

# Long-term Antithrombotic Protection by In Vivo Depletion of Platelet Glycoprotein VI in Mice

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## Abstract

Coronary artery thrombosis is often initiated by abrupt disruption of the atherosclerotic plaque and activation of platelets on the subendothelial layers in the disrupted plaque. The extracellular matrix protein collagen is the most thrombogenic constituent of the subendothelial layer; therefore, a selective inhibition of the collagen activation pathway in platelets may provide strong antithrombotic protection while preserving other platelet functions. Here we demonstrate that treatment of mice with a monoclonal antibody against the activating platelet collagen receptor glycoprotein VI (GPVI; JAQ1) results in specific depletion of the receptor from circulating platelets and abolished responses of these cells to collagen and collagen-related peptides (CRPs). JAQ1-treated mice were completely protected for at least 2 wk against lethal thromboembolism induced by infusion of a mixture of collagen (0.8 mg/kg) and epinephrine (60  $\mu$ g/ml). The tail bleeding times in JAQ1-treated mice were only moderately increased compared with control mice probably because the treatment did not affect platelet activation by other agonists such as adenosine diphosphate or phorbol myristate acetate. These results suggest that GPVI might become a target for long-term prophylaxis of ischemic cardiovascular diseases and provide the first evidence that it is possible to specifically deplete an activating glycoprotein receptor from circulating platelets in vivo.

Key words: thrombosis • immunotherapy • collagen • receptor • mouse

## Introduction

Platelet aggregation is a key mechanism for normal hemostasis limiting blood loss after tissue trauma (1, 2) but may lead to arterial occlusion in the setting of atherosclerosis and precipitate diseases such as myocardial infarction (3, 4). Arterial thrombosis is often initiated by abrupt disruption of the atherosclerotic plaque and deposition and activation of platelets on the subendothelial layers (4, 5). Although several of the macromolecular components of the subendothelial layer such as laminin, fibronectin, and von Willebrand factor (vWf)<sup>1</sup> all provide a suitable substrate for platelet ad-

hesion, fibrillar collagen is considered the most thrombogenic constituent of the vascular subendothelium, as it not only supports platelet adhesion but is also a strong activator of platelets (6, 7). The interaction between platelets and collagen involves first adhesion and, subsequently, activation leading to second phase adhesion, secretion, and ultimately aggregation (8, 9). Besides glycoprotein (GP)Ib-IX-V, which indirectly interacts with collagen via vWF (10), several collagen receptors have been identified on platelets, including integrin  $\alpha_2\beta_1$  (11), and the nonintegrin GPVI (12). It is presently accepted that integrin  $\alpha_2\beta_1$  is the major receptor supporting platelet adhesion to collagen, whereas GPVI mediates activation (13–15). The recent cloning of human and mouse GPVI showed that this receptor is a 60–65-kD type I transmembrane glycoprotein belonging to the immunoglobulin superfamily (16, 17) that forms a complex with the FcR  $\gamma$ -chain at the cell surface in human and mouse platelets (14, 15, 18). Signaling through GPVI oc-

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<sup>1</sup>Abbreviations used in this paper: ADP, adenosine diphosphate; CRP, collagen-related peptide; ECL, enhanced chemiluminescence; GP, glycoprotein; prp, platelet-rich plasma; RT, room temperature; vWF, von Willebrand factor.

curs via a similar pathway to that used by immunoreceptors (19) as revealed by the tyrosine phosphorylation of the FcR  $\gamma$ -chain immunoreceptor tyrosine-based activation motif (ITAM) by an src-like kinase (20, 21). GPVI-deficient patients suffer from a mild bleeding diathesis, and their platelets show severely impaired responses to collagen (12, 22). Furthermore, platelets from FcR  $\gamma$ -chain-deficient mice, which lack GPVI (15), also fail to aggregate in response to collagen (14, 19), but major bleeding has not been reported to occur in these mice.

In this study we investigated the antithrombotic effect of the first anti-(mouse) GPVI Ab (JAQ1; [15]). We show that pretreatment with JAQ1 leads to irreversible depletion of GPVI on circulating platelets resulting in abolished responses of the cells to collagen and, consequently, profound and long-lasting protection from mortality in a model of collagen-dependent intravascular thrombosis. The tail bleeding times in GPVI-depleted mice were only moderately increased compared with the controls, probably because other activation pathways were preserved in these platelets. These results provide the first evidence that GPVI might serve as an attractive target for long-term prophylaxis of cardiovascular diseases.

## Materials and Methods

**Animals.** Specific pathogen-free mice (NMRI) 6–10 wk of age were obtained from Charles River Laboratories and kept in our animal facilities.

**Chemicals.** Anesthetic drugs xylazine (Rompun®) and ketamine (Imalgene 1000®) were from Bayer and Merial, respectively. Immobilized papain (Pierce Chemical Co.), high molecular weight heparin, adenosine diphosphate (ADP), phorbol 12-myristate 13-acetate (PMA; all from Sigma-Aldrich), FITC-labeled annexin V (Boehringer), and collagen (Kollagenreagent Horn; Nycomed) were purchased. Collagen-related peptide (CRP; GKO-(GPO)<sub>10</sub>-GKOG; single letter amino acid code where O = hydroxyproline) and convulxin were provided by S.P. Watson (University of Oxford, Oxford, UK). FITC-labeled convulxin was a gift from M. Jandrot-Perrus (INSERM, Paris, France).

**Abs.** The rat anti-mouse P-selectin mAb RB40.34 was provided by D. Vestweber (University of Münster, Münster, Germany). Polyclonal rabbit Abs to human fibrinogen and vWF were purchased from Dako and were modified in our laboratories. Rabbit anti-FITC-horseradish peroxidase (HRP) was from Dako. mAbs against the integrin  $\alpha$ 2 and  $\beta$ 1 subunits were from BD PharMingen. All other Abs were generated, produced, and modified in our laboratories and have been described (23, 24). Modification of Abs: Fab fragments from JAQ1 were generated by 12-h incubation of 10 mg/ml mAb with immobilized papain (Pierce Chemical Co.), and the preparations were then applied to an immobilized protein A column followed by an immobilized protein G column (Amersham Pharmacia Biotech) to remove Fc fragments and any undigested IgG. The purity of the Fab fragments was checked by SDS-PAGE and silver staining of the gel. F(ab')<sub>2</sub> fragments from JON/A (anti-mouse GPIIb/IIIa) were generated as described (24).

**Platelet Preparation and Counting.** Mice were bled under ether anesthesia from the retroorbital plexus. Blood was collected in a tube containing 10% (vol/vol) 0.1 M sodium citrate or 7.5 U/ml

heparin, and platelet-rich plasma (prp) was obtained by centrifugation at 300 g for 10 min at room temperature (RT). For determination of platelet counts, blood (20  $\mu$ l) was obtained from the retroorbital plexus of anesthetized mice using siliconized microcapillaries and immediately diluted 1:100 in Unopette kits (Becton Dickinson). The diluted blood sample was allowed to settle for 20 min in an Improved Neubauer haemocytometer (Superior), and platelets were counted under a phase contrast microscope at  $\times$ 400 magnification.

