

Involvement of Glycoprotein VI in Platelet Thrombus Formation on Both Collagen and von Willebrand Factor Surfaces Under Flow Conditions

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Background—We studied the role of glycoprotein (GP) VI in platelet adhesion and thrombus formation on the immobilized collagen and von Willebrand factor (vWF) surface under flow conditions.

Methods and Results—Whole blood obtained from 2 patients with GP VI-deficient platelets and the effects of the Fab of anti-GP VI antibody (Fab/anti-GP VI) were tested. Blood containing platelets rendered fluorescent by mepacrine was perfused on immobilized type I collagen or vWF under controlled wall shear rate. Platelet adhesion and thrombus formation were detected by epifluorescent videomicroscopy. The percentage of surface coverage by the platelets was calculated. Fc receptor γ -chain and spleen tyrosine kinase (Syk) were immunoprecipitated from the lysate of platelets stimulated by vWF plus ristocetin and then analyzed by antiphosphotyrosine immunoblotting. No platelet attachment was seen on the surface of collagen even after 9 minutes of perfusion of blood at relatively low (100 s^{-1}) or high (1500 s^{-1}) wall shear rate, either in the case of blood containing GP VI-deficient platelets or in the presence of Fab/anti-GP VI, whereas significant platelet thrombus formation was noted after control blood perfusion. Such interference with the actions of GP VI also reduced firm platelet adhesion on immobilized vWF. vWF-induced tyrosine phosphorylation of GP VI-associated Fc receptor γ -chain followed by Syk activation occurred in normal platelets, but little activation of Syk occurred in GP VI-deficient platelets.

Conclusions—GP VI plays crucial roles in platelet thrombus formation on the surface of collagen under flow conditions in humans and is also involved in the process of firm platelet adhesion on the surface of vWF. (*Circulation*. 2002;106:266-272.)

Key Words: platelets ■ von Willebrand factor ■ glycoproteins ■ thrombosis

Platelet adhesion and subsequent thrombus formation on subendothelial matrix at the site of vascular damage play a crucial role in the arrest of posttraumatic bleeding^{1,2} and also in pathological thrombotic events, such as acute coronary syndrome.^{3,4} Recent clinical experience has clearly demonstrated that integrins, especially integrin $\alpha_{\text{IIb}}\beta_3$ (GP IIb/IIIa), are intimately involved in occlusive thrombus formation at the site of endothelial damage caused by interventional treatments, such as PTCA.^{5,6} Because initial platelet contact is thought to be mediated by platelet interaction with components of the subendothelial matrix, including collagen⁷ and immobilized and self-associated soluble von Willebrand factor (vWF),^{8,9} at the site of endothelial damage, this interaction could provide a suitable target for specific antiplatelet agents. Recently, Nieswandt et al¹⁰ performed functional studies

using β_1 -null or GP VI-deficient mouse platelets under flow conditions and reported that GP VI rather than integrin $\alpha_2\beta_3$ plays a more important role in platelet interaction with immobilized collagen under various flow conditions. They further proposed, on the basis of a revised model, that GP VI binding to collagen after GP Ib α -vWF interactions is an essential prerequisite for upregulation of the activity of integrins such as $\alpha_2\beta_3$ and $\alpha_{\text{IIb}}\beta_3$, which mediate firm platelet adhesion that is followed by platelet thrombus formation.

In the present study, we investigated the role of GP VI in platelet thrombus formation on the collagen surface under flow conditions in humans and further attempted to clarify the involvement of GP VI in platelet adhesion on the immobilized vWF surface in the absence of collagen.

