A Crucial Role of Glycoprotein VI for Platelet Recruitment to the Injured Arterial Wall In Vivo

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Abstract

Platelet adhesion and aggregation at sites of vascular injury is crucial for hemostasis but may lead to arterial occlusion in the setting of atherosclerosis and precipitate diseases such as myocardial infarction. A current hypothesis suggests that platelet glycoprotein (GP) Ib interaction with von Willebrand factor recruits flowing platelets to the injured vessel wall, where subendothelial fibrillar collagens support their firm adhesion and activation. However, so far this hypothesis has not been tested in vivo. Here, we demonstrate by intravital fluorescence microscopy of the mouse carotid artery that inhibition or absence of the major platelet collagen receptor, GPVI, abolishes platelet–vessel wall interactions after endothelial denudation. Unexpectedly, inhibition of GPVI by the monoclonal antibody JAQ1 reduced platelet tethering to the subendothelium by ~89%. In addition, stable arrest and aggregation of platelets was virtually abolished under these conditions. Using different models of arterial injury, the strict requirement for GPVI in these processes was confirmed in GPVI-deficient mice, where platelets also failed to adhere and aggregate on the damaged vessel wall. These findings reveal an unexpected role of GPVI in the initiation of platelet attachment at sites of vascular injury and unequivocally identify platelet–collagen interactions (via GPVI) as the major determinant of arterial thrombus formation.

Key words: arterial thrombosis • collagen • receptor • GPVI • mouse

Introduction

Platelet adhesion and aggregation is essential to limit blood loss at sites of vascular injury but may also lead to arterial occlusion and irreversible tissue damage after disruption of the atherosclerotic plaque. The first platelet response to vascular injury is adhesion to the exposed subendothelial matrix, which triggers subsequent platelet aggregation. A current hypothesis supported by numerous in vitro studies suggests that the interaction of glycoprotein (GP)*Ib-V-IX with von Willebrand factor (vWf) recruits flowing platelets to the injured vessel wall (1), where they interact with exposed extracellular matrix proteins resulting in firm adhesion and thrombus growth (2, 3). Although several of the macromolecular components of the subendothelial layer such as laminin, fibronectin, and vWf all provide a suitable substrate for platelet adhesion, fibrillar collagen is considered the most thrombogenic constituent of the vascular subendothelium as it not only supports platelet adhesion but also acts as a strong activator of platelets in vitro (3, 4). However, the in vivo relevance of platelet–collagen interactions in the setting of arterial thrombosis has not been established. This might be explained by the complexity of the platelet–collagen interaction, which involves a variety of different receptors and signaling pathways making the in vivo inhibition of this process very difficult. Besides GPIb-V-IX and integrin $\alpha_{IIb}\beta_3$, which interact indirectly with collagen via vWf (5), a large number of collagen re-

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**Abbreviations used in this paper:* DCF, 5-carboxyfluorescein diacetate succinimidyl ester; GP, glycoprotein; vWf, von Willebrand factor.

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ceptors have been identified on platelets, including most importantly integrin $\alpha_2\beta_1$ (6), GPV (7), and GPVI (8).

Only recently, GPVI has been established as the central platelet collagen receptor that is essential for platelet adhesion and aggregation on immobilized collagen in vitro, as it mediates the activation of different adhesive receptors, including integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ (9–12). GPVI is a 60–65-kD type I transmembrane GP belonging to the immunoglobulin superfamily (13, 14) that forms a complex with the FcR γ chain at the cell surface in human and mouse platelets (9, 10). Signaling through GPVI occurs via a pathway similar to that used by immunoreceptors as revealed by the tyrosine phosphorylation of the FcR γ chain immunoreceptor tyrosine-based activation motif by a src-like kinase (15). The mAb JAQ1 (10) blocks the major collagen binding site on mouse GPVI and inhibits firm platelet adhesion to collagen under low and high shear flow conditions (12). In vivo application of JAQ1 induces virtually complete internalization and degradation of GPVI on mouse platelets resulting in a "GPVI knockout"–like phenotype for at least 2 wk. Such GPVI-depleted mice have significantly prolonged bleeding times and their platelets fail to respond to collagen but not to other agonists (11). Despite its essential role in collagen-induced activation of platelets, there has been only very limited evidence for a role of GPVI as a direct adhesion receptor (14, 16).

In this study we investigated the in vivo significance of platelet–collagen interactions in the dynamic process of platelet adhesion and aggregation at sites of arterial injury. We show that inhibition or deletion of GPVI virtually abrogates stable platelet adhesion and aggregation after endothelial denudation of the carotid artery in mice. Very unexpectedly, we found that tethering/slow surface translocation of platelets was also strongly inhibited in the absence of functional GPVI. These findings reveal a crucial role of GPVI in the initiation of platelet recruitment at sites of vascular injury and provide the first in vivo evidence that platelet–collagen interactions are of paramount importance during arterial thrombus formation.

Materials and Methods

Animals. Specific pathogen-free C57BL/6J mice were obtained from Charles River Laboratories. For experiments, 12-wk-old male mice were used. All experimental procedures performed on animals were approved by the German legislation on protection of animals.

mAbs. mAbs against GPVI (JAQ1) and GPIbα (p0p/B) were generated as previously described (10). Fab fragments from JAQ1 and p0p/B were also generated as previously described (11). Irrelevant control rat IgG and FITC-conjugated hamster anti–β1 integrin (Ha31/8) was obtained from BD Biosciences. The following antibodies were produced and modified in our laboratory (17) and used for flow cytometry: JON1-PE (anti-GPIIb/IIIa), p0p4-PE (anti-GPIbα), p0p6-FITC (anti-GPIX), DOM1-FITC (anti-GPV), and LEN1-FITC (anti-GPIa).