**Immunoblotting.** Platelets ( $3 \times 10^8$ ) were washed three times with PBS and subsequently solubilized in 0.3 ml lysis buffer (Tris-buffered saline containing 20 mM Tris/HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml aprotinin, 0.5  $\mu$ g/ml leupeptin, and 0.5% Nonidet P-40; all from Boehringer) for 30 min at 4°C. Cell debris was removed by centrifugation (15,000 g, 10 min) and the whole-cell extract was run on an SDS-PAGE gel under nonreducing conditions and transferred onto a PVDF membrane. The membrane was first incubated with 5  $\mu$ g/ml FITC-labeled primary Ab followed by rabbit anti-FITC-HRP (1  $\mu$ g/ml). Proteins were visualized by enhanced chemiluminescence (ECL).

**Two-dimensional Electrophoresis.** Washed platelets were pelleted and resuspended in 20 mM Tris, pH 7.5, 2 mM EDTA, and 0.25 M sucrose. Platelets were solubilized by addition of 4 vol of 8.75 M urea, 2.5 M thiourea, 25 mM dithiothreitol (DTT), 1.25% Triton X-100, and 0.75% ampholytes 3–10. Two-dimensional gel electrophoresis (2D-E) was carried out as described (25). In brief, IEF was carried out with commercially available immobilized pH gradient (linear pH gradient 3–10, 7 cm length), using the Protean IEF Cell apparatus (Bio-Rad Laboratories). The gels were rehydrated in the presence of the samples (platelet lysates corresponding to  $5 \times 10^7$  platelets) for 16 h and focused for 20,000 Vh. After IEF, the gel strips were incubated at RT in solutions containing DTT and then iodoacetamide, as described (26). The gels were then subjected to the second-dimensional run and silver stained.

**Aggregometry.** To determine platelet aggregation, light transmission was measured using prp (200  $\mu$ l with  $0.5 \times 10^6$  platelets/ $\mu$ l). Transmission was recorded on a Fibrinometer 4 channel aggregometer (APACT Laborgeräte und Analysensysteme) over 10 min and was expressed as arbitrary units with 100% transmission adjusted with plasma. Platelet aggregation was induced by addition of collagen (5–50  $\mu$ g/ml), PMA (50 ng/ml), or ADP (10  $\mu$ M).

**Flow Cytometry.** Heparinized whole blood was diluted 1:30 with modified Tyrodes-Hepes buffer (134 mM NaCl, 0.34 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.9 mM KCl, 12 mM NaHCO<sub>3</sub>, 20 mM Hepes, 5 mM glucose, and 1 mM MgCl<sub>2</sub>, pH 6.6) and left for 30 min at 37°C before stimulation. Samples were stimulated with the indicated concentrations of ADP or CRP for 2 min at RT, stained with fluorophore-labeled mAbs for 10 min at RT, and directly analyzed on a FACScan™ (Becton Dickinson). Flow cytometric analysis of annexin V-FITC binding to resting and activated (combination of 50  $\mu$ g/ml collagen and 0.01 U/ml thrombin) platelets was measured according to the instructions of the manufacturer.

**In Vivo Experiments.** Abs (in 200  $\mu$ l PBS) were injected intraperitoneally. Thromboembolism induced by collagen and epinephrine: mice were anesthetized by intraperitoneal injection of 150  $\mu$ l of a mixture of 0.08% xylazine base (Rompun; Bayer) and 1.6% ketamine (Imalgene 1000; Merial). Anesthetized mice received a mixture of collagen (0.8 mg/kg) and epinephrine (60  $\mu$ g/kg) injected into the jugular vein (27). The incisions of sur-

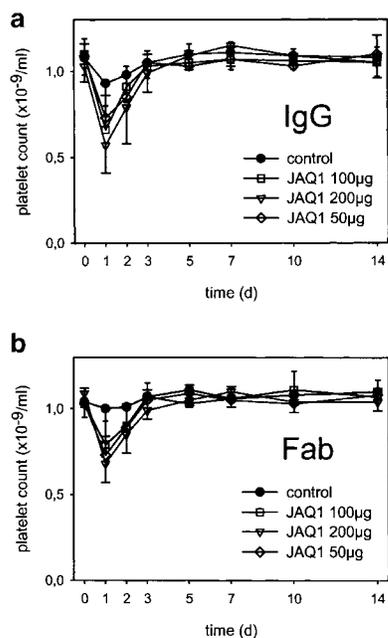
viving mice were stitched, and they were allowed to recover. Necropsy and histological studies were performed on lungs fixed in 4% formaldehyde and paraffin sections were stained with hematoxylin/eosin. Bleeding time experiments: mice were anesthetized and 3 mm of tail tip was amputated with a scalpel. The tail was then blotted with filter paper every 15 s until the paper was no longer blood stained (28). Where necessary, bleeding was manually stopped at the 10 min time point to prevent death. Experiments were conducted in accordance to the regulations of the local authorities.

**Immunohistochemistry.** Acetone-fixed cryosections (6  $\mu\text{m}$ ) were blocked (5% normal goat serum, 5 mg/ml BSA in PBS) for 30 min at RT. HRP-conjugated p0p1 (anti-mouse GPIIb-IX [23]) was added at a final concentration of 2  $\mu\text{g}/\text{ml}$  for 90 min and the 3-amino-9-ethyl-carbazole (AEC) substrate was added after the three washing steps. The sections were then counterstained with hematoxylin.

**Platelet Adhesion.** Collagen (2  $\mu\text{g}$ ) in 100  $\mu\text{l}$  PBS was immobilized on F96-MaxiSorp plates (Nunc) at 4°C overnight. The plates were then saturated with 1 mg/ml BSA in PBS for 3 h at 37°C and washed with PBS. Washed platelets in Tyrode's-albumin buffer (10<sup>6</sup>/well) were incubated in the wells for up to 45 min. The plates were washed three times with PBS and then incubated with HRP-labeled anti-GPIIb-IX (p0p1) for 30 min at RT, and 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well after three washing steps. The reaction was stopped by addition of 2 N H<sub>2</sub>SO<sub>4</sub> after 10 min. Absorbance at 450 nm was recorded on a Multiskan MCC/340 (Labsystems).

## Results

We recently reported the generation of the first mAb against mouse GPVI (JAQ1, rat IgG2a) and demonstrated



**Figure 1.** JAQ1 induces transient thrombocytopenia. Mice received purified IgG (a) or Fab fragments (b) of the indicated mAb intraperitoneally in 200  $\mu\text{l}$  sterile PBS. Platelet counts were determined at the indicated times using an improved Neubauer hemocytometer. Results are expressed as the mean platelet count  $\pm$  SD for groups of each six mice.

