPIb interaction at high-shear[24,25]; anti-α_2 mAb 6F1, whose epitope within the I-domain is separate from the MIDAS motif and lies between residues 173 and 259[36]; and the GFOGER-GPP peptide, which inhibits collagen binding through the α_2 MIDAS.[37]

The importance of GPIb in primary adhesion[4] was reconfirmed under the shear rate conditions used here. Using 12G1 Fab_2 at a maximally effective dose of 40 μg/mL, the overall surface coverage was reduced by 50% ± 14% (Figure 6), which confirms that at (arterial) shear rates of approximately 1000 s^{-1}, platelet adhesion to collagen is only partially dependent on VWF-GPIb interaction.[38,39] Although in 2 donors surface coverage was strongly inhibited (63% and 67%), 2 gave a weaker reduction in thrombus formation (38% and 45%), but the differential response did not correlate with −5C/T polymorphism. For all donors, blocking of GPIb strongly influenced platelet tethering. GPIbα-blocked platelets moved swiftly over the collagen surface, but, once adherent, they became fully activated. Adding 12G1 Fab_2 reduced the PS exposure of platelets, but this simply reflected the decrease in surface coverage (Figure 6A) without a change in Pi. Like 12G1, saratin, which blocks the binding of VWF to collagen,[40] caused only a partial reduction of platelet surface coverage (68% ± 14.5%) and annexin V staining (27% ± 0.3%; n = 2).

Inhibition of $\alpha_2\beta_1$ integrin by mAb 6F1, at a saturating concentration of 10 to 20 μg/mL, reduced surface coverage in all donors by approximately 30% ± 11% (Figure 6A). The tethering platelets reluctantly anchored to collagen fibers and, on arrest, formed only small, dense aggregates (Figure 1F). Image analysis indicated a smaller mean aggregate size of 117 ± 9 μm^2 with a shift toward round structures ($P < .001$). Aggregate sizes showed a kurtotic distribution ($P < .0001$), with a 50% increase in mid-sized features (Figure 3E). Similarly, GFOGER-GPP significantly reduced surface coverage by approximately 45% ± 13% at a maximally effective dose of 500 μg/mL (Figure 6A). Changes in aggregate morphology closely matched those obtained with 6F1 mAb—that is, aggregates appeared as round structures (Figure 1G), of smaller, average size (Figure 3F).

Inhibition of $\alpha_2\beta_1$ with either mAb 6F1 or GFOGER-GPP had a small but significant effect ($P < .01$) on the exposure of PS (Figure 6A). The Pi was reduced from 0.43 ± 0.03 to 0.30 ± 0.04 ($P < .05$) and 0.37 ± 0.08 (not significant), respectively. To ensure maximal blocking of $\alpha_2\beta_1$, we tested combinations of antagonists binding different epitopes. The most effective of 3 anti-β_1 mAbs tested, 4B4—against the activatory hinge region 207 to 218[22]—caused less inhibition than GFOGER or 6F1 (data not shown). Slight further inhibition was seen when 6F1 was combined with 4B4 or GFOGER, but these reductions were not significant (Figure 6B). A role of $\alpha_2\beta_1$ in platelet signaling was also supported by the decreased Ca^{2+} response of the collagen-adherent platelets. With both 6F1 (Figure 5E) and GFOGER-GPP (Figure 5F), the average Ca^{2+} response was decreased, and the responses of most single platelets showed transient or spiking increases in intracellular Ca^{2+}. These findings reconfirm the contribution of GPIb and $\alpha_2\beta_1$