Generation of GPVI-deficient Mice. To generate mice lacking GPVI, C57BL6/J wild-type mice were injected with 100 μg JAQ1 intracardially (11). Animals were used for in vivo assessment of platelet adhesion on day 5 after mAb injection. Absence of GPVI expression on platelets was verified by Western blot analysis and flow cytometry.

Flow Cytometry. Heparinized whole blood, obtained from wild-type C57BL6/J or GPVI-depleted mice was diluted 1:30 with modified Tyrodes-Hepes buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM Hepes, 5 mM glucose, and 1 mM $CaCl_2$, pH 6.6). The samples were incubated with fluorophore-labeled antibodies for 10 min at room temperature and directly analyzed on a FACScalibur™ (Becton Dickinson).

Preparation of Platelets for Intravital Microscopy. Wild-type or GPVI-deficient platelets were isolated from whole blood as previously described (18) and labeled with 5-carboxyfluorescein diacetate succinimidyl ester (DCF). The DCF-labeled platelet suspension was adjusted to a final concentration of 200×10^6 platelets/250 μl. Where indicated, fluorescent wild-type platelets were preincubated with 50 μg/ml anti-GPVI (JAQ1) Fab fragments, which do not induce any detectable platelet signaling (19). In a separate set of experiments, platelets were preincubated with 50 μg/ml anti-GPIbα (p0p/B) Fab fragments for 10 min to examine the role of GPIbα for platelet recruitment after endothelial denudation. The pretreated platelets together with the Fab fragments were infused into wild-type recipient mice and platelet adhesion was assessed before and after carotid injury by in vivo video microscopy, as described below.

Carotid Ligation and Assessment of Platelet Adhesion and Aggregation by Intravital Microscopy. Wild-type C57BL6/J or GPVI-deficient mice were anesthetized by intraperitoneal injection of a solution of midazolame (5 mg/kg body weight; Ratiopharm), medetomidine (0.5 mg/kg body weight; Pfizer), and fentanyl (0.05 mg/kg body weight; CuraMed Pharma GmbH). Polyethylene catheters (Portex) were implanted into the right jugular vein and 200×10^6/250 μl fluorescent platelets were infused intravenously. The right common carotid artery was dissected free and ligated vigorously near the carotid bifurcation for 5 min to induce vascular injury. Before and after vascular injury, the fluorescent platelets were visualized in situ by in vivo video microscopy of the right common carotid artery. Platelet–vessel wall interactions were monitored using a Zeiss Axiotech microscope (20× water immersion objective, W 20×/0.5; Carl Zeiss MicroImaging, Inc.) with a 100-W HBO mercury lamp for epi-illumination. All videotaped images were evaluated using a computer-assisted image analysis program (Cap Image 7.4; provided by Dr. Zeintl; references 18 and 20). Tethered platelets were defined as all cells establishing initial contact with the vessel wall, followed by slow surface translocation at a velocity significantly lower than the centerline velocity, or by firm adhesion. Their numbers are given as cells per mm^2 endothelial surface. The number of adherent platelets was assessed by counting the cells that did not move or detach from the endothelial surface within 10 s. The number of platelet aggregates at the site of vascular injury was also quantified and is presented per mm^2.

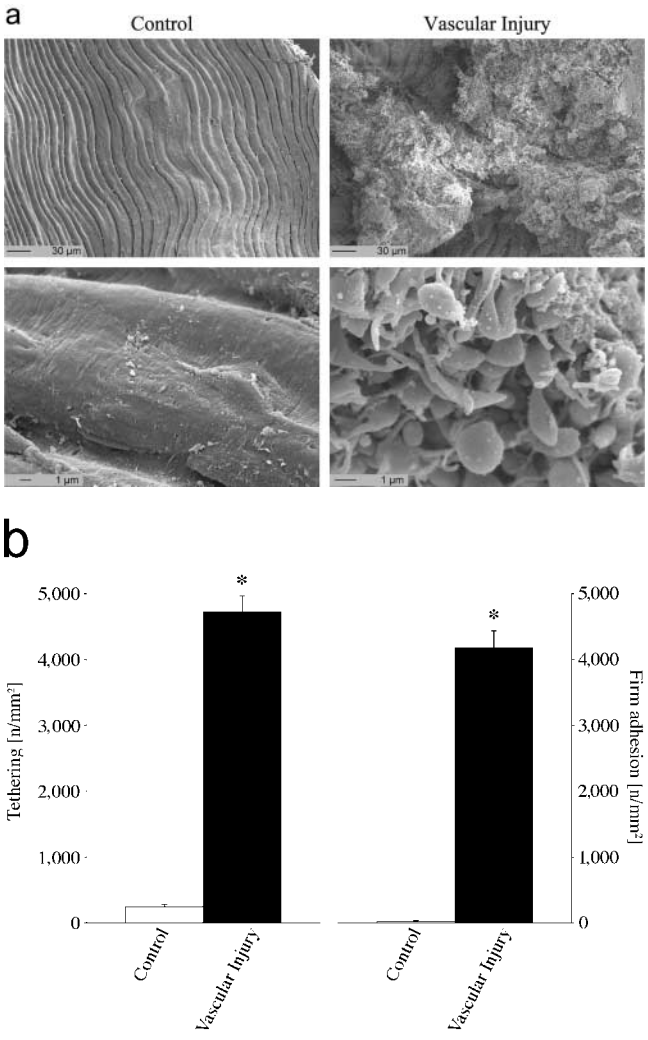
Scanning Electron Microscopy. After intravital video fluorescence microscopy, the carotid artery was perfused with PBS at 37°C for 1 min, followed by perfusion fixation with phosphate-buffered glutaraldehyde (1% vol/vol). The carotid artery was excised, opened longitudinally, additionally fixed by immersion in 1% PBS-buffered glutaraldehyde for 12 h, dehydrated in ethanol, and processed by critical point drying with CO_2. The carotid artery specimens were then oriented with the lumen exposed, mounted with carbon paint, sputter coated with platinum, and examined using a field emission scanning electron microscope (JSM-6300F; Jeol Ltd.).