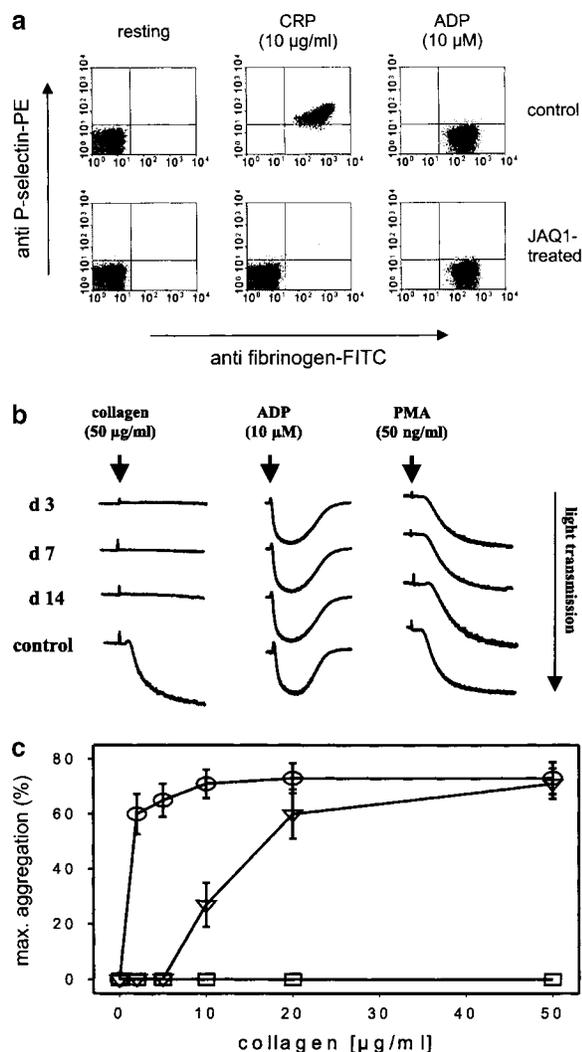
that JAQ1 has a significant but limited inhibitory effect on collagen-induced platelet aggregation *in vitro* (15, 29). In this study, we investigated the antithrombotic effects of JAQ1 *in vivo*. Injection of JAQ1 (100  $\mu\text{g}$ ) caused mild and transient thrombocytopenia with a maximum drop of platelet counts of  $\sim 34 \pm 7.4\%$  on day 1 and a return to normal after 72 h, where they remained for at least 11 more days (Fig. 1 a). Injection of higher (200  $\mu\text{g}$ ) or lower (50  $\mu\text{g}$ ) doses had comparable effects on platelet counts (Fig. 1 a). The transient drop of platelet counts was not Fc dependent, as Fab fragments of JAQ1 had similar effects (Fig. 1 b). JAQ1-treated mice did not show any signs of anaphylactic reactions as known to be induced by anti-GPIIb/IIIa mAbs (30) and did not develop spontaneous bleeding for at least 3 wk. JAQ1 was immunohistochemically detectable on splenic and bone marrow-derived megakaryocytes 3 h after Ab injection, demonstrating that the mAb reached these cells in both organs (not shown).

**JAQ1 Treatment Abolishes Platelet Responses to Collagen and CRPs Ex Vivo for at Least 2 Wk.** The effect of JAQ1 on circulating platelets was studied *ex vivo* at different time points after Ab injection. The basal surface expression of the major GP receptors GPIIb/IIIa and GPIb-IX-V, CD9 and integrin  $\alpha_2\beta_1$  was unchanged compared with control platelets at 3, 7, and 14 d after Ab injection (Table I). At no time after Ab injection did circulating platelets show any signs of activation, as demonstrated by the lack of surface bound fibrinogen and surface-expressed P-selectin (Table I). On days 3, 7, and 14, platelets from JAQ1-treated mice were resistant towards activation with the CRP (up to 30  $\mu\text{g}/\text{ml}$ ), which is known to be a strong GPVI-specific platelet agonist (31; Fig. 2 a). In contrast, ADP induced normal activation (fibrinogen binding) of these platelets. Furthermore, platelets from JAQ1-treated mice were completely resistant to activation with collagen at concentrations of up to 50  $\mu\text{g}/\text{ml}$  *ex vivo*, and this profound inhibitory effect also lasted for at least 14 d upon a single injection of 100  $\mu\text{g}$  JAQ1 (Fig. 2 b). In contrast to collagen, ADP and PMA induced normal aggregation of these

**Table I.** Expression of Glycoproteins and Surface-bound Fibrinogen on Platelets from JAQ1-treated Mice

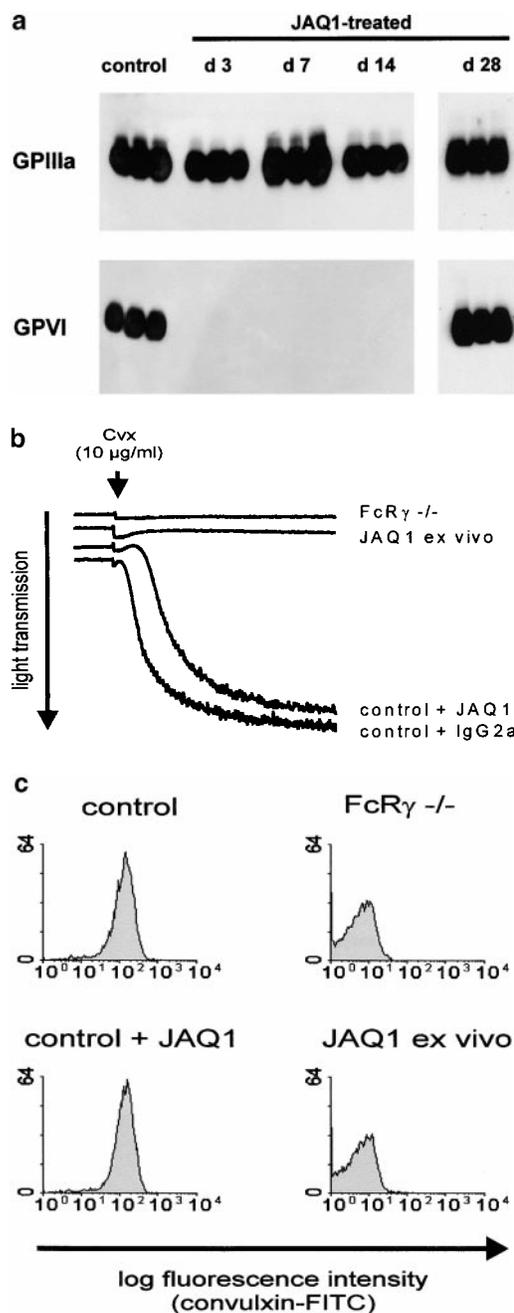
|                     | Control          | JAQ1 3 d         | JAQ1 7 d         | JAQ1 14 d        |
|---------------------|------------------|------------------|------------------|------------------|
| GPIIb/IIIa          | 321.3 $\pm$ 9.7  | 318.1 $\pm$ 9.4  | 328.7 $\pm$ 9.1  | 325.3 $\pm$ 9.8  |
| GPIb-IX             | 278.9 $\pm$ 16.8 | 275.4 $\pm$ 18.0 | 269.5 $\pm$ 15.9 | 273.1 $\pm$ 11.4 |
| GPV                 | 165.4 $\pm$ 10.9 | 163.3 $\pm$ 14.1 | 169.1 $\pm$ 15.3 | 158.1 $\pm$ 10.5 |
| CD9                 | 543.8 $\pm$ 15.8 | 554.3 $\pm$ 14.6 | 549.5 $\pm$ 19.6 | 557.0 $\pm$ 13.0 |
| GPIa ( $\alpha_2$ ) | 38.2 $\pm$ 6.7   | 40.3 $\pm$ 6.5   | 35.2 $\pm$ 7.8   | 36.7 $\pm$ 6.2   |
| Fibrinogen          | 14.1 $\pm$ 1.7   | 15.0 $\pm$ 1.4   | 14.3 $\pm$ 1.5   | 14.4 $\pm$ 1.5   |
| P-selectin          | 6.2 $\pm$ 0.8    | 6.5 $\pm$ 0.8    | 6.7 $\pm$ 0.8    | 6.0 $\pm$ 1.1    |

Diluted whole blood from the indicated mice was incubated with FITC-labeled Ab at saturating concentrations for 15 min at RT and platelets were analyzed directly. Results are expressed as mean log fluorescence  $\pm$  SD for six mice per group.



