Principal Role of Glycoprotein VI in $\alpha 2\beta 1$ and $\alpha IIb\beta 3$ Activation During Collagen-Induced Thrombus Formation

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Objective—High-shear perfusion of blood over collagen results in rapid platelet adhesion, aggregation, and procoagulant activity. We studied regulation of $\alpha 2\beta 1$ and $\alpha IIb\beta 3$ integrin activation during thrombus formation on collagen.

Methods and Results—Blockade of glycoprotein (GP) VI by 9O12 antibody or of P2Y purinergic receptors permitted platelet adhesion but reduced aggregate formation, fibrinogen binding, and activation of $\alpha 2\beta 1$ and $\alpha IIb\beta 3$, as detected with antibodies IAC-1 and PAC1 directed against activation-dependent epitopes of these integrins. Combined blockade of GPVI and P2Y receptors and thromboxane formation abolished integrin activation but still allowed adhesion of morphologically unstimulated, nonprocoagulant platelets. Exogenous ADP partly restored the suppressive effect of GPVI blockade on integrin $\alpha 2\beta 1$ and $\alpha IIb\beta 3$ activation. Adhesion was fully inhibited only with simultaneous blocking of GPVI and $\alpha 2\beta 1$, indicating that the integrin can support platelet–collagen binding in the absence of its activation. Blockade or absence of GPIbα only moderately influenced integrin activation and adhesion unless GPVI was inhibited.

Conclusions—GPVI- and autocrine-released ADP induce affinity changes of $\alpha 2\beta 1$ and $\alpha IIb\beta 3$ during thrombus formation on collagen under flow. These integrin changes are dispensable for adhesion but strengthen platelet–collagen interactions and thereby collagen-induced platelet activation. **(*Arterioscler Thromb Vasc Biol*. 2004;24:1727-1733.)**

Key Words: ADP ■ collagen ■ glycoprotein VI ■ integrins ■ platelets ■ thrombus

Platelet integrins are critical in hemostasis. Abundantly expressed at the platelet surface, integrins are required for platelet interactions with subendothelial matrix components and for platelet–platelet interactions leading to aggregate and thrombus formation.[1] Integrin $\alpha 2\beta 1$ plays a role in platelet adhesion to collagen under static[2,3] and flow conditions.[4,5] Integrin $\alpha IIb\beta 3$ allows platelets to bind to fibrinogen and von Willebrand factor (vWF) present on collagen and other platelets.[6] This leads to stable platelet adhesion and aggregate formation.[7]

On resting platelets, these integrins are considered to be present in a low-affinity state. Intracellular signaling or ligand binding results in conformational changes of the integrins with a switch to higher-affinity states.[6] Agonists such as thrombin, collagen, ADP, and vWF induce $\alpha IIb\beta 3$ activation and platelet aggregation.[8,9] Full integrin activation with ADP requires the $P2Y_1$ and $P2Y_{12}$ purinergic receptors.[10,11] Recent studies show that integrin $\alpha 2\beta 1$ can also be activated by inside-out signaling.[12,13] Thrombin and collagen turn this integrin into a high-affinity form, whereas ADP changes it to intermediate affinity.[13] Although much is known of the affinity and avidity changes of $\alpha IIb\beta 3$ on isolated platelets especially,[8,14] regulation of integrin activation during thrombus formation is incompletely understood.

In vivo studies as well as ex vivo experiments, in which blood was allowed to flow over collagen under arterial shear conditions, have indicated that glycoprotein (GP) VI is a principal receptor responsible for collagen-induced activation of platelets during thrombus formation.[5,15–21] The $\alpha 2\beta 1$contribution to platelet–collagen interaction has been debated extensively.[16] Current evidence with murine and human platelets shows that this integrin functions to reinforce the activating effect of GPVI to produce stable, nonembolizing thrombi.[17,22–24] Integrin $\alpha 2\beta 1$, putatively in its activated form, synergizes with GPVI to stimulate Ca^{2+} signaling, granule secretion, and subsequent aggregate formation.[4,15,22,24,26] It also assists GPVI in triggering of the procoagulant platelet response (ie, by stimulating surface exposure of phosphatidylserine [PS]).[3,5,17,22] This procoagulant phospholipid strongly potentiates local formation of thrombin and, hence, coagulation.[27] In flowing human (but less clearly so in murine) blood, GPVI blockade still allows $\alpha 2\beta 1$-dependent platelet adhesion to collagen.[5,22] This raises the question of how the activation state of this integrin relates