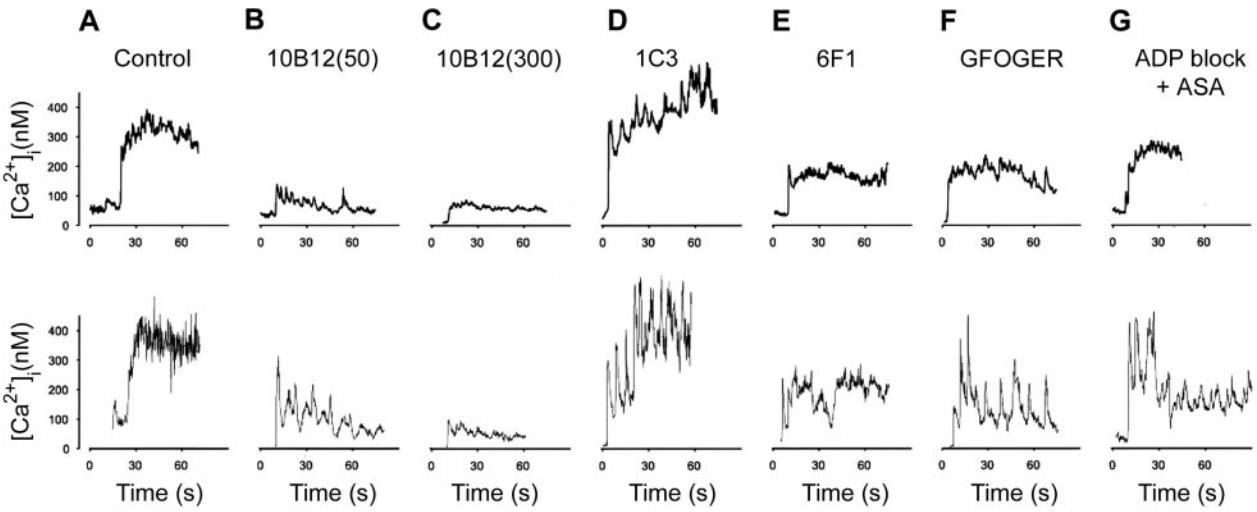

Figure 5. Effects of inhibitory antibodies and receptor antagonists on Ca^{2+} responses of platelets interacting with collagen under flow. Blood was spiked with 10% Fluo-3–loaded autologous platelets, incubated with antagonists, and perfused over collagen, as indicated for Figure 1. Changes in $[Ca^{2+}]_i$ were recorded during perfusion in single collagen-adherent platelets. Traces above are averaged curves from 15 to 25 platelets (3 donors); traces below are representative curves from single platelets. (A) Control. (B) 10B12 at 50 μg/mL. (C) 10B12 at 300 μg/mL. (D) 1C3 at 100 μg/mL. (E) 6F1 at 20 μg/mL. (F) GFOGER-GPP at 500 μg/mL. (G) ADP-receptor and TxA_2 blockade, as in Figure 1.