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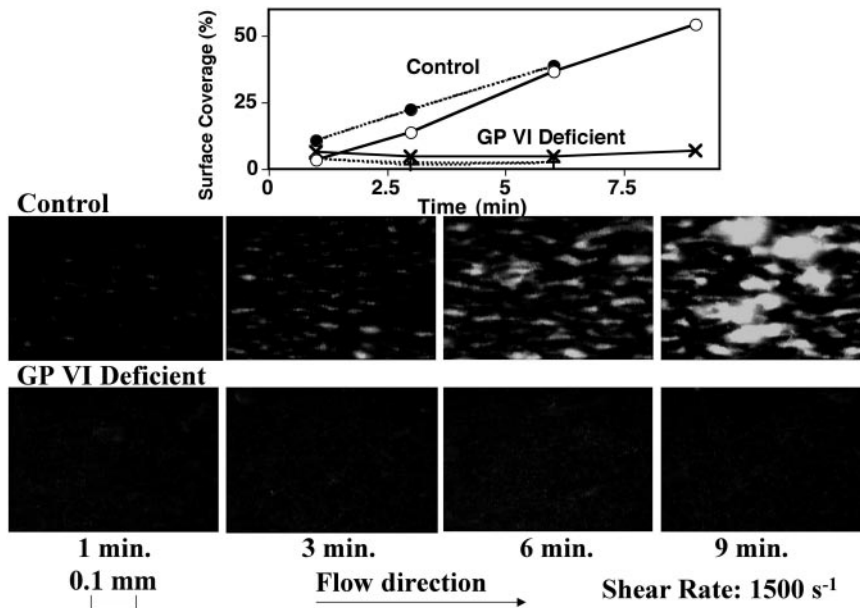


Figure 1. Platelet thrombus formation on immobilized collagen under a shear rate of 1500 s⁻¹. Blood samples obtained from control subjects and patients with GP VI-deficient platelets were perfused onto an immobilized collagen surface at a wall shear rate of 1500 s⁻¹. Representative results shown at bottom were obtained by perfusing 20 mL of blood obtained from 1 patient with GP VI-deficient platelets (Kyoto patient) and control subject over 9 minutes and 15 mL of blood obtained from other patient with GP VI-deficient platelets (Tokyo patient) and other control subject over 6 minutes. Top, Percentage of surface area coverage by platelets in 2 experiments. X (Kyoto patient) and + (Tokyo patient) indicate results obtained with blood from 2 patients with GP VI-deficient platelets; open and closed circles indicate results obtained with blood from control subjects.

Methods

Materials

Convulxin was purified, as described by Polgár et al,¹¹ from lyophilized *Crotalus durissus terrificus* venom. Collagen-related peptide was synthesized as described previously.¹² Human anti-GP VI IgG and its Fab fragment (Fab/anti-GP VI) was prepared as previously reported^{13,14} by use of the serum of a patient with GP VI deficiency. Specific inhibitory effects of Fab/anti-GP VI on GP VI-mediated platelet activation have been documented previously,^{13,14} as follows: pretreatment of platelets with 80 to 100 µg/mL of Fab/anti-GP VI almost completely abolished platelet aggregation in response to collagen (5 to 20 µg/mL); a similar effect was noted with 2 well-known GP VI-specific agonists, convulxin (10 ng/mL)¹¹ and collagen-related peptide (1 µg/mL),¹⁵ but not in response to other platelet agonists, such as ADP and epinephrine.

Sample Preparation

Blood was obtained from 2 patients whose platelets lacked GP VI [one followed up at Kyoto University Hospital (Kyoto patient¹⁴) and

the other at Tokyo Medical University Hospital (Tokyo patient¹⁶)], as well as from 2 normal volunteers, a 40-year-old man and a 30-year-old woman, at the same time and by the same method. The blood was then anticoagulated by the addition of 1/10 volume of 1 mmol/L of a specific antithrombin agent, argatroban (Mitsubishi Kagaku, Inc), and used for the following experiments, in the presence of physiological concentrations of divalent cations.¹⁷ Plasma-free reconstituted blood was obtained from the Tokyo patient and the 40-year-old male control subject as described previously, with slight modification.⁹ Briefly, blood cells were resuspended in modified HEPES-Tyrode's solution (mmol/L: 10 HEPES, 140 NaCl, 27 KCl, 0.4 NaH₂PO₄, 10 NaHCO₃, 5 dextrose, and 1 CaCl₂, and 5 mg/mL BSA, pH 7.4) after the washing procedures.⁹ These blood donors had not taken any drugs known to interfere with platelet functions, such as NSAIDs, for at least 4 weeks before the study.