**Figure 2.** Platelets from JAQ1-treated mice do not respond to CRP and collagen. (a) Two-color flow cytometric analysis of platelets from JAQ1-treated or control mice 3 d after Ab injection. Diluted whole blood was stimulated with 10  $\mu$ M ADP or 10  $\mu$ g/ml CRP for 2 min and subsequently incubated with antifibrinogen<sup>FITC</sup> and anti-P-selectin<sup>PE</sup> Abs for 10 min at RT and analyzed directly. Platelets were gated by FSC/SSC characteristics and F13 intensity (anti-mouse GPIb  $\alpha^{PE/Cy5}$ ). The data shown are representative of six mice per group. Similar results were obtained on days 7 and 14 after Ab injection. (b) Heparinized prp from the indicated mice was stimulated with collagen (50  $\mu$ g/ml), ADP (10  $\mu$ M), or PMA (50 ng/ml). Light transmission was recorded on a FibrinTimer 4 channel aggregometer. (c) Heparinized prp from control mice was incubated with stirring in the presence of irrelevant rat IgG2a (20  $\mu$ g/ml;  $\circ$ ) or JAQ1 (20  $\mu$ g/ml;  $\nabla$ ) for 5 min before the addition of the indicated concentrations of collagen. In parallel, prp from JAQ1-treated mice was tested ( $\square$ ). Results are expressed as the maximum (max.) platelet aggregation  $\pm$  SD for groups of each six mice.

platelets, indicating that JAQ1 specifically blocked GPVI-dependent platelet activation pathways whereas other functions were not affected. In vitro, saturating concentrations of JAQ1 (20  $\mu$ g/ml) only displayed a limited inhibitory effect on collagen-induced platelet aggregation which could be overcome when collagen concentrations higher than  $\sim$ 7  $\mu$ g/ml were used (Fig. 2 c), confirming earlier results (29).



**Figure 3.** GPVI is not detectable in platelets from JAQ1-treated mice for at least 2 wk. (a) Whole platelet proteins were separated by SDS-PAGE under nonreducing conditions and immunoblotted with FITC-labeled JAQ1 (anti-GPVI) or EDL1 (anti-GPIIIa). Bound mAb was detected by HRP-labeled rabbit anti-FITC and ECL. (b) Washed platelets from control, FcR  $\gamma$ -chain-deficient (FcR $\gamma$ <sup>-/-</sup>) and JAQ1-treated (day 7) mice were stimulated with 10  $\mu$ g/ml convulxin (Cvx). Control platelets were preincubated with irrelevant rat IgG2a or JAQ1 (20  $\mu$ g/ml) for 5 min before the addition of Cvx. (c) Washed platelets from the indicated mice were incubated with FITC-labeled convulxin (5  $\mu$ g/ml) for 15 min at RT and then analyzed on a FACScan<sup>TM</sup> (Becton Dickinson). The data shown are representative of six mice per group.

*JAQ1 Induces the Loss of GPVI on Circulating Platelets In Vivo.* The discrepancy between the inhibitory effect of JAQ1 on collagen-induced aggregation in vitro and ex vivo was surprising and suggested that mechanisms other

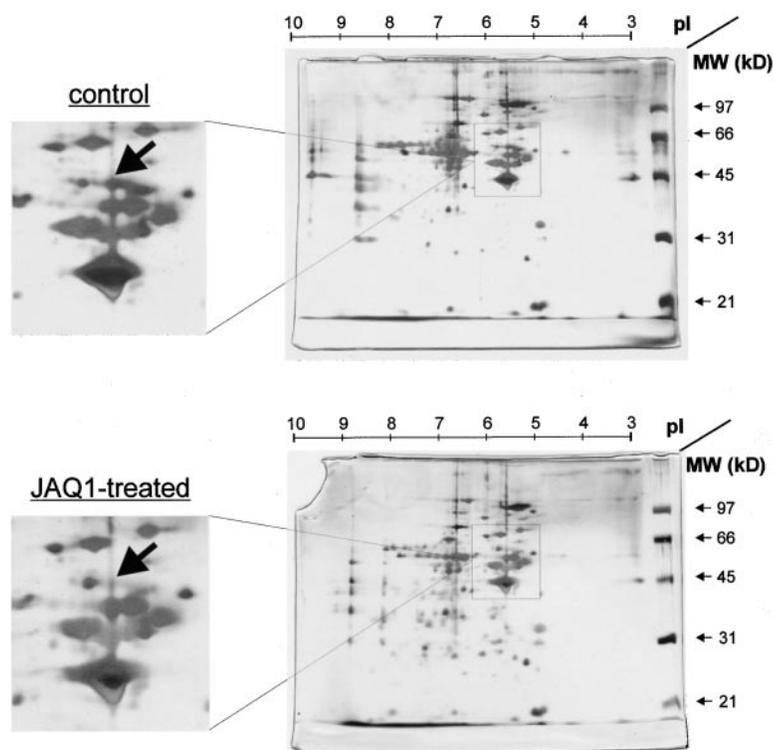
than pure blockage of an epitope on GPVI must be involved. Therefore, the next step was to test platelets from JAQ1-treated mice for the presence of GPVI in a Western blot analysis of whole cell lysates. As shown in Fig. 3 a, GPVI was not detectable in platelets from JAQ1-treated mice for at least 14 d upon a single injection of JAQ1, whereas GPIIIa was present in normal amounts at any time point. In contrast, in all mice tested, new platelets expressing functional GPVI were detectable after 28 d. To further assess the absence of GPVI on platelets from JAQ1-treated mice, we used the GPVI-specific snake venom toxin convulxin (32). As shown in Fig. 3 b, convulxin did not induce aggregation of platelets from JAQ1-treated mice on day 3, 7, and 14, whereas it induced aggregation of control platelets in the presence or absence of saturating amounts of JAQ1. Furthermore, flow cytometric analysis demonstrated that FITC-labeled convulxin did not bind to platelets from JAQ1-treated mice (Fig. 3 c). Finally, the absence of an ~60 kD protein with an isoelectric point of 5.6 in the platelets from JAQ1-treated mice was confirmed by two-dimensional gel electrophoresis (Fig. 4). Together, these results strongly suggested that GPVI had been irreversibly inactivated and removed from these platelets in vivo.

