Platelet Glycoprotein VI Dimerization, an Active Process Inducing Receptor Competence, Is an Indicator of Platelet Reactivity

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Objective—The immune receptor homologue glycoprotein VI (GPVI)/FcR receptor γ chain complex is primarily responsible for platelet activation by collagen. There is growing evidence that optimal binding of GPVI to collagen depends on the assembly of GPVI dimers. The valence of GPVI on resting platelets needs to be clearly established because platelet avidity for collagen would be greater if GPVI is constitutively expressed as a dimer than as a monomer.

Methods and Results—Using a monoclonal antibody (9E18) that preferentially binds to GPVI dimers, we found that GPVI was maintained in a monomeric form on human resting platelets under the control of intraplatelet cAMP concentration. Activation by soluble agonists or von Willebrand factor induced a shift toward GPVI dimerization related to increased platelet adhesion to collagen. A correlation between platelet binding of 9E18 and P-selectin exposure was observed in patients experiencing coronary artery disease, and antagonists of the ADP receptor P2Y12 limited ADP-induced GPVI dimerization.

Conclusion—The rapid assembly of highly competent dimers of GPVI at sites of vascular lesion represents an important step in the sequence of events leading to platelet activation by collagen. GPVI dimers could represent a new marker to analyze platelet reactivity. (Arterioscler Thromb Vasc Biol. 2012;32:778-785.)

Key Words: antiplatelet drugs ■ collagen ■ monoclonal antibodies ■ platelets ■ glycoprotein VI

Platelet adhesion at sites of vascular damage is required for normal hemostasis. Circulating platelets adhere to proteins of the subendothelial matrix exposed by vessel injury in a process involving several receptors. Collagen fibers are highly thrombogenic, and the platelet glycoprotein VI (GPVI) predominantly mediates collagen-induced platelet responses.

GPVI is a platelet-specific receptor of the immunoglobulin (Ig) superfamily containing 2 extracellular Ig domains, a single transmembrane domain, and a short cytoplasmic tail. GPVI shares with other receptors of the same family the particularity that the extracellular ligand-binding domain and the intracellular signaling domain are on separate subunits. GPVI signals through the noncovalently associated immune receptor adaptor FcRγ. The receptor is assembled via a transmembrane interaction between Asp11 in the FcRγ homodimer and Arg273 of GPVI. On stimulation, the Tyr residues of the immunoreceptor tyrosine-based activation motif of FcRγ are phosphorylated in an early, obligatory event triggering the signaling cascade.

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There is growing evidence that optimal binding of GPVI to collagen depends on the formation of GPVI dimers at the platelet surface. Miura et al., using recombinant proteins, were the first to report that collagen binds to the dimeric form but not to the monomeric form of GPVI and that only the former was able to inhibit collagen-induced platelet activation. Crystallographic data showing dimerization of GPVI ectodomains, studies using synthetic peptides with differentially spaced GPVI binding motifs to activate the receptor in platelets, and studies using chemical cross-linking agents have strongly reinforced the notion that GPVI functions as a dimer. However, the valence of GPVI on resting and on activated platelets is still a matter of debate. It was recently proposed that at least some copies of GPVI may exist in a dimeric conformation at the surface of resting platelets. The extent of GPVI dimerization is important to determine because it is expected that platelet avidity for collagen would be

Received on: July 5, 2011; final version accepted on: November 27, 2011.
The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.111.241067/-/DC1.
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© 2012 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.111.241067

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greater if GPVI was constitutively expressed as a dimer than as a monomer.

We have obtained an anti-human GPVI monoclonal antibody 9E18 that binds to dimeric GPVI with an affinity at least 200-fold higher than to monomeric GPVI. Using 9E18, we provide evidence that most of GPVI is maintained in a monomeric form on resting platelets under the control of intraplatelet eCaMP and that GPVI dimerization occurs in response to soluble agonists and to von Willebrand factor (vWF)-induced platelet activation. Interestingly, in a population of coronary artery disease (CAD) patients, a good correlation between 9E18 binding and P-selectin exposure was observed. Furthermore, ADP-induced 9E18 binding to platelets was prevented in patients treated with thienopyridines. In conclusion, binding of 9E18 to platelets is an indicator of GPVI dimerization and could represent a new biomarker of early platelet reactivity and responsiveness to P2Y12 antagonists.

**Materials and Methods**

Detailed methods available in the online-only Data Supplement.

**Antibodies**

The anti-human GPVI monoclonal antibodies 3J24 and 9E18 were obtained as previously described. They are not activating antibodies, as purified IgGs do not trigger P-selectin exposure on platelets. 3J24 and 9E18 bind to different epitopes. The 9E18 IgG is able to inhibit GPVI binding to collagen, but its Fab is far less inhibitory than the 9O12 Fab on collagen-induced platelet activation.