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to its adhesive and signaling function. Under flow over collagen, this may involve the GPIb-V-IX complex, which is another receptor implicated in integrin αIIbβ3 activation on interaction with vWF.[28,29]

By using novel antibodies against GPVI and against activation-dependent epitopes on $\alpha2\beta1$, we investigated $\alpha2\beta1$ and αIIbβ3 activation during human platelet interaction with collagen and subsequent thrombus formation under flow. We found that GPVI and P2Y receptor stimulation caused activation of either integrin, whereas GPIb contributed to a lesser extent. Surprisingly, we found significant integrin-dependent adhesion in the absence of its activation.

Methods

Materials, blood preparation, and experimental design are available online at http://atvb.ahajournals.org.

Thrombus Formation Under Flow Conditions

Flow experiments over collagen were performed with D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK)-anticoagulated blood, as described.[22] Briefly, whole blood was perfused for 4 minutes over a collagen-coated coverslip through a parallel-plate transparent flow chamber at a wall-shear rate of 150 to 1000 s^{-1}. PS exposure was detected by postperfusion with rinsing Hepes buffer, pH 7.45 (in mmol/L: 136 NaCl, 10 glucose, 5 Hepes, 2.7 KCl, 2 $MgCl_2$, and 2 $CaCl_2$, plus 0.1% BSA and 1 U/mL heparin), containing 1 μg/mL OG488-annexin-A5. Integrin activation was monitored by adding fluorescein isothiocyanate (FITC)-coupled IAC-1 (10 μg/mL) or PAC1 (1:50) to blood before perfusion. Where indicated, probes were added to the rinsing buffer.

High-resolution phase-contrast and fluorescent images were recorded in real time with intensified cameras as described.[17] Using Quanticell software (Visitech), fluorescence images (average 32 camera images) were corrected for background fluorescence by subtraction of the mean gray level from an adjacent site of the coverslip. Area coverage by fluorescent platelets was determined with Quanticell software. Area coverage from phase-contrast images was analyzed offline using ImagePro software (Media Cybernetics) by automated threshold settings and application of double masks. Area coverage data were used to determine platelet deposition on collagen because they could be collected simultaneously with, and thus compared with, fluorescence data.

Confocal and 2-Photon Laser Scanning Microscopy

For confocal microscopy, coverslips with thrombi stained with FITC-IAC-1, Gi9-FITC, or MOPC-21-FITC were observed with a Bio-Rad laser-scanning microscope. Where indicated, thrombi were fixed with 2% formaldehyde and blocked with 15% BSA in PBS, pH 7.5. After permeabilization with 0.005% sodium dodecyl sulfate, actin cytoskeletal fibers were labeled with Texas-red phalloidin. An MRC600 laser-scanning microscope system was used, equipped with argon–krypton and red diode lasers. Optical sections (4 to 8 scans) were recorded in Kalman filtering mode.

Two-photon laser scanning microscopy (TPLSM) was with a Bio-Rad 2100 multiphoton system. PPACK blood containing 30% calcein-labeled platelets[17] was perfused over collagen, and z-series of scans were recorded during perfusion. Alternatively, blood was supplemented with OG488-fibrinogen (0.25 mg/mL). Excitation was by a Spectra-Physics Tsunami Ti:Sapphire laser, tuned and mode-locked at 800 nm, producing pulses of $\approx$100 fs wide (repetition rate 82 MHz). Fluorescence was detected at 508 to 523 nm.

Results

Suppressed Aggregation and PS Exposure by GPVI and ADP Receptor Blockade but Unchanged Platelet Adhesion to Collagen

The monoclonal antibody (mAb) 9O12, directed against the GPVI collagen-binding site, acts as an efficient GPVI antagonist in vitro.[30] At 25 μg/mL, antigen-binding fragments (Fabs) of 9O12 specifically blocked platelet adhesion to collagen under static conditions. The fragments also inhibited collagen-induced platelet aggregation and granule secretion. These effects were accompanied by suppression of collagen-induced protein tyrosine phosphorylation (Figure I, available online at http://atvb.ahajournals.org). Surprisingly, 9O12 stimulated phosphorylation of unknown protein bands at 25 and 36 kDa. The mAb binds to the first Ig domain of GPVI, distal from the membrane (C. Lecut and M. Jandrot-Perrus, unpublished data, 2003).

