The Fab Fragment of a Novel Anti-GPVI Monoclonal Antibody, OM4, Reduces In Vivo Thrombosis Without Bleeding Risk in Rats

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**Background**—Inhibition of GPVI has been proposed as a useful antithrombotic strategy; however, in vivo proof-of-concept animal studies targeting GPVI are lacking. We evaluated a novel anti-human GPVI monoclonal antibody OM4 Fab in rats.

**Methods and Results**—OM4 Fab specifically inhibited collagen-induced aggregation of rat platelets in vitro with an IC₅₀ of 20 to 30 μg/mL but not ADP and AA-induced platelet aggregation. After intravenous administration of OM4 Fab, a rapid inhibition of ex vivo platelet aggregation was observed with a gradual recovery within 60 to 90 minutes which corresponded to the decline in OM4 Fab plasma concentration and time-dependent decrease in platelet-bound OM4 Fab. In contrast to previous reports in mice, intravenous OM4 Fab did not deplete platelet GPVI. Injection of OM4 IgG caused acute thrombocytopenia. In a modified Folds model of cyclic flow reduction in rat carotid artery, the number of complete occlusions was significantly reduced by intravenous administration of OM4 Fab (20 mg/kg) before or after mechanical injury to the vessel, without prolongation of bleeding time.

**Conclusion**—Fab fragment of the monoclonal antibody OM4 effectively inhibits collagen induced platelet aggregation in vitro and ex vivo, and in vivo thrombosis in rats without prolonging bleeding time. Antibodies against GPVI may have therapeutic potential, inhibiting thrombosis without prolonging bleeding time. (Arterioscler Thromb Vasc Biol. 2007;27:1199-1205.)

**Key Words:** glycoprotein VI ■ platelet ■ aggregation ■ thrombosis ■ bleeding time

Arterial thrombosis resulting from platelet aggregation is one of the leading causes of myocardial infarction, stroke, and peripheral occlusive arterial disease. Thus, it is not surprising that antiplatelet and antithrombotic treatments have become standard therapies for these life-threatening and debilitating conditions. Currently prescribed drugs include aspirin, antagonists of ADP, and GPIIIb/IIa receptors. Although these treatments are usually effective, they are associated with an increased risk of bleeding.

Recent evidence suggests that direct inhibition of the platelet collagen receptor glycoprotein VI (GPVI) may be an effective means of preventing thrombosis without increasing the bleeding risk. Arterial thrombosis is often initiated by the interaction of platelets with components of the blood vessel wall that have been exposed either by damage, or by a pathological process, such as atherosclerosis. Fibrillar collagen is the most thrombogenic component of the subendothelium, which, in addition to supporting platelet adhesion, is also a potent platelet activator. The interaction between platelets and collagen first involves adhesion and subsequently activation, leading to a second phase of adhesion, secretion, and, ultimately, aggregation involving GPIIb binding to collagen via von Willebrand Factor (vWF) followed by platelet collagen receptors GPVI and GPIa/IIa. The potential for blocking GPVI as a novel antithrombotic strategy has been demonstrated by both clinical and experimental evidence that platelets lacking GPVI are unable to respond to collagen and that mice, or humans with GPVI deficiency, exhibit only a modest elevation of bleeding time. Several attempts have been made to develop antibodies that block the interaction of platelet and GPVI, but the antibodies described so far have low potency and in vivo evaluation is lacking, with only a few reports using mice. We have recently developed 3 monoclonal antibodies (OM1, OM2, and OM4) by the immunization of GPVI knockout mice with Chinese hamster ovary (CHO) cells expressing high amounts of human GPVI. Although the Fab fragments from all 3 antibodies inhibit collagen-induced aggregation of human platelets in vitro, only OM2 Fab effectively inhibits collagen-induced aggregation of monkey platelets. Administration of OM2 Fab in Cynomolgus monkeys causes protracted inhibition of ex vivo collagen-induced platelet aggregation, with only a slight transient elevation of template
bleeding time.\(^{19}\) Although it is essential to examine the anti-thrombotic efficacy of drugs in vivo, such types of experiments are difficult and expensive to perform in large animals.

Here we report that OM4 Fab cross-reacts with rat platelets, allowing the study of this antibody in rats. We evaluated the effect of OM4 Fab on agonist-induced platelet aggregation in vitro and the inhibition of ex vivo collagen-induced platelet aggregation after intravascular administration in rats. In addition, we examined the effect of OM4 Fab in a cyclic flow reduction (CFR) model, or Folts-type model.\(^{20,21}\) Moreover, the effect of OM4 Fab on hemostasis was examined by measuring tail- and nail-bleeding times. We used the F(ab\(^{-}\))\(^{2}\), fragment of 7E3, a murine anti-human integrin \(\alpha_{IIb}\beta_{3}\) (GPIIb/IIIa) antibody for comparison.

Materials and Methods
For detailed description of the methods, please see the supplemental materials available online at http://atvb.ahajournals.org.

Chemicals
All the chemicals were obtained from commercial sources.

Antibody Preparation and Labeling
Anti-GPVI antibody-producing clones were isolated from GPVI knockout mice inoculated with CHO cells coexpressing human GPVI and FcR\(\gamma\) using conventional hybridoma methods.\(^{18}\)

Platelet Aggregation
For in vitro platelet aggregation studies, OM4 Fab, or 7E3 F(ab\(^{-}\))\(^{2}\), was added to the diluted whole blood 5 minutes before collagen addition, and comparisons were made side-by-side with saline, OM4 Fab-, and 7E3 F(ab\(^{-}\))\(^{2}\)-treated samples from the same animal; samples from 3 to 4 rats were evaluated for each treatment. To determine the agonist specificity, we determined the effect of a high concentration of OM4 Fab (100 \(\mu\)g/mL) on aggregation induced by ADP (2.5 \(\mu\)mol/L), or arachidonic acid (0.75 \(\mu\)mol/L), using similar protocols as with collagen.