**JAQ1-induced GPVI Loss Occurs Rapidly In Vivo and Is Fc Independent.** To examine the mechanisms underlying the loss of GPVI, mice were injected with biotinylated JAQ1 and the amount of surface-bound mAb was determined flow cytometrically ex vivo at early time points after injection. Interestingly, as soon as 6 h after injection only very low levels of surface-bound JAQ1 were detectable and the signals further decreased to control after 24 and 48 h (Fig. 5 a), whereas JAQ1<sup>FITC</sup> and Cvx<sup>FITC</sup> bound to the platelets at

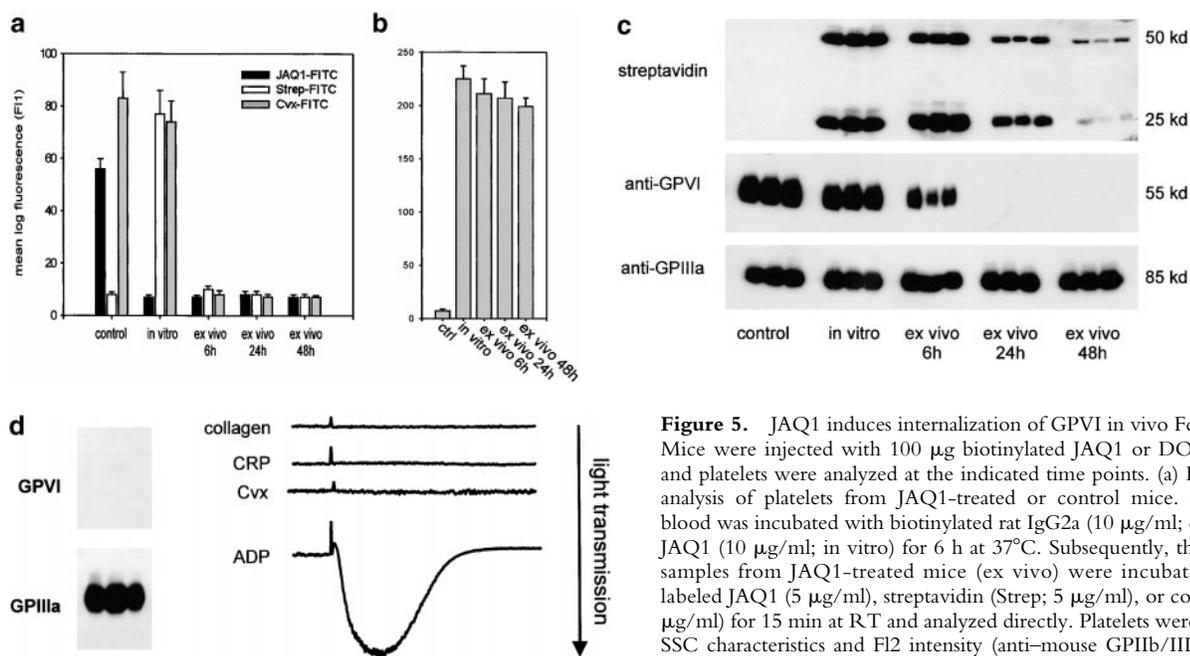
no time point. These data suggested that the JAQ1–GPVI complex had been cleared from the surface of those platelets within 6 h. In contrast, platelets from mice injected with a biotinylated mAb against GPV (24) constantly yielded positive staining with FITC-labeled streptavidin (Fig. 5 b). In the next step, we tested whole cell lysates from platelets of JAQ1-treated mice for the presence of GPVI and the biotinylated mAb. As shown in Fig. 5 c, JAQ1 was strongly detectable in platelets 6 h after injection, whereas signals markedly decreased at 24 h and even more at 48 h. A similar picture was found for GPVI, strongly suggesting that the JAQ1–GPVI complex had become internalized and was degraded within 2 d. In contrast to its in vivo effects, JAQ1 did not induce any detectable downregulation of surface GPVI within 6 h incubation at 37°C on washed platelets or in whole blood (heparinized or citrated), indicating that a second signal may be required to induce this effect and that this signal is absent in vitro (Fig. 5 a).

To determine whether the Fc part of JAQ1 or its divalent form is required for internalization/degradation of GPVI, mice received 100 µg Fab fragments of the mAb and the platelets were tested for the presence of GPVI after 48 h. As shown in Fig. 5 d, the Fab fragments, like the intact IgG, induced the complete loss of GPVI from circulating platelets and the cells were completely resistant towards activation with CRP, collagen, or convulxin.

**GPVI-depleted Platelets Display Reduced Adhesion to Collagen and Abolished Collagen-dependent Procoagulant Activity.** It is currently thought that GPVI is the platelet collagen receptor for activation, whereas integrin  $\alpha_2\beta_1$  and GPIb–V–IX (via vWF) mediate adhesion. As shown before



**Figure 4.** Absence of a ~60-kD protein in platelets from JAQ1-treated mice. Whole cell lysates of platelets from control and JAQ1-treated (day 7) mice were subjected to two-dimensional gel electrophoresis and the gels were silver stained. First dimension: IEF (linear pH gradient 3–10). Second dimension: SDS-PAGE under reducing conditions. The molecular weight marker is shown on the right. The position of the ~60-kD protein (pI: ~5.6) that is absent in platelets from JAQ1-treated mice is indicated by arrows.