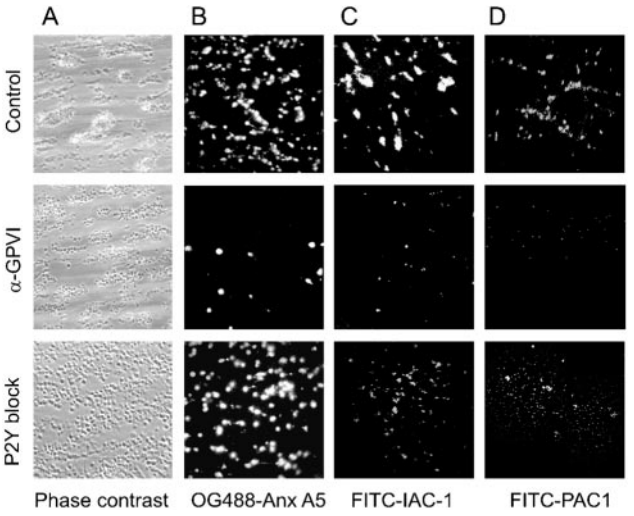

Figure 1. Effect of GPVI and P2Y receptor blockade on platelet aggregation, PS exposure, and integrin activation. Whole blood was perfused over collagen at 1000 s^{-1} for 4 minutes; preincubation with vehicle (control condition), anti-GPVI 9O12 (50 μg/mL), or P2Y blockers (40 μmol/L MRS2179 and 20 μmol/L AR-C69931MX). A, Representative phase-contrast images (120$\times$120 μm) after perfusion. Note the presence of collagen fibers. Representative fluorescence images (150$\times$150 μm) after perfusion with: B, OG488-annexin-A5 to measure PS exposure; C, FITC-IAC-1 to detect activated $\alpha2\beta1$; or D, FITC-PAC1 to detect activated αIIbβ3.

The ability of 9O12 Fab to block GPVI in blood flowing over type-I collagen fibers was investigated at intermediate shear stress (1000 s^{-1}). Under control conditions, perfusion resulted in rapid platelet deposition on collagen, which increased linearly in time for at least 5 minutes, as recorded from phase-contrast images.[17,22] In blood supplemented with calcein-loaded platelets, the 3D build-up of thrombus formation was followed by recording of stacks of fluorescence images using the high-resolution technique of TPLSM, which provides higher sensitivity and less bleaching than conventional microscopy. After an initial phase of 2 minutes of adhesion to the collagen, flowing platelets preferentially incorporated into aggregates (Figure II, available online at http://atvb.ahajournals.org). In the presence of 9O12 Fab (50 μg/mL), aggregate formation but not initial adhesion was severely impaired, leaving only 1 to 2 layered platelet groups. As a result, thrombus volume was reduced greatly with 9O12.

Phase-contrast images, recorded after an arbitrary end point of 4 minutes of flow (Figure 1), indicated that surface area covered by aggregated platelets under control conditions was 16.4$\pm$1.3% on average (n=11 subjects), with a variation between donors