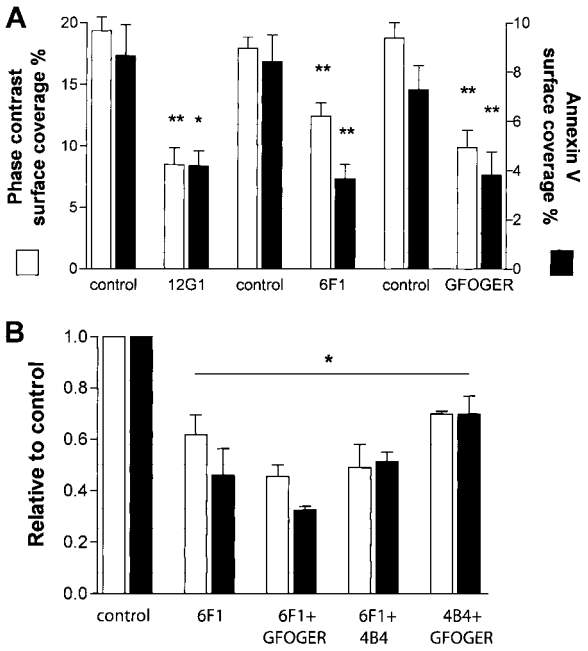

Figure 6. Blocking of GPIbα or $\alpha_2\beta_1$ partially reduces surface coverage on collagen, but blocking only of $\alpha_2\beta_1$ affects PS exposure. (A) Blood was treated with 12G1 Fab_2 at 40 μg/mL and 6F1 at 20 μg/mL or GFOGER-GPP at 500 μg/mL and was perfused over collagen, as described for Figure 1. Data present surface coverage of platelet deposition or OG488-labeled annexin V binding (mean ± SE, from 4-6 experiments). $^{**}P < .01$, and $^{*}P < .05$ compared with matched controls. (B) Blood was treated with combinations of anti-α_2 6F1 or GFOGER and anti-β_1 4B4 (10 μg/mL). Data (mean ± SE) present the proportion of phase- or annexin V–positive surface coverage relative to matched controls. All combinations significantly inhibited platelet responses compared with respective controls ($^{*}P < .05$; n = 2) but not with each other.

to collagen-induced thrombus formation but underscore their different roles. Blocking GPIb interfered with platelet tethering and initial anchorage and dispersed aggregates with little effect on PS exposure, but $\alpha_2\beta_1$ blockade reduced primary adhesion (partially by prolonging firm anchorage) and restricted thrombus size, platelet activation, Ca^{2+} signaling, and PS exposure.

Combined inhibition of GPVI with either GPIb or $\alpha_2\beta_1$ integrin completely abolishes thrombus formation

Coinhibition of GPVI with 10B12 (100 μg/mL) and of GPIb with 12G1 Fab_2 (40 μg/mL) resulted in almost complete abolition of platelet adhesion, with remaining single platelets covering not more than 2% of the surface; PS-exposing platelets were hardly detected (Figure 7A-B). Similarly, coinhibition of GPVI and $\alpha_2\beta_1$ with 6F1 (20 μg/mL) or GFOGER-GPP (500 μg/mL) completely inhibited stable platelet adhesion and all subsequent activation events, eradicating thrombus formation (Figure 7A-B). Calcium responses, measured in platelets adhering in the presence of 6F1 and 10B12, resembled the responses measured in the presence of 10B12 alone (data not shown).

In contrast, combined blocking of GPIb and $\alpha_2\beta_1$ had a less dramatic effect on platelet deposition and aggregation because significant numbers of small aggregates could still be observed. Surface coverage after combined blockade of GPIb with $\alpha_2\beta_1$ was reduced by approximately 70% (Figure 7A), a greater effect than achieved by blocking either receptor individually ($P < .05$). The remaining platelets were in an activated state, judged by the relatively high surface coverage of PS-exposing platelets and the apparent increase in Pi to 0.72. In conclusion, our data show that GPIb and $\alpha_2\beta_1$ have partially overlapping functions in mediating human platelet adhesion and GPVI-dependent thrombus formation.

Blocking of ADP receptors further inhibits thrombus formation restricted by GPVI or $\alpha_2\beta_1$ blockade

ADP regulation of thrombus size has been attributed to the platelet $P2Y_{12}$ purinergic receptor; $P2Y_1$ regulates the onset of thrombus formation.[41] To obliterate any effects of ADP, we used apyrase in conjunction with specific $P2Y_{12}$ and $P2Y_1$ inhibitors. Full inhibition of ADP- or TxA_2-mediated (inhibited by ASA) effects reduced the surface coverage by 43% ($P < .001$). The morphology of platelet deposition was similar to that under GPVI blockade: a layer of single platelets remained on the collagen (Figure 1H), and aggregates were eradicated (Figure 3E). However, combined blockade of ADP and TxA_2 actions had no effect on Pi (0.53 ± 0.15 [not significant]) or platelet blebbing, though it slightly reduced the Ca^{2+} response (Figure 5G).