For ex vivo platelet aggregation, a small sample of blood was withdrawn from the carotid artery before and after administration of the drug for determination of platelet count and the degree of platelet aggregation elicited by 1 \(\mu\)g/mL collagen (determined in triplicate). Dose escalation studies were performed with both OM4 Fab and 7E3 F(ab\(^{-}\))\(^{2}\), to find the amount of antibodies needed to inhibit ex vivo collagen-induced platelet aggregation to the same extent. It was found that 20 minutes after the injection of 20 mg/kg OM4 Fab, or 10 mg/kg 7E3 F(ab\(^{-}\))\(^{2}\), resulted in a similar inhibition (~80%).

Using 20 mg/kg OM4 Fab, we then performed a time-course study of ex vivo platelet aggregation.

Determination of Plasma OM4 Concentration
Plasma OM4 Fab concentration was measured by sandwich ELISA.

Determination of GPVI Level and Platelet-Bound OM4 Fab Using Western Blotting
Platelet proteins were resolved on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. GPVI and OM4 Fab bands were visualized with HRP-conjugated OM4 IgG/ECL reagent and HRP-conjugated anti-mouse IgG/ECL reagent respectively.

Cyclic Flow Reduction Model
To examine the effect of OM4 Fab, 2 treatments were used: pre- and post-injury administration, as shown in supplemental Figure II. In the post-injury protocol (n=6), CFRs were initiated by mechanical injury and 50% flow reduction. Blood flow was observed for 15 minutes to confirm the reproducibility of CFRs, and animals were then given an intravenous bolus injection of saline (Saline-Post), or OM4 Fab (20 mg/kg; OM4-Post) and CFRs were recorded for an additional 30 minutes. Comparison of OM4 Fab with an anti-GPIIb/IIIa antibody was performed in the same rat by injecting 7E3 F(ab\(^{-}\))\(^{2}\); 5 mg/kg) 1 hour after the injection of OM4 Fab. Five mg/kg of 7E3 F(ab\(^{-}\))\(^{2}\) was chosen because this dose produced a similar reduction in CFR as that induced by OM4 Fab at 20 mg/kg in preliminary studies. In the pre-injury protocols (n=8), either saline (Saline-Pre) or OM4 (OM4-Pre) was given by an intravenous bolus injection 2 minutes before the mechanical injury was applied. CFRs were recorded for 30 minutes after the injection of saline or OM4 Fab.

Bleeding Time Determination
Tail-bleeding time was determined by cutting a hind limb toenail at a point that transected the nail pulp and absorbing the blood every 15 seconds using a piece of filter paper. To minimize the variation between animals, nail-bleeding time was normalized as the percentage change in duration before and after treatment. Tail-bleeding time was also determined 30 minutes after the administration of antibody or saline by removing the terminal 3 mm of the tail, using a sharp scalpel blade and immersing the distal 2 cm into 37°C saline. Three groups of 9 rats per group were evaluated after the injection of saline, OM4 Fab (20 mg/kg), or 7E3 F(ab\(^{-}\))\(^{2}\) (5 mg/kg).

Statistics
Data are expressed as mean±SD. The level of statistical significance was set at \(P<0.05\). Data were analyzed with one-way ANOVA (analysis of variance), followed by a post hoc Tukey test, using Sigma Stat 2.03 (Jandel Corporation). Data for aggregation and bleeding time were not normally distributed, and thus were ranked before analysis.

Results
In Vitro and Ex Vivo Platelet Aggregation
The detailed characterization of OM1 and OM2 Fabs is described in a recent publication.\(^{18}\) The comparison of species cross-reactivity of OM4 Fab with that of OM1 and OM2 Fabs is summarized supplemental Table I, and the specificity of OM4 Fab against collagen-induced aggregations is demonstrated in supplemental Figure I.

In vitro inhibitory activity of OM4 Fab was compared with 7E3 F(ab\(^{-}\))\(^{2}\), a thoroughly characterized and a widely used antiaggregatory antibody: collagen-induced rat platelet aggregation was similarly inhibited by OM4 Fab and 7E3 F(ab\(^{-}\))\(^{2}\), (Figure 1A). The IC\(_{50}\) values for inhibition ranged 20 to 30 \(\mu\)g/mL for both antibodies.

Intravenous administration of OM4 Fab (20 mg/kg) in rats resulted in a rapid inhibition of ex vivo collagen-induced platelet aggregation that recovered with time, corresponding to a 50% recovery in 30 to 45 minutes (Figure 1B). The short-lived effect of OM4 Fab correlates with its rapid clearance from plasma. At the 5-minute time point, OM4 Fab plasma concentration has the highest value, which declined with time reaching 5% at 90 minutes after administration (Figure 1C).