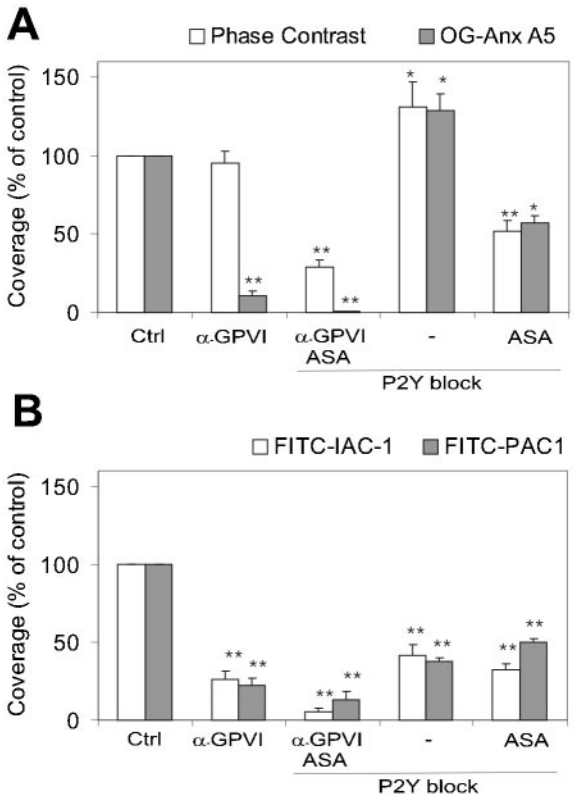

Figure 2. Quantitative effect of GPVI and P2Y receptor blockade on platelet aggregation, PS exposure, and integrin activation. Whole blood was perfused over collagen, and platelets were stained with fluorescent annexin-A5, IAC-1, or PAC-1 (Figure 1). Blood was untreated (control) or treated with anti-GPVI Fab 9O12 (50 μg/mL). Alternatively, blood was treated with P2Y blockers (40 μmol/L MRS2179 and 20 μmol/L AR-C69931MX) in combination with anti-GPVI Fab and ASA; with P2Y blockers alone; or with P2Y blockers and ASA. A, Surface area coverage of all platelets and PS-exposing platelets. B, Surface area coverage of FITC-IAC-1 and PAC-1 staining detecting activated $\alpha 2\beta 1$ and $\alpha IIb\beta 3$, respectively. Per parameter, data were expressed as percentages of respective control condition set at 100% (mean±SEM; n=4 to 5; *$P<0.05$; **$P<0.01$ vs control).

from 9.9% to 25.6%. With 9O12 present, only small aggregates were observed together with numerous single platelets, whereas the area covered by platelets remained unchanged (94.9±7.5% of control condition; Figure 2A). This is an underestimate of the platelet deposition reduction, bearing in mind the abolishment of aggregate formation. Higher concentrations of 9O12 up to 200 μg/mL gave similar results.

GPVI-induced PS exposure of the platelets on collagen was monitored by staining with OG488-annexin-A5.[22,31] Under control conditions, many platelets bound annexin-A5, giving an area coverage with fluorescence of 10.5±1.5% (n=8). These platelets had a round, blebbing structure, as described previously.[17] With 50 μg/mL 9O12 Fab present, annexin-A5 staining reduced greatly to 15% of the control situation (Figures 1B and 2A), along with platelet blebbing. Thus, 9O12 suppressed collagen-induced aggregate formation and PS exposure under flow (both effects of GPVI-induced platelet activation) but not platelet adhesion to collagen. This was in agreement with earlier results obtained with the anti-GPVI scFv antibody 10B12.[17]

Blockers of $P2Y_1$ (MRS2179) and $P2Y_{12}$ (AR-C6991MX) receptors[25] were used to evaluate the contribution of autocrine ADP in platelet deposition under flow. With the P2Y blockers present, platelets deposited as single cells or as small 2-layered aggregates (Figure 1A). Surface area covered with platelets was increased slightly to 131.0±19.9% of control (Figure 2A), likely because of increased contacts of platelets with collagen fibers. Annexin-A5 fluorescence was increased similarly to 128.4±29.5% of control (Figures 1B and 2A). In the presence of P2Y blockers, adhesion and PS exposure were about halved when the blood was also pretreated with acetylsalicylic acid (ASA) to block thromboxane formation (Figure 2A), indicating that released thromboxane is involved in platelet adhesion.

Platelet deposition was reduced further when 9O12 was combined with P2Y blockers and ASA. This resulted in adhesion of merely single platelets (28.9±4.8% of control), whereas annexin-A5 staining was abolished completely (Figure 2A). This extends earlier observation[14] and indicates that human GPVI, together with the secondary mediators ADP and thromboxane, is responsible for aggregate formation and PS exposure but is dispensable for platelet adhesion.

Suppressed $\alpha 2\beta 1$ and $\alpha IIb\beta 3$ Activation by Blockade of GPVI or ADP Receptors

To study $\alpha 2\beta 1$ activation, a new antibody IAC-1 was used, which specifically recognizes high-affinity forms of this integrin.[32] IAC-1 does not bind to resting platelets but readily binds to a neoepitope in the $\alpha 2$ I-domain that become exposed during platelet activation with ADP, thromboxane, or thrombin. Activation uncovers an I-domain region at amino acids 199 to 201, which is located at the opposite site of the metal ion-dependent adhesion site domain that is involved in binding to collagen. IAC-1 is thus of little effect on collagen binding.[32] FITC-labeled IAC-1 binds to platelets on immobilized convulxin (data not shown), indicating that $\alpha 2\beta 1$is activated after platelet adhesion via GPVI.