**Reagents**

Collagen from equine tendon (Horm collagen) was from Nycomed (Munich, Germany). Convulxin was purified from the venom of Crotalus durissus terrificus.

Recombinant soluble GPVI was obtained as the extracellular domain of human GPVI fused to the Fc domain of human IgG (GPVI-Fc that is dimeric) or fused to a poly-His tag (GPVI-His that is monomeric). Their Kd app for collagen were respectively 1.13±0.03 μg mL⁻¹ (5±0.2 nmol/L) and 14.7±3.3 μg mL⁻¹ (270±54 nmol/L), in agreement with published data. Monomeric GPVI was also obtained by reduction of GPVI-Fc.

**Binding Assays Using Purified Proteins**

Experiments were conducted mainly as previously described. Increasing amounts of purified GPVI-Fc and GPVI-His were incubated in 9E18-coated wells and detected using horseradish peroxidase–coupled anti-human IgG and anti-6XHis, respectively.

**Isolation of Platelets**

Blood was obtained from healthy volunteers who had not taken medication for 10 days, after full informed consent was obtained according to the Declaration of Helsinki. Platelet-rich plasma (PRP) and platelet poor plasma were obtained by centrifugation of citrated blood. Alternatively, blood was collected on acid-citrate-dextrose anticoagulant to prepare washed platelets as previously reported.

**Platelet Adhesion and Activation**

Platelet adhesion to immobilized collagen in static conditions was measured as described. Platelets activated by ADP, thrombin receptor activating peptide (TRAP), epinephrine, or phorbol 12-myristate 13-acetate (PMA) were analyzed by flow cytometry after incubation with fluorescein isothiocyanate–coupled anti-GPVI or P-selectin antibodies. Alternatively, shear-induced platelet activation was performed as previously described. Phosphorylation of vasodilator–stimulated phosphoprotein was measured by quantitative flow cytometry. Resistance to clopidogrel was defined by a P2Y₁₂ reactivity ratio >50%.

**Immunoblotting**

Proteins from whole platelet lysates were separated by electrophoresis in SDS-polyacrylamide gels and analyzed by immunoblotting using anti-GPVI antibodies and secondary horseradish peroxidase–coupled IgG.

**Patients**

Blood samples were obtained from a cohort of patients with CAD referred to the department of cardiology of Bichat hospital for percutaneous coronary intervention (BIOCORE-2 study; clinical trial registration: http://clinicaltrials.gov/ct2/show/NCT01186666?term=NCT01186666&rank=1). The study complies with the Declaration of Helsinki and was approved by the institutional ethics committee. All of the patients provided written informed consent. The patients were treated with dual antiplatelet therapy consisting of aspirin and an antagonist of the P2Y₁₂ receptor, at the discretion of the cardiologist. Blood was drawn on 0.109 mol/L citrate the day after percutaneous coronary intervention. Fluorescence-activated cell sorting analysis was performed on PRP prepared less than 1 hour after blood sampling using fluorescein isothiocyanate–coupled 9E18, 3J24, or anti-P-selectin. Controls were healthy subjects who had taken no medication during the 2 previous weeks.

**Statistics**

Data were analyzed using PRISM (GraphPad, San Diego, CA). Box plots represent means, minimal, and maximal values. Statistical analysis included the paired Student t test (2-tailed) and the Mann-Whitney test (2-tailed). Correlations were determined using linear regression and Spearman 2-tailed analysis.

**Results**

**9E18 Discriminates Dimeric From Monomeric GPVI**

In preliminary experiments, 9E18 IgG were found to bind poorly to GPVI-His (monomeric GPVI) as compared with another anti-GPVI IgG, 3J24. We thus characterized the interaction of dimeric and monomeric GPVI with 9E18 in binding experiments (Figure 1A). Binding of GPVI-Fc to immobilized 9E18 was saturable, allowing calculation of an affinity constant (Kₐ=0.36 nmol/L). In contrast, GPVI-His hardly bound to 9E18, and saturation was not reached. The best estimated Kₐ was 78 nmol/L, indicating that the affinity of dimeric GPVI for 9E18 is at least 200-fold higher than the affinity of monomeric GPVI. Furthermore, in competition experiments, soluble GPVI-Fc efficiently inhibited 9E18 binding to immobilized GPVI-Fc, whereas GPVI-His and reduced GPVI-Fc were much less efficient (Figure 1 in the online-only Data Supplement), confirming that the affinity of 9E18 for monomeric GPVI is far lower than for dimeric GPVI.

Immunoblotting confirmed the poor capacity of 9E18 to detect GPVI-His or reduced GPVI-Fc (Figure 1B and Figure 1 in the online-only Data Supplement). 9E18 thus permits discrimination between GPVI dimers and monomers.