Assessment of Arterial Thrombosis after Ferric Chloride Exposure. Vascular injury of the carotid artery was induced by local application of ferric chloride ($FeCl_3$) essentially as described by Fay et al. (21). In brief, control or GPVI-depleted C57BL/6J mice were anesthetized and fluorescence-tagged control or GPVI-deficient platelets were infused intravenously into the jugular vein of untreated or GPVI-depleted recipients, respectively. Thereafter, the common carotid artery was dissected free and a filter paper (0.5×1.0 mm) saturated with 10% $FeCl_3$ was applied to the adventitial surface of the vessel, as previously described (21). The time to thrombotic occlusion of the carotid artery downstream of the site of injury ($n = 10$ per group) was defined as the time required for complete arrest of blood flow in the center of the vessel (platelet flow velocity 0 m/s) after removal of the filter paper.

Determination of Platelet Recruitment after Wire-induced Arterial Denudation. Wire-induced endothelial disruption was performed according to a method described by Lindner et al. (22). In brief, GPVI-depleted C57BL/6J mice were generated as described above ($n = 12$). Mice pretreated with 100 μg irrelevant control IgG ($n = 6$) or PBS ($n = 12$) served as controls. 5 d after mAb injection, the animals were anesthetized and platelets were isolated from control or GPVI-depleted mice and labeled with DCF (see above). In the recipient mice, the right carotid artery was exposed via a midline neck incision. The common, external, and internal carotid arteries were identified, the right internal carotid artery was looped proximally and tied off distally with 8–0 silk suture (Ethicon). Additional 8–0 silk ties were looped round the common and external carotid arteries for temporary vascular control during the procedure. A transverse arteriotomy was made in the right internal carotid artery and a 0.014-in flexible angioplasty guidewire was introduced and advanced 1 cm to the aortic arch. Endothelial denudation injury of the right common carotid artery was performed by wire withdrawal with rotating motion to ensure uniform and complete endothelial denudation. After removal of the wire, the right internal carotid artery was tied off and platelet–vessel wall interactions were visualized at the site of injury by in vivo video fluorescence microscopy as described above.

Results and Discussion

To test the biological significance of platelet–collagen interactions in the processes of adhesion and aggregation in vivo, we assessed platelet–vessel wall interactions after vas-



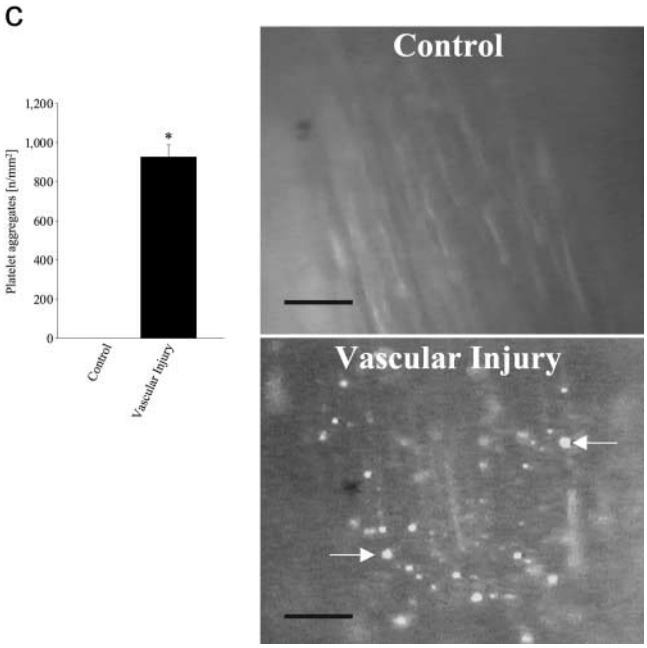
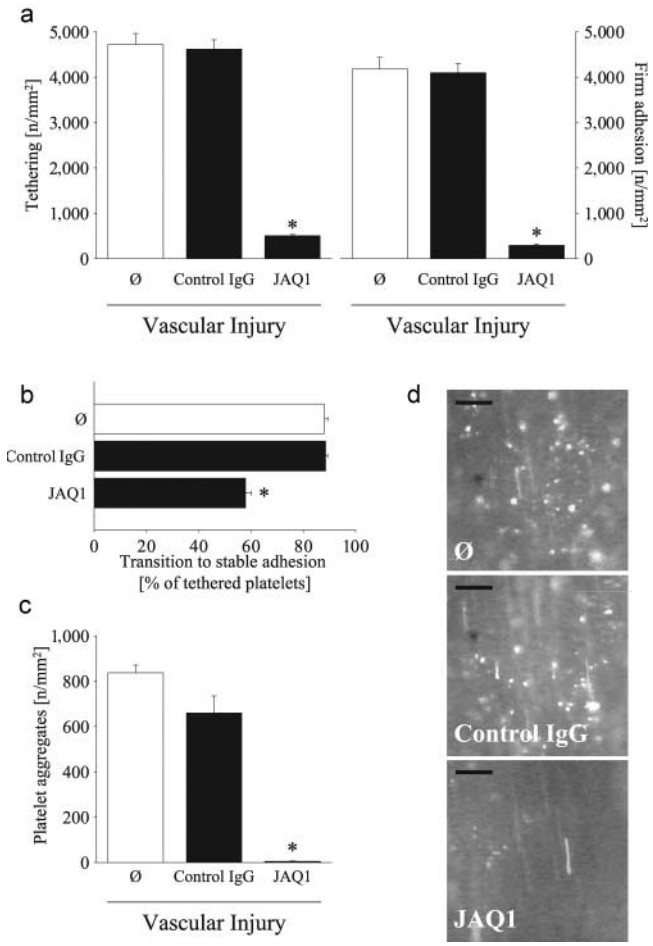