Platelets were rendered fluorescent by the addition of mepacrine at a final concentration of 10 µmol/L (Sigma). Although mepacrine is known to affect platelet function through inhibition of phospholipid hydrolysis,¹⁸ previous studies have shown that it does not affect

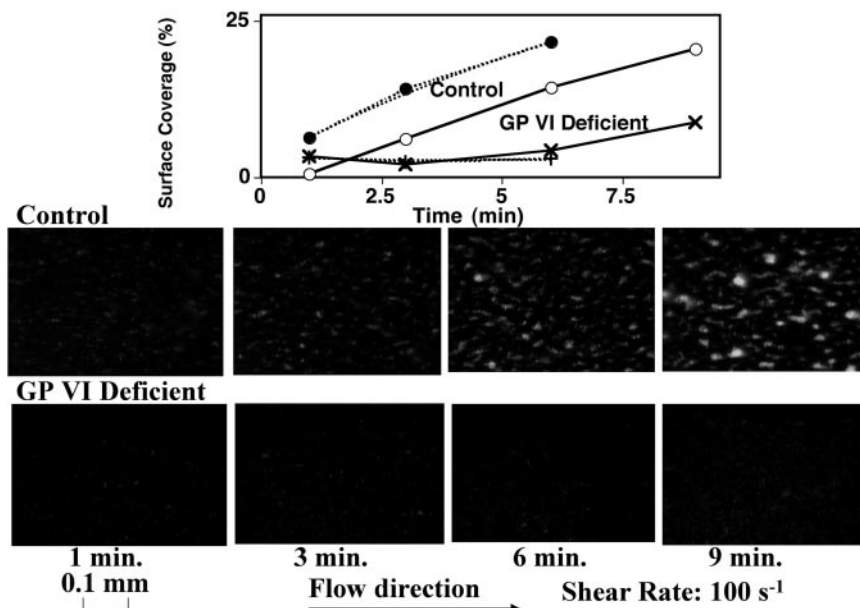


Figure 2. Platelet thrombus formation on immobilized collagen under a shear rate of 100 s⁻¹. Experiments were performed as explained in legend for Figure 1, but blood was perfused at a wall shear rate of 100 s⁻¹. Top, Percentage of surface area coverage by platelets. X and + indicate results with blood obtained from 2 patients with GP VI-deficient platelets; open and closed circles indicate results in control subjects.

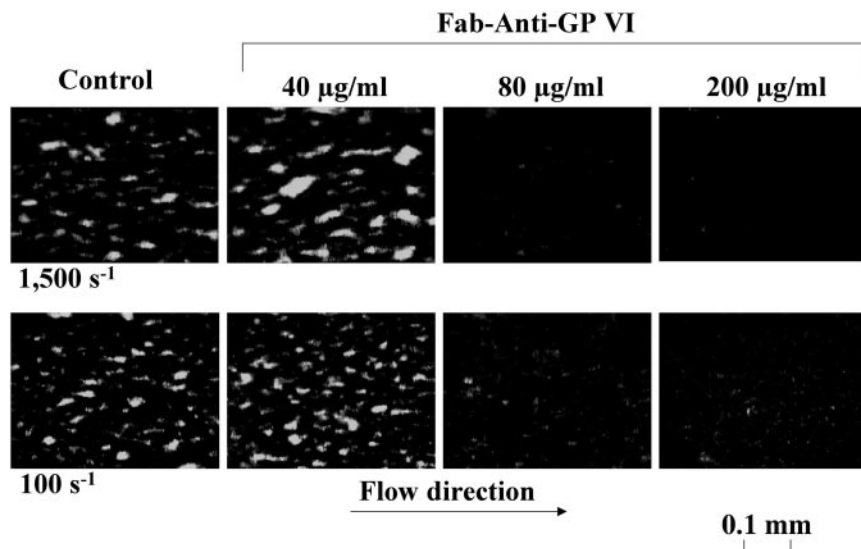


Figure 3. Effect of Fab/anti-GP VI on platelet thrombus formation on immobilized collagen under flow conditions. Blood (15 mL) drawn from apparently healthy adult donors was mixed with 1/100 volume of HEPES-NaCl solution (10 mmol/L HEPES, 140 mmol/L NaCl, pH 7.4) with or without Fab/anti-GP VI to achieve final concentrations shown. Platelet thrombi that formed on collagen surface after 6 minutes of blood perfusion at a relatively low (100 s^{-1}) and a high (1500 s^{-1}) wall shear rate are shown. Each panel shows results representative of duplicate experiments.