**Binding of 9E18 to Resting Platelets**

By flow cytometry, a very low level of 9E18 binding to platelets was observed in whole blood as compared with the level of GPVI labeled by 3J24 (Figure 1C). Interestingly, the basal level of 9E18 binding to platelets increased progres-
Together, these data indicate that 9E18 recognizes an epitope exposed by GPVI dimers whose formation is an activation-dependent event.

Shear-Induced Binding of the 9E18 Antibody

The initial interaction of GPIb with vWF, assumed to slow down platelets and to favor GPVI interaction with collagen, was triggered in vitro by high shear stress (Figure 3A). 9E18 binding increased from 3.6±1.6% after 5 minutes at 0 s⁻¹ to 7±2% at 4000 s⁻¹ (P<0.05 n=3). Interestingly, although the number of 9E18-positive events was low in the gate corresponding to isolated platelets (2.7±0.4%), it was increased to 26±7.7% in the gate focused on shear-induced aggregates. Experiments performed with a control mouse IgG ruled out any nonspecific trapping in shear-induced platelet aggregates. When experiments were repeated on washed platelets, the addition of vWF induced a parallel increase in shear-induced platelet aggregation and in the level of 9E18 binding (Figure 3B). P-selectin exposure was also observed on platelet aggregates.

Platelet Adhesion to Collagen

To investigate whether binding of 9E18 was related to the capacity of platelets to interact with collagen, 9E18 binding and GPVI-specific platelet attachment to collagen were measured in parallel, after αIIBβ₃ and α₂β₁ integrins were blocked by the addition of RGDS and the absence of Mg²⁺. A significant linear correlation was observed between binding of 9E18 and the adhesion to collagen (r²=0.87, P=0.0002, n=9), indicating that the conformation of GPVI recognized by 9E18 is favorable for GPVI-dependent platelet adhesion to collagen (Figure 3C).

Mechanisms Leading to Activation-Dependent Changes in GPVI

As binding of 9E18 to platelets was induced by receptor activation, we examined whether it could be induced independently of a cell surface receptor and found that PMA increased the level of 9E18 binding to platelets up to 31.5±4% (P<0.001) (Figure 4).

cAMP is established as limiting platelet activation. Adenylate cyclase is inhibited by ADP activation of its receptor P2Y₁₂ and activated by prostacyclin. Whereas ADP increased the binding of 9E18, PGE₁ reduced it on washed resting platelets, as well as on TRAP-, ADP-, or PMA-activated platelets (Figure 4A). Forskolin and IBMX induce a receptor-independent elevation of cAMP via the direct activation of adenylate cyclase and the inhibition of phosphodiesterase, respectively. Forskolin plus IBMX (Figure 4A) and even forskolin alone (data not shown) inhibited 9E18 binding to resting, or ADP-, TRAP-, or PMA-activated platelets.

NO donors that increase intraplatelet cGMP concentration are also inhibitors of platelet activation; the NO donor SNAP inhibited 9E18 binding to resting platelets, as well as to TRAP-, ADP-, or PMA-activated platelets (Figure 4A).

The role of endogenous platelet agonists in 9E18 binding to platelets was analyzed. Indomethacin had no effect on the level of 9E18 binding to stimulated platelets, ruling out a role of TXA₂. In contrast, apyrase inhibited both ADP- and
TRAP-induced binding of 9E18 by 85% and 75%, respectively, indicating that released ADP contributes to GPVI changes. The P2Y12 antagonist 2MeSAMP also inhibited 9E18 binding to ADP- or TRAP-stimulated platelets (data not shown).

Phenylarsine oxide, a reagent that binds to vicinal sulfhydryls and that was previously reported to inhibit GPVI-dependent platelet activation19,20 is commonly used at low concentrations as an inhibitor of phosphotyrosine phosphatases. It fully prevented PMA-induced binding of 9E18 to platelets and GPVI-dependent platelet adhesion to immobilized collagen but was without effect on P-selectin exposure (Figure III in the online-only Data Supplement).

In contrast, PMA-induced binding of 9E18 remained unchanged in the presence of 20 μmol/L of the Tyr kinase inhibitor PP1 (28.4 versus 27.7%). FcRγ was not phosphorylated in conditions leading to GPVI dimerization (PMA- or ADP-activated platelets), indicating that binding of 9E18 to platelets occurs independently of signals downstream of GPVI.