Figure 1. Platelet adhesion and aggregation after vascular injury of the common carotid artery in C57BL/6J mice in vivo. (a) Scanning electron micrographs of carotid arteries before (left) and 2 h after vascular injury (right). Endothelial denudation induces platelet adhesion and aggregation, resulting in the formation of a platelet-rich (lower right) thrombus. (b) Platelet–endothelial cell interactions 5 min after vascular injury were investigated by in vivo fluorescence microscopy of the common carotid artery in situ (solid columns). Animals without vascular injury served as controls (open columns). The left and right panels summarize platelet tethering and firm platelet adhesion, respectively, of eight experiments per group. Platelets were classified according to their interaction with the endothelial cell lining as previously described (refer to Materials and Methods) and are given per mm^2 of vessel surface. Mean ± SEM. *, significant difference compared with control, $P < 0.05$. (c) Platelet aggregation after vascular injury was determined by fluorescence microscopy in vivo (solid columns). Animals without vascular injury served as controls (open columns). Mean ± SEM and $n = 8$ each group. *, significant difference compared with wild-type mice, $P < 0.05$. The microphotographs (right) show representative in vivo fluorescence microscopy images in control animals (top) or after vascular injury (bottom). White arrows indicate adherent platelets. Bars, 50 μm.

cular injury of the mouse carotid artery. Vigorous ligation of the carotid artery consistently caused complete loss of the endothelial cell layer and initiated platelet adhesion at the site of injury, as assessed by scanning electron microscopy (Fig. 1 a). Next, we used in vivo fluorescence microscopy (18, 20) to directly visualize and quantify the dynamic process of platelet accumulation after vascular injury. Numerous platelets were tethered to the vascular wall within the first minutes after endothelial denudation (4725 ± 239 platelets/mm^2). Virtually all platelets establishing contact with the subendothelium initially exhibited a slow surface translocation of the "stop-start" type (23). As we observed transition from initial slow surface translocation to irreversible platelet adhesion in 88% of all platelets (4.182 ± 253 platelets/mm^2; Fig. 1 b), platelet arrest remained transient in only 12% (543 ± 32 platelets/mm^2). Once firm arrest was established, adherent platelets recruited additional platelets from the circulation, resulting in aggregate formation (Fig. 1 c). Similar characteristics of platelet recruitment have been obtained earlier with immobilized collagen in vitro (5). In contrast, only few platelets were tethered to the intact vascular wall under physiological conditions ($P < 0.05$ vs. vascular injury) and ~100% of these platelets were displaced from the vascular wall without firm arrest ($P < 0.05$ vs. vascular injury; Fig. 1, a–c).

Subendothelial fibrillar collagen has been proposed to be of major importance for platelet adhesion and aggregation at sites of vascular injury (2, 4, 24) as in vitro it strongly supports platelet activation and adhesion. However, this hypothesis has not been tested in vivo where various other agonists and adhesion molecules might be involved in thrombus formation. To directly test the in vivo relevance of platelet–collagen interactions in arterial thrombus formation, we inhibited or deleted GPVI in vivo. The mAb JAQ1 (10, 25) blocks the major collagen-binding site on mouse GPVI and almost completely inhibits firm platelet adhesion to immobilized fibrillar collagen under high shear flow conditions in vitro (12). To study the effect of GPVI inhibition in arterial thrombus formation, mice received syngeneic, fluorescence-tagged platelets preincubated with JAQ1 Fab fragments or isotype-matched control IgG and carotid injury was induced as described above. Very unexpectedly, platelet tethering/slow surface translocation at sites of endothelial denudation, a process thought to be mediated solely by GPIbα interaction with immobilized vWf (1, 3, 26, 27), was reduced by 89% ($P < 0.05$ vs. control IgG; Fig. 2 a) in the presence of JAQ1 Fab fragments. In addition, stable platelet arrest was reduced by 93% in the presence of JAQ1 (Fig. 2 a). We observed transition from initial tethering/slow surface translocation to irreversible