Similar inhibition was observed on ex vivo collagen-induced platelet aggregation, 20 minutes after the administration of 20 mg/kg OM4 Fab (17±7% of the control) and 10 mg/kg 7E3 F(ab\(^{-}\))\(^{2}\) (22±3% of the control). Neither OM4 Fab nor 7E3 F(ab\(^{-}\))\(^{2}\) administration affected platelet count. Platelet counts were 853±193×10\(^{3}\)/\(\mu\)L before versus 833±96×10\(^{3}\)/\(\mu\)L after saline treatment, 842±178×10\(^{3}\)/\(\mu\)L before versus 872±176×10\(^{3}\)/\(\mu\)L after 7E3 F(ab\(^{-}\))\(^{2}\) treatment, and 810±102×10\(^{3}\)/\(\mu\)L before versus 811±56×10\(^{3}\)/\(\mu\)L after OM4 Fab treatment. Hemodynamics were not altered by OM4 Fab or 7E3 F(ab\(^{-}\))\(^{2}\); (data not shown).

To exclude the possibility of anti-GPVI antibody-induced acute thrombocytopenia reported previously by several investigators,\(^{3,11,22,23}\) we examined the effect of OM4 IgG and...
OM4 Fab administration on the time-dependent (0 to 90 minutes) changes in platelet counts and bleeding time (90 minutes after antibody administration) in a separate set of animals. Administration of OM4 IgG (20 mg/kg) to rats resulted in a precipitous fall in platelet count as early as 5 minutes with no sign of recovery until 90 minutes, whereas OM4 Fab (20 mg/kg) administration had minimal effect on platelet count (Figure 1D). The tail bleeding time was prolonged over 30 minutes in OM4 IgG–treated rats while saline and OM4 Fab–administered rats exhibited essentially similar tail bleeding times (table inset, Figure 1D). A rapid and severe thrombocytopenia and prolongation of tail bleeding time in OM4 IgG–treated but not in OM4 Fab–treated rats may be attributable to activation/aggregation of platelets by OM4 IgG.

Platelet GPVI Content and OM4 Fab Levels
Western blot analysis of recovered washed platelets at various time points after administration of OM4 Fab (20 mg/kg) revealed that the GPVI content of the circulating platelets remains essentially unchanged over time (Figure 2). However, during the same period platelet-bound OM4 Fab content declined rapidly (Figure 2). These results agree with our earlier observation that a single administration of OM2 Fab in monkeys does not deplete platelet GPVI, contrary to previous observations in mice.

In Vivo Thrombosis
OM4 Fab at 20 mg/kg was effective in reducing thrombosis when administered either pre- or post-injury. Representative blood flow measurements from saline- (Saline-Post and Saline-Pre) and OM4 Fab- (OM4-Post and OM4-Pre) treated rats are shown in Figure 3. In some cases occlusion of the artery was completely prevented by OM4 Fab.

Flow data were quantified by counting CFRs (defined by a reduction of blood flow to zero) during the 30-minute observation period. OM4 Fab, given either after or before the
establishment of CFRs, significantly reduced the number of CFRs compared with saline treated animals (Figure 4). A similar attenuation was observed following the administration of 5 mg/kg 7E3 F(ab')2 in the post-injury group.

**Figure 3.** Representative carotid artery mean blood flow recordings are shown for the 4 groups. The arrow indicates when the injection of the drug was performed. The y-axis is the averaged carotid blood flow (mL/min), and the x-axis is the time elapsed since the start of the recording. In the post-injury treatment groups (Saline-Post and OM4-Post), CFRs were established and observed for 15 minutes. The animal then received either a bolus intravenous injection of saline or OM4 Fab (20 mg/kg). In the Pre-injury treatment groups (Saline-Pre and OM4-Pre), rats were administered either a bolus intravenous injection of saline or OM4 Fab (20 mg/kg), followed by mechanical injury and the initiation of CFRs, which were observed and recorded for 30 minutes.

**Bleeding Time Changes**
OM4 Fab (20 mg/kg) did not prolong either nail- or tail-bleeding time, whereas 7E3 F(ab')2 (5 mg/kg) caused a doubling of both parameters (Figure 4, right panels). The
differences in tail bleeding times seen in Figure 1D and Figure 4 in controls and OM4 Fab administered animals are probably attributable to the fact that the measurements were performed at different time points (30 minutes in Figure 4 versus 90 minutes post treatment in Figure 1D), and variation between two operators as they were performed by two individuals.

**Discussion**

GPVI has now been recognized as a major collagen receptor, crucial for platelet adhesion and thrombus formation. Recognition of GPVI as a promising therapeutic target is supported by several observations: platelets from GPVI-deficient humans and mice do not respond to collagen, the most thrombogenic component of the subendothelium. Collagen-induced platelet aggregation and lethal thromboembolism in mice was inhibited by the administration of an anti-mouse GPVI antibody (JAQ1). Furthermore, platelet adhesion and aggregation on the injured vessel wall in mice was inhibited by the administration of an anti-mouse GPVI antibody (JAQ1). The detailed characterization of OM1 and OM2 has been described in a recent publication. Fab fragments obtained from 3 antibodies potently inhibited collagen-induced human platelet aggregation. However, only OM4 Fab cross-reacted with rat platelets, providing a suitable tool to test the effectiveness of an anti-GPVI antibody in vivo. Interestingly, intact OM4 IgG administration in rats causes rapid and severe thrombocytopenia resulting in an excessive prolongation of bleeding time. This led us to use Fab fragment for the ensuing in vitro human and rat studies.