platelet activation and thrombus formation on the surface of collagen and immobilized vWF under flow conditions at the dose we used.^{8,9,19–21}

Preparation of the Flow Chamber System and Platelet Thrombus Visualization by Epifluorescent Videomicroscopy

Acid-insoluble fibrillar type I collagen from bovine Achilles tendon (Sigma) was immobilized on a glass coverslip (Corning, Inc; $24 \times 50 \text{ mm}$) in a parallel plate flow chamber, as described previously.^{19–21} Similarly, human vWF purified from the factor VIII/vWF concentrate of Fhandi was also immobilized on a glass coverslip in the chamber as previously reported.²² The blood samples were then introduced into the chamber with a syringe pump (Holliston, MA 01746, Harvard Apparatus Co) at a constant flow rate to achieve the intended wall shear rates on the surface of collagen or vWF.

Platelet thrombi forming on the surface of collagen or vWF were visualized with an inverted-stage epifluorescence videomicroscope system equipped with a 480-nm excitation light source (DM IRB, IRB-FLUO, Leica) as described previously.²¹ The microscopic images were digitized online with a photosensitive color CCD camera (L-600, Leica) and stored as digital images in a personal

computer (Power Macintosh G3, Apple). To quantify the percentage of surface coverage by the platelets, the digital color images were converted into black-and-white images with NIH Image software (public domain software by Dr Wayne Rasband, National Institutes of Health, version 1.62), and the percent area covered by the platelets was calculated.

The effect of GP VI deficiency on platelet-vWF surface interaction was qualitatively demonstrated by overlaying 30 consecutive frames of the video images (corresponding to 1 second) so that firmly attached platelets that did not move appeared as thick images and moving (eg, rolling) platelets, which appeared in only some of the frames, appeared as thinner images. The movement of every single platelet appearing in consecutive image frames was quantified. The effect of GP VI deficiency on platelet-vWF surface interaction was also tested in plasma-free reconstituted blood.

Immunoprecipitation and Immunoblotting

Washed platelets (1.0×10^9 cells/mL) prepared as previously described²³ were preincubated with or without 100 µg/mL Fab/anti-GP VI for 3 minutes and stimulated with 10 µg/mL vWF in the presence of 1 mg/mL ristocetin for the indicated times at 37°C in an aggregometer under continuous stirring. Unstimulated or stimulated