When added to blood at 10 μg/mL, FITC-labeled IAC-1 did not inhibit platelet deposition on collagen under flow (surface area coverage with all platelets 13.2±1.4% versus 13.6±1.1% in the absence of IAC-1; n=7). Yet, FITC-IAC-1 avidly bound to the platelets adhering to collagen under control conditions but not in the presence of platelet inhibitors (Figure III, available online at http://atvb.ahajournals.org). Other experiments were performed with FITC-Gi9, a mAb recognizing all $\alpha 2\beta 1$ forms, and with FITC-MOPC-21, a mAb that does not bind to platelets. FITC-Gi9 stained control and inhibited platelets, but FITC-MOPC-21 failed to give detectable staining (Figure III).

During perfusions under control condition, fluorescence staining of FITC-IAC-1 increased in time; it covered 9.5±2.1% (n=8 subjects) of the surface area after 4 minutes. Addition of 9O12 (50 μg/mL) greatly reduced staining to 23±1.9% of the control condition, despite the presence of many adherent platelets (Figures 1C and 2B). Addition of P2Y blockers also reduced IAC-1 staining to 41.2±22.1% of control. Blockade of GPVI and P2Y and presence of ASA resulted in almost complete suppression of IAC-1 fluorescence (Figure 2B).

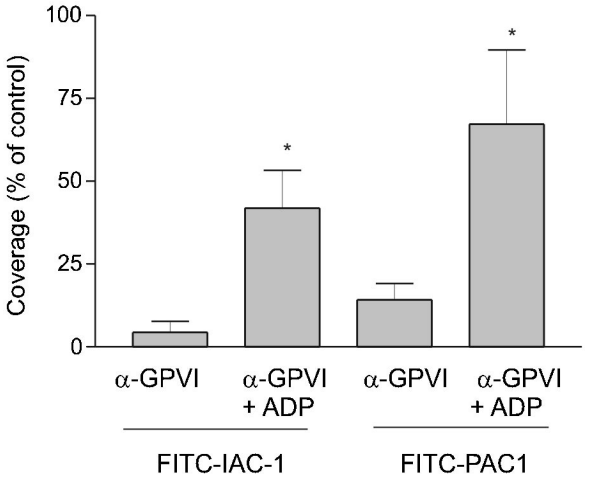

Figure 3. Effect of ADP addition on integrin activation of GPVI-inhibited platelets. Blood containing 50 μg/mL 9O12 was perfused over collagen, and FITC-PAC1 or FITC-IAC-1 binding was measured. Thereafter, vehicle buffer or ADP (10 μmol/L) with same fluorescent antibody was superfused at 1000 s^{-1} for 1 minute. Percentages of area coverage with fluorescence are given at respective control condition (mean±SEM; n=3; *$P<0.05$ vs 9O12 alone).

To ensure that the reduced IAC-1 binding was not caused by a low detection level of the fluorescence camera, platelets were counterstained for actin with fluorescent phalloidin and examined by confocal microscopy. Under control conditions, individual platelets in aggregates were strongly labeled with FITC-IAC-1, as apparent from overlays of IAC-1 and phalloidin images (Figure IV, available online at http://atvb.ahajournals.org). With 9O12 present, FITC-IAC-1 fluorescence of collagen-adherent platelets was reduced greatly, in contrast to the still bright phalloidin staining.

Because 9O12 inhibited platelet aggregation, its effect was studied on signaling toward integrin αIIbβ3 using fluorescent-labeled PAC1, which is a mAb against activated αIIbβ3.[33] Under control conditions, addition of FITC-PAC1 to blood or postperfusion with FITC-PAC1 gave fluorescent-labeled platelet aggregates on collagen (Figure 1D). After 4 minutes of perfusion, staining with FITC-PAC1 was 7.5±2.1% (n=8) of the surface. Blocking of GPVI or P2Y receptors decreased PAC1 staining (Figure 2B). Similarly, as observed with IAC-1, the combination of GPVI and P2Y blockers plus ASA almost completely suppressed PAC-1 staining.