Using rat platelets, we demonstrated that the in vitro potency for the inhibition of collagen-induced aggregation was similar for OM4 Fab and 7E3 F(ab')2. After an intravenous administration of OM4 Fab (20 mg/kg) in rats, collagen-induced ex vivo platelet aggregation was significantly inhib-
ited. The magnitude of the inhibition was similar to that achieved by 7E3 F(ab')2 at 10 mg/kg. Abciximab, the human/mouse chimeric Fab fragment of 7E3, is not effective in the rat and 7E3 IgG causes thrombocytopenia; thus it is necessary to use the F(ab')2 fragment as an alternative.26

Folts model20,21 is probably the most often used in large laboratory animals (rats, rabbits, monkeys, and dogs) because of its close mimicking of clinical thrombus formation. An advantage of this model is that the effectiveness of a drug can be monitored over time; in addition, a comparison of the effectiveness of different drugs is possible in the same animal, if the half-lives are suitably short. By creating a severe stenosis, with 50% flow reduction, we were able to produce CFR in the rat carotid artery. This stenosis is different from most other studies using this model in which a so-called “critical” stenosis was applied causing no basal flow reduction, but with all vasodilatory reserves exhausted.21 The successful establishment of this model allowed us to evaluate the effect of OM4 Fab and compare it with that of 7E3 (Fab')2. OM4 Fab at 20 mg/kg significantly reduced the number of complete occlusions during the 30-minute observation period. 7E3 F(ab')2 is also effective, as shown in this study and in a previous study using dogs.27 Based on direct observations and blood flow recordings, it appears that platelets initially aggregated at the site of the injury, but often broke free spontaneously in OM4 Fab–treated rats. This suggests that the attachment of platelet aggregates to the exposed collagen may be weakened in the presence of OM4 Fab. Moreover, because collagen is a potent platelet activator and causes release of secondary mediators such as ADP and thromboxane A2,18 blocking GPVI with OM4 Fab may reduce the size and cohesiveness of the aggregates. Interestingly, OM4 Fab seems to be equally effective when given either before or after the injury. Future studies are required to explain the effectiveness of OM4 Fab with post-injury administration.

We observed that ex vivo collagen-induced aggregation and in vivo CFRs returned to initial values by about 40 minutes after the OM4 Fab injection. This is likely attributable to the fast renal clearance and rapid dissociation of OM4 Fab from platelet GPVI, as evident from OM4 Fab plasma concentration (Figure 1C) and Western blot analysis (Figure 2). Reasons for the rapid dissociation of platelet-bound OM4 Fab may include its small size, low affinity for rat GPVI, and a rapid renal clearance from circulation, as shown for other Fab fragments.28 The half-life of murine monoclonal antibodies in rats is short and is size-related: IgG >F(ab')2 >Fab.28 However, the short half-life of OM4 Fab fortuitously allowed us to compare it with 7E3 F(ab')2 in the same animal.

Ex vivo studies clearly showed that administration of OM4 Fab at 20 mg/kg and 7E3 F(ab')2 at 10 mg/kg had similar inhibitory effect on collagen-induced aggregation of rat platelets. On the other hand, a comparable inhibitory effect by 7E3 F(ab')2 on CFRs was seen at half that amount. One likely explanation is that in ex vivo studies collagen is the only aggregatory ligand, whereas under in vivo conditions platelets are exposed to multiple adhesion ligands and possibly locally generated thrombin under flow conditions. OM4 Fab inhibits only the collagen-platelet GPVI interaction, whereas 7E3 F(ab')2 prevents aggregate formation by inhibiting bridging of GPIIb/IIIa molecules on adjoining platelets through fibrinogen or vWF, the ultimate step of platelet aggregation. Thus, it is not unexpected that whereas OM4 Fab and 7E3 F(ab')2 may have similar potency on collagen-induced in vitro platelet aggregation, 7E3 F(ab')2 is more potent in vivo.

OM4 Fab did not increase bleeding time, as measured by nail and tail bleeding times, which is consistent with the known function of GPVI in human and mice. In contrast, 7E3 F(ab')2 at 5 mg/kg significantly prolonged bleeding time. Rats injected with a relatively large dose of OM4 Fab (20 mg/kg) did not exhibit any evidence of thrombocytopenia. Furthermore, CFRs returned in 40 minutes after a bolus injection, suggesting that functional GPVI remained on the platelet surface in these animals. To confirm this observation, we performed Western blotting analysis of GPVI expression on/in platelet after OM4 Fab injection. As suggested by the results of the ex vivo platelet aggregation study, the platelet GPVI level was not affected by the injection of OM4 Fab (Figure 2). These results are at variance with the results obtained in JAQ1-treated mice, which experienced a profound depletion of GPVI on circulating platelets for at least 2 weeks subsequent to transient thrombocytopenia after a single bolus injection of anti-GPVI antibody.3 In a recent study, nonactivating Fab fragments of anti-GPVI antibody were shown to induce GPVI shedding from human platelets in vivo using NOD/SCID mouse.23 We should emphasize that other anti-GPVI antibodies apart from OM4 also failed to induce GPVI depletion: for example, OM2 Fab showed a clear inhibitory effect on ex vivo collagen-induced platelet aggregation in monkeys without causing thrombocytopenia, or GPVI depletion 24 hours after a single intravenous injection.19 It is not clear whether the route of administration is responsible for difference as we injected OM2 and OM4 intravenously, whereas the other two studies used intraperitoneal route. Alternative explanations for the discrepant results may be the differences in the experimental species, or the properties of the antibody, ie, affinity, or the target epitope(s). Further studies may resolve these issues. Although, we made observations only up to 90 minutes, we do not anticipate any GPVI depletion in extended time periods because of a rapid clearance of OM4 Fab from plasma (Figure 1C) and its dissociation from platelets (Figure 2).