Inhibiting the ADP pathway further decreased surface coverage when combined with GPVI blockade. Irrespective of the 10B12 concentration (50 or 300 μg/mL), ADP receptor antagonism caused an additional reduction in platelet surface coverage (55%; $P < .001$) (Figure 8A), suggesting that the presence of ADP was not solely derived from GPVI-dependent secretion. Because GPVI inhibition itself abolished aggregate formation, the additional blockade of ADP receptors had no further effect on the mean feature size: 24 ± 2.6 μm^2 (ADP + GPVI blockage) and 23 ± 1.8 μm^2 (GPVI blockade alone) (Figure 8B). When applied together with 10B12, ADP receptor blockade reduced the number of PS-exposing platelets by attenuating platelet deposition because the Pi did not significantly decrease.

When the ADP receptors were coinhibited with $\alpha_2\beta_1$ (20 μg/mL 6F1), surface coverage decreased by 41% (Figure 8A), and the mean aggregate size decreased from 121 ± 8 to 44 ± 4 μm^2 ($P < .0002$) (Figure 8B). Again, this was accompanied by a small decrease in PS-exposing platelets and an unaltered Pi value. These

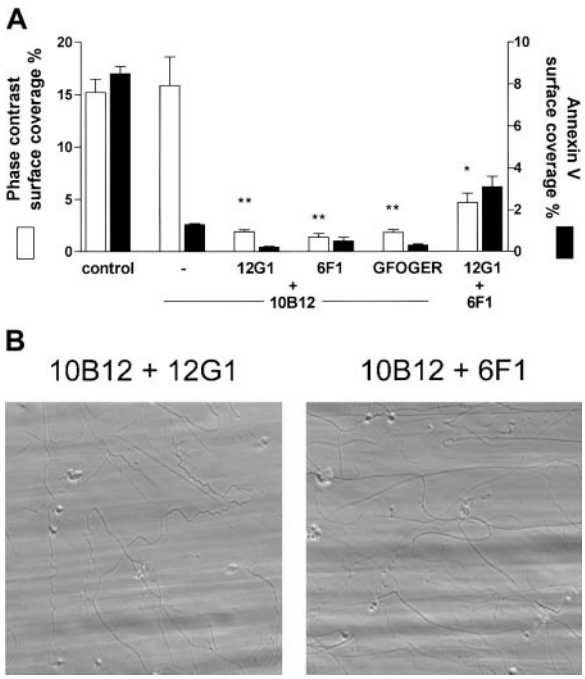

Figure 7. Combined inhibition of GPVI with GPIb or $\alpha_2\beta_1$ abolishes collagen-induced thrombus formation. Blood was treated with the indicated combination of antagonists before perfusion over collagen: 10B12 at 100 μg/mL and 6F1 at 10 to 20 μg/mL, GFOGER-GPP at 500 μg/mL or 12G1 Fab_2 at 40 μg/mL perfused over collagen as in Figure 1. (A) Surface coverage of platelet deposition or OG488-labeled annexin V binding (mean ± SE, from 4-6 donors). $^{**}P < .001$, and $^{*}P < .05$ compared with single antibody controls. (B) Representative phase-contrast images after perfusion, also showing visible collagen fibers. Original magnification, × 60.