To verify that ADP could activate integrins on collagen-adherent platelets in the absence of GPVI activity, 9O12-treated platelets were postperfused with 10 μmol/L ADP. Perfusion with ADP but not with vehicle gave a substantial ≈5-fold increase in IAC-1-labeling or PAC1-labeling of adherent platelets (Figure 3).

Addition of OG488-labeled human fibrinogen to the blood provided another means to measure αIIbβ3 activation on flow. TPLSM with low detection threshold showed that platelet aggregates on collagen were labeled diffusely with OG488-fibrinogen (Figure 4A and 4B). In the presence of 9O12, only some of the single, collagen-adherent platelets showed fluorescence. Total surface area coverage with

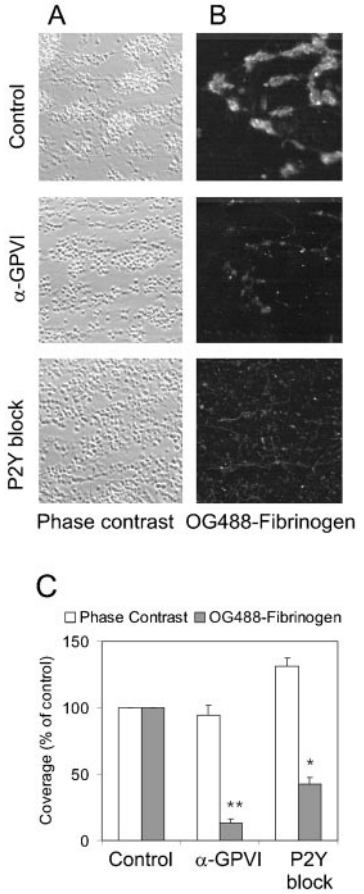

Figure 4. Effect of GPVI and P2Y receptor blockade on platelet–fibrinogen binding. Blood with OG488-labeled fibrinogen (0.25 mg/mL) was perfused for 4 minutes over collagen in the presence of vehicle (control), anti-GPVI 9O12, or P2Y blockers (Figure 1). A, Phase-contrast images (120×120 μm) and B, merged stacks of TPLSM images (110×110 μm) after perfusion. C, Quantitative effect of receptor blockade on OG488-fibrinogen fluorescence. Data are percentages of control (mean±SEM; n=3; **$P<0.01$).

OG488-fibrinogen decreased greatly to 13.0±2.8% of control (Figure 4C). P2Y blockers were less effective in reducing OG488-fibrinogen binding (ie, to 42.4±5.0% of control); many individual platelets still bound fibrinogen (Figure 4B). With 9O12, ASA, and P2Y blockers present, fibrinogen binding was no longer detected (data not shown).

These results indicate that antagonism of GPVI or the P2Y receptors severely impaired exposure of activation-dependent epitopes on α2β1 and αIIbβ3. Only combined blockade of GPVI and secondary mediators resulted in full inhibition of integrins. This confirms that autocrine ADP and thromboxane play key roles in thrombus build-up[24,25] and reveals that these mediators, along with GPVI, mediate α2β1 and αIIbβ3 activation.

GPIb Involved in Collagen Platelet Adhesion but not in Integrin Activation

GPIb-IX-V mediates platelet adhesion to collagen under shear. Its role in integrin activation was studied using the anti-GPIbα mAb 12G1, which specifically hinders shear-dependent adhesion to vWF.[34] At maximally effective con-