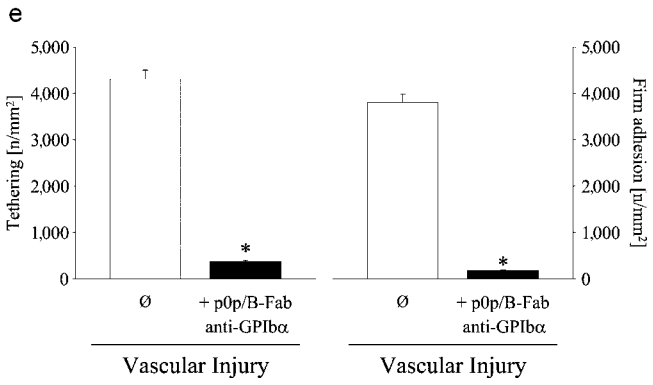


Figure 2. Inhibition of GPVI abrogates platelet adhesion and aggregation after vascular injury. (a) Platelet adhesion after vascular injury was determined by intravital video fluorescence microscopy. Fluorescent platelets were preincubated with 50 μg/ml anti-GPVI (JAQ1) Fab fragments or control rat IgG. Platelets without mAb preincubation served as control. The left and right panels summarize transient and firm platelet adhesion, respectively. Mean ± SEM and $n = 8$ each group. *, significant difference compared with control, $P < 0.05$. (b) The percentage of platelets establishing irreversible adhesion after initial tethering/slow surface translocation is illustrated. (c) Platelet aggregation after vascular injury in vivo. Aggregation of platelets preincubated with tyrode, irrelevant rat IgG, or anti-GPVI (JAQ1) was assessed by fluorescence microscopy as previously described. Mean ± SEM and $n = 8$ each group. *, significant difference compared with control, $P < 0.05$. (d) The photomicrographs show representative in vivo fluorescence microscopy images illustrating platelet adhesion in the absence or presence of anti-GPVI Fab (JAQ1) or control IgG. Bars, 30 μm. (e) Inhibition of GPIbα abrogates platelet recruitment after vascular injury. Platelets were incubated with 50 μg/ml anti-GPIbα Fab fragments (p0p/B) for 10 min. Platelets without mAb preincubation served as control. The left and right panels summarize transient and firm platelet adhesion, respectively. Mean ± SEM and $n = 6$ each group. *, significant difference compared with control, $P < 0.05$.

platelet adhesion in only 58% of those platelets establishing initial contact with the subendothelial surface compared with 89% with control IgG–pretreated platelets ($P < 0.05$; Fig. 2 b). Aggregation of adherent platelets was virtually absent after pretreatment of platelets with JAQ1 Fab fragments but not in the controls ($P < 0.05$ vs. control; Fig. 2, c and d). The unanticipated inhibitory effect of GPVI blockade on tethering/slow surface translocation prompted us to examine the role of GPIbα in this process. Mice received fluorescence-tagged platelets preincubated with Fab fragments of a function blocking antibody against GPIbα (p0p/B) and carotid injury was induced as described above. As shown in Fig. 2 e, this treatment resulted in a similarly profound reduction in platelet tethering and firm adhesion (and consequently also in aggregate formation) as anti-GPVI treatment (see above) confirming the crucial role of GPIbα for platelet attachment to the damaged vascular wall under conditions of arterial shear. This finding strongly suggested that both GPVI and GPIbα are required to recruit platelets to the injured arterial wall in vivo.

Together, the results described above demonstrated for the first time that direct platelet–collagen interactions are essential for initial platelet tethering and subsequent stable platelet adhesion and aggregation at sites of arterial injury. In addition, these data identify GPVI as a key regulator in this process whereas other surface receptors, most impor-