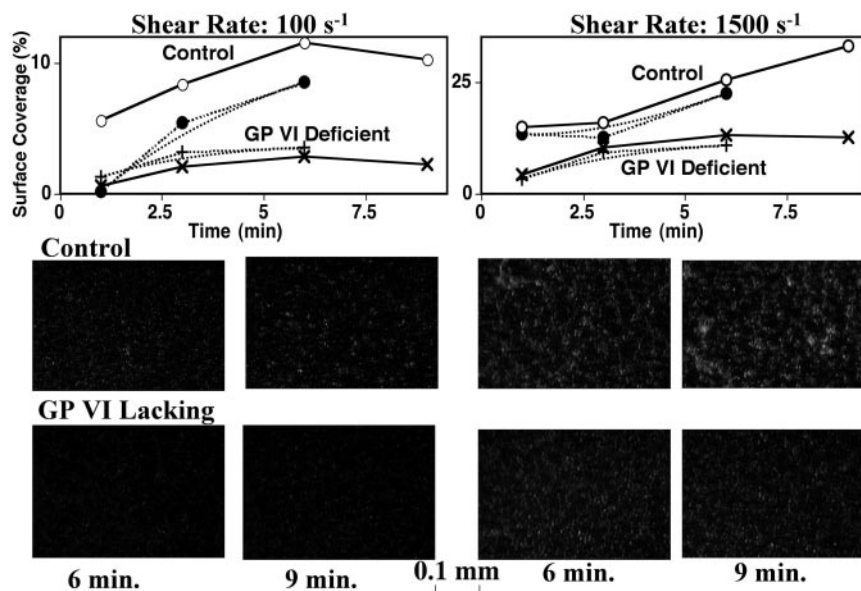


Figure 4. Platelet thrombus formation on immobilized vWF under flow conditions. Blood specimens obtained from control subjects and patients with GP VI-deficient platelets were perfused onto an immobilized collagen surface. Bottom, Representative results from perfusion of 20 mL of blood obtained from 1 patient with GP VI-deficient platelets (Kyoto patient) and from control subject on an immobilized vWF surface over 9 minutes at wall shear rate of 100 s^{-1} (left) and 1500 s^{-1} (right), respectively. Additional experiments were performed with 15 mL of blood obtained from other patient with GP VI-deficient platelets (Tokyo patient) and from other control subject. Top, Percentage of surface area coverage by platelets in 2 experiments performed under wall shear rate of 100 s^{-1} and 1500 s^{-1} , respectively. X (Kyoto patient) and + (Tokyo patient) indicate results obtained with blood from 2 patients with GP VI-deficient platelets; open and closed circles indicate results with blood obtained from control subjects.

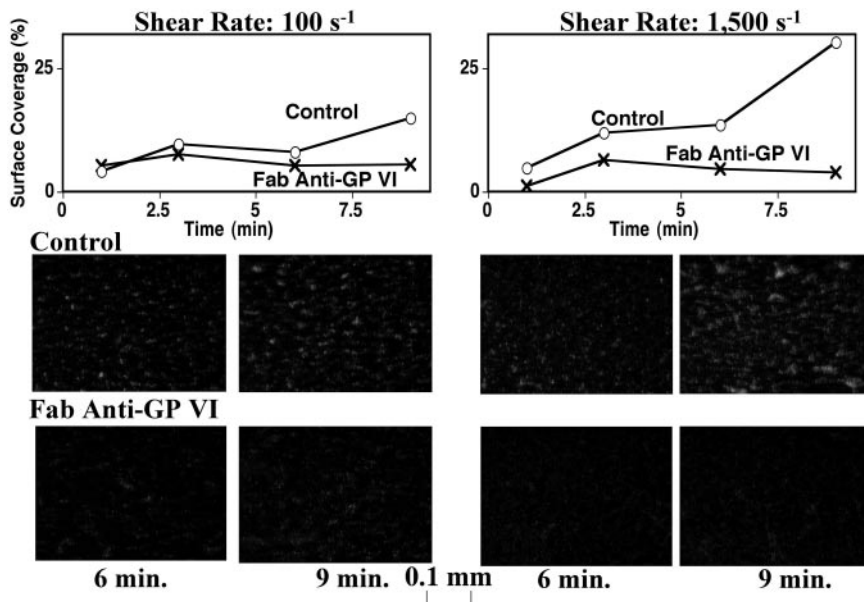


Figure 5. Effect of Fab/anti-GP VI on platelet thrombus formation on immobilized vWF under flow conditions. Blood (20 mL) drawn from apparently healthy adult donors was mixed with 1/100 volume of HEPES-NaCl solution (10 mmol/L HEPES, 140 mmol/L NaCl, pH 7.4), with or without Fab/anti-GP VI, to achieve final concentration of 120 $\mu\text{g/mL}$. Blood specimens were then perfused onto immobilized vWF surface at a wall shear rate of 100 s^{-1} or 1500 s^{-1} . Results shown are representative of duplicate experiments.

platelets were lysed in an ice-cold lysis buffer, and immunoprecipitation of each specified protein, followed by immunoblot analysis, was performed as described previously.²⁴

Results

Platelet Thrombus Formation on Collagen Surface Under Flow Conditions

Virtually no platelet attachment could be seen on the collagen surface even after 9 minutes of blood perfusion at either relatively low (100 s^{-1}) or high (1500 s^{-1}) wall shear rate in the case of blood containing GP VI-deficient platelets, whereas significant platelet thrombus formation

was noted after control blood perfusion (Figures 1 and 2). Similar results were obtained with blood containing Fab/anti-GP VI at a concentration of $>80 \mu\text{g/mL}$ (Figure 3), lending support to the notion that GP VI plays crucial roles in platelet thrombus formation on the surface of collagen under flow conditions.