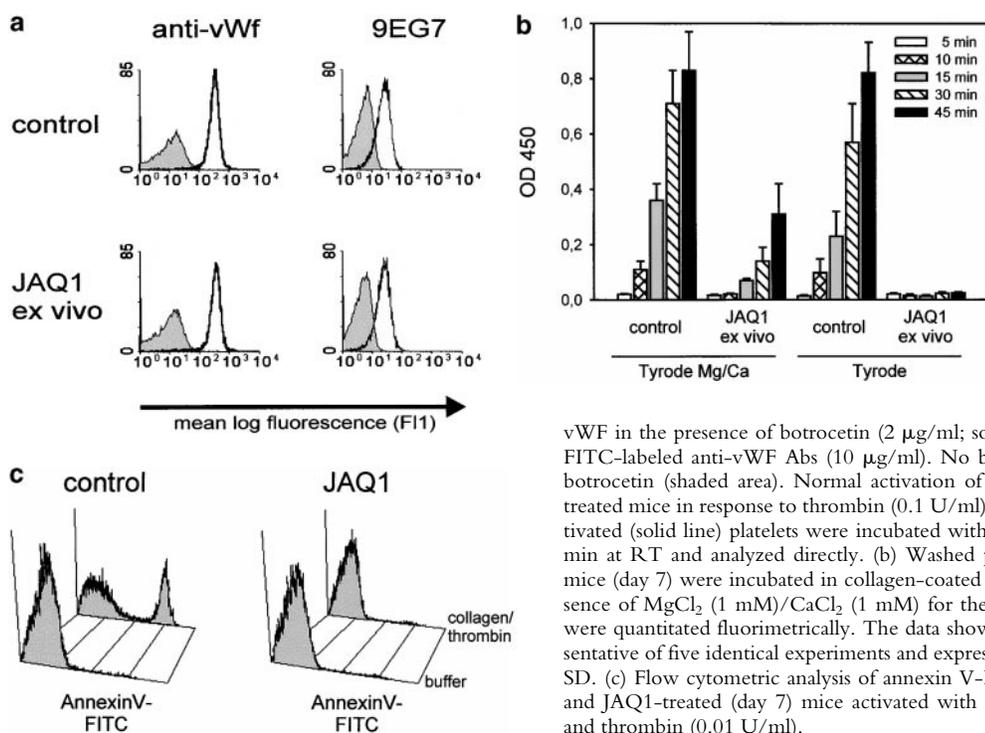


**Figure 5.** JAQ1 induces internalization of GPVI in vivo Fc independently. Mice were injected with 100  $\mu\text{g}$  biotinylated JAQ1 or DOM1 (anti-GPV) and platelets were analyzed at the indicated time points. (a) Flow cytometric analysis of platelets from JAQ1-treated or control mice. Diluted control blood was incubated with biotinylated rat IgG2a (10  $\mu\text{g}/\text{ml}$ ; control [ctrl]) or JAQ1 (10  $\mu\text{g}/\text{ml}$ ; in vitro) for 6 h at 37°C. Subsequently, these samples and samples from JAQ1-treated mice (ex vivo) were incubated with FITC-labeled JAQ1 (5  $\mu\text{g}/\text{ml}$ ), streptavidin (Strep; 5  $\mu\text{g}/\text{ml}$ ), or convulxin (Cvx; 5  $\mu\text{g}/\text{ml}$ ) for 15 min at RT and analyzed directly. Platelets were gated by FSC/SSC characteristics and F12 intensity (anti-mouse GPIIb/IIIa<sup>PE</sup>). (b) Detection of surface-bound biotinylated DOM1 (anti-GPV) ex vivo. The staining was performed as described for biotinylated JAQ1. Results in panels a and b are expressed as mean log F11  $\pm$  SD ( $n = 6$ ). (c) Top: whole platelet proteins were separated by SDS-PAGE under reducing conditions and biotinylated JAQ1 was detected with HRP-labeled streptavidin/ECL. For detection of GPVI and GPIIIa, the proteins were separated under nonreducing conditions and immunoblotted with FITC-labeled JAQ1 or EDL1 followed by HRP-labeled rabbit anti-FITC/ECL. (d) Mice were injected with 100  $\mu\text{g}$  Fab fragments of JAQ1 and platelets were analyzed in a Western blot for the presence of GPVI and GPIIIa after 48 h. These platelets did not aggregate in response to collagen (50  $\mu\text{g}/\text{ml}$ ), CRP (30  $\mu\text{g}/\text{ml}$ ), or Cvx (10  $\mu\text{g}/\text{ml}$ ), whereas ADP (10  $\mu\text{M}$ ) induced normal aggregation.

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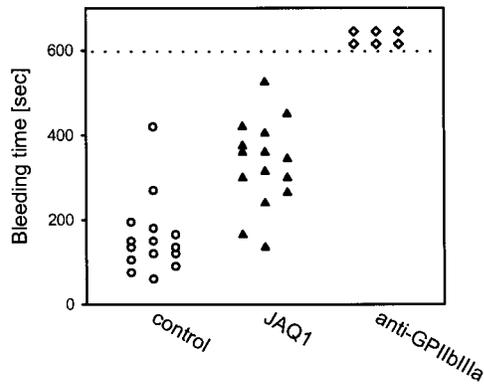
(Table I), the basal surface expression of both receptors was not influenced by the JAQ1 treatment. Further experiments demonstrated that platelets from JAQ1-treated mice bound normal levels of vWF in the presence of botrocetin,

and thrombin induced normal activation of  $\beta_1$ -integrins, as assessed with the mAb 9EG7, which specifically recognizes the activated form of the  $\beta_1$  subunit (33; Fig. 6 a). In the next step, the adhesion of platelets from JAQ1-treated mice



**Figure 6.** Reduced adhesion to collagen and abolished procoagulant response of GPVI-depleted platelets. (a) Platelets from JAQ1-treated mice (day 7) bind normal amounts of plasma

vWF in the presence of botrocetin (2  $\mu\text{g}/\text{ml}$ ; solid line). Bound vWF was detected by FITC-labeled anti-vWF Abs (10  $\mu\text{g}/\text{ml}$ ). No binding was detected in the absence of botrocetin (shaded area). Normal activation of  $\beta_1$ -integrins on platelets from JAQ1-treated mice in response to thrombin (0.1 U/ml). Resting (shaded area) or thrombin activated (solid line) platelets were incubated with FITC-labeled 9EG7 (5  $\mu\text{g}/\text{ml}$ ) for 15 min at RT and analyzed directly. (b) Washed platelets from control or JAQ1-treated mice (day 7) were incubated in collagen-coated microtiter plates in the presence or absence of  $\text{MgCl}_2$  (1 mM)/ $\text{CaCl}_2$  (1 mM) for the indicated times and adherent platelets were quantitated fluorimetrically. The data shown are from a single experiment, representative of five identical experiments and expressed as the mean of triplicate readings  $\pm$  SD. (c) Flow cytometric analysis of annexin V-FITC binding to platelets from control and JAQ1-treated (day 7) mice activated with a combination of collagen (50  $\mu\text{g}/\text{ml}$ ) and thrombin (0.01 U/ml).