**Binding of 9E18 Is a Potential New Marker of Platelet Activation and Efficacy of P2Y12 Antagonists**

Because binding of 9E18 is the consequence of an active GPVI modification that favors platelet interaction with fibrillar collagen, it could represent a new circulating biomarker of platelet reactivity in patients. To test this hypothesis, 9E18 binding and P-selectin exposure were measured in PRP from CAD patients (n=45) participating in the BIOCORE-2 study. All patients were under dual antiplatelet therapy, aspirin (75–160 mg/day), and clopidogrel (75 mg/day). A positive correlation was observed between basal 9E18 binding and P-selectin exposure (Spearman r=0.568; P<0.0001) (Figure 5A).
We hypothesized that antagonists of P2Y₁₂ could inhibit ADP-induced 9E18 binding to platelets. To test this hypothesis, 9E18 binding to platelets was measured before and after PRP incubation with ADP in normal subjects and patients treated with clopidogrel (75 mg) (Figure 5B). A highly significant reduction in the response was observed in clopidogrel-treated patients. A good correlation was found between ADP-induced 9E18 binding and vasodilator–stimulated phosphoprotein phosphorylation, which is one of the biological markers of P2Y₁₂ antagonists (Figure 5C).

**Discussion**

The 9E18 antibody that was raised against dimeric GPVI is characterized by its extremely poor capacity to bind to monomeric GPVI, whereas it binds to dimeric GPVI with a nanomolar affinity. This observation indicates that the antibody recognizes a specific conformational epitope on dimeric GPVI.

Several groups have reported that GPVI may form a dimer that has functional significance.¹⁻²⁻²¹⁻²² X-ray crystallography of soluble GPVI revealed a dimeric conformation.⁶ Furthermore, the GPVI-Fc fusion protein has demonstrated effective inhibition of thrombus formation, consistent with its ability to bind fibrillar collagen with high specificity and reasonable affinity.¹⁻²⁻³ The very large discrepancy between the apparent affinities of monomeric and dimeric GPVI for fibrillar collagen suggests that the GPVI dimer acquires a specific conformation suitable for binding to collagen. The similarly large discrepancy between the apparent affinities of monomeric and dimeric GPVI for fibrillar collagen suggests that the GPVI dimer acquires a specific conformation suitable for binding to collagen. The capacity of 9E18 to bind to resting platelets was at the level of detection in fresh whole blood, indicating that GPVI is mostly monomeric on circulating platelets; the level of 9E18 binding to platelets is correlated with their capacity to adhere to collagen 30 minutes. The increase in adherent platelets (ratio of adherent platelets in activated vs resting conditions) is plotted as a function of the increase of 9E18 binding. R²=0.848, P=0.0012.
in the resting state. More recently, Jung et al. provided evidence for the existence of a specific dimer conformation of GPVI at the platelet surface. The monoclonal antibody (204-11) is activating platelets; its monovalent recombinant m-Fab-F did not bind to GPVI monomers and bound to GPVI-Fc with a very low affinity. The authors observed a low level of m-Fab-F binding to washed platelets, suggesting the presence of some preformed dimers at the platelet surface. Here, using fluorescein isothiocyanate–9E18 with good affinity for GPVI-Fc, we showed that GPVI dimers are scarce on unstimulated platelets, particularly in whole blood. Furthermore, the fact that the binding of 9E18, and thereby the proportion of dimeric GPVI, increased after isolation of platelets suggests that the equilibrium between the monomeric and dimeric forms is easily shifted toward the latter.

We indeed observed that stimulating platelets with a weak (ADP) or a strong (TRAP) soluble agonist significantly increased the binding of 9E18, indicating that platelet stimulation brought GPVI monomers together to form dimers. The observation of a double population in ADP- or TRAP-stimulated platelets but not in PMA-treated platelets indicates that regulation of GPVI dimerization depends on the agonist and possibly on the level of intracellular calcium mobilization. In agreement with the increased affinity of GPVI dimers for collagen, the initial interaction of platelets with collagen was favored in relation to the presence of dimers of GPVI at the platelet surface.

In the sequence of events assumed to result in stable platelet adhesion and aggregation, the interaction of GPIb with collagen-bound vWF is thought to precede GPVI binding to collagen. Application of a high shear to PRP triggered platelet aggregation and GPVI dimerization. Furthermore, on washed platelets, shear-induced 9E18 binding and aggregation were dependent on the presence of vWF. We thus showed for the first time that platelet activation by the vWF/GPIb axis has a priming effect on GPVI, resulting in a direct enhancement of GPVI interaction with collagen. The rapid assembly of highly competent dimers of GPVI at sites of vascular lesion represents an important and heretofore unrecognized step in the sequence of events leading to platelet activation by collagen.