Platelet Thrombus Formation on Immobilized vWF

Platelet thrombus formation on immobilized vWF was also inhibited, but not completely, after perfusion of blood containing platelets lacking GP VI (Figure 4). The percentage surface coverage by platelets was inhibited by $>50\%$ after perfusion of blood containing platelets lacking GP VI. Involvement of GP VI in platelet attachment on the surface of vWF was also demonstrated by the results obtained with blood containing Fab/anti-GP VI (Figure 5).

To understand the role of GP VI in platelet attachment on the surface of vWF in the absence of collagen under flow conditions, platelet movement on the surface of immobilized vWF was also investigated. Most of the control platelets became firmly attached to the vWF surface without exhibiting continuous movement, whereas the majority of GP VI-deficient platelets, especially under the relatively high shear rate of 1500 s^{-1} , exhibited continuous movements on the vWF surface (Figure 6). Control platelets, once they adhered to the vWF surface, moved only $0.25 \pm 0.71 \mu\text{m}$ (mean \pm SD) during a period of 1 second, whereas GP VI-deficient platelets moved $6.18 \pm 6.40 \mu\text{m}$ ($P=0.000022$). Virtually no platelet interaction with the vWF surface could be seen in GP VI-deficient platelets, whereas stable platelet adhesion on the vWF surface could still be seen in the control platelets in the absence of plasma proteins (Figure 7).

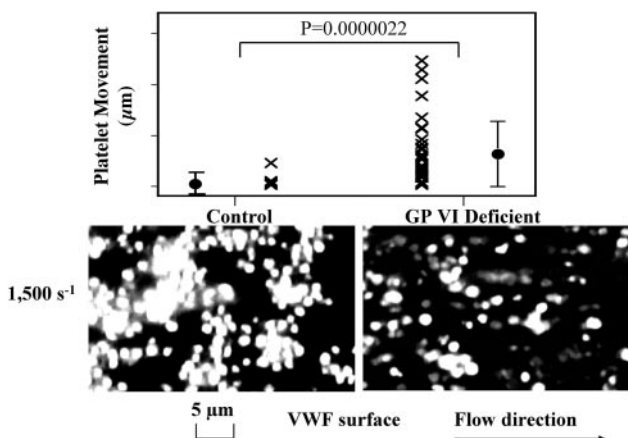


Figure 6. Behavior of GP VI-deficient platelets on vWF surface. Experiments were performed as described in legend for Figure 4. Images of single platelets, rather than platelet thrombi, were magnified with $\times 100$ objective lens. Thirty consecutive video images, converted to black and white with NIH Image software, were overlaid on 256-scale gray-scale images (bottom). Thick particles represent platelets firmly attached to surface; thinner particles represent platelets continuously moving over surface. Results shown are representative of duplicate experiments. To quantify platelet movements, total distance moved by platelets during 1 second was measured for each platelet (top). Circles and error bars represent mean and SD values, respectively, of all platelet movements measured.

vWF Stimulation Accompanies Tyrosine Phosphorylation of the Fc Receptor γ -Chain Associated with GP VI

It has been well established that GP VI is coupled to Fc receptor γ -chain (FcR γ) and signals tyrosine phosphorylation