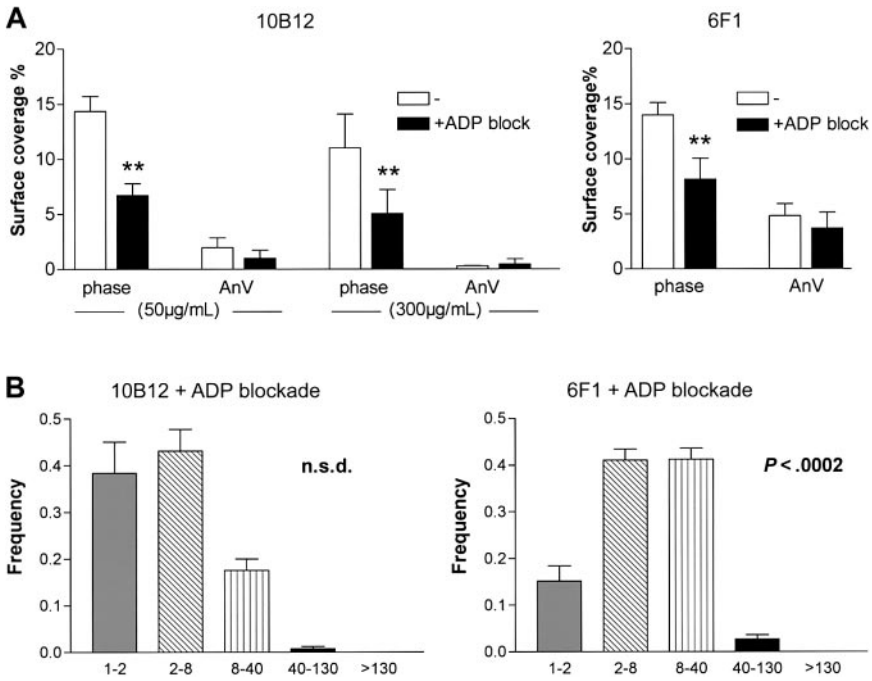

Figure 8. ADP antagonism further reduces thrombus formation after the inhibition of GPVI or $\alpha_2\beta_1$. Blood was treated with 10B12 at 50 or 300 μg/mL, 6F1 at 20 μg/mL with or without ADP receptor antagonism (40 μM MRS2179, 20 μM AR-C69 931MX, and 1 U/mL apyrase). (A) Total surface coverage of platelets and of annexin V–positive platelets was measured (mean ± SE from 4-6 donors). $^{**}P < .01$ compared with control condition. (B) Histograms of feature sizes from phase-contrast micrographs, determined as in Figure 2, where individual antibody effects are shown. Estimated numbers of platelets per feature were 1 to 2 (▤), 2 to 8 (▧), 8 to 40 (▥), 40 to 130 (■), and more than 130 platelets (□). Data are means ± SE from 3 donors.

results let us conclude that ADP (from various sources) stimulates GPVI- and $\alpha_2\beta_1$-dependent adhesion and contributes to aggregate formation but is insufficient to promote PS exposure. Thus, platelets respond to ADP by inducing one of the thrombus end points, aggregation, in contrast to full activation through GPVI.

Discussion

The present study shows that, in humans, collagen-induced thrombus formation under high shear rate requires the orchestrated interplay of several platelet receptors. Using antibodies and peptides allowed clarification of the complementary and overlapping roles of the GPIb/V/IX complex, $\alpha_2\beta_1$ integrin, GPVI, and the platelet purinergic receptors in the processes of platelet adhesion, aggregation, and procoagulant activity. Our results, though formally in line with the original 2-step adhesion-activation model, also revealed its oversimplification. Although the main role of GPIb is to provide initial contact with collagen-bound VWF, it stabilizes the anchorage of thrombi on collagen together with $\alpha_2\beta_1$. GPVI is involved in GPIb- and $\alpha_2\beta_1$-mediated adhesion through activation, and it is the main signaling receptor during thrombus formation. Conversely, $\alpha_2\beta_1$ assists GPVI in signaling. Complete abrogation of adhesion and subsequent activation thus requires simultaneous inhibition of GPVI together with either GPIb or with $\alpha_2\beta_1$.