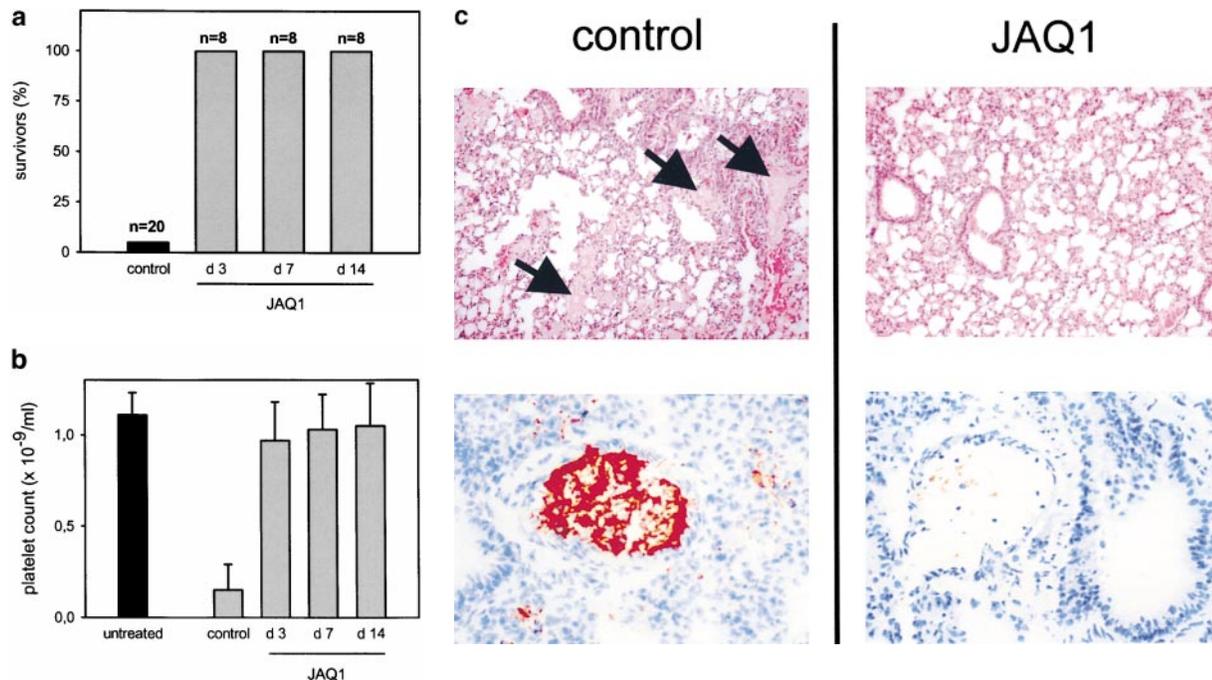


**Figure 7.** Bleeding time of JAQ1-treated mice. Bleeding times were determined in mice 7 d after injection of 100  $\mu\text{g}$  nonimmune IgG2a or JAQ1 ( $n = 15$  per group). As a control, mice received 100  $\mu\text{g}$   $\text{F}(\text{ab}')_2$  fragments of JON/A (anti-GPIIb/IIIa) 24 h before the experiment ( $n = 6$ ). Where necessary, bleeding was manually stopped at the 10 min time point to prevent death. Each point represents one individual.

to collagen was tested in a static assay. As shown in Fig. 6 b, the adhesion of platelets from JAQ1-treated mice was strongly reduced as compared with control platelets and was abolished in the absence of extracellular free magnesium/calcium, strongly suggesting it to be mediated predominantly by integrin  $\alpha_2\beta_1$  (34). It is well known that GPVI is critically involved in the procoagulant response of platelets where stimulated platelets expose negatively

charged phosphatidylserine (PS) at the plasma membrane which facilitates thrombin generation (35). Indeed, platelets from JAQ1-treated mice did not expose PS in response to a combination of collagen and thrombin on day 3, 7, and 14 after Ab injection, as demonstrated by the lack of annexin V binding (Fig. 6 c).

*Anti-GPVI Treatment Induces Long-term Antithrombotic Protection but Only Moderately Increased Bleeding Times.* The results of the previous experiments suggested that JAQ1 specifically induced complete depletion of GPVI in platelets in vivo. To examine to which extent this specific defect influenced normal hemostasis, we determined the tail bleeding times on day 7 after a bolus injection of JAQ1 (100  $\mu\text{g}$ ). As shown in Fig. 7, the bleeding times were significantly increased in GPVI-depleted mice compared with control mice ( $330 \pm 103$  vs.  $158 \pm 89$  s, respectively), but consistently lower than in mice pretreated with 100  $\mu\text{g}$  blocking  $\text{F}(\text{ab}')_2$  fragments against GPIIb/IIIa (reference 24;  $>600$  s). In the next step, we examined the protective effect of JAQ1 in a model of lethal pulmonary thromboembolism induced by infusion of a mixture of collagen (0.8 mg/kg body weight) and epinephrine (60  $\mu\text{g}/\text{kg}$  body weight; reference 27). Among control mice pretreated with irrelevant rat IgG2a, 95% (19 of 20) died within 5 min from widespread pulmonary thrombosis and cardiac arrest. In contrast, all mice pretreated with JAQ1 (100  $\mu\text{g}$ ) survived, irrespective of whether they had received the mAb 3, 7, or 14 d before challenge ( $n = 8$  per group; Fig. 8 a).



**Figure 8.** JAQ1 induces long-term protection from intravascular thrombosis. Thromboembolism in response to a bolus injection of a mixture of collagen (0.8 mg/kg body weight) and epinephrine (60  $\mu\text{g}/\text{kg}$  body weight). (a) Mortality in control mice and mice treated with 100  $\mu\text{g}$  JAQ1 at the indicated times before challenge. (b) Platelet counts in control and JAQ1-treated mice 3 min after infusion of collagen/epinephrine ( $n = 8$  per group). (c) Top: representative histology of the lungs (original magnification:  $\times 100$ ); obstructed vessels are indicated by arrowheads. Bottom: immunohistochemical detection of platelets in the thrombi (original magnification:  $\times 400$ ). Acetone-fixed frozen sections were reacted with a platelet-specific Ab (anti-GPIb-IX) and counterstained with hematoxylin. The red HRP reaction product shows high density of platelets in the thrombus.

Although the platelet counts in JAQ1 pretreated mice had not been influenced significantly by the infusion of collagen/epinephrine, there was a sharp decrease detectable in control mice ( $n = 8$ ) which was determined 3 min after induction of thromboembolism in a separate group (Fig. 8 b). For histological examination, control and JAQ1 pretreated (3, 7, and 14 d) mice received the same treatment in parallel experiments but the lungs were removed after 3 min. Although the vast majority of large and small vessels were obstructed by platelet rich thrombi in the lungs of control mice, there were only very few thrombi detectable in the lungs of JAQ1 pretreated mice (Fig. 8 c).

## Discussion

In this study we demonstrate that treatment of mice with mAb against GPVI results in profound long-term anti-thrombotic protection against collagen-dependent thromboembolism. These results confirm the proposed critical role of GPVI in collagen-induced activation of platelets in vivo and indicate that anti-GPVI agents might be effective in preventing arterial thrombosis induced by atherosclerotic plaque rupture, where platelets are thought to become activated mainly by the subendothelium under conditions of high shear stress (4, 5, 36). Among the matrix proteins which support platelet adhesion and subsequent activation, collagen has a critical role, at least in normal hemostasis, as patients with defects in collagen receptors display mild bleeding disorders (12, 37, 38). Although the role of GPIb, GPIIb/IIIa, and their respective ligands vWF and fibrinogen in thrombosis are well documented (for a review by Z.M. Ruggeri, see reference 39), the finding that vWF and fibrinogen double-knockout mice are still able to form occlusive thrombi suggests that collagen and its platelet receptors might also have a critical role in thrombosis (40).

The profound inhibitory effect of JAQ1 in vivo was unexpected, as it was based on clearing of GPVI from circulating platelets and no such specific depletion of a platelet receptor has been described to date. The complete loss of functional GPVI on circulating platelets in JAQ1-treated mice was confirmed by different approaches. First, the protein was not detectable in a Western blot analysis of platelet lysates for at least 2 wk (which exceeds the normal life span of platelets [41]). Second, the GPVI-specific snake venom toxin convulxin, which binds to a different epitope than JAQ1 (Fig. 3, b and c), did not bind to platelets from JAQ1-treated mice, strongly suggesting the absence of GPVI from the platelet membrane. Third, an  $\sim 60$ -kD protein with an isoelectric point of  $\sim 5.6$  (which is similar to that described for human GPVI [42]) is absent in the lysate of platelets from JAQ1-treated mice (Fig. 4), and the same protein is absent in platelets from FcR  $\gamma$ -chain-deficient mice (not shown) which are known to lack GPVI (15). Most importantly, the functional platelet responses to collagen were completely abolished by JAQ1 in vivo, whereas the mAb only has limited inhibitory effects in vitro (29; Fig. 2 c). These results demonstrate that JAQ1 induced the clearing of GPVI from the surface of circulating plate-