The role of GPVI in arterial thrombus formation has not been supported in all the studies. FeRγ-chain null (FeRγ−/−) lacking platelet GPVT29,30 and GPVI-deficient mice24 have been used to investigate the importance of GPVI in arterial thrombosis. Using 3 distinct arterial thrombosis models, namely electrical, mechanical, and laser injury models involving deep vascular injury, Mangin et al showed that GPVI deficiency alone does not provide significant protection.29 However, a coadministration of thrombin inhibitor hirudin in GPVI-deficient mice results in a profound reduction in thrombus formation.29 In addition, the results of another study using experimentally-induced vascular injury in FeRγ−/− mesentery and cremaster muscle showed reduced thrombosis in FeCl3-induced vascular injury model but not in laser-induced injury model.30 In the contrary, platelet tethering and thrombus formation on the injured carotid artery was almost abolished in GPVI-deficient mice (induced by JAQ1 injection) as compared with wild-type mice.24 Contrasting obser-
vations made by various investigators probably reflect variations in the technique, most likely the severity of the vascular injury. A severe injury may generate an overwhelming amount of local thrombin resulting in the formation of fibrin-rich thrombus, thus masking the protective effect of GPVI-deficiency. Importantly, all these studies were performed in mice. It is imperative to validate GPVI as a therapeutic target and function blocking anti-GPVI antibody as an antithrombotic agent in other species. The current study is the first to verify the potential of GPVI inhibitors as novel antithrombotic agents in rats. Studies are on-going to evaluate the efficacy of our antibodies in other species.

In summary, the Fab fragment of a novel monoclonal anti-human GPVI antibody, OM4, inhibits in vitro and ex vivo collagen-induced rat platelet aggregation. More importantly, OM4 Fab inhibits thrombosis in vivo in a CFR model without the prolongation of bleeding time that is seen with anti-GPIIb/IIIa antibodies. The potential antithrombotic activity of anti-GPVI antibodies has now been shown in rats (this study) and in previous studies in mice. Further studies are needed to investigate whether OM4 and the other anti-GPVI antibodies we generated can be developed for future clinical use.

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**Disclosures**

All the authors are the employees of Otsuka Maryland Medicinal Laboratories, Inc.

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Materials and Methods

The present study was conducted in accordance with the “Guide for the Care and Use of Laboratory Animals”, published by the National Research Council, 1996, Washington DC, and approved by the Institutional Animal Care and Use Committee. The protocol to draw human blood was approved by the institutional review committee according to the Helsinki convention.

Chemicals:

Collagen was obtained from Nycomed Arzneimittel, Munich, Germany. Protein G-Sepharose, Protein A-Sepharose, MonoQ (Source 15 beads), Fast Flow Q-Sepharose and size exclusion matrix Superdex75 were purchased from Amersham. ADP, arachidonic acid, papain, pepsin, iodoacetamide, and cysteine were purchased from Sigma Chemicals. Ultra low IgG fetal calf serum and monoclonal antibody to V5 were purchased from Invitrogen (Carlsbad, CA). HRP (horseradish peroxidase)-conjugated κ-chain specific anti-mouse IgG and affinity purified rabbit anti-(6-His) antibody were purchased from Bethyl (Montgomery, TX). Trimethylbenzine (TMB) peroxidase-substrate was purchased from KPL (Gaithersburg, MD).

Antibody preparation:

Anti-GPVI antibody-producing clones were isolated from GPVI knockout mice inoculated with Chinese hamster ovary (CHO) cells co-expressing human GPVI and FcRγ by conventional hybridoma methods. Briefly, clones showing a strong positive reaction with GPVI-transfected CHO cells were identified by FACS analysis, expanded
and cultured on a large scale. Hybridomas producing target antibodies were grown in DMEM/F12 medium containing 5% fetal calf serum (containing negligible amounts of bovine IgG: <1 µg/mL). OM4 IgG was purified by protein G-Sepharose affinity chromatography on a Waters 650 protein separation system as described earlier.1 Protein G-bound IgG was eluted by glycine-HCl (pH 2.75), collected directly into basic solution and dialyzed against saline for the preparation of Fab fragments.

**Preparation of OM4-HRP:** OM4-HRP conjugate was obtained by coupling NHS-HRP to free NH₂ groups in OM4 IgG using a micro-spin column HRP-labeling kit, as described by the manufacturer (Dojindo Molecular Technologies Inc, Gaithersburg, MD).

**OM4 Fab preparation:** Papain digestion was used to prepare OM4 Fab fragment: a solution of IgG (5 mg/mL) in 100 mM citric acid, pH 6.5 and 5.0 mM EDTA was digested for 15 hrs at 37 °C with cysteine-free pre-activated papain at an enzyme:IgG ratio of 1:50 (w/w). Digestion was quenched with freshly prepared iodoacetamide. The digest was extensively dialyzed against 10 mM Tris.HCl, pH 8.0 and OM4 Fab was separated from Fc and undigested IgG by ion exchange chromatography on MonoQ (SourceQ-15 Pharmacia). Fab fragment was usually eluted from the column at low salt concentration (30-50mM) while elution of undigested IgG and Fc required 80-100 and 120-150 mM salt, respectively. Fractions containing Fab and a small amount of F(ab’)2 were pooled and concentrated using an Amicon Ultra filtration assembly, using a PM30 filter. Fab and F(ab’)2 were resolved by size exclusion chromatography on Superdex75 and dialyzed into normal saline for further use. The resulting Fabs did not activate human platelets when tested at ≤ 300 µg/mL.
Preparation of F(ab’)2 fragment of 7E3, a monoclonal antibody against GPIIb/IIIa: A 7E3-producing mouse hybridoma cell line was purchased from ATCC (Clone HB-8832). These cells were expanded and grown on a large scale to produce 7E3 IgG. IgG was purified from the culture supernatant as described above, but using a Protein A-Sepharose affinity chromatography. Bivalent 7E3 fragment was prepared from the intact IgG by digestion with pepsin (1:50 enzyme:IgG w/w) for 4 hrs at 37 °C, pH 3.5. The reaction was terminated by increasing the pH of the reaction mix to 8.0 with Tris.HCl (3 M, pH 8.6). The digest was extensively dialyzed against 20 mM Tris.HCl pH 8.0 and F(ab’)2 was resolved from other components by ion-exchange chromatography on Fast-flow Q Sepharose. Fractions containing F(ab’)2 were pooled, concentrated and dialyzed into normal saline. The purity of F(ab’)2 fragment ranged from 92-95% as determined by protein assay chip in a 2100 Bioanalyzer (Agilent Technologies).