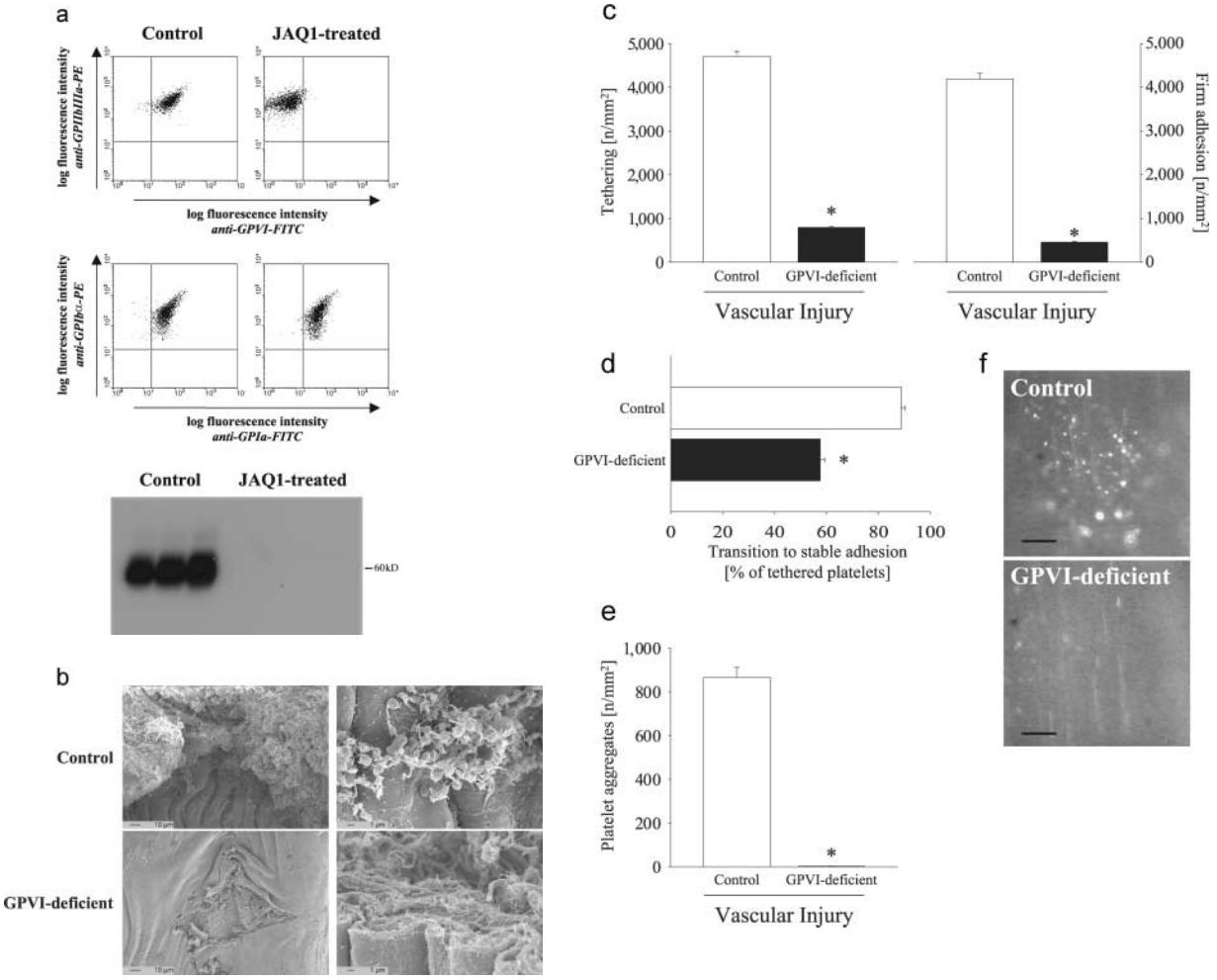


Figure 3. Platelet adhesion after endothelial denudation in GPVI-deficient mice. (a) JAQ1-treated mice lack GPVI. On the top, platelets from mice pretreated with irrelevant control IgG or anti-GPVI (JAQ1) were stained for GPVI and GPIIb/IIIa (top) or GPIa and GPIbα (bottom) and directly analyzed on a FACScalibur™ is shown. Representative dot plots of six mice per group are presented. The expression levels of GPIIb/IIIa, GPIb-V-IX, and GPIa/IIa were not significantly different between the two groups of mice (refer to Table I). On the bottom, whole platelet lysates from three control IgG or JAQ1-treated mice separated by SDS-PAGE under nonreducing conditions and immunoblotted with FITC-labeled JAQ1, followed by incubation with horseradish peroxidase–labeled rabbit anti–FITC antibody is shown. (b) Scanning electron micrographs of carotid arteries 2 h after vascular injury in control animals or GPVI depleted. Endothelial denudation induced platelet adhesion and platelet aggregation in control animals. In contrast, only very few platelets attached along the damaged vessel wall in GPVI-depleted mice. Subendothelial collagen fibers are visible along the denuded area. (c) Platelet tethering and firm platelet adhesion, (d) transition from initial tethering to stable arrest (percentage of tethered platelets), and (e) platelet aggregation after vascular injury of the carotid artery was determined in GPVI-deficient (JAQ1-pretreated mice) or control IgG–pretreated mice (for details refer to Materials and Methods). The panels summarize platelet adhesion (tethering and firm adhesion) and platelet aggregation in eight experiments per group. Mean ± SEM. *, significant difference compared with control IgG, $P < 0.05$. (f) The photomicrographs show representative in vivo fluorescence microscopy images illustrating platelet adhesion in GPVI-deficient (JAQ1) and control IgG–treated mice. Bars, 30 μm.

tantly GPIb-V-IX and $\alpha_2\beta_1$, are not sufficient to initiate platelet adhesion and aggregation on the subendothelium in vivo.

The profound inhibition of platelet tethering by GPVI blockade was surprising and suggested a previously unrecognized function of this receptor in the very initial phase of thrombus formation. To exclude the possibility that this effect was based on steric impairment of other receptors, e.g. GPIb-V-IX, by surface-bound JAQ1, we generated GPVI-deficient mice by injection of JAQ1 5 d before vascular injury. As reported previously, such treatment induces virtually complete internalization and proteolytic degradation of GPVI in circulating platelets, resulting in a GPVI knockout–like phenotype for at least 2 wk (11). As illustrated in Fig. 3 a, GPVI was undetectable in platelets from JAQ1-treated mice on day 5 after injection of 100 μg/mouse JAQ1 but not control IgG, whereas surface expression and function of all other tested receptors, including GPIb-V-IX, $\alpha_{IIb}\beta_3$, and $\alpha_2\beta_1$ was unchanged in both groups of mice, confirming earlier results (Fig. 3 a and Table I; reference 11).

As shown by scanning electron microscopy, platelet adhesion and aggregation after endothelial denudation of the common carotid artery were virtually absent in GPVI-deficient, but not in IgG-pretreated, mice (Fig. 3 b). Next, in vivo video fluorescence microscopy was used to define platelet adhesion dynamics after vascular injury in GPVI-deficient mice (Fig. 3, c–f). The loss of GPVI profoundly reduced tethering/slow surface translocation of platelets at the site of vascular injury by 83% compared with IgG-pretreated mice ($P < 0.05$). This GPVI-independent slow surface translocation required vWf-GPIbα–interaction as it was abrogated by preincubation of the platelets with Fab fragments of p0p/B (anti-GPIbα), confirming the critical role of GPIbα in this process (not depicted). In the absence of GPVI, stable platelet adhesion was reduced by ~90% compared with the (IgG-treated) control, whereas aggregation of adherent platelets was virtually absent (Fig. 3, b–f). We saw transition from platelet tethering to stable platelet adhesion in only 58% of all platelets initially tethered to the site of injury compared with 89% with control mAb–pretreated platelets ($P < 0.05$; Fig. 3 d), indicating that GPIbα-dependent surface translocation is not sufficient to promote stable platelet adhesion and subsequent aggregation.

To further substantiate the role of GPVI in the process of platelet recruitment after endothelial disruption, we next examined platelet adhesion/aggregation using two additional models of arterial thrombosis. First, arterial injury was induced in control or GPVI-depleted mice by local administration of ferric chloride to the adventitial surface of the carotid artery as previously described (21). Time to arterial occlusion was monitored by in vivo fluorescence microscopy. As shown in Fig. 4, $FeCl_3$ exposure resulted in a rapid thrombotic response in control animals. 9 out of 10 carotid arteries showed complete occlusion after 235 ± 33 s. In contrast, arterial thrombus formation was dramatically retarded in GPVI-deficient mice ($P < 0.05$ vs. control mice). In fact, 6 out of 10 GPVI-deficient mice did not show arterial occlusion until 600 s after removal of the $FeCl_3$-saturated filter paper. In the remaining vessels, occlusion was markedly delayed (356 ± 55 s). These results further support a crucial role of GPVI in the process of arterial thrombus formation.