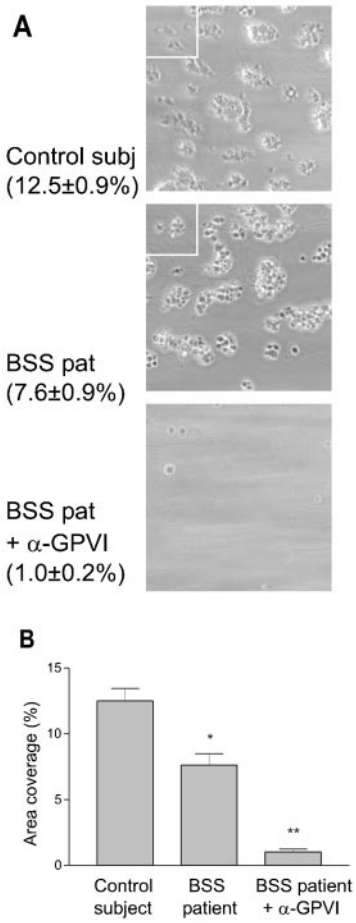

Figure 5. Abolished adhesion by blockade of GPVI of platelets from Bernard-Soulier (BSS) patient. Blood from a healthy control subject or a patient displaying BSS was perfused over collagen with/without 9O12 (50 μg/mL). A, Representative phase-contrast images (120×120 μm) of deposited platelets; inserts are 3-fold magnifications showing giant size of BSS platelets. B, Quantitative effect on platelet deposition. Values are percentage of surface area coverage (mean±SEM; n=3; *P<0.05; **P<0.01 vs absence of 9O12).

centration of 40 μg/mL, 12G1 F(ab′)$_2$ completely blocked adhesion of platelets to immobilized vWF. However, the F(ab′)$_2$ only partially reduced adhesion to collagen fibers at 1000 s^{-1} (Figure V, available online at http://atvb.ahajournals.org). Although less platelet-collagen contacts were formed, aggregate formation was not prevented; the area covered by platelets remained 72.2±10.6% (n=7) of control. GPIb blockade with 12G1 inhibited staining with OG488-annexin-A5 to 41.7±3.3% of control (Figure V). This intervention reduced staining with FITC-PAC1 and FITC-IAC-1 only moderately to 70.7±11.4% and 73.8±16.3% of control, respectively. However, with 9O12 present, 12G1 markedly inhibited platelet deposition to 12.0±5.3% of control.

The results with blocking antibody were corroborated by studies with blood from a patient with Bernard-Soulier syndrome, displaying GPIb-deficient platelets (Figure 5A). After 4 minutes of perfusion, surface area covered with patient platelets was 7.6±0.9% (ie, slightly lower than the averaged value for healthy subjects [16.4±1.3%; n=11]). With 9O12, adhesion of patient platelets was almost abolished to 8.2±1.8% of control conditions (Figure 5A and 5B). Thus, at this shear rate, GPIb and GPVI together determine adhesion to collagen, but GPIb does not have a major role in integrin activation.

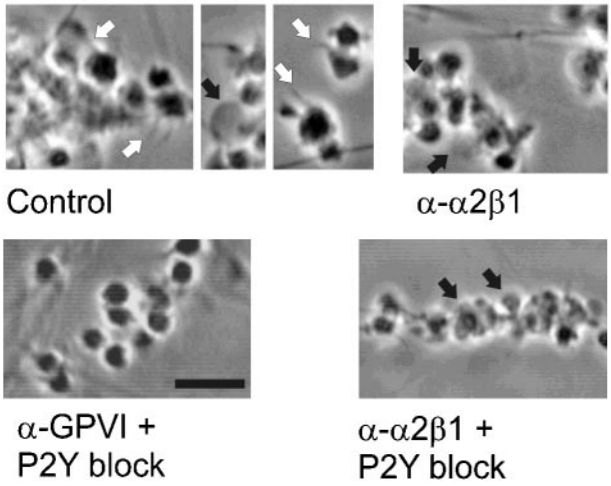

Figure 6. Platelet shape and activation of integrin $\alpha 2\beta 1$. Blood was perfused over collagen (Figure 2). Blood was treated with anti-$\alpha 2\beta 1$ 6F1 (10 μg/mL), anti-GPVI 9O12 (50 μg/mL), or P2Y blockers (MRS2179+AR-C69931MX) plus ASA, as indicated. Shown are representative phase-contrast images (bar=10 μm). White arrows indicate pseudopods; black arrows, blebbing platelets.

Functional Importance of $\alpha 2\beta 1$ Activation

Under control conditions, most platelets on collagen displayed pseudopods, lamellipods, or blebs. When 9O12 was combined with ASA and P2Y blockers, platelets adhered individually (coverage 28.9±4.8% of control) and did not show morphological signs of activation (Figure 6). The remaining adhesion was integrin-dependent because blocking anti-$\alpha 2\beta 1$ mAb 6F1 (10 μg/mL) severely abrogated adhesion with 9O12 (18.0±3.5% versus control; n=4), and extra addition of αIIbβ3-blocking arg-gly-asp-ser (400 μmol/L) eliminated all platelet deposition (<5%). Thus, integrins participated in platelet adhesion in the absence of detectable binding of IAC-1, PAC1, or fibrinogen.