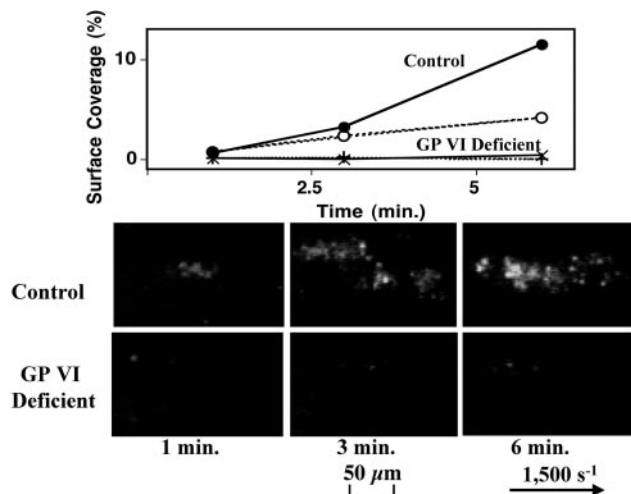


Figure 7. Platelet-vWF surface interaction in plasma-free reconstituted blood. Plasma-free reconstituted blood obtained from Tokyo patient and control subject were perfused on immobilized vWF surface at a wall shear rate of 1500 s^{-1} for 6 minutes. Top, Percentage of surface area coverage by platelets in duplicate experiments. X and + indicate results obtained with blood from GP VI-deficient platelets; open and closed circles indicate results obtained with blood from control subjects. Bottom, Results of representative duplicate experiments.

of FcR γ , followed by activation of spleen tyrosine kinase (Syk).^{24,25} Therefore, we examined biochemically whether GP VI-associated FcR γ was tyrosine-phosphorylated in platelets stimulated by vWF. FcR γ was immunoprecipitated from the lysate of washed platelets stimulated by vWF plus ristocetin in the absence or presence of Fab/anti-GP VI and was analyzed by antiphosphotyrosine immunoblotting. As shown in Figure 8A, stimulation of platelets with vWF plus ristocetin resulted in tyrosine phosphorylation of FcR γ , but much less in the presence of Fab/anti-GP VI. Reprobing of the immunoblot with anti-GP VI antibody confirmed that the tyrosine-phosphorylated FcR γ was associated with GP VI.

The degree of tyrosine phosphorylation of Syk was compared between vWF plus ristocetin-stimulated normal and GP VI-deficient platelets and also between similarly stimulated normal platelets in the absence and presence of Fab/anti-GP VI. The results revealed that tyrosine phosphorylation of Syk in GP VI-deficient or Fab/anti-GP VI-pretreated normal platelets stimulated by vWF plus ristocetin was much weaker than that in normal platelets similarly stimulated by the same substances (Figure 8B).

Discussion

To date, the following has been widely accepted as the model of platelet thrombus formation: GP Ib α -vWF mediates initial tethering at relatively high shear rates,^{8,26} followed by $\alpha_2\beta_1$ -collagen²⁷ and $\alpha_{IIb}\beta_3$ -vWF-mediated firm attachment,^{8,28} which arrests platelets and enables collagen to interact with GP VI, resulting in platelet activation and thrombus formation.^{10,27-29} Recently, this concept was challenged by Nieswandt et al,¹⁰ who showed complete loss of attachment of mouse platelets to the surface of immobilized collagen under flow conditions in

the absence of GP VI. Because species-specific differences cannot be ruled out in their study, we first attempted to test the behavior of human platelets deficient in GP VI on immobilized collagen surface under flow conditions. Our results revealed that platelet attachment on the surface of collagen was completely inhibited in the presence of GP VI deficiency and also by the addition of Fab/anti-GP VI, corroborating the results of Nieswandt et al¹⁰ obtained with mouse platelets.

Because previous studies have suggested a crucial role for the interaction between vWF bound to collagen and its receptor GP Ib α on the initial tethering of platelets under high shear rates,^{19,26,29} we were further prompted to investigate whether interference with GP VI function would affect platelet thrombus formation on immobilized vWF in the absence of collagen under flow conditions. Surprisingly, the results showed that firm platelet attachment and subsequent thrombus formation on immobilized vWF in the absence of collagen were also inhibited in the presence of GP VI deficiency and Fab/anti-GP VI. This suggested the involvement of GP VI, not only in collagen-mediated but also in the vWF-mediated process of platelet thrombus formation. Moreover, our results showing complete loss of platelet-vWF interaction with GP VI-deficient platelets suggest the crucial role of GP VI even in transient platelet-vWF interaction, which is presumably mediated exclusively by vWF-GP Ib α interaction.²⁶ These results cannot be explained by the revised model proposed by Nieswandt et al.¹⁰ However, the question arises as to how the GP VI/FcR γ complex is involved in vWF-mediated thrombus formation under flow conditions. Two groups have recently reported that FcR γ was tyrosine-phosphorylated when platelets were stimulated by vWF-GP Ib α interaction induced by ristocetin or botrocetin.^{30,31} Because we previously suggested that FcR γ was intimately associated with GP VI in human platelets,²⁵ their studies also favor the possibility that the GP VI/FcR γ complex is activated in response to vWF-GP Ib α interaction. In this study, we have shown the occurrence of tyrosine phosphorylation of GP VI-associated FcR γ , followed by Syk activation in association with vWF-GP Ib α interaction induced by ristocetin. Thus, our data from the present study support the possibility of involvement of the GP VI/FcR γ complex pathway in the process of platelet activation, leading to firm platelet adhesion on the surface of vWF through activation of $\alpha_{IIb}\beta_3$ initiated by vWF-GP Ib α interaction but not by GP VI-collagen interaction. It is important to note, however, that the inhibitory effects of interference with GP VI function on platelet attachment on immobilized vWF were less significant than those observed on collagen surface, especially in the presence of plasma proteins. This suggests that the activation of the GP VI/FcR γ complex pathway during vWF-GP Ib α interaction is not an absolute requirement for subsequent thrombus formation on immobilized vWF and may suggest the possibility of the existence of GP VI/FcR γ complex-independent mechanisms for the activation of $\alpha_{IIb}\beta_3$ by vWF-GP Ib α interaction. Another important question is how the GP VI/FcR γ complex is activated during vWF-GP