Next, we assessed platelet recruitment in the carotid artery after wire-induced endothelial disruption (22). As reported earlier by Zhu et al. (28) and Lindner et al. (22), vascular injury with a flexible wire consistently caused complete endothelial denudation (unpublished data). In untreated control animals and mice pretreated with irrelevant control IgG, disruption of the endothelial surface initiated platelet tethering and adhesion as assessed in vivo by video fluorescence microscopy (Fig. 5). Numerous platelets were tethered to the vascular wall within the first minute after endothelial denudation (11.495 ± 1.283 tethered platelets/mm^2). ~46% of all platelets establishing contact with the subendothelium showed transition from initial slow surface translocation to irreversible platelet adhesion (5.266 ± 915 firmly adherent platelets/mm^2). Platelet adhesion at the site of injury was associated with the formation of platelet aggregates attached to the site of injury. Platelet adhesion dynamics in mice pretreated with irrelevant IgG did not differ significantly from untreated control animals (13.521 ± 2.519 and 5.474 ± 1.575 tethered and

Table I. *Surface Expression of GPs on Platelets from JAQ1-treated Mice*

	Control	JAQ1-treated (d5)
GPVI	58.8 ± 6.4	5.7 ± 0.5
GPIbα	298.1 ± 11.5	293.5 ± 12.7
GPIX	326.9 ± 14.7	323.4 ± 13.9
GPV	176.9 ± 15.0	169.9 ± 16.4
GPIIb/IIIa	370.9 ± 19.5	381.4 ± 14.5
GPIa (α2)	60.0 ± 9.3	57.4 ± 8.4
GPIIa (β1)	223.9 ± 15.4	230.8 ± 18.3

Diluted whole blood from the indicated mice was incubated with fluorophore-labeled antibodies at saturating concentrations for 10 min at room temperature and platelets were analyzed directly. Results are expressed as mean log fluorescence ± SD for six mice per group.

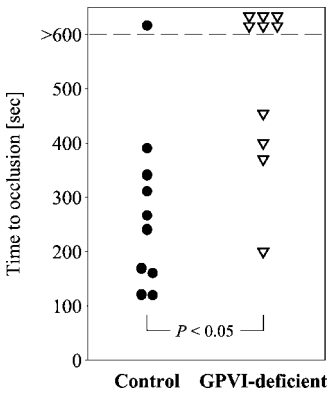


Figure 4. Role of GPVI in arterial thrombosis after ferric chloride exposure. Vascular injury of the carotid artery was induced by local application of ferric chloride on the carotid artery in GPVI-deficient or control mice. The time to thrombotic occlusion of the carotid artery downstream of the site of injury ($n = 10$ per group) was assessed in vivo by video fluorescence microscopy. Each symbol represents one experiment.

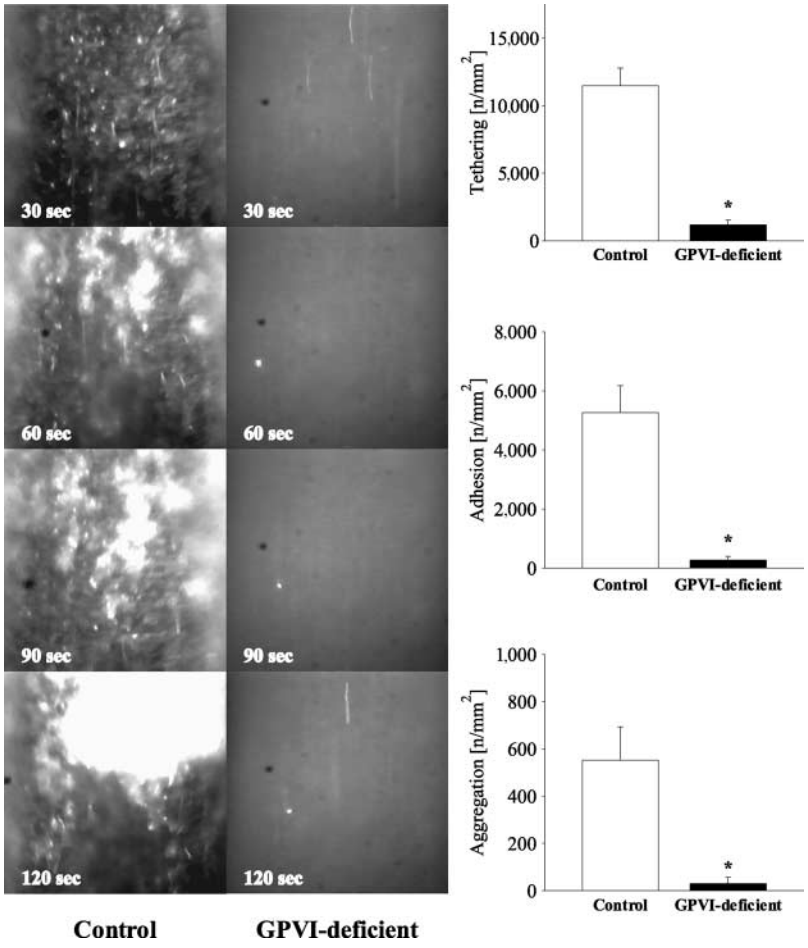


Figure 5. Role of GPVI in the regulation of platelet recruitment after wire injury of the carotid artery. Wire-induced endothelial denudation of the carotid artery was induced in GPVI-deficient mice. Untreated animals served as controls. The left shows representative in vivo fluorescence microscopy images illustrating the time course of platelet recruitment to the site of injury in control animals or GPVI-deficient mice (×500). The right summarizes platelet tethering, firm adhesion, and aggregate formation. Mean ± SEM. *, significant difference compared with control, $P < 0.05$.