The mechanism leading to GPVI dimerization is clearly distinct from the signaling cascade downstream of GPVI: it requires neither FcγR phosphorylation nor the activation of downstream Tyr kinases. Because the assembly of a GPVI dimer is not sufficient to trigger downstream signaling, clusters of at least 2 GPVI-dimers appear to be the minimal

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Figure 5. 9E18 as a marker of platelet reactivity. A, 9E18 binding to platelets and P-selectin exposure were measured in blood samples (platelet-rich plasma [PRP]) obtained from a cohort of patients with coronary artery disease (CAD) admitted for percutaneous coronary intervention (n=45). B, ADP-induced binding of 9E18 to platelets with or without shear was measured on blood samples (PRP) from normal subjects and patients with CAD treated by clopidogrel (n=26). Results are expressed as the ratio between the levels of 9E18 binding to ADP-stimulated/nonstimulated samples×100. C, ADP-induced 9E18 binding to platelets of patients treated by clopidogrel was plotted against corresponding data of the vasodilator-stimulated phosphoprotein (VASP) phosphorylation assay. PRI indicates platelet reactivity index.
Figure 6. Schematized representation of the mechanism of glycoprotein VI (GPVI) dimerization (adapted from20). On resting platelets, 1 GPVI chain is associated with an FcRy homodimer. Complexes assemble as dimers in response to von Willebrand factor and to soluble agonists by a mechanism involving Ser/Thr kinases PKA(G) and Tyr phosphatase(s) and prevented by elevated cAMP or cGMP concentrations. These dimers are competent to bind collagen, leading to the clustering of receptors and consequently to the phosphorylation of the FcRy immunoreceptor tyrosine-based activation motif by constitutively GPVI-bound preactivated Lyn and to the downstream signaling cascade. PRD indicates proline rich domain; PY, phosphotyrosine.

signaling unit.22,24 Recent studies have provided evidence that Lyn, a Src family kinase constitutively bound to the Pro-rich motif of the GPVI cytoplasmic domain, is in an activated state25 and that CD148, a Tyr phosphatase, maintains a pool of active Src family kinases.26 Consistent with the inhibition of collagen-induced platelet activation,19 GPVI dimerization was blocked by PAO, a commonly used inhibitor of Tyr phosphatases.26 Protein disulfide isomerase inhibition by PAO was ruled out by the absence of effect of bacitracin (3 μmol/L) on 9E18 binding (data not shown).

Interestingly, GPVI dimerization is controlled by the intracellular cAMP concentration, as indicated by the inhibition of 9E18 binding to resting as well as to ADP-, TRAP-, or PMA-activated platelets by PGE1 or forskolin. Increased concentrations of intracellular cAMP induce potent inhibition of platelet functions.27 The activity of adenylate cyclase is modulated by classical and the functional efficiency of P2Y12 antagonists. Although numerous platelet function tests have been proposed to identify low responders to P2Y12 antagonists, there is currently no consensus regarding the most appropriate method. GPVI dimerization could be of great interest because it integrates both the initial activation state and the degree of platelet inhibition. Additional studies are now required to determine whether GPVI dimers represent a reliable predictive marker for the risk of arterial thrombosis, or a reliable marker of the reactivity to P2Y12 antagonists, by analyzing 9E18 binding to platelets of age- and sex-matched patients.

In this study, using the monoclonal antibody 9E18 as a specific tracer of dimeric GPVI, we have demonstrated that GPVI is maintained in a monomeric form on resting platelets and that GPVI dimerization is an active process that primes platelet interaction with fibrillar collagen and is controlled by cAMP. Consequently, GPVI dimers could represent a new, sensitive, and early marker of platelet responsiveness.

Acknowledgments

The authors thank Samira Benada and the Institut Claude Bernard (Universite Paris Denis Diderot) for imaging facilities and Florence Tubach and Estelle Marcault (Département d’Épidémiologie et Recherche Clinique, Hôpital Bichat) for their help in monitoring the BIOCORE-2 study. Drs Lotfi Menaoui, Valérie Duchatelle, and Emmanuel Sorbets (Département de Cardiologie, Hôpital Bichat) are acknowledged for recruiting patients in the BIOCORE-2 study; Julien Gaudas for handling patients’ samples; and Dr Mary Osborne-Pellegrin for help in editing the manuscript.
Sources of Funding
This work was supported by Institut National de la Santé et de la Recherche Médicale, the University of Paris Denis Diderot, and the Fondation de France (Grant 2007/001960). Dr Feldman is the recipient of research grants from the Institut de l’Atherothrombose et la Recherche Clinique et au Développement de l’Assistance Publique–Hôpitaux de Paris. The BIOCORE-2 study was sponsored by the Département à la Recherche Clinique et au Développement de l’Assistance Publique–Hôpitaux de Paris.

Disclosures
None.