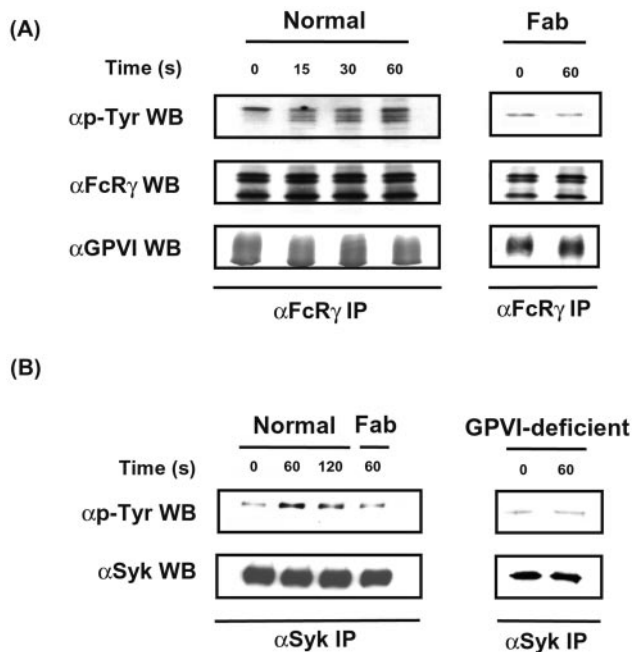


Figure 8. vWF plus ristocetin-induced tyrosine phosphorylation of FcR γ followed by Syk activation downstream of GP VI. Anti-FcR γ (α FcR γ) or anti-Syk (α Syk) immunoprecipitates (IP) were prepared from lysate of normal platelets stimulated by 10 μ g/mL vWF plus 1 mg/mL ristocetin in absence (Normal) or presence (Fab) of 100 μ g/mL Fab/anti-GP VI and from that of GP VI-deficient platelets similarly stimulated by same substances for indicated times, and analyzed by antiphosphotyrosine (α p-Tyr) or anti-GP VI (α GPVI) immunoblotting (WB).

Ib α interaction in the absence of collagen. Studies are currently under way to resolve these remaining questions.

Because GP VI appears to possess more profound roles in platelet thrombus formation under flow conditions than previously thought, one would consider that antiplatelet agents targeting GP VI would be attractive antiplatelet agents to inhibit platelet thrombus formation at the site of the exposed subendothelial matrix in the event of atheroma rupture in coronary arteries. A recent report of the existence of a relationship between genetic polymorphism of GP VI and the onset of myocardial infarction lends support to this notion.³² Our present findings should motivate researchers to develop an anti-GP VI agent to control such clinical events.

In conclusion, we have shown that GP VI plays a crucial role in platelet thrombus formation on the surface of collagen under flow conditions in humans. Furthermore, GP VI is also involved in the process of firm platelet adhesion to the surface of immobilized vWF in the absence of collagen.

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