Experiments in which 6F1 was added to the blood informed on the functional importance of (activated) $\alpha 2\beta 1$. Blocking of $\alpha 2\beta 1$ with 6F1 notably reduced pseudopod formation but still allowed bleb formation (Figure 6). The 6F1 effect was complete with P2Y blockers present as well (only blebs formed because of GPVI activation). Pseudopod and lamellipod formation was restored when 9O12-treated platelets were postperfused with ADP (data not shown). This suggested that $\alpha 2\beta 1$-dependent pseudopod formation, which correlated with IAC-1 binding, was responsible for increased platelet–collagen contact.

Discussion

In this study, we used newly developed tools to determine the role of human GPVI and ADP in integrin activation during collagen-induced thrombus formation under flow. The O12 mAb, directed against the collagen-binding site of human GPVI, was used to block GPVI activity. This inhibited

formation of platelet aggregates and staining with annexin-A5 (detecting PS exposure) and IAC-1 (detecting activated $\alpha 2\beta 1$), as well as fibrinogen and PAC1 (detecting activated $\alpha IIb\beta 3$). FITC-IAC-1 is the first described mAb to specifically recognize high activation states of $\alpha 2\beta 1$.[32] Blockade of the $P2Y_1$ and $P2Y_{12}$ receptors partially inhibited binding of IAC-1, PAC1, and fibrinogen to platelets, but blockade of GPVI and P2Y receptors in combination with ASA treatment was needed to abolish all binding completely. Conversely, postperfusion with ADP resulted in increased IAC-1 and PAC1 binding to GPVI-inhibited platelets. Together, these results provide direct evidence for a role of GPVI and autocrine ADP in inside-out integrin signaling. The inhibitory effects of 9O12 are consistent with studies using isolated platelets showing that stimulation with GPVI agonists results in integrin activation[6,13] and exposure of procoagulant PS.[27] In addition, they extend recent evidence that GPVI and $\alpha 2\beta 1$ contribute to human thrombus formation.[17,23]

Although anti-GPVI 9O12 efficiently suppressed aggregation, procoagulant activity, and integrin activation under flow, it did not abolish platelet adhesion to collagen, even not at a high dose of 200 μg/mL. This is remarkably similar to the effects of anti-human GPVI scFv, 10B12, which is also directed against the collagen-binding domain of GPVI.[17] Thus, 2 different antibodies against human GPVI appear to suppress platelet activation under shear but not adhesion to collagen. However, we find that combined blockade of human GPVI and ADP/thromboxane effects does lower the adhesion. For mouse blood, this is less clear because blockade or absence of murine GPVI has been found to either abolish[5,18,22] or still permit[21] platelet–collagen adhesion under flow. This discrepancy is probably attributable to different experimental conditions.

At the moderately high shear rate of 1000 s^{-1} used, the anti-GPIb mAb 12G1 only partially reduced platelet adhesion to collagen/vWF. When given alone, 12G1 inhibited adhesion slightly and hardly influenced binding of IAC-1 and PAC1. However, in combination with GPVI blockade, GPIb antagonism or absence of GPIb (in a Bernard-Soulier patient) completely abolished platelet adhesion to collagen. This indicates that GPIb-V-IX only partially contributes to integrin activation under conditions in which GPVI and P2Y receptors are also signaling.

Adhesion of platelets treated with GPVI and P2Y antagonists was mostly blocked when anti-$\alpha 2\beta 1$ mAb 6F1 was added. Furthermore, staining of platelets with IAC-1 or PAC1 correlated with $\alpha 2\beta 1$-dependent pseudopod formation of the platelets on collagen. These observations suggest that platelet adhesion to collagen can occur under conditions in which the IAC-1/PAC1 epitopes are not or only partially exposed (ie, with no or local integrin activation), basically in agreement with earlier suggestions by Inoue et al.[35] In conclusion, our results indicate that GPVI is responsible for integrin affinity regulation on platelet adhesion to collagen under high shear. Furthermore, autocrine released ADP and subsequent engagement of P2Y receptors play assisting roles. Thus GPVI- and P2Y-coupled signaling act synergistically to achieve full integrin activation and thereby stable thrombus formation.

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