References
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Arterioscler Thromb Vasc Biol. 2012;32:778-785; originally published online December 8, 2011;
doi: 10.1161/ATVBAHA.111.241067

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Material and Methods

Reagents: Mouse IgG1 and HRP-coupled goat anti-mouse IgG were from Jackson ImmunoResearch (Suffolk, UK), mouse anti-P selectin from Ancell (Bayport, MN), Alexa-Fluor 568 goat anti-mouse IgG from Invitrogen (Cergy, France), FITC-coupled mouse anti-P-selectin and mouse non-immune IgG1 from Beckman Coulter (Villepintes, France), and rabbit polyclonal anti-FcRγ subunit from Upstate (Lake Placid, NY). Apyrase (grade VII), prostaglandin E1 (PGE1), bovine serum albumin (BSA), Ortho-Phenylenediamine dihydrochloride (OPD), Phorbol 12-myristate 13-acetate (PMA), Phenylarsineoxide (PAO), forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (St Louis, MO), S-Nitroso-Nacetyl penicillamine (SNAP) from Calbiochem (San Diego CA), the thrombin-receptor activating peptide (SFLLRN, TRAP-1) from Polypeptides (Strasbourg, France), RGDS peptide from Bachem (Weil am Rhein, Germany), ADP from Stago (Asnières, France) and human vWF from LFB (les Ulis, France).

GPVI-Fc and GPVI-His were produced in HEK cells and affinity purified on Protein A and Ni columns respectively. GPVI-Fc was reduced by 20 mM dithiothreitol and alkylated by 20 mM iodoacetamide. Efficacy of reduction was ~100% as indicated by a single 75-kDa band in SDS-PAGE. Subunits tend to non-covalently reassociate in non-denaturing conditions, as indicated by the presence of two peaks at 70 and 140 kDa in mass spectrometry, the proportion of reassociated dimers being of ~30%.

Binding assays using purified proteins: Microtitration wells were coated with 9E18 IgG (0.2 µg/well) and saturated with BSA (10 mg/mL). Increasing amounts of purified GPVI-Fc or GPVI-His in PBS, containing 1 mg.mL\(^{-1}\) BSA and 0.1% Tween 20, were incubated in coated wells and detected using HRP-coupled anti-human IgG.
or anti 6xHis respectively and OPD. Competition experiments were performed on wells coated with GPVI-Fc (0.2 µg/well). 9E18 IgG (0.6 µg/mL) was added to the wells in the presence of increasing amounts of soluble recombinant GPVI-Fc or GPVI-His and detected using HRP-coupled anti-mouse IgG and OPD. Five washes were performed between each step. Data were analyzed and graphs were produced using PRISM GraphPad, (San Diego, CA). Affinity was determined as $K_{D_{app}} = \frac{B_{max} \cdot X}{K_d + X}$ where $B_{max}$ is the maximum specific binding, $K_d$ the equilibrium binding constant and $X$ the ligand concentration.

*Isolation of platelets:* Blood was obtained from healthy volunteers, who had not taken medication for 10 days, after full informed consent according to the Helsinki declaration. Blood was collected in vacutainers containing 0.109 M sodium citrate; platelet-rich plasma (PRP) was obtained by centrifugation at 120 g for 15 min and platelet-poor plasma by centrifugation at 1200 g 12 min. Alternatively, blood was collected on acid-citrate-dextrose anticoagulant (ACD-A) to prepare washed platelets in the presence of apyrase (25 µg.mL$^{-1}$) and prostaglandin E1 (100 nM) as previously reported$^1$. Platelets were resuspended in 5 mM HEPES, 137 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 12 mM NaHCO$_3$, 0.3 mM NaH$_2$PO$_4$, 5.5 mM glucose, 2 mM CaCl$_2$, pH 7.4, containing 1mg.mL$^{-1}$ BSA.

*Flow cytometry:* Resting and activated platelets (5x10$^7$.mL$^{-1}$) were incubated with FITC-coupled anti-GPVI or P-selectin antibodies for 20 min and, when needed, fixed with 1% PFA. Samples were analyzed by flow cytometry using an Epics XL (Beckman Coulter Villepinte, France) or an LSRII (BD Biosciences, Le Pont de Claix, France) apparatus. Platelets were gated on forward and side scatter and >95% of events in the gate were positive for CD41. A mouse IgG1 (isotypic control) was used to define negative events in the population of gated platelets. Phosphorylation of
vasodilator–stimulated phosphoprotein (VASP) was measured by quantitative flow cytometry assay (Platelet VASP, Diagnostica Stago, Biocytex, Marseille, France) and using a Coulter EPICS XL cytometer, (Beckman Coulter Inc., Fullerton, California). Blood samples were incubated with prostaglandin E1(PGE1) alone or PGE1+ADP. After cellular permeation, VASP in its phosphorylated state was labelled by indirect immunofluorescence using a specific monoclonal antibody. The P2Y12 reactivity ratio was calculated as [mean fluorescence intensity PGE1-(mean fluorescence intensity PGE1+ADP)/ mean fluorescence intensity PGE1] x 100. Resistance to clopidogrel was defined by P2Y12 reactivity ratio > 50%.

Platelet adhesion: Microtiter plates were coated overnight with type I collagen (20 µg/mL in PBS). After blocking with BSA, platelets (10^8/mL) in Mg^{2+}-free reaction buffer, with or without 300 µM RGDS, were allowed to adhere for 30 min. After gentle washing, adherent platelets were quantified using p-nitrophenyl phosphate as described^2.