Platelet aggregation:

Adult male Sprague-Dawley rats (300-400 g) were anesthetized with a mixture of 80 mg/kg ketamine and 12 mg/kg xylazine intramuscularly.

For in vitro platelet aggregation studies, the right carotid artery was exposed by blunt dissection and a catheter attached to a collection syringe containing heparin (10 U/mL, final) inserted. Blood was withdrawn to the required volume, maintained at room temperature and used within 3 hours. Blood was diluted 2:1 v/v with saline and incubated at 37°C for 5-10 min in the aggregometer before aggregation was initiated. Collagen (Horm, Nycomed, Germany; 1–4 µg/mL) was used as agonist; aggregation was monitored using a whole blood aggregometer (Chrono-log, Corporation, PA). The
collagen dose that elicited 70% aggregation was determined for each blood sample and used for all subsequent determinations, unless the response diminished, in which case the dose was increased accordingly. OM4 Fab, or 7E3 F(ab’)2 was added to the diluted whole blood 5 minutes prior to collagen addition. The predetermined dose of collagen was then added and aggregation monitored for an additional 6 min (Aggro/link v 4.75, Chronolog). Comparisons were made side-by-side with saline, OM4 Fab- and 7E3 F(ab’)2-treated samples from the same animal; samples from 3-4 rats were evaluated for each treatment. To determine the agonist specificity, we determined the effect of a high concentration of OM4 Fab (100 µg/mL) on aggregation induced by ADP (2.5 µM) and arachidonic acid (0.75 mM). Diluted blood was preincubated with OM4 Fab (5 min) in the aggregation cuvette and agonist added; aggregation was monitored throughout.

For ex vivo platelet aggregation, heparin-filled (10 U/mL) catheters were inserted into the femoral vein and femoral and carotid arteries for drug administration, blood pressure/heart rate recording and blood sampling, respectively. Blood pressure and heart rate data were continuously recorded electronically with a blood pressure and heart rate measuring device (Stoelting 50110, Wood Dale, IL) via a pressure transducer attached to the femoral artery catheter. During the course of the experiment, the animal’s core temperature was maintained at 37 °C using a heating pad and a thermostatically controlled infrared lamp. A small sample of blood (~1.2 mL, anticoagulated with 10 U/mL heparin) was withdrawn from the carotid artery before and after drug administration for determination of platelet count (Unopette 365855, BD, Franklin Lakes, NJ) and the degree of platelet aggregation elicited by 1 µg/mL collagen (determined in triplicate), using whole blood (diluted 2:1 v/v with saline) aggregometry (Chrono-log).
Dose escalation studies were performed with both OM4 Fab and 7E3 F(ab’)_2 to find the amounts of antibody needed to inhibit ex vivo collagen-induced platelet aggregation to the same extent. It was found that 20 min after the injection of 20 mg/kg OM4 Fab, or 10 mg/kg 7E3 F(ab’)_2 resulted in a similar inhibition (~80%). Using 20 mg/kg OM4 Fab, we then performed a time-course study of ex vivo platelet aggregation in which blood samples were withdrawn before and 5, 15, 30, 45, 60 and 90 min after administration. Platelet aggregation is expressed as a percentage of the value prior to each treatment. Three to 6 animals were evaluated for each time points. Blood from other laboratory animals was collected in 1/10th volume of 3.8% trisodium citrate and platelet aggregation experiments and FACS analysis were performed as described earlier.\(^1\)

**Determination of plasma OM4 concentration using ELISA:**

To produce recombinant extracellular domain of human GPVI (soluble GPVI, sGPVI) to be used in ELISA, we cloned the extracellular domain of GPVI encoding 22-219 amino acid residues into plasmid pEF1/SecTagV5/HisA using EcoRI/NotI sites (Invitrogen). The resulting plasmid was transfected into CHO-K1 cells using LF2000 as recommended by the manufacturer (Invitrogen). A single clone producing sGPVI was selected in the presence of G418 (G418 resistant cell). sGPVI-expressing CHO cells were cultured in DMEM/F12 (1:1) containing 2.5% FCS and G418 (100 µg/ml) at 37°C in 5% CO₂-containing atmosphere in roller bottles.

The culture supernatant was harvested once the cells reached confluence. The culture medium was spun at 3800 g for 30 minutes at 4°C to remove carryover cells. The supernatant was further clarified by filtration (0.4 µm) and concentrated to 1/100th
original volume using Pellicon XL (Millipore). The concentrated supernatant was loaded
onto an OM1-coupled Sepharose column (HiTrap-NHS HP obtained from Amersham
Pharmacia was used to couple OM1 IgG to Sepharose beads) equilibrated in PBS. The
column was washed extensively with PBS. The column-bound sGPVI was eluted with
3M KSCN in PBS. The fractions absorbing at 280 nm were pooled, dialyzed against
PBS, concentrated and stored at -20 °C.

The authenticity of sGPVI was confirmed by demonstrating its reactivity with
OM1 and OM4 IgGs on Western blots and by ELISA assay. Furthermore, the presence of
X6His and V5 tags was confirmed by showing positive reactivity with anti-His and anti-
V5 antibodies by Western blotting.

Plasma OM4 Fab concentration was measured by sandwich ELISA. Briefly, wells
of a microtiter plate were coated overnight with 6-His antibody (100ng/well) at 4 °C.
The unoccupied sites were blocked with BSA (1%) in PBS for 2 hours at room
temperature. A solution of sGPVI (250 ng/ml in PBS) was added to the wells and
incubated overnight at 4 °C to immobilize sGPVI to the plate via the 6-His sequence
located in its C-terminal domain. Solutions of known amounts of OM4 Fab and
appropriately diluted rat plasma samples were added to the wells. After one hour at room
temperature, wells were washed three times with PBS. HRP-coupled anti-mouse κ-chain
specific antibody (1:50,000) was added to each well and incubated for 1 hour at room
temperature. Peroxidase activity was measured with TMB substrate solution as described
by the manufacturer (KPL).
**Determination of platelet GPVI levels and platelet-bound OM4 Fab by Western blotting:**

Rat blood (0.5 ml) was diluted 1:1 with platelet wash buffer (10 mM Tris. HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.35 % BSA, 0.1 % azide, 100 ng/ml PGE₁) and centrifuged for 20 seconds at 3400 rpm in SeroFuge (Becton Dickenson) to recover diluted platelet rich plasma. Platelet rich plasma was centrifuged at 3400 rpm for 10 minutes, the resulting platelet pellet was washed twice with platelet wash buffer and finally suspended in platelet wash buffer without BSA at ~1x10⁹ platelets/ml. Washed platelet suspensions were mixed with an equal volume of 2x platelet lysis buffer [2% Triton X-100 and protease inhibitor cocktail (Halt- PIERCE) in platelet wash buffer without BSA] and incubated for 1 hour at 4 °C to solubilize the platelets. Samples were centrifuged at 15,000 xg for 15 minutes in a refrigerated centrifuge. A clarified aliquot of each platelet lysate, containing 10 µg protein, was resolved by SDS-PAGE on a 4-20 % NuPage Bis-Tris gel (Invitrogen) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in SuperBlock T20 (TBS) Blocking Buffer (PIERCE) for 30min. The PVDF membrane was incubated with HRP-conjugated OM4 IgG (0.1 µg/ml) for the detection of rat GPVI, or with HRP-anti mouse κ-chain specific antibody (1:5,000 dilution) to detect platelet bound OM4 Fab, for 30 minutes at room temperature. The membranes were washed extensively with PBS-Tween 20 (0.05%) and immune reactive bands were visualized using an enhanced chemiluminescence detection system (ECL, Amersham Biotech).

**Cyclic flow reduction model:**
Adult male Sprague-Dawley rats (300-400 g) were anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and mechanically ventilated via tracheal intubation, using a small animal ventilator (Harvard Apparatus). Body temperature was maintained at approximately 38 °C using an electric blanket (Homeothermic Blanket Control Unit; Harvard Apparatus). A segment of the femoral vein was exposed for drug administration. The carotid artery was exposed via a midline incision in the ventral cervical area and carefully dissected free of connective tissue. A small probe (Transonic 1RB; Transonic Systems Inc., Ithaca, NY) was placed distally on the artery to measure blood flow and average flow recorded using a PowerLab/4SP data acquisition system (ADIstruments Inc., Colorado Spring, CO). A 2-step process was used to induce thrombosis: (i) three applications of a needle holder forceps to the middle of the exposed arterial segment, each of 10 sec duration (to standardize the injury the forceps were tightened each time until one click of the locking mechanism was engaged) and (ii) immediately following injury, inflation of a C-shaped vascular occluder (2 mm inner diameter; In Vivo Metric, Healdsburg, CA) to cause a 50% flow reduction. Blood flow gradually declined to zero over about 1-4 minutes due to the formation of a platelet-rich thrombus/thrombi. Subsequent deflation of the balloon allowed embolization of the thrombus to occur whereupon the blood flow immediately returned to the pre-injury level. Reinflation of the occluder resulted in formation of another thrombus.

To examine the effect of OM4 Fab, two treatments were used: pre- and post-injury administration, as shown in Fig. 1. In the post-injury protocol (n=6), CFRs were initiated by mechanical injury and 50% flow reduction; blood flow was observed for 15 min to confirm the reproducibility of CFRs and animals were then given an intravenous
bolus injection of saline (Saline-Post), or OM4 Fab (20 mg/kg) (OM4-Post) and CFRs were recorded for an additional 30 min. Since OM4 Fab has a short half-life in rats, we compared the effect of OM4 Fab with that of an anti-GPIIb/IIIa antibody in the same rat by injecting 7E3 F(ab’)2 (5 mg/kg) 1 hr after the injection of OM4 Fab. Five mg/kg of 7E3 F(ab’)2 was chosen because this dose produced a similar reduction in CFR as that induced by OM4 Fab at 20 mg/kg in preliminary studies. Injection of 10 mg/kg of 7E3 F(ab’)2 completely prevented CFR (data not shown). In the pre-injury protocols (n=8), either saline (Saline-Pre) or OM4 (OM4-Pre) was given by an intravenous bolus injection 2 min before the mechanical injury was applied. CFRs were recorded for 30 min after the injection of saline or OM4 Fab.

In pilot experiments, we were able to induce CFRs in the majority of animals using the procedures described above, but in a small number (approximately 10%) the phenomenon could not be elicited. In animals in which OM4 Fab or saline was administered post-injury, the ability to induce CFRs was confirmed in the pre-administration monitoring period. However, when OM4 Fab, or saline was administered pre-injury this was not the case; an additional 2 animals (total n = 8 vs. n = 6 in post-injury group) were included in this group to allow for the possibility that CFRs would not be inducible in some animals.

**Bleeding time determination:**

Bleeding time was determined in a separate set of rats because the extensive blood loss observed in some animals may have affected hemodynamics and CFRs. Adult male Sprague-Dawley rats (300-400 g) were anesthetized with pentobarbital (50 mg/kg,
intraperitoneally) and mechanically ventilated, as described above. Nail-bleeding time was determined by cutting a hind limb toenail at a point that transected the nail pulp and absorbing the blood every 15 seconds using a piece of filter paper, without touching the cut surface of the nail. Nail-bleeding time was defined as the time elapsed between cutting the nail and the point at which no further blood was absorbed onto the filter paper. Nail bleeding time was determined on two separate toenails before and 30 min after the vehicle or drug treatment. To minimize the variation between animals, nail bleeding time was normalized as the percentage change before and after treatment. Tail-bleeding time was also determined 30 min after the administration of antibody or saline by removing the terminal 3 mm of the tail using a sharp scalpel blade and immersing the distal 2 cm into 37 °C saline. Tail-bleeding time was defined as the time elapsed between cutting the tail and the point at which no more blood issued from the cut surface of the tail. Three groups of 9 rats per group were evaluated following the injection of saline, OM4 Fab (20 mg/kg) or 7E3 F(ab’)2 (5 mg/kg).
<table>
<thead>
<tr>
<th>Fab ID</th>
<th>IC$_{50}$ (µg/ml)</th>
<th>Rat</th>
<th>Guinea pig</th>
<th>Rabbit</th>
<th>Pig</th>
<th>dog</th>
<th>Monkey</th>
<th>Human</th>
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<tr>
<td>OM1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25.8 ± 40.5</td>
<td>0.60 ± 0.50</td>
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<tr>
<td>OM2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.00 ± 0.67</td>
<td>1.69 ± 0.51</td>
</tr>
<tr>
<td>OM4</td>
<td>20-30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.10 ± 5.51</td>
<td></td>
</tr>
<tr>
<td>ReoPro or 7E3 F(ab’)$_2$</td>
<td>20-30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.05 ± 0.9</td>
<td>1.70 ± 0.34</td>
</tr>
</tbody>
</table>

Each antibody Fab was tested with 3-4 platelet samples. In case of rat whole blood aggregometry was performed, while in other cases light transmittance aggregometry with platelet rich plasma was performed. ReoPro [7E3 F(ab’)$_2$ in rats] was used as a positive control in these experiments. OM1, OM2, and OM4 Fabs inhibited collagen-induced aggregation of human platelets with rank order of potency OM1>OM2>OM4. Intact IgGs (OM1, OM2 and OM4) bound to human platelets with high affinity and aggregated human platelets at relatively low IgG concentration (< 1µg/mL). However, Fab fragments of these antibodies did not activate the platelets. While both OM1 and OM2 Fabs showed complete lack of binding to rat platelets (unpublished data) and failed to inhibit collagen-induced aggregation, OM4 Fab concentration-dependently inhibited in vitro collagen-induced platelet aggregation and bound to rat platelets as demonstrated by FACS (data not shown). Additionally, the specificity of OM4 IgG was confirmed by demonstrating its reactivity with a ~60kDa band, expected to be rat GPVI by Western blotting (Fig. 2).
Fig. I
Protocol

15min  30min  30min  30min

Saline-Post

OM4 Fab (20mg/kg)  7E3 F(ab’)2 (5mg/kg)

Saline-Pre

OM4 Fab

OM4-Pre

Fig. II
Reference List


Figure Legends

Fig. I: OM4 Fab specifically inhibits *in vitro* rat platelet aggregation induced by collagen but not by ADP and arachidonic acid. Diluted rat blood was incubated with varying amounts of OM4 Fab (10, 25, 50 and 100 µg/mL) and then challenged with collagen (A) and aggregation was followed for 8 minutes. Maximal dose of OM4 (100 µg/mL) was used to examine the effect of OM4 Fab on platelet aggregation induced by ADP (B) and arachidonic acid (C). Note a concentration-dependent inhibition of collagen-induced platelet aggregation but not ADP and arachidonic acid induced aggregation, suggesting that OM4 Fab blocks the collagen-binding site in rat GPVI. Representative traces are shown (n = 3).

Fig. II: Panel (A) shows the experimental protocol for *in vivo* thrombosis using a modified Folts model. In the post-injury treatment groups (Saline-Post and OM4-Post), CFRs were established and observed for 15 minutes. The animal then received either a bolus intravenous injection of saline, or OM4 Fab (20 mg/kg). CFRs were observed and recorded for an additional 30 minutes. Taking advantage of the short half-life of OM4 Fab (CFRs returned 40 min after OM4 Fab injection), the CFRs were observed for another 30 min before injection of 7E3 F(ab’)_2 (5 mg/kg) and patterns of CFRs were recorded for an additional 30 min. In the Pre-injury treatment groups (Saline-Pre and OM4-Pre), rats were administered either a bolus intravenous injection of saline, or OM4 Fab (20 mg/kg), followed by mechanical injury and the initiation of CFRs, which were observed and recorded for 30 min.