firmly adherent platelets/mm^2, respectively). In contrast to control animals, platelet tethering/slow surface translocation and firm adhesion at sites of wire-induced endothelial denudation were reduced by 90 and 95% in GPVI-depleted mice ($P < 0.05$ vs. control mice; Fig. 5). We observed transition from initial tethering/slow surface translocation to irreversible platelet adhesion in only 24% of those platelets establishing initial contact with the subendothelial surface compared with 46% with control animals ($P < 0.05$). Aggregation of adherent platelets was virtually absent in GPVI-deficient mice ($P < 0.05$ vs. control; Fig. 5). Together, these data add additional strong evidence to the concept that GPVI-mediated direct platelet–collagen interactions are essential for initial platelet tethering and subsequent stable platelet adhesion and aggregation at sites of arterial injury.

Fibrillar collagen is a major constituent of the normal vessel wall but also of atherosclerotic lesions (29). In the process of atherogenesis, enhanced collagen synthesis by intimal smooth muscle cells and fibroblasts has been shown to significantly contribute to luminal narrowing (30). Plaque rupture or fissuring, either spontaneously or after balloon angioplasty, results in exposure of collagen fibrils to the flowing blood but their contribution to arterial thrombus formation has been elusive. Platelets express a large number of different collagen receptors, which made it very difficult to identify the role of each of these receptors in the processes of adhesion and activation in vitro. In addition, reagents suitable for specific inhibition of individual collagen receptors in vivo have not been available. Only recently has GPVI been identified as the central platelet receptor that is essential for both adhesion and activation of platelets on collagen in vitro (12). In contrast, the absence of other major collagen receptors such as integrin $\alpha_2\beta_1$ or GPV only results in more subtle defects in the platelet–collagen interaction (7, 12, 31), suggesting that inhibition or deletion of GPVI, but no other collagen receptor, is required to abrogate platelet collagen–interactions in vivo.

The results of this study provide the first definitive evidence that subendothelial collagens are the major trigger of arterial thrombus formation and reveal an unexpected function of GPVI in platelet recruitment to the injured vessel wall. The processes of platelet tethering and slow surface translocation under conditions of elevated shear are known to largely depend on GPIbα interaction with immobilized vWf (1). In addition, a number of studies have shown that GPIbα or even its NH_2-terminal 45-kD domain, which carries the binding site for vWf, mediates tethering of cells or coated beads, respectively, to a vWf-coated surface under high shear flow conditions (32, 33).

Together, these findings suggested that GPIbα–vWf interactions might be sufficient to establish the initial contact and slow surface translocation of platelets at sites of vascular injury. However, the results presented here demonstrate that tethering/slow surface translocation of platelets at sites of arterial injury in vivo is largely inhibited in the absence of functional GPVI although expression and function of GPIb-V-IX is not altered under these experimental conditions (Figs. 2 and 3; reference 11). On the other hand, inhibition of the vWf binding site on GPIbα by Fab fragments of the p0p/B mAb also virtually abrogated platelet adhesion to the injured vessel wall, confirming the strict requirement for this interaction under conditions of high shear in mice (Fig. 2 e). Thus, it appears that GPIbα and GPVI act in concert to recruit platelets to the subendothelium in vivo by yet undefined mechanisms. This strongly suggests that presentation of vWf on the extracellular matrix of the damaged vessel wall may differ significantly from the conditions found in vitro when it is homogeneously coated to a glass surface. At sites of vascular injury, vWf is thought to become immobilized mostly on fibrillar collagen (1, 5). Based on our results, one may speculate that the vWf layer on collagen fibrills might be inhomogeneous and frequently interrupted making efficient interactions between GPIbα and vWf impossible unless a second receptor interacts with the "gaps," i.e., collagen not covered with vWf. GPVI is known to be a low affinity collagen receptor mediating loose, but not firm adhesion that may support this hypothesis (14, 16). Another point in favor of the idea that GPIb and GPVI act in concert is the recent identification of different snake venom–derived proteins that interact with platelets specifically through both GPIb and GPVI, indicating that these two receptors might be physically and functionally linked (34, 35).

During platelet tethering, ligation of GPVI can shift $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrins from a low to a high affinity state (12). Both $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ then act in concert to promote subsequent stable arrest of platelets on collagen (5, 12) whereas $\alpha_{IIb}\beta_3$ is essential for subsequent aggregation of adherent platelets. Thus, ligation of GPVI during the initial contact between platelets and subendothelial collagen provides an activation signal that is essential for subsequent stable platelet adhesion and aggregation. Our results suggest that occupation or lateral clustering of GPIbα (during GPIbα-dependent surface translocation), which has been shown to induce low levels of $\alpha_{IIb}\beta_3$ integrin activation in vitro (32), may not be sufficient to promote platelet adhesion in vivo.

This revised model of platelet attachment to the subendothelium highlights a central role of GPVI–collagen interactions in all major phases of thrombus formation, i.e., platelet tethering, firm adhesion, and aggregation at sites of arterial injury (e.g., during acute coronary syndromes). Although the data obtained in mice cannot be directly extrapolated to the situation in humans, the profound antithrombotic protection that was achieved by inhibition or depletion of GPVI strongly indicates that a selective pharmacological modulation of GPVI–collagen interactions may become a promising strategy to control the onset and progression of pathological arterial thrombosis.

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