Shear-induced platelet activation: Shear-induced platelet activation was performed as previously described^3. PRP, containing FITC-coupled 9E18 (4 µg/mL) or isotypic IgG, was submitted to 0 or 4000 s^{-1} during 5 min in a rotative shearing device and immediately analysed by flow cytometry. Alternatively, experiments were performed on washed platelets (3x10^8/mL) supplemented or not with vWF (50 µg/mL)


Supplemental Figures
Figure I: **Binding of 9E18 to GPVI monomers and dimers**

A: Microtitration wells were coated with GPVI-Fc (2 µg/well). Purified 9E18 IgG (0.6 µg/mL) was incubated in the well in the presence of increasing amounts of soluble GPVI-Fc or GPVI-His or reduced and alkylated GPVI-Fc. Data are from one representative experiment out of 3.

B: 1 µg non-reduced or reduced alkylated GPVI-Fc were immunoblotted with 9E18 or 3J24 (2 µg/mL).

Figure II: **Binding of 9E18 to platelets does not require FcγRIIA**

PRP was preincubated with the FcγRII-blocking antibody IV.3 (10 µg/mL) before activation by PMA (0.2 µM) for 20 min. Binding of 9E18 to platelets was analyzed by flow cytometry. In grey, resting platelets, in black, PMA-activated platelets, in red,
IV.3 pretreated, PMA-activated platelets. Data are from one representative experiment out of 3.

Figure III: **PMA-induced GPVI dimerization and platelet adhesion to collagen are inhibited by PAO**

A: Washed platelets pretreated or not with PAO (1 µM, 2 min at 37°C) were incubated with PMA (0.2 µM) for 20 min before being analyzed for GPVI expression (FITC-3J24), GPVI dimerization (FITC-9E18) and P-selectin exposure. Results are from 9 independent experiments.

B: Washed platelets (10⁸/mL in reaction buffer without magnesium and containing 300 µM RGDS) were treated with buffer or with PAO, activated by PMA and allowed to adhere on immobilized collagen. Results are from 6 independent experiments.
“Supplement Material”

Material and Methods

Reagents: Mouse IgG1 and HRP-coupled goat anti-mouse IgG were from Jackson ImmunoResearch (Suffolk, UK), mouse anti-P selectin from Ancell (Bayport, MN), Alexa-Fluor 568 goat anti-mouse IgG from Invitrogen (Cergy, France), FITC-coupled mouse anti-P-selectin and mouse non-immune IgG1 from Beckman Coulter (Villepintes, France), and rabbit polyclonal anti-FcRγ subunit from Upstate (Lake Placid, NY). Apyrase (grade VII), prostaglandin E1 (PGE1), bovine serum albumin (BSA), Ortho-Phenylenediamine dihydrochloride (OPD), Phorbol 12-myristate 13-acetate (PMA), Phenylarsineoxide (PAO), forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma (St Louis, MO), S-Nitroso-Nacetyl penicillamine (SNAP) from Calbiochem (San Diego CA), the thrombin-receptor activating peptide (SFLLRN, TRAP-1) from Polypeptides (Strasbourg, France), RGDS peptide from Bachem (Weil am Rhein, Germany), ADP from Stago (Asnières, France) and human vWF from LFB (les Ulis, France).

GPVI-Fc and GPVI-His were produced in HEK cells and affinity purified on Protein A and Ni columns respectively. GPVI-Fc was reduced by 20 mM dithiothreitol and alkylated by 20 mM iodoacetamide. Efficacy of reduction was ~100% as indicated by a single 75-kDa band in SDS-PAGE. Subunits tend to non-covalently reassociate in non-denaturing conditions, as indicated by the presence of two peaks at 70 and 140 kDa in mass spectrometry, the proportion of reassociated dimers being of ~30%.

Binding assays using purified proteins: Microtitration wells were coated with 9E18 IgG (0.2 µg/well) and saturated with BSA (10 mg/mL). Increasing amounts of purified GPVI-Fc or GPVI-His in PBS, containing 1 mg.mL⁻¹ BSA and 0.1% Tween 20, were incubated in coated wells and detected using HRP-coupled anti-human IgG
or anti 6xHis respectively and OPD. Competition experiments were performed on wells coated with GPVI-Fc (0.2 µg/well). 9E18 IgG (0.6 µg/mL) was added to the wells in the presence of increasing amounts of soluble recombinant GPVI-Fc or GPVI-His and detected using HRP-coupled anti-mouse IgG and OPD. Five washes were performed between each step. Data were analyzed and graphs were produced using PRISM GraphPad, (San Diego, CA). Affinity was determined as $K_{D_{app}} = B_{max} \times X/(K_d+X)$ where $B_{max}$ is the maximum specific binding, $K_d$ the equilibrium binding constant and $X$ the ligand concentration.