We find that GPVI has a crucial role in regulating human thrombus growth. Inhibiting GPVI resulted in the complete loss of platelet aggregates and the full inhibition of collagen-induced Ca^{2+} mobilization and PS exposure. In contrast, the adhesion of single, nonaggregated platelets was not significantly diminished unless ADP was also antagonized, probably eliminating the contribution of $\alpha_2\beta_1$. These results corroborate findings from earlier flow studies with GPVI-deficient platelets in which the surface coverage equaled that obtained with $\alpha_{IIb}\beta_3$ antagonists.[6] A recent study using the blood of patients with GPVI deficiency also showed the obliteration of collagen-induced thrombus formation though a layer of single platelets was still visible in the PDF-format micrographs,[42] supporting our conclusions. In contrast, these results clearly deviate from those obtained in the first flow study using GPVI-deficient mice,[13] which showed the total abolition of platelet-collagen adhesion. In mice, GPVI function was severely impaired when more than 90% of receptors are blocked. At the highest level of the scFv 10B12 used here (300 μg/mL), calculated receptor occupancy was 97%, supporting the idea that unaltered platelet adhesion was not caused by the inadequate inhibition of GPVI. Although it is possible that antibody dissociation or displacement by ligand may allow a proportion of GPVI to remain functional during multireceptor interaction under flow, 10B12 caused the near-complete, concentration-dependent abolition of platelet activation processes such as PS exposure, Ca^{2+} response, and aggregate formation. While this paper was in preparation, a true GPVI knockout model was generated in mice in which thrombus formation was similar to that observed in our study.[43] The reasons for the discrepancies regarding the different mouse data remain to be elucidated.

Both GPIb and $\alpha_2\beta_1$ were implicated in the primary adhesive step at high shear rate, as others suggested after using recombinant receptors in liposomes.[44] Inhibiting GPIb or $\alpha_2\beta_1$ on its own, but not GPVI, dramatically reduced the number of collagen-adherent platelets, though those remaining became activated. The interplay of GPVI with both receptors is implicit in the near-complete suppression of adhesion using the combined blockade of GPIb and GPVI or $\alpha_2\beta_1$ and GPVI. This indicates that all 3 receptors are required for optimal adhesion. As a first step, GPIb tethers the platelet, allowing other receptors to interact, which perhaps further regulates GPIb function, as recently reported.[42,45] Adhesion through $\alpha_2\beta_1$ follows closely, with GPVI and $\alpha_2\beta_1$ acting interdependently. Initial $\alpha_2\beta_1$ binding to high-affinity collagen sequences, not requiring activation, supports the weak GPVI-collagen interaction, which, in turn, mediates the switch of the integrin to the high affinity required for stable adhesion. In line with this, blocking GPVI with 10B12 caused a more dramatic (but not full) reduction in surface coverage on collagen type III containing only low-affinity xxxGER sequences, but not on collagen type I containing the high-affinity GFOGER sequence used in this study (P.A.S., unpublished data, 2002). Collagen-dependent differences in GPVI

inhibition were also observed elsewhere.[6] The failure of $\alpha 2\beta 1$ blockade to cause further reduction in Ca^{2+} response and PS exposure in the presence of 10B12 implies that $\alpha_2\beta_1$ signaling occurs downstream from GPVI. This conclusion was reinforced by almost complete loss of binding to adherent platelets, in the presence of 300 μg/mL 10B12, of an antibody that recognizes only the fully activated state of α_2-integrin, IAC-1 (7F6).[46] However, the signaling end points of this study (Ca^{2+}, PS exposure, and aggregate size) were measured to identify the contribution of specific receptors rather than to study the detail of the signaling processes, which will be subject to further study.