lets in vivo. This finding is also supported by the observation that biotinylated JAQ1 was detectable in the lysates, but not on the surface, of platelets 6 h after injection and the same was found for GPVI. Furthermore, the decreasing signals for both GPVI and JAQ1 after 24 and 48 h strongly suggest that the internalized complex was degraded in the intracellular compartments. GPVI belongs to the immunoglobulin superfamily and is closely related to immunoreceptors, some of which may become internalized when stimulated appropriately (43, 44). In the case of JAQ1-GPVI, it was difficult to define what the appropriate stimulus is, but it seems clear that the Fc part of the mAb is not required to induce internalization, as Fab fragments produced the same effect, thereby also excluding the requirement for GPVI clustering. In vitro, JAQ1 did not induce the downregulation of GPVI from the platelet membrane (Fig. 5 a), suggesting that a second signal may be required for the induction of this process that is provided by other cells in vivo. This assumption may be supported by the observation that JAQ1 and Fab fragments of the mAb induced transient thrombocytopenia. The reason for this is not clear, but it might be due to weak activation of GPIIb/IIIa leading to formation of loose aggregates and their temporary sequestration to the spleen where the actual loss of GPVI may occur. Recent evidence indicates that JAQ1 recognizes an epitope identical with or in close vicinity to the CRP binding site on GPVI (29), which is regarded as the major binding site for collagen on the receptor. So far, very little is known about the cellular regulation of GPVI, but in the light of the current data it seems possible that occupancy of this epitope provides a signal that finally results in downregulation of the receptor.

Irrespective of the underlying mechanism, platelets from JAQ1-treated mice were completely unresponsive towards activation with high concentrations of CRP or collagen, whereas they were normally activatable with ADP or PMA. This strongly suggests that JAQ1 selectively induced a transient GPVI deficiency in mice, whereas other membrane glycoproteins, including GPIIb/IIIa, GPIb-IX-V, CD9, and integrin  $\alpha_2\beta_1$  were not affected in expression and/or function. JAQ1-treated mice had prolonged bleeding times, which confirms the important role of GPVI in normal hemostasis and correlates well with the bleeding diathesis in GPVI-deficient patients (12, 22). Very interestingly, one GPVI-deficient patient developed highly specific Abs against the absent receptor (45) which may be difficult to explain. However, based on the results presented here it is feasible to speculate that this patient may suffer from an acquired GPVI deficiency, based on autoantibody-induced clearing of GPVI from her circulating platelets.

Besides its pivotal role in collagen-induced platelet activation, GPVI is also critically involved in the procoagulant reaction of platelets (46), which was confirmed by the abolished collagen-dependent procoagulant activity of platelets from JAQ1-treated mice. This result strongly suggests that anti-GPVI treatment also modulates coagulation at sites of vascular injury. Such an anticoagulant activity has been demonstrated for GPIIb/IIIa antagonists (47), which are

currently considered the most powerful inhibitors of platelet participation in thrombosis (48), as they inhibit the final common pathway of platelet aggregation irrespective of the agonist that stimulates the cells. It has been suggested that this more or less complete inhibition of platelet function may come with a potential safety risk, as platelet aggregation is also required for normal hemostasis (49). We found that JAQ1 induced significantly shorter bleeding times than blocking Abs against GPIIb/IIIa in mice, indicating that GPVI-depleted platelets still contributed significantly to normal hemostasis *in vivo*. Although there is no clear correlation between the bleeding time and bleeding risk (50), it is tempting to speculate on the grounds of these results that anti-GPVI therapy might be associated with a relatively low risk of clinical hemorrhage.

The mechanisms of collagen-platelet interactions are complex and involve direct or indirect binding of collagen to several platelet receptors, including the GPIb-IX-V complex, integrin  $\alpha_2\beta_1$ , GPIV, GPVI, and 65- and 85-kD proteins (51). Despite its essential role in collagen-induced activation of platelets, there has been only very limited evidence for a role of GPVI in adhesion to collagen (17), which is mainly thought to be mediated by GPIb-IX-V (via vWf) and integrin  $\alpha_2\beta_1$ . In mice, GPVI-depleted platelets expressed normal amounts of integrin  $\alpha_2\beta_1$ , and  $\beta_1$ -integrins were normally activatable, which has been reported to be a prerequisite for effective binding of collagen (52; Fig. 6 b). Indeed, GPVI-depleted platelets adhered to collagen through  $\alpha_2\beta_1$ , but the extent of adhesion was strongly reduced compared with control platelets. A similar observation has been reported with platelets from GPVI-deficient patients (12, 45), indicating that GPVI may be required for normal adhesion to collagen probably by supporting the activation of  $\alpha_2\beta_1$  (53). The expression of GPIb-IX-V was not affected by the JAQ1 treatment and the receptor bound normal levels of vWF in the presence of botrocetin (Fig. 6 a). Together, these results suggest that platelet adhesion to collagen at sites of vascular injury may be reduced, but not blocked, by anti-GPVI treatment.

Very recent evidence suggests that GPVI is exclusively expressed in platelets and mature megakaryocytes (17, 54), and this is confirmed by immunohistochemical studies with JAQ1 (not shown). Therefore, the effects of anti-GPVI agents (like JAQ1) should be restricted to platelets and, very importantly, megakaryocytes. JAQ1 was detectable on megakaryocytes in spleen and bone marrow 3 h after Ab injection (not shown), suggesting that the next generation of platelets was also affected by the mAb. This assumption may be confirmed by the fact that GPVI was not detectable in platelets for at least 2 wk, although the normal life span of mouse platelets is only  $\sim 4$ –5 d (41). Based on the estimated number of  $\sim 2 \times 10^9$  platelets/mouse ( $10^9$ /ml blood) and a life span of the cells of 5 d, the GPVI molecules of  $6 \times 10^9$  platelets must be depleted to result in the absence of the receptor for 15 d. The amount of 100  $\mu\text{g}$  JAQ1 (mol wt: 150 kD) represents  $\sim 6.7 \times 10^{13}$  Ab molecules. Therefore,  $\sim 1.1 \times 10^4$  Ab molecules per platelet are available to bind and deplete GPVI. As the estimated ex-

pression rate of GPVI is only  $1$ – $2 \times 10^3$  copies/platelet (55), 100  $\mu\text{g}$  JAQ1 is sufficient to induce the observed effect.

Preliminary results show that a second injection of JAQ1 2 wk after the first injection has no influence on platelet counts, but prolongs the absence of GPVI on circulating platelets (not shown). This indicates that the second dose of the mAb affects newly differentiated megakaryocytes, but has no effect on circulating (GPVI-depleted) platelets. Thus, JAQ1 can be used to induce a GPVI knock out-like phenotype in mice for several weeks, allowing studies on platelet function in the absence of this critical activating receptor *in vitro* and *in vivo*.

Taken together, the results presented here indicate that GPVI might become an interesting target for long-term prophylaxis of ischemic cardiovascular diseases and provide the first evidence that it is possible to specifically deplete an activating glycoprotein receptor from circulating platelets *in vivo*. These findings may open the way for the development of a new generation of powerful, yet safe, antithrombotics.

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