**Isolation of platelets:** Blood was obtained from healthy volunteers, who had not taken medication for 10 days, after full informed consent according to the Helsinki declaration. Blood was collected in vacutainers containing 0.109 M sodium citrate; platelet-rich plasma (PRP) was obtained by centrifugation at 120 g for 15 min and platelet-poor plasma by centrifugation at 1200 g 12 min. Alternatively, blood was collected on acid-citrate-dextrose anticoagulant (ACD-A) to prepare washed platelets in the presence of apyrase (25 µg.mL$^{-1}$) and prostaglandin E1 (100 nM) as previously reported$^1$. Platelets were resuspended in 5 mM Hepes, 137 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 12 mM NaHCO$_3$, 0.3 mM NaH$_2$PO$_4$, 5.5 mM glucose, 2 mM CaCl$_2$, pH 7.4, containing 1mg.mL$^{-1}$ BSA.

**Flow cytometry:** Resting and activated platelets ($5 \times 10^7$.mL$^{-1}$) were incubated with FITC-coupled anti-GPVI or P-selectin antibodies for 20 min and, when needed, fixed with 1% PFA. Samples were analyzed by flow cytometry using an Epics XL (Beckman Coulter Villepinte, France) or an LSRII (BD Biosciences, Le Pont de Claix, France) apparatus. Platelets were gated on forward and side scatter and >95% of events in the gate were positive for CD41. A mouse IgG1 (isotypic control) was used to define negative events in the population of gated platelets. Phosphorylation of
vasodilator–stimulated phosphoprotein (VASP) was measured by quantitative flow cytometry assay (Platelet VASP, Diagnostica Stago, Biocytex, Marseille, France) and using a Coulter EPICS XL cytometer, (Beckman Coulter Inc., Fullerton, California). Blood samples were incubated with prostaglandin E1 (PGE1) alone or PGE1+ADP. After cellular permeation, VASP in its phosphorylated state was labelled by indirect immunofluorescence using a specific monoclonal antibody. The P2Y12 reactivity ratio was calculated as \([\text{mean fluorescence intensity PGE1} - \text{mean fluorescence intensity PGE1+ADP}] / \text{mean fluorescence intensity PGE1}] \times 100\). Resistance to clopidogrel was defined by P2Y12 reactivity ratio > 50%.

**Platelet adhesion:** Microwell plates were coated overnight with type I collagen (20 µg/mL in PBS). After blocking with BSA, platelets (10⁸/mL) in Mg²⁺-free reaction buffer, with or without 300 µM RGDS, were allowed to adhere for 30 min. After gentle washing, adherent platelets were quantified using p-nitrophenyl phosphate as described².

**Shear-induced platelet activation:** Shear-induced platelet activation was performed as previously described³. PRP, containing FITC-coupled 9E18 (4 µg/mL) or isotypic IgG, was submitted to 0 or 4000 s⁻¹ during 5 min in a rotative shearing device and immediately analysed by flow cytometry. Alternatively, experiments were performed on washed platelets (3x10⁸/mL) supplemented or not with vWF (50 µg/mL)


**Supplemental Figures**
Figure I: **Binding of 9E18 to GPVI monomers and dimers**

A: Microtitration wells were coated with GPVI-Fc (2 μg/well). Purified 9E18 IgG (0.6 μg/mL) was incubated in the well in the presence of increasing amounts of soluble GPVI-Fc or GPVI-His or reduced and alkylated GPVI-Fc. Data are from one representative experiment out of 3.

B: 1 μg non-reduced or reduced alkylated GPVI-Fc were immunoblotted with 9E18 or 3J24 (2 μg/mL).

Figure II: **Binding of 9E18 to platelets does not require FcγRIIA**

PRP was preincubated with the FcγRII-blocking antibody IV.3 (10 μg/mL) before activation by PMA (0.2 μM) for 20 min. Binding of 9E18 to platelets was analyzed by flow cytometry. In grey, resting platelets, in black, PMA-activated platelets, in red,
IV.3 pretreated, PMA-activated platelets. Data are from one representative experiment out of 3.

Figure III: **PMA-induced GPVI dimerization and platelet adhesion to collagen are inhibited by PAO**

A: Washed platelets pretreated or not with PAO (1 µM, 2 min at 37°C) were incubated with PMA (0.2 µM) for 20 min before being analyzed for GPVI expression (FITC-3J24), GPVI dimerization (FITC-9E18) and P-selectin exposure. Results are from 9 independent experiments.

B: Washed platelets (10^8/mL in reaction buffer without magnesium and containing 300 µM RGDS) were treated with buffer or with PAO, activated by PMA and allowed to adhere on immobilized collagen. Results are from 6 independent experiments.