The present model of human thrombus formation deviates in certain respects from that derived from murine data. Although murine thrombus formation seems to rely more on GPVI, it was recently shown that the interplay of $\alpha_2\beta_1$ with GPVI is required for full GPVI-induced platelet activation and procoagulant activity, using β_1-deficient mouse platelets.[28] In addition, in another $\alpha_2(-/-)$ mouse flow model, platelet-collagen interaction was severely impaired.[47] Thus, the main deviation between human and mouse concerns the role of primary receptors in platelet adhesion to collagen under flow. There may be several nonexclusive reasons for discrepancy. First, human $\alpha_2\beta_1$ may be competent to bind GFOGER sequences in type I collagen but not those of lower affinity, such as GASGER,[48] before GPVI involvement. Second, human $\alpha_2\beta_1$ may become activated independently of GPVI, perhaps caused by GPIb activity. It has been proposed that GPIb uses the same Fcγ-mediated signaling pathway as GPVI,[49] and it reportedly induces rapid Ca^{2+} signaling under flow.[50] Similarly, the GPVI-independent presence of ADP in the blood may activate $\alpha_2\beta_1$ at an early stage. Third, the threshold for $\alpha_2\beta_1$ activation of human platelets may be low, that is, small signals from the slight percentage of GPVI not blocked by 10B12 may still be sufficient to trigger $\alpha_2\beta_1$ for adhesion. Fourth, the human integrin-collagen contact—though dependent on GPIb and GPVI—may be more prone to autocrine signaling than that in mouse platelets. Finally, the considerable differences in human and murine GPVI structures may influence their contribution to the platelet-collagen interaction. Murine GPVI shares only 64% homology with the human receptor, and its cytoplasmic domain, half the length of the human 51-amino acid tail, lacks signaling motifs.[51,52] A human-to-mouse mutation, K59E, was shown to decrease GPVI binding to CRP, indicating important differences in the function of human and murine receptors.[19]

After the primary platelet-collagen contact, platelets form aggregates or become procoagulant; both processes contribute to full-blown thrombus formation. We defined 2 conditions in which the formation of thrombi was inhibited: blocking GPVI and blocking ADP- and TxA_2-mediated events. Inhibiting GPVI suppressed aggregate formation and signaling processes, detected as the greatly reduced Ca^{2+} and procoagulant response. However, ADP/TxA_2 antagonism reduced Ca^{2+} signaling only slightly, though no aggregates were formed, with no effect on Pi. GPVI regulates aggregate formation through autocrine ADP/TxA_2 secretion, in part through synergism with $G_{i/q}$-coupled receptors.[53] We found that ADP contributed to aggregation and primary adhesion but that its influence was not eliminated by GPVI inhibition. This implies that ADP was derived from sources other than GPVI-stimulated platelets.

Platelet aggregation was mostly accompanied by high Ca^{2+} signaling and PS exposure, even when blocking shear-dependent adhesion through GPIb. The platelets that escaped inhibition were activated, as demonstrated by an unchanged Pi. A notable exception occurred with the blocking of $\alpha_2\beta_1$ function. Ca^{2+} signaling, PS exposure, and aggregate formation were all affected. This implies that $\alpha_2\beta_1$ has an activatory effect on these processes, in synergy with GPVI. Most, if not all, integrins exhibit inside-out and outside-in signaling,[54] suggesting that outside-in signaling from activated $\alpha_2\beta_1$ can participate in platelet activation. We have previously shown that integrin-mediated adhesion to fibrinogen or collagen increases the responsiveness of platelets toward GPVI agonists.[55] Similar cross-talk between GPVI and $\alpha_2\beta_1$ also likely controls platelet activation in the present experiments. Previously, others have linked $\alpha_2\beta_1$ activation with $\alpha_{IIb}\beta_3$ up-regulation[56,57] and established cross-talk between $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$.[58]

Together, the results presented here advance the idea that human thrombus formation occurs through the concerted action of several receptors: the interplay of GPIb/V/IX, integrin $\alpha_2\beta_1$ and GPVI enables platelet deposition, and GPVI in cross-talk with $\alpha_2\beta_1$ mediates subsequent thrombus formation, additionally supported by ADP. Yet these receptors may not be the only determinants of human platelet collagen interaction because other collagen receptors[35,57,59,60] may participate in the early stages of platelet contact with collagen, and new receptors are being discovered.[61,62] Ultimately, these other receptors may participate in the interaction and may be shown to allow further fine-tuning of the receptor cross-talk identified in this study.

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