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Research Article   **Hematology**

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Platelet adhesion to vascular subendothelium, mediated in part by interactions between collagen and glycoprotein VI (GPVI) complexed with Fc receptor γ-chain, is crucial for thrombus formation. Antiplatelet therapy benefits patients with various thrombotic and ischemic diseases, but the safety and efficacy of existing treatments are limited. Recent data suggest GPVI as a promising target for a novel antiplatelet therapy, for example, GPVI-specific Abs that deplete GPVI from the surface of platelets. Here, we characterized GPVI-specific autoAbs (YA-Abs) from the first reported patient with ongoing platelet GPVI deficiency caused by the YA-Abs. To obtain experimentally useful human GPVI–specific mAbs with characteristics similar to YA-Abs, we generated human GPVI–specific mouse mAbs and selected 2 representative mAbs, mF1201 and mF1232, whose binding to GPVI was inhibited by YA-Abs. In vitro, mF1201, but not mF1232, induced human platelet activation and GPVI shedding, and mF1232 inhibited collagen-induced human platelet aggregation. Administration of mF1201 and mF1232 to monkeys caused GPVI immunodepletion with and without both significant thrombocytopenia and GPVI shedding, respectively. When a human/mouse chimeric form of mF1232 (cF1232) was labeled with a fluorescent endocytosis probe and administered to monkeys, fluorescence increased in circulating platelets and surface GPVI was lost. Loss of platelet surface GPVI mediated by cF1232 was successfully reproduced in vitro in the presence of a cAMP-elevating agent. Thus, we have characterized cAMP-dependent endocytosis of GPVI mediated by a human GPVI–specific mAb as what we believe to be a novel antiplatelet therapy.

Introduction

Antiplatelet therapy provides significant clinical benefits in patients with a variety of thrombotic or ischemic diseases such as myocardial infarction, unstable angina, and stroke (1). However, the safety and efficacy of existing treatments are limited, and there remains a clinical need for novel antiplatelet interventions to reduce pathological thrombus formation without promoting bleeding (2).

Over the last decade, considerable progress has been made in identifying and describing the molecular events regulating the initiation of thrombus formation. At sites of vascular damage, subendothelial matrix proteins, primarily collagen, are exposed, promoting platelet adhesion and activation through interactions with specific cell-surface receptors, leading to thrombus formation.

Several cellular collagen receptors mediate this response, and, among these, glycoprotein VI (GPVI) plays a critical role in the platelet response to collagen. GPVI is a member of the Ig superfamily (3–5), and it is constitutively associated and expressed with the Fc receptor γ-chain (FcRγ), an immunoreceptor tyrosine-based activating motif–bearing (ITAM-bearing) receptor (6). Upon GPVI engagement, phosphorylation of the ITAM motif of FcRγ initiates a signaling cascade, leading to platelet activation (6–10). The extracellular domain of GPVI contains 2 Ig-like loops of the C2 type (3–5). The extracellular domain 1 (D1) N-terminal loop is of the IgC2-1 type, and the extracellular D2 loop is of the IgC2-2 type (11). Deficiency or blockade of GPVI slightly prolongs bleeding time in both humans and mice (12–14), and this produces antithrombotic effects in several different experimental conditions (14–17). These data suggest that GPVI is a promising target for the development of novel antiplatelet therapies, but such therapies have yet to be successfully developed and/or clinically deployed.

Platelet GPVI deficiency and unresponsiveness to collagen have been first reported in a patient (YA) who developed anti-GPVI Abs in the plasma (12). Monovalent Fab fragments prepared from YA's serum IgG blocked collagen-induced platelet aggregation, but divalent F(ab')$_2$ fragments caused GPVI cross-linking and platelet activation. This report also suggested the possibility of specific Ab-induced GPVI immunodepletion. This assumption was confirmed by later studies in mice (14, 18), and an additional patient with Ab-induced GPVI deficiency was identified (19). Short-term antiplatelet effects may be safely generated by the infusion of anti-GPVI Fab fragments to block the GPVI/collagen interaction. The F(ab')$_2$ fragments and intact Ab have a longer half-life in the serum than the Fab fragments, but their divalent nature would likely activate platelets by cross-linking GPVI. Alternatively, specific Ab-induced GPVI immunodepletion could be clinically useful. However, metalloproteinase-dependent proteolytic shedding of GPVI has been demonstrated in vitro to cause considerable loss of GPVI following activation of platelets exposed to GPVI-specific Abs or ligands (20, 21). On the other hand, GPVI internalization and endocytosis may
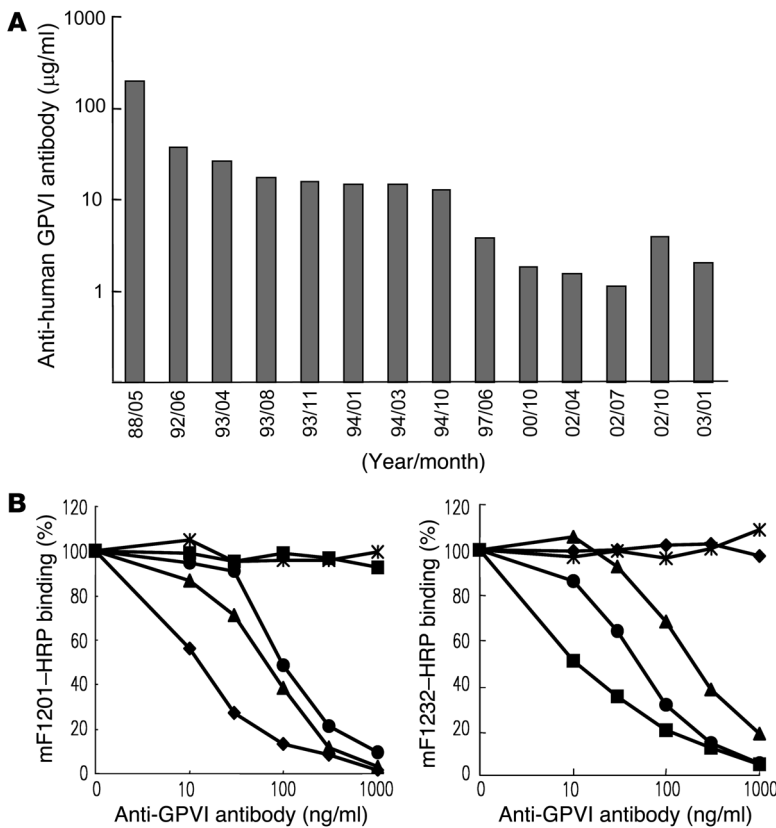
Figure 1
Characterization of YA-Abs over 15 years. (**A**) YA's serially diluted plasma stored over 15 years or control solutions containing known amounts of a human/mouse chimera anti-GPVI mAb were incubated with biotin-labeled hGPVI-Fc on streptavidin-coated plates. After washing, the bound Abs were detected using peroxidase-labeled anti-kappa and -lambda chain Abs and subsequent colorimetric reaction. The content of anti-GPVI auto-Abs in the plasma was expressed as an equivalent content of a human/mouse chimera anti-GPVI mAb bound to hGPVI-Fc. Each bar represents the mean of duplicate determinations. (**B**) Both YA-Abs-88 and -03 were purified from the patient's plasma stored in 1988 and 2003, respectively, and quantified as described in Methods. Serially diluted YA-Abs-88 (triangles), YA-Abs-03 (circles), mF1201 (diamonds), mF1232 (squares), or mouse IgG (asterisks) as the competing Ab was applied to hGPVI-Fc–immobilized plates followed by the incubation with the peroxidase-labeled mF1201 (left panel) or mF1232 (right panel). After washing, the bound labeled Abs were detected by colorimetric reaction. Data are expressed as the percent of total binding of each peroxidase-labeled mAb without the competitor and are the mean of triplicate determinations.

not be denied as another mechanism for Ab-induced GPVI immunodepletion. However, no data exist about a GPVI-specific Ab that does induce GPVI internalization and endocytosis but not GPVI shedding. Most importantly, specific Ab-induced GPVI internalization and endocytosis remains to be completely demonstrated.

With these backgrounds in mind, we aimed to obtain clinically useful mAbs against human GPVI (hGPVI) with characteristics similar to YA's auto-Abs (YA-Abs) and investigated the mechanisms underlying GPVI immunodepletion. In addition to our first presentation of nonactivating hGPVI-specific mAb-induced GPVI deficiency without significant thrombocytopenia (22), the present work reports the establishment of clinically applicable induction of the Ig-D2–specific mAb-mediated GPVI deficiency and also what we believe to be the first clarification of the mechanisms underlying such induction.

Results

Our initial approach was based upon 2 assumptions: (a) the plasma of patient YA contains auto-Abs that downregulate GPVI expression, and (b) mouse mAbs that bind the same epitope(s) on hGPVI will function similarly to YA-Abs. We first analyzed frozen plasma isolated from patient YA for the presence of anti-GPVI auto-Abs using recombinant hGPVI fused to human Fc (hGPVI-Fc) as a target. As seen in Figure 1A, the concentration of anti-GPVI Abs in the plasma of YA was highest in 1988 and decreased more than 100-fold over the course of 15 years, plateauing at 1 to 5 μg/ml after 2002. However, during this time, the patient's platelets remained unresponsive to collagen stimulation due to complete GPVI deficiency (23), and the number of platelets was maintained at normal levels (data not shown).

In order to characterize YA-Abs more in detail, we purified YA-Abs from the patient's plasma stored in 1988 (YA-Abs-88) and in 2003 (YA-Abs-03) using sequential affinity chromatography on protein A–column and recombinant hGPVI-Fc–column and examined cross-reactivities of those YA-Abs with mouse anti-GPVI mAbs for binding to hGPVI-Fc as described in Methods. YA-Abs efficiently competed with several mAbs for binding to hGPVI-Fc, and we selected 2 representative mAbs, mF1201 and mF1232, for further study. These 2 mAbs did not cross-inhibit one another, indicating that they bind different epitopes of hGPVI. As shown in Figure 1B, mF1201 binding to hGPVI-Fc was inhibited by both YA-Abs-88 and YA-Abs-03 (IC_{50}, 58 ng/ml versus 89 ng/ml). In contrast, mF1232 binding to hGPVI-Fc was inhibited by YA-Abs-03 more strongly than YA-Abs-88 (IC_{50}, 45 ng/ml versus 136 ng/ml). These data suggest that YA-Abs-03 likely shares more epitopes with mF1232 than mF1201, despite the relatively low titer.

We next examined the ability of the isolated mAbs to modulate platelet function either through cross-linking–induced activation or by inhibiting collagen-induced platelet aggregation. Treatment of platelets with up to 10 μg/ml of mF1201 (in excess of reported plasma concentrations of YA-Abs isolated after 1997) induced both platelet aggregation as well as expression of the activation marker CD62P in a dose-dependent manner. In contrast to this finding, mF1232 neither induced aggregation nor significant CD62P expression but dose dependently inhibited collagen-induced platelet aggregation (Figure 2, A–C). However, 10 μg/ml mF1232 did not inhibit platelet aggregation in response to collagen-related peptide (CRP), a synthetic peptide that acts as a GPVI-specific agonist (24), adenosine diphosphate (ADP), ristocetin and vWF, or thrombin receptor-agonist peptide (TRAP) (Figure 2C and data not shown).
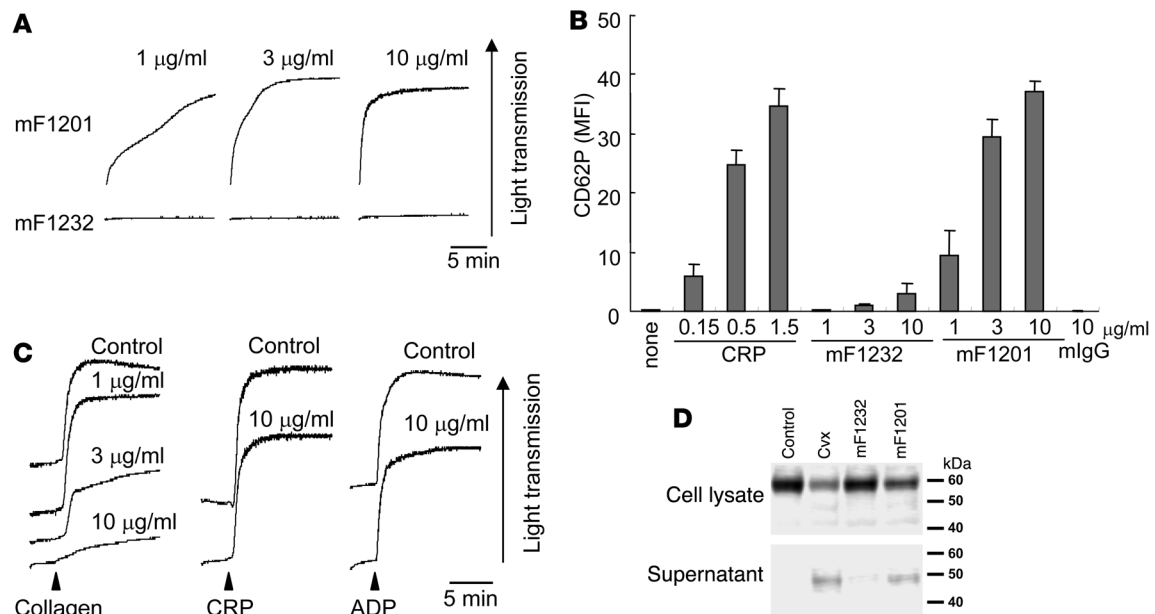
Figure 2
mF1201, but not mF1232, induces both platelet aggregation and shedding of surface GPVI, while mF1232 inhibits collagen-induced, but not CRP- or ADP-induced, platelet aggregation. (**A**) Platelet aggregation was monitored after the addition of the indicated concentrations of mF1201 or mF1232 into human PRP. (**B**) After human PRP was incubated without (none) or with the indicated concentrations of CRP, mF1232, mF1201, or mIgG for 30 minutes at 37°C, the platelets were fixed and stained with PE-labeled anti-human CD62P Ab for flow cytometry analysis. Data represent mean ± SEM of triplicate determinations. MFI, mean fluorescence intensity. (**C**) Human PRP was preincubated with the indicated concentrations of mF1232 for 5 minutes at 37°C and stimulated with 1 μg/ml collagen, 4 μg/ml CRP, or 5 μM ADP as indicated by arrows. Traces are representatives of 2 or 3 separate experiments from different donors. (**D**) Washed human platelets were treated without (control) or with 0.15 μg/ml convulxin (Cvx), 10 μg/ml mF1232, or 10 μg/ml mF1201 for 60 minutes at 37°C, and centrifuged to isolate platelet pellets from supernatants. Platelet pellets (cell lysate) and supernatants were analyzed by western blotting using rabbit anti-GPVI polyclonal Abs. Data are representatives of 3 separate experiments from different donors.

Metalloproteinase-dependent proteolytic shedding is thought to mediate the loss of GPVI expression from platelets exposed to GPVI-specific mAb or ligands (20, 21). Therefore, we examined whether either mF1201 or mF1232 induced GPVI-shedding from the platelet surface. Incubation of washed human platelets with 10 μg/ml mF1201 resulted in the loss of surface GPVI, accompanied by the appearance of its soluble cleavage product, but similar changes were not seen in platelets exposed to mF1232 (Figure 2D).

YA-Abs have been used to probe only hGPVI (6–8, 10, 12, 13, 17, 23, 25), and we tested both mF1201 and mF1232 for their ability to react with GPVI-Fc obtained from different species. We cloned cynomolgus GPVI (cGPVI), and based on amino acid sequence alignment, it is 90% identical to hGPVI. In contrast, mouse GPVI (mGPVI) is only 63% identical to hGPVI over the same region (Figure 3A). Using a competition assay, in which a signal is only obtained if there is no cross reactivity, binding of mF1201 or mF1232 to hGPVI-Fc was not inhibited by mGPVI-Fc (Figure 3, B and C). In contrast, cGPVI-Fc efficiently competed with hGPVI-Fc for binding to either mAb. These data indicate that both generated anti-GPVI mAbs did not recognize mGPVI but cGPVI was bound. To further characterize the epitopes recognized by mF1201 and mF1232, we mutated different regions of hGPVI-Fc to contain the corresponding sequence as indicated in Figure 3A. Using the same competition assay, mutation of loop 9 of the Ig-D2 and mutation of either loop 4 or 5 of the Ig-D1 were found to lose their inhibitory effects on mF1232 and mF1201 binding to hGPVI-Fc, respectively (Figure 3, B and C).

These results were confirmed by a direct binding assay using WT and mutant hGPVI-Fc coated on a plate (Figure 3, D and E). Previous studies identified 2 distinct collagen-binding sites on hGPVI. One binding site includes Lys59 and is recognized by the 10B12 single-chain phage Ab (26). The other collagen-binding site is located in Ig-D1 and includes residues Gly30, Val34, and Leu36, and this site is bound by the 9O12 mAb (27). In our study, either mF1201 or mF1232 binding to hGPVI-Fc was inhibited by the mutant at loop 3, including Gly30, Val34, and Leu36 (Figure 3, B and C). However, since Lys59 is included within loop 5, we also generated a hGPVI-Fc point mutant containing the corresponding mouse residue (K59E) at this location. There was no binding of mF1201 to K59E mutant hGPVI-Fc (data not shown), suggesting that mF1201 recognizes the same collagen-binding site as 10B12 (26).

We next wished to investigate the ability of both mAbs to immunodeplete GPVI in vivo using cynomolgus monkeys as a model. We first confirmed that both mAbs affected cynomolgus platelets in a similar manner to human platelets (data not shown). When either mAb was i.v. injected into monkeys at a dose of 0.3 mg/kg, an amount estimated to be sufficient to saturate the total surface GPVI according to previously reported GPVI expression data (28), expression of both GPVI and FcRγ was decreased within 1 to 2 days, and this was accompanied by platelet unresponsiveness to collagen but not to ADP, ristocetin and vWF, and TRAP (Figure 4, A–C, and data not shown). Additionally, significant thrombocytopenia occurred following injection of mF1201 but not mF1232 (Figure 4D).
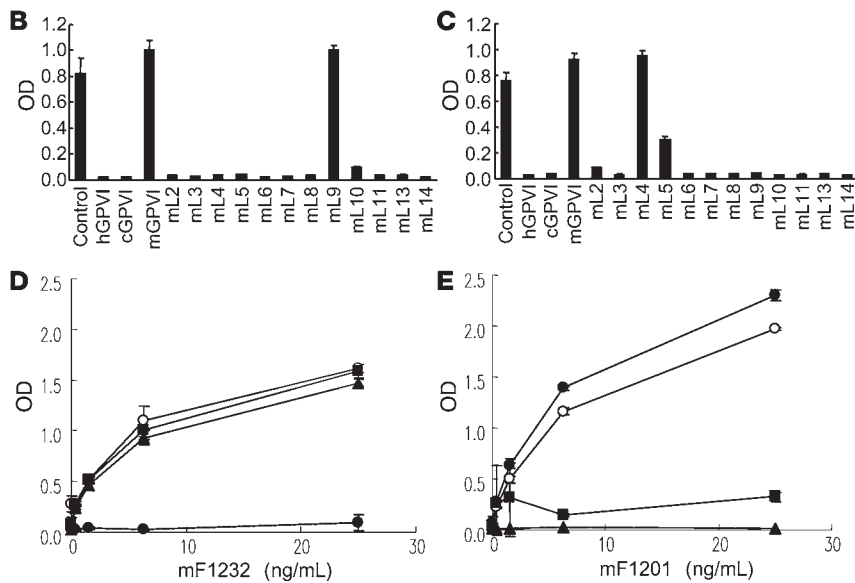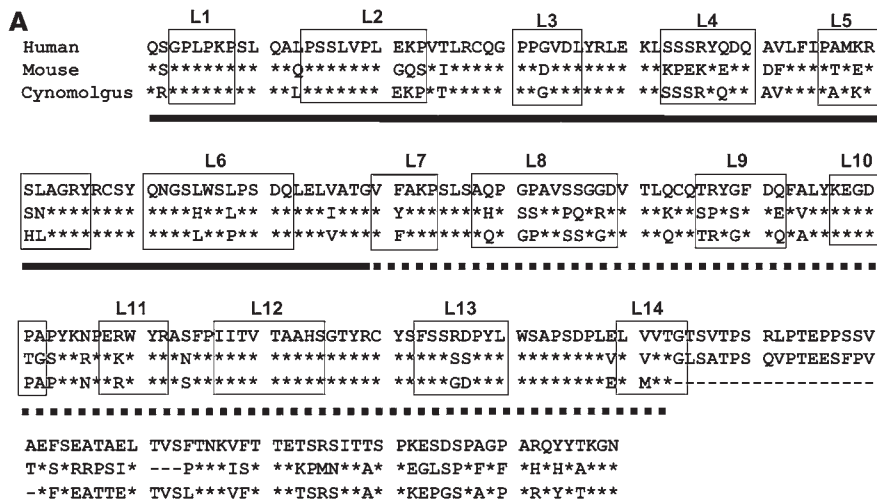
Figure 3
Binding of mF1232 and mF1201 to WT GPVI and to hGPVI mutants. (**A**) Amino acid alignment of the extracellular domains of GPVI from 3 species are shown. Conserved amino acids among the 3 species are indicated by stars. The Ig-D1 and -D2 regions of GPVI are underlined with solid lines and dotted lines, respectively. To construct of the extracellular portion of GPVI, a comparative molecular modeling was carried out with "Homology" (Accelrys Software Inc.) using PDB 1UGN_A and PDB 1P6F_A as a template. The regions boxed with solid lines were defined as the loop structures. The abbreviation over the box denotes each loop number. (**B** and **C**) Fifty ng/ml of peroxidase-labeled mF1232 (**B**) or mF1201 (**C**) was incubated in an hGPVI-Fc–coated immunoplate for 60 minutes at 37°C with 1 μg/ml each of the absorption antigens hGPVI, mGPVI, cGPVI, or human/mouse chimera GPVI mutants. Human/mouse chimera GPVI mutant protein is represented as "mL plus number" meaning that the amino acid sequence of loop number on hGPVI is substituted for the counter loop region of mGPVI. (**D** and **E**) Peroxidase-labeled mF1232 (**D**) or mF1201 (**E**) were incubated with hGPVI-Fc– (open circles), mL9- (filled circles), mL4- (triangles), or mL5-coated (squares) plates. After incubation, the bound peroxidase-labeled mAb was measured. Data are presented as mean ± SD of triplicate determinations.

these 2 reagents (data not shown). Importantly, cF1232 did not induce substantial activation of human platelets such as aggregation and CD62P expression, even at 3 mg/ml, the dose maximally tested here (data not shown).

In order to monitor the behavior of cF1232 after injection into monkeys and binding to platelet surface GPVI, we used CypHer5E, a fluorescent endocytosis probe. This dye is nonfluorescent at neutral pH, but its fluorescence increases exponentially as it passes through the acidic environment of endosomes and lysosomes, with maximum fluorescence at pH 5, the luminal lysosomal pH (29, 30). After CypHer5E-labeled cF1232 was i.v. injected into monkeys, we monitored the fluorescence of CypHer5E in circulating platelets and surface GPVI expression by flow cytometry as well as the expression of GPVI/FcRγ in whole cell lysates by immunoblotting. As shown in Figure 5A, CypHer5E fluorescence in circulating platelets rapidly increased within 1 hour after injection, and this was maintained at a maximum level thereafter. Both surface GPVI (Figure 5B) and total cellular GPVI/FcRγ complex expression (Figure 5C) began to concomitantly decrease, and surface GPVI was reduced by approximately 80% at 6 hours after injection. We wished to confirm that Ab binding and uptake was dependent upon the surface expression of GPVI, and we injected monkeys with unlabeled cF1232, at a dose of 1 mg/kg, to immunodeplete surface GPVI. Five days after this initial injection, monkeys were then treated with CypHer5E-labeled cF1232, and very low levels of fluorescence were seen at 3 hours after injection. This was much lower than the fluorescence seen in nonimmunodepleted monkeys (Figure 5, A and B).

We next used monkey platelet-rich plasma (PRP) to study the behavior of cF1232 and GPVI in vitro. Incubation of PRP with CypHer5E-labeled cF1232 at 37°C led to a time-dependent increase in fluorescence corresponding to Ab endocytosis, and little increase in fluorescence was seen when samples were incubated at 4°C (Figure 6A). When CypHer5E-labeled cF1232 was incubated at 37°C with GPVI-deficient PRP that was obtained from monkeys treated with unlabeled cF1232 for 5 days as described above, CypHer5E fluorescence

Taken together, our data suggest that mF1232 is a more promising therapeutic agent than mF1201, an activating mAb, and we constructed a human/mouse chimera of mF1232 (cF1232) for further study. When we compared cF1232 with mF1232 in assays described in Figures 2, 3, and 4, there were no differences between was not detected in platelets (data not shown), confirming that the in vitro cF1232 internalization and endocytosis was strictly dependent on surface GPVI. However, in contrast to what was seen in vivo, there was no change in surface GPVI expression during incubation at 37°C (Figure 6A).
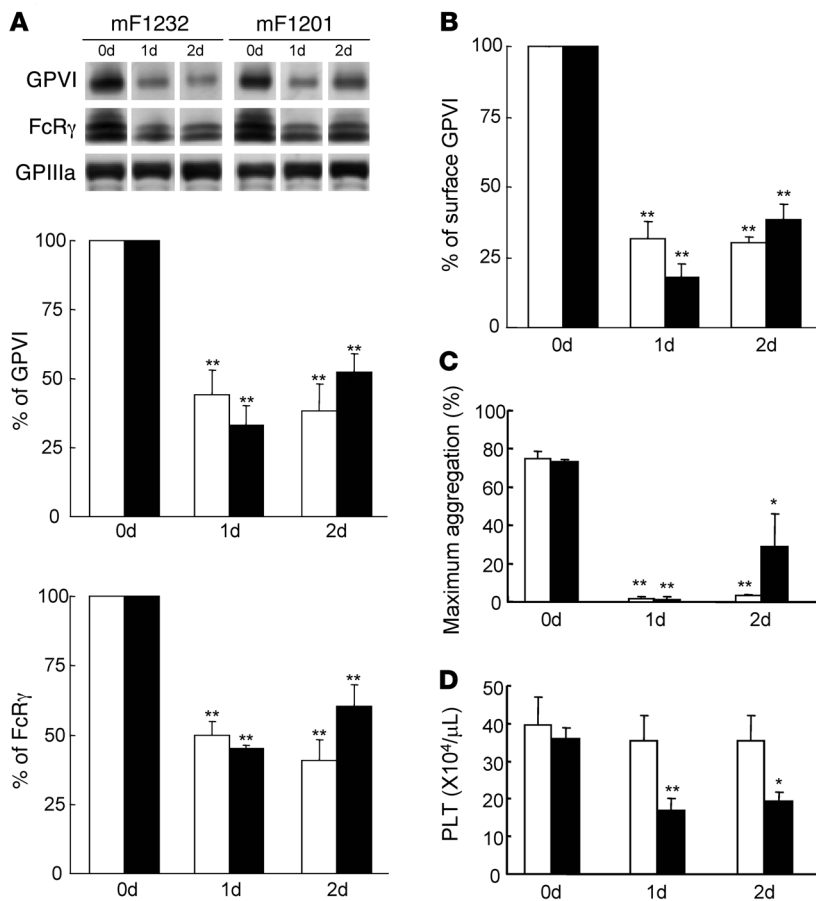
Figure 4
Effects of mF1232 or mF1201 injected into cynomolgus monkeys on GPVI immunodepletion, collagen-induced platelet aggregation, and platelet counts. mF1232 (white bars) or mF1201 (black bars) was i.v. injected at a dose of 0.3 mg/kg into monkeys. Blood was collected on day 0 (0d), day 1 (1d), and day 2 (2d) after injection. The level of GPVI, FcRγ, and GPIIIa in platelet lysates (**A**) and the expression of surface GPVI (**B**) were analyzed by western blotting and by flow cytometry, respectively, using each specific Ab. (**A**) Representative data of western blotting from 3 separate experiments are shown at the top of the panel. The band density of GPVI (middle panel) or FcRγ (bottom panel) was quantified by a Typhoon Scanner 9410 and shown as percentage of that on day 0. (**B**) Mean fluorescence intensity was normalized to 100 for that on day 0. Maximal platelet aggregation was monitored using PRP stimulated with 2 μg/ml collagen (**C**), and platelet (PLT) counts in whole blood (**D**) were determined. Data are presented as mean ± SEM of 3 separate experiments. *$P < 0.05$, **$P < 0.01$ versus day 0.

We next examined whether cF1232 could be internalized and endocytosed in a platelet activation-independent manner, and PRP was pretreated with 3 μM PGE_1, a well-known potent inhibitor of platelet activation that acts by elevating intracellular cAMP. In PGE_1-treated platelets, as cF1232 was endocytosed into platelets, surface GPVI expression was lost in parallel and it decreased to approximately 30% untreated levels after 2 hours of cF1232 treatment (Figure 6B). The same experiment was also performed by replacing cF1232 with mF1201. As shown in Figure 6C, mF1201 was endocytosed and significant decrease in surface GPVI expression was observed as with mF1232. Since we found that mF1201 induced shedding of surface GPVI in the absence of PGE_1 as described in Figure 2D, we performed experiments to compare Ab endocytosis and the loss of surface GPVI between PGE_1-treated and -untreated platelets, and washed monkey platelets were incubated with CypHer5E-labeled or -unlabeled mAbs in the presence or absence of PGE_1 at 37°C (Figure 6, D and E). As shown in Figure 6D, both CypHer5E-labeled mF1232 and mF1201 were endocytosed whether or not PGE_1 was included in the reaction mixtures. As was seen with PRP, there was a significant decrease in surface GPVI expression in washed platelets incubated with CypHer5E-labeled mF1232 in the presence, but not absence, of PGE_1. In contrast, regardless of PGE_1 treatment, surface GPVI expression of washed platelets incubated with CypHer5E-labeled mF1201 was significantly decreased. We also examined the supernatants of washed platelet suspensions incubated with either mAb in the presence or absence of PGE_1. Once again, cleaved GPVI was

detected by immunoblotting in the supernatants of the samples incubated with mF1201, but not with mF1232, and PGE_1 did not significantly affect this (Figure 6E).

We wished to measure an increased amount of soluble GPVI in the plasma from monkeys after mF1232 or mF1201 treatment. For this purpose, we developed a sandwich ELISA assay that could quantitatively detect injected mAb-bound and -unbound soluble GPVI. The increased amounts of soluble GPVI in the plasma from monkeys after mF1232 or mF1201 treatment at a dose of 0.3 mg/kg were assessed as described in the Methods and compared between 2 groups. As shown in Figure 7, most of plasma samples from monkeys after mF1201 treatment revealed detectable increase in soluble GPVI content above the limit of ELISA sensitivity until 4 hours after treatment but not thereafter, and the highest levels of increase in soluble GPVI content were observed 1 hour after treatment. In contrast, little increase in soluble GPVI content over the limit of sensitivity was observed in the plasma from monkeys over 24 hours after mF1232 treatment. Statistical analyses revealed that soluble GPVI content in the plasma significantly increased 1 hour after mF1201, but not mF1232, treatment and that the difference of increased amounts of soluble GPVI between those 2 groups was significant. Interestingly, surface GPVI expression levels on platelets from mF1201-treated monkeys appeared to decrease a little more rapidly than those from mF1232-treated monkeys, but those levels did not differ significantly from each other 24 hours after treatment, confirming the finding shown in Figure 4B (Figure 7, top left panel). Furthermore, although the
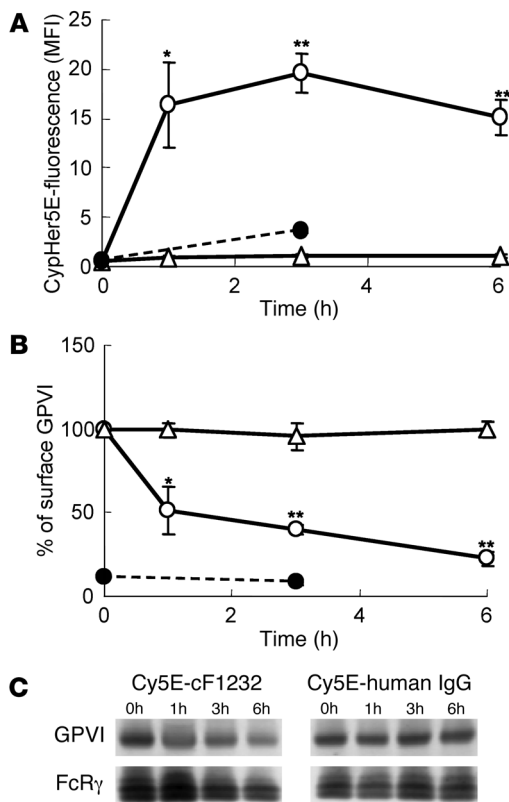
Figure 5
CypHer5E-labeled cF1232 injected into cynomolgus monkeys is endocytosed into circulating platelets correlating with GPVI depletion. CypHer5E-labeled cF1232 or human IgG was i.v. injected at a dose of 1 mg/kg into cynomolgus monkeys. The fluorescence intensity (**A**) in circulating platelets and platelet surface GPVI expression (**B**) at the indicated time points after injection of CypHer-5E–labeled cF1232 (open circles) or human IgG (open triangles) were analyzed by flow cytometry. The levels of surface GPVI expression are normalized to 100 for day 0. The filled circles with dotted lines represent monkeys pretreated with 1 mg/kg unlabeled cF1232. GPVI and FcRγ expression in circulating platelets at the indicated time points after injection were analyzed by western blotting (**C**). All data are presented as mean ± SEM of 3 separate experiments. *$P < 0.05$, **$P < 0.01$ versus control groups at the same time points.

maximal plasma concentration of either injected mAb was less than 10 μg/ml, plasma concentrations of mF1201 were found to be lower than those of mF1232 at the indicated time points after injection, suggesting more rapid clearance of mF1201-forming immune complexes with activated platelets/microaggregates or soluble GPVI (Figure 7, top right panel).

In patient YA, sustained anti-GPVI Ab concentrations around 1 μg/ml were sufficient to maintain decreased GPVI surface expression, and we wished to maintain the same concentration of cF1232. Monkeys were injected with a single dose of cF1232 of 1 mg/kg either i.v. or s.c., and we followed the plasma Ab concentration over time (Figure 8A). Subcutaneous injection of cF1232 led to a sustained plasma concentration over 1 μg/ml for more than 3 days, but i.v. injection achieved this concentration for less than 2 days. Platelets isolated from injected monkeys lost surface GPVI expression within the first day, and this decrease was maintained for 7 to 10 days without significant thrombocytopenia and accompanied unresponsiveness to collagen but not ADP stimulation (Figure 8B and data not shown).

Discussion

The first patient with platelet GPVI deficiency has been followed up by our department for more than 20 years and has platelets still lacking GPVI, without thrombocytopenia, or any signs of bleeding. Although the patient has been suffering from chronic atrial fibrillation for the last 7 years, she has not received any antithrombotic medications for fear of inducing bleeding events. Additionally, she denies any signs or symptoms of thrombosis in the brain and heart (unpublished observations). The patient's serum IgG, isolated and described in our original report, has been an invaluable tool for

both the immunological detection of GPVI and studies of GPVI function. However, serum isolated more recently is much less reactive and of limited utility. These clinical and laboratory observations hinted to us that the low titer YA-Abs have generated a long-term state of GPVI immunodepletion without serious episodes of bleeding or thrombosis. Then, we characterized YA-Abs over 15 years to identify conditions compatible with long-term GPVI depletion. The concentration of anti-GPVI YA-Abs-03 was less than 10 μg/ml and 100-fold lower than comparable YA-Abs-88, suggesting that YA-Abs-03 might contain anti-GPVI Ab immunodepleting the target in low titer without causing thrombocytopenia.

We generated mouse anti-hGPVI mAbs with the hope of replicating the performance characteristics of YA-Abs. Indeed, 2 representative mAbs, mF1201 and mF1232, were effectively prevented from binding to hGPVI-Fc by YA-Abs. These mAbs did not cross-inhibit one another, and both recognized distinct epitopes on hGPVI. These data also confirm the oligoclonal nature of YA-Abs.

Upon further characterization of mF1201 and mF1232, we found striking functional differences between these mAbs tested at the concentrations similar to those of anti-GPVI Abs in the recent plasma of YA, namely at less than 10 μg/ml. Treatment of platelets with mF1201 alone resulted in platelet aggregation, activation, and the shedding of surface GPVI. GPVI shedding is thought to be mediated by metalloproteinase-dependent proteolysis, and this may occur in an activation-dependent manner (20, 21). In contrast, mF1232 induced neither significant platelet activation such as aggregation and CD62P expression nor the shedding of surface GPVI.

When we mapped the binding sites of mF1201 and of mF1232 using a systematic mutagenesis approach, we demonstrated that mF1201 recognized a previously reported collagen-binding site on hGPVI (26). In contrast, mF1232 recognized what we believe to be a novel epitope on loop 9 (Thr116-Gln122) of the Ig-D2 of hGPVI. It is interesting to consider the contrasting functional data for these 2 mAbs in the context of their binding sites. Treatment of platelets with collagen induces aggregation and activation, and it is likely that the agonistic nature of mF1201 leads to cross-linking of the collagen-binding sites of GPVI. In contrast, mF1232 inhibits collagen-induced aggregation but does not affect CRP-induced aggregation. Interestingly, Dumont et al. reported that Ig-D2 plays a specific and significant role in GPVI binding to collagen but CRP does not. Additionally, Horii et al. determined the crystal structure of the collagen-binding domain of hGPVI and suggested that Ig-D2 of the 2 GPVI molecules formed a parallel, back-to-back dimer on the platelet membrane, matching the dimensions of a collagen fiber
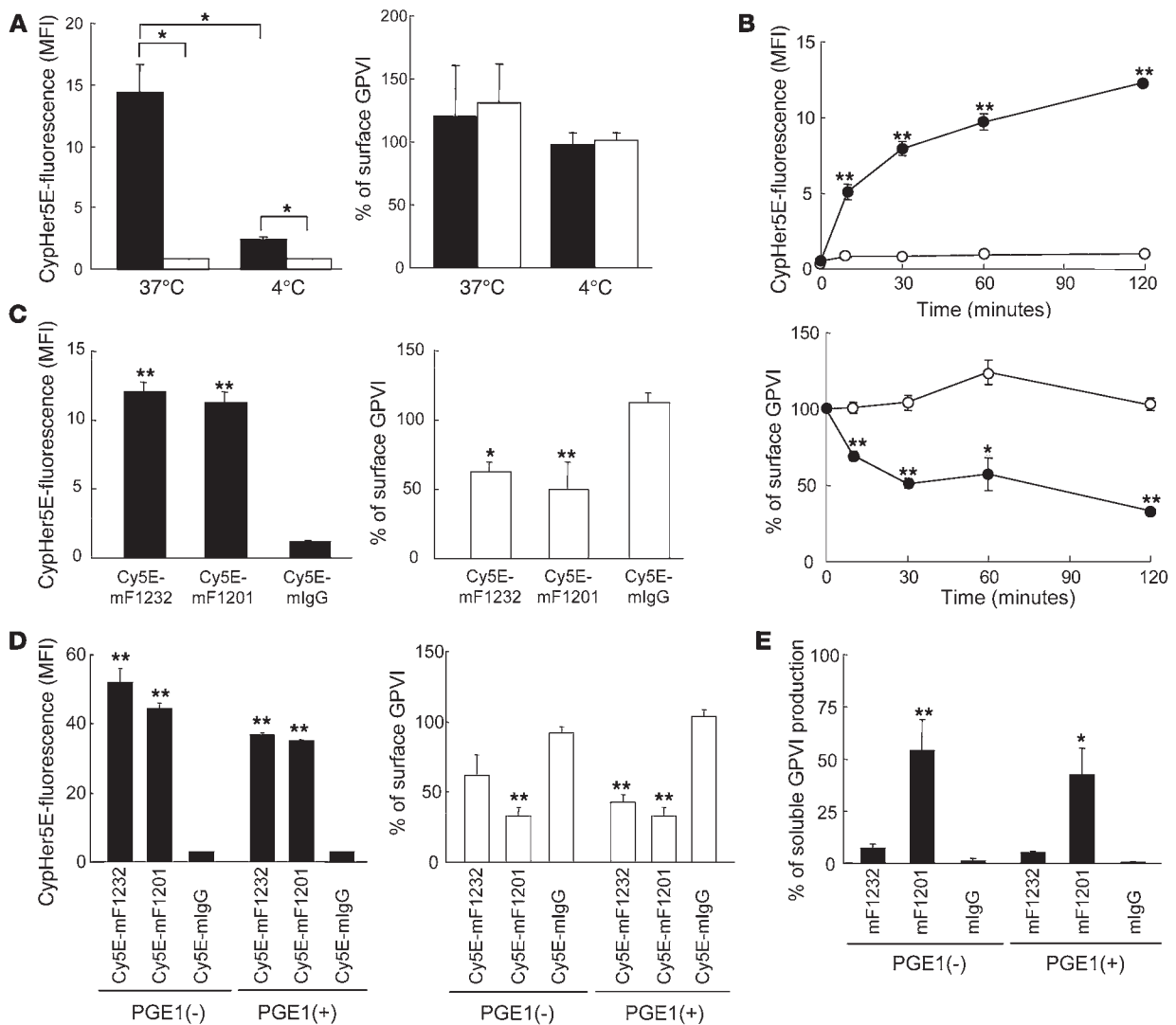
Figure 6
In vitro internalization and endocytosis of GPVI-specific Abs with or without GPVI immunodepletion under various experimental conditions. Cynomolgus PRP was incubated with 10 μg/ml CypHer5E-labeled (Cy5E-labeled) Ab in vitro under various experimental conditions as follows: 60 minutes incubation with cF1232 (black bars) or human IgG (white bars) at 37°C or 4°C (**A**), incubation with cF1232 (filled circles) or human IgG (open circles) for up to 120 minutes at 37°C in the presence of 3 μM PGE$_1$ (**B**), 60 minutes incubation with mF1232, mF1201, or mouse IgG (mIgG) at 37°C in the presence of 3 μM PGE$_1$ (**C**). Washed cynomolgus platelets were treated with the indicated Abs in the absence or presence of 3 μM PGE$_1$ for 60 minutes at 37°C (**D**). (**A–D**) The fluorescence in platelets (left panels) and surface GPVI expression (right panels) were analyzed by flow cytometry. Surface GPVI expression before incubation was normalized to 100. (**E**) Washed cynomolgus platelets were treated with 0.15 μg/ml convulxin, 10 μg/ml mF1232, 10 μg/ml mF1232, or 10 μg/ml mouse IgG for 60 minutes at 37°C in the absence or presence of PGE$_1$ and sedimented to separate supernatants. The supernatants were analyzed by western blotting with rabbit anti-human GPVI polyclonal Ab. The band density corresponding to soluble GPVI was quantified by a Typhoon Scanner 9410, and the value for convulxin treatment in the absence of PGE$_1$ was normalized to 100. All results represent mean ± SEM of 3 (**A**, **D**, and **E**) or 4 (**B** and **C**) separate experiments. *$P < 0.05$, **$P < 0.01$ versus control groups.

(32). Taken together with these reports, it is tempting to speculate the following mechanisms by which mF1232 binding to loop 9 of Ig-D2 interferes with collagen-mediated, but not CRP-mediated, platelet interaction. Loop 9 of Ig-D2 might contain a heretofore undefined collagen-binding site but not a CRP-binding site. If the site of mF1232 binding in loop 9 does not participate in the back-to-back dimer formation described by Horii et al. (32), mF1232 binding to GPVI might be able to disrupt the adoption of the appropriate dimer structure. Because CRP binds monomeric GPVI, its ability

to induce platelet activation might be unaffected by mF1232. These reports and our present findings suggest a novel series of experiments to better clarify the mechanism of collagen and CRP binding to GPVI that should be undertaken in the future.

The generation of 2 mAbs that bind GPVI with distinct effects on platelet activation allowed us to further investigate the mechanisms underlying Ab-induced GPVI immunodepletion using cynomolgus monkeys as a model system. First, we showed that both mAbs bound cGPVI, a protein highly homologous to hGPVI,
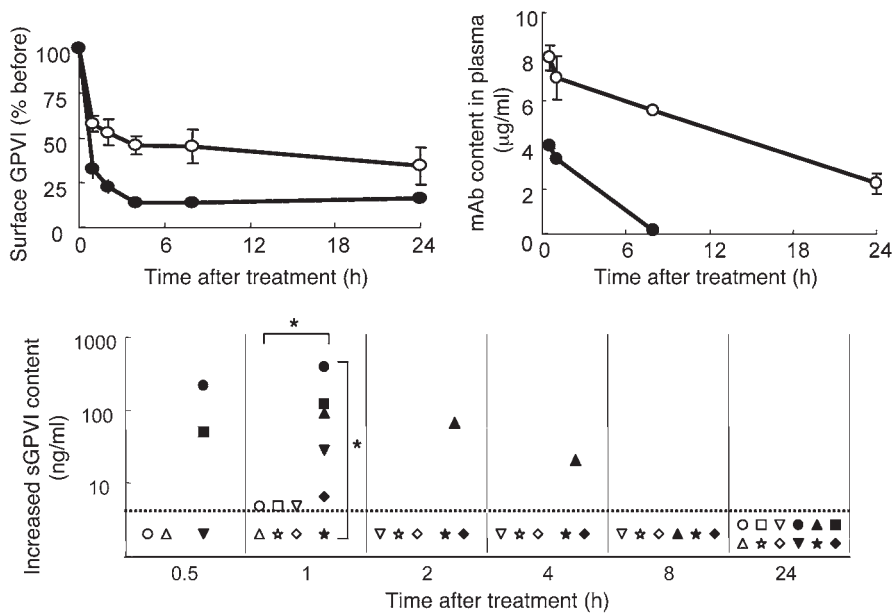
Figure 7
Comparison of increased amounts of soluble GPVI in the plasma from monkeys after F1232 or F1201 treatment. mF1232 (open symbols) or mF1201 (filled symbols) was i.v. injected at a dose of 0.3 mg/kg into monkeys. Blood was collected before injection and at the indicated time points after injection. The increased amounts of soluble GPVI in the plasma at the indicated time points after each treatment (bottom panel), the expression of surface GPVI (top left panel), and plasma concentrations of each mAb (top right panel) were assessed as described in the Methods and compared between 2 groups. The number of tested monkeys ranged from 2 to 6 at each time point. Data are expressed as the amount of cGPVI-Fc, a standard, or as mean ± SEM. The dotted bar represents the limit of sensitivity of the assay (4.9 ng/ml). *$P < 0.05$ versus 0 ng/ml or mF1232 versus mF1201.

and they affected platelet function in monkeys in a manner similar to that seen for humans. Notably, both mAbs induced GPVI immunodepletion when i.v. injected into monkeys at a low dose of 0.3 mg/kg. However, injection of mF1201 caused significant thrombocytopenia that might reflect shortened platelet life span. Most of previous studies provided evidence for the metalloproteinase-dependent cleavage of surface GPVI both in vitro and in vivo (20, 21, 33, 34). We clearly showed that 10 μg/ml mF1201, but not mF1232, induced shedding of surface GPVI in vitro, and it is likely that mF1201-induced shedding is at least partly responsible for the reductions in GPVI surface expression seen in vivo. However, there was no shedding seen in vitro following mF1232 treatment, but GPVI was rapidly lost from the platelet surface following mF1232 injection. This suggested that intracellular degradation might be responsible for reductions in GPVI expression following mF1232 treatment.

We wished to obtain direct evidence for the internalization and endocytosis of GPVI using a fluorescent endocytosis probe, CypHer5E, which becomes fluorescent in the acidic environment of intracellular organelles such as endosomes and lysosomes (29, 30). We also used a chimeric Ab based on mF1232 (cF1232) for these studies. The behavior of cF1232 toward both human and cynomolgus platelets was the same as mF1232. Importantly, cF1232 did not activate human platelets in vitro, even at a maximal test concentration of 3 mg/ml. Therefore, it is very likely that

cF1232 binding to loop 9 of Ig-D2 on GPVI does not result in GPVI-mediated activation of human platelets, not only in vitro but also when injecting cF1232 into humans. When CypHer5E-labeled cF1232 was injected into monkeys, there was a dramatic GPVI-dependent increase in fluorescence in circulating platelets and there was a corresponding decrease in surface GPVI expression. These data indicate that injected cF1232 bound to surface GPVI is internalized and endocytosed into circulating platelets. A similar surface GPVI-dependent endocytosis of cF1232 was also observed when platelets were incubated with CypHer5E-labeled cF1232 at 37°C but not 4°C in vitro. However, in contrast to the results seen in vivo, there was sustained GPVI expression at the platelet surface. These results suggest that in vitro GPVI was internalized and rapidly recycled back to the platelet surface without degradation in order to maintain surface GPVI level, but an unknown mechanism was operating to downregulate surface GPVI level in vivo.

The solution came from the experiment to see whether in vitro internalization and endocytosis of CypHer5E-labeled cF1232 into platelets could occur when a cAMP-elevating agent, PGE₁, was included in the reaction mixture. In the presence of PGE₁, cF1232 was endocytosed, and there was a corresponding reduction in surface GPVI that was not shed in vitro. In vivo, the vasculature produces prostacyclin, a potent endogenous inhibitor of platelet activation that increases intracellular cAMP levels analogous to PGE₁. It is very likely that prostacyclin constitutively released from the vas-

Figure 8
Pharmacokinetics and long-term antiplatelet effects after cF1232-treatment. (**A**) cF1232 was i.v. (filled circles) or s.c. (open circles) injected at a dose of 1 mg/kg into cynomolgus monkeys, and plasma concentrations of cF1232 were measured by ELISA. (**B**) After s.c. injection of cF1232, maximal platelet aggregation in response to 2 μg/ml collagen (filled circles) or 20 μM ADP (filled squares) was periodically monitored. The aggregation rate was normalized to 100% for those on day 0. The experiments were carried out with 3 monkeys and the data represent mean ± SEM.
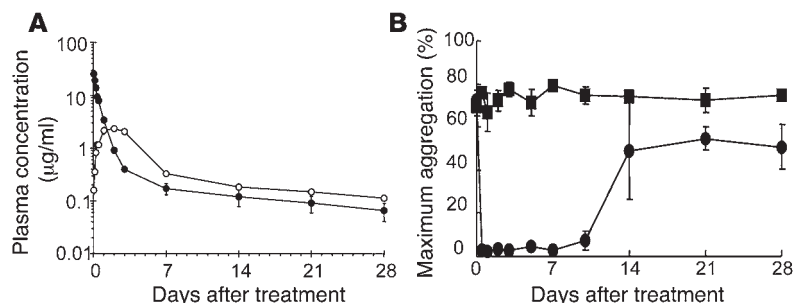
culature is essential for the observed downregulation of GPVI seen in vivo, and we must recreate this in vivo effect to observe a similar phenomena in vitro. Importantly, we found a statistically significant increase in plasma concentrations of soluble GPVI in mF1201-treated, but not mF1232-treated, monkeys and also a significant difference of increase in those between 2 groups. Taken together, this is what we believe to be the first direct evidence demonstrating specific Ab-induced GPVI internalization and endocytosis both in vitro and in vivo, and it resolves the questions regarding acquired GPVI deficiency caused by specific auto-Abs (12, 19). Interestingly, mF1201-induced proteolytic shedding occurred even in the presence of PGE$_1$, indicating that the elevation of intracellular cAMP cannot completely inhibit the proteolytic shedding. Nevertheless, mF1201 was endocytosed to the same extent as cF1232 or mF1232 during incubation at 37°C in vitro, suggesting that mF1201 could deplete surface GPVI through both proteolytic shedding and endocytosis. With regard to receptor internalization, Nieswandt et al. showed that decreased surface GPVI expression preceded reductions in GPVI in whole platelet lysates within 24 hours after injection of the anti-mGPVI mAb JAQ-1 into WT mice (14). However, the presence of a large, internal pool of GPVI has been proven (23) and could explain their findings. Indeed, they confirmed that JAQ-1 caused proteolytic shedding of surface GPVI that was associated with the occurrence of thrombocytopenia (34). In contrast to what we directly demonstrated here using CypHer5E-labeled mAbs and WT platelets or monkeys both in vivo and in vitro, they used biotin-labeled JAQ-1 and mutant mice lacking GPVI downstream signaling molecules to show JAQ-1–induced GPVI internalization in vivo but not in vitro (34). Without using such mutant animals, we definitely showed that the Ig-D2–specific mAbs were endocytosed into platelets irrespective of GPVI downregulation in a surface GPVI-dependent manner but caused GPVI internalization and endocytosis without significant GPVI shedding both in vivo and in vitro when platelets were also exposed to a cAMP-elevating agent. Taken together with our previous report that GPVI downstream signals are cAMP-insensitive (7), the mechanisms underlying specific Ab-induced depletion of surface GPVI through internalization and endocytosis could not be simply explained by GPVI downstream signals themselves. It was of great interest that GPVI immunodepletion could be safely induced without thrombocytopenia following injection of mF1232 into WT monkeys (22). In view of the recent report that JAQ-1 treatment caused significant thrombocytopenia in WT mice but did not in the mutant mice, revealing no shedding of surface GPVI (34), it is noteworthy that injection of mAbs recognizing what we believe to be a novel epitope on Ig-D2 of hGPVI did not cause significant thrombocytopenia in WT monkeys as shown here. Clearly, anti-GPVI mAbs such as mF1201 that strongly cause thrombocytopenia are unsuitable for clinical application.

In a clinical application of cF1232, its injection via s.c. route could be more practical and useful than that via i.v. route to maintain a longer effect of GPVI immunodepletion. In fact, we found that a single s.c. injection of cF1232 into monkeys led to sustained low titer plasma concentrations and long-term antiplatelet effects due to GPVI immunodepletion without significant thrombocytopenia. Present work warrants further studies to emphasize the therapeutic promise of the cF1232 initially characterized here.

Methods

Reagents. CRP was synthesized as described previously (35). cDNA of hGPVI was prepared as described previously (5). Rabbit anti-GPVI polyclonal Ab was produced by immunizing rabbits with a synthetic peptide corresponding to amino acids Glu148-Arg162 of hGPVI. CypHer5E mono NHS ester and R-PE Labeling Kit-NH$_2$ were purchased from GE Healthcare and Dojindo Laboratories, respectively. Conjugation of Abs with CypHer5E or PE was performed according to the manufacturer's instructions. All other reagents were obtained as previously reported (6–8, 10, 17, 23, 25).

Recombinant GPVI proteins and mutant proteins. The cDNA encoding the extracellular region of hGPVI (Met-1 of the signal peptide to Asn-269) and the Fc regions of mouse IgG$_{2a}$ or human IgG$_1$ H chain were cloned by genomic PCR, and these fragments were inserted to downstream of the human elongation promoter in the pEF-expression vector (36). An expression plasmid encoding a mutant-hGPVI protein was constructed using site-specific mutagenesis by overlap extension with mGPVI as a template (37). The cDNA encoding cGPVI was cloned by PCR using primer pairs based on the hGPVI cDNA and cynomolgus monkey genomic DNA as a template. The cDNA encoding the extracellular region of mGPVI was cloned by genomic PCR using primer pairs based on the mGPVI cDNA sequence submitted to GENBANK with accession number XM145298. The cDNA encoding mutated GPVI was cloned into the pEF-expression vector to allow fusion with the human Fc region. COS-1 or CHO cells were transfected with expression plasmids using FuGENE6 (Roche) according to the manufacturer's instructions and cultured in serum-free medium in 5% CO$_2$ at 37°C. Recombinant GPVI proteins fused to human Fc or mouse Fc regions were purified from culture supernatants by protein A–Sepharose affinity chromatography (Prosep-A; Millipore). The extracellular domain of cGPVI or mGPVI was also fused to the human Fc region and each fusion protein was prepared as described above. The nucleotide sequence of cGPVI has been submitted to the DDBJ/EMBL/GenBank databases with accession number AB292416 (http://www.ddbj.nig.ac.jp).

Generation of mouse anti-GPVI mAbs. Female ddY mice (Japan SLC Inc.) at 8 weeks of age were primed and boosted at 11-day intervals with 20 μg of the recombinant extracellular domain of hGPVI fused to the Fc region of mouse IgG$_{2a}$ with adjuvant. Three days later, lymphocytes were harvested from lymph nodes and fused with mouse myeloma cells (P3U1; ATCC) by the polyethylene glycol method. Hybridomas secreting anti-GPVI mAbs were identified by ELISA using hGPVI-Fc–coated plates. Mouse anti-GPVI mAbs were purified by Prosep-A column. In some experiments, those mAbs were conjugated with peroxidase by a periodate method (38).

Construction of human/mouse chimera anti-GPVI mAb. Single-stranded cDNAs were prepared from the hybridoma secreting mouse anti-GPVI mAb using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen). The Ig-variable region was amplified by a PCR using the obtained single-stranded cDNA as a template and Mouse Ig-Primer Set (Merck). The extreme 3′-primer was designed with a restriction site allowing fusion with human H chain Cγ4 cDNA. Using these primers, PCR was performed, amplifying both the H- and L-chain variable regions followed by subcloning into the pT7BlueT vector (Merck). The gene fragment encoding H chain ligated to Cg4 cDNA and encoding L chain were introduced into appropriate pEF-expression plasmids. COS-1 cells or CHO cells were transfected with the expression plasmids using FuGENE6, and cells were cultured in serum-free medium in 5% CO$_2$ at 37°C. Human/mouse chimera anti-GPVI mAb was purified from the supernatant by protein A–column affinity chromatography.

Quantification of anti-GPVI Abs in the patient plasma. hGPVI-Fc was biotinylated with D-biotinoyl-ε-aminocaproic acid-*N*-hydroxysuccinimide ester (Roche) according to the manufacturer's instructions. Sample plasma was obtained from patient YA and stored at –70°C during our previously reported studies (6–8, 10, 17, 23). Fifty microliters of appropriately diluted plasma or control solutions, containing known amounts of a human/mouse chimera anti-GPVI mAb, were incubated with 50 μl of biotinylated

hGPVI-Fc (2 μg/ml) for 2 hours at 37°C. The mixture was then applied to a 96-well plate (Nunc-Immuno Module C8 Maxisorp; Nunc) coated with streptavidin (10 μg/ml). After 20-minutes incubation at 37°C, the wells were washed 5 times with saline containing 0.05% Tween 20. The bound Abs were colorimetrically quantified using peroxidase-labeled anti-human kappa and lambda chain Abs (Dako) and tetramethyl benzidine (TMB) (TMB 1 Component HRP Microwell Substrate; BioFX Laboratories Inc.).

Cross-reactivities of YA-Abs with mouse anti-GPVI mAbs for the binding to hGPVI-Fc. Sample plasma from patient YA was applied on a Prosep-A column equilibrated with D-PBS. The IgG fraction was eluted with 0.1 M Glycine-HCl (pH 3.0) and neutralized in 1 M Tris-HCl (pH 8.5), and this was subsequently applied onto the hGPVI-Fc column prepared with formyl-cellulofine (Seikagaku Corporation) according to the manufacturer's instructions. Anti-GPVI Abs were eluted with 10 mM HCl and neutralized with 10x D-PBS. The concentration of anti-GPVI Abs was determined by ELISA as described above.

Fifty microliters of the serially diluted anti-GPVI Abs was applied to a 96-well plate (Nunc-Immuno Module C8 Maxisorp; Nunc) coated with 0.5 μg/ml hGPVI-Fc and incubated overnight at 4°C. Fifty microliters of a peroxidase-labeled anti-GPVI mAb (50 ng/ml) was then added to each well and incubated for 45 minutes at 37°C. The bound peroxidase-labeled mAbs were colorimetrically quantified by reaction with TMB substrate.

Binding of anti-GPVI mAbs to WT and mutant GPVI-Fc. Two types of ELISA were conducted. First, 25 μl of 50 ng/ml peroxidase-labeled anti-GPVI mAb and 1 μg/ml WT, mutant GPVI-Fc, or a control buffer were added to wells of a 96-well plate coated with hGPVI-Fc and incubated for 1 hour at 37°C. After incubation, the bound peroxidase-labeled mAbs were quantified by ELISA. Additionally, serially diluted anti-GPVI mAbs were added to a 96-well plate coated with WT or mutant GPVI-Fc. After 1 hour incubation at 37°C, the bound mAbs were colorimetrically quantified by reaction with peroxidase-labeled anti-mouse Igs (Dako) and TMB substrate.

Blood sample preparation. Venous blood was collected from the patient and healthy volunteers after obtaining informed consent, and blood was collected from cynomolgus monkeys after approval by the ethics committees at Kyoto University and Mochida Pharmaceutical Co. Ltd. Anticoagulation of blood with 0.1 vol of 3.8% sodium citrate and preparation of PRP, platelet-poor plasma, or washed platelets were performed as described previously (12, 39).

Platelet aggregation. The ability of anti-GPVI mAbs to activate platelets was tested by incubating PRP (3×10^5 platelets/μl) with 1–10 μg/ml mAbs under stirring conditions at 208 g in an aggregometer (MCM Hema Tracer 313M; MC Medical). The ability of anti-GPVI mAbs to inhibit platelet activation was examined by preincubating PRP with or without 10 μg/ml mAbs for 8 minutes followed by stimulation with either 1 μg/ml collagen, 4 μg/ml CRP, 5 μM ADP, 1.5 mg/ml ristocetin, or 50 μM TRAP. Maximal platelet aggregation was monitored over 12 minutes.

In vitro CD62P expression assay. PRP (3×10^5 platelets/μl) was incubated with 1–10 μg/ml anti-GPVI mAbs or 1.5 μg/ml CRP as a positive control for 30 minutes at 37°C. Incubated samples were fixed with 1% paraformaldehyde for 30 minutes at 4°C and washed 2 times with D-PBS washing buffer (D-PBS, 2.5% heat inactivated FBS, 2.5 mM EDTA). CD62P expression on platelets was determined by flow cytometry.

In vitro shedding assay. In vitro shedding assay was performed as described previously with slight modifications (20, 21). Washed platelets (3×10^8 platelets/μl), suspended in Tyrode-HEPES buffer (137 mM NaCl, 2.7 mM KCl, 3 mM NaH$_2$PO$_4$, 10 mM HEPES, 5.5 mM glucose, pH 7.4), were pretreated with or without 3 μM PGE$_1$ for 10 minutes at 37°C before incubation with anti-GPVI mAbs or convulxin (0.15 μg/ml) as a positive control for 60 minutes at 37°C. The reaction was stopped by the addition of one-fifth vol of 25 mM EDTA, containing 6× complete protease inhibitor cocktail

(Complete, EDTA free; Roche). Platelets were sedimented by centrifugation at 15,000 × g for 1 minute to separate the platelet pellets from supernatant. The platelet pellets and supernatant were analyzed by western blotting.

In vivo experiments. All experiments using cynomolgus monkeys were approved by the animal ethics committee at Mochida Pharmaceutical Co. Ltd. Anti-GPVI mAbs, CypHer5E-labeled or -unlabeled anti-GPVI mAbs, or human IgG were i.v. injected into male monkeys (2.5~4.5 kg, from HAMRI Co. Ltd.). Citrated blood was collected at time periods indicated in the legends for Figures 4, 5, 7, and 8, and the platelet count was determined by a hematology analyzer (F-820; Sysmex).

Assessment of the increased amounts of soluble GPVI in the plasma from monkeys after anti-GPVI mAb treatment. Either the plasma from mF1232-treated monkeys or control plasma containing known amounts of cGPVI-Fc as a standard was mixed with 50 μg/ml mF1232 and diluted 5-fold with PBS containing 0.05% Tween20 and 0.1% BSA. These mixtures were transferred to a 96-well plate coated with a human/mouse chimera anti-hGPVI mAb, cF1232-10-1, that did not cross-react with mF1232. After incubation overnight at 4°C, peroxidase-labeled anti-mouse IgG Fc Ab (Bethyl) was added, followed by incubation for 90 minutes at 37°C. The bound was colorimetrically detected by a reaction with TMB substrate. When dealing with the plasma from mF1201-treated monkeys, the same procedure was also performed by replacing mF1232 and cF1232-10-1 with mF1201 and cF1232-18-3, respectively. Since the plasma before treatment was equivalent to control plasma with respect to undetectable soluble GPVI content and both were diluted 5-fold for the assay, potential trace amounts of soluble GPVI in those plasmas, if any, were considered almost equally negligible. Therefore, the levels of soluble GPVI content in both the plasma before treatment and control plasma were assumed to be 0 ng/ml, and the limit of sensitivity of the assay for nondiluted plasma samples was determined to be 4.9 ng/ml of cGPVI-Fc. In order to assess increased amounts of soluble GPVI in the plasma after treatment, optical densities of diluted plasma before treatment and control plasma were subtracted from the values of diluted plasma after treatment and control plasma containing cGPVI-Fc, respectively.

Measurement of plasma concentrations of anti-GPVI mAb. Appropriately diluted plasma or control plasma containing known amounts of anti-GPVI mAb was added to a 96-well plate coated with hGPVI-Fc. After 1 hour incubation at 37°C, the bound mAbs were colorimetrically quantified by the reaction with peroxidase-labeled anti-mouse or -human kappa (Dako) and TMB substrate.

In vitro internalization and endocytosis assay. Washed platelets or PRP were pretreated with or without 3 μM PGE$_1$ for 10 minutes at 37°C before incubation with 10 μg/ml CypHer5E-labeled mAbs at 37°C or on ice for the time periods indicated in the legend for Figure 6. After incubation, surface GPVI expression and the fluorescence derived from the endocytosed CypHer5E were determined by flow cytometry.

Western blot analysis. The platelet pellets, the supernatants, or the washed platelet suspensions, prepared as described above, were lysed in the SDS sample buffer containing 2-mercaptoethanol and boiled for 5 minutes. Samples were subjected to SDS-PAGE using 5%–20% gradient Tris-HCl gels (ATTO), electrotransferred to PVDF membranes (Millipore), and immunoblotted with rabbit anti-human GPIIIa polyclonal Abs (Santa Cruz) or anti-GPVI polyclonal Abs. For the detection of FcRγ, samples were subjected to SDS-PAGE using 10%–20% gradient tricine gels (Invitrogen) and immunoblotted with rabbit anti-human FcεRI γ-subunit polyclonal Abs (Millipore). All blots were probed with HRP-conjugated goat anti-rabbit Abs (Dako), developed with ECL-Plus (GE Healthcare), and imaged using a Typhoon Scanner 9410 (GE Healthcare). The band density was quantified by the analysis software ImageQuant 5.2 (GE Healthcare).

Flow cytometry. Washed platelet suspensions or PRP were diluted to a final concentration of 1×10^4 or 3×10^4 platelets/μl with D-PBS washing buffer.

Diluted samples were then divided into 2 parts. The first part was diluted more than 25-fold with HEPES buffer (pH 8.0) containing 2.5 mM EDTA and analyzed in a CYTOMICS FC500 flow cytometer (Beckman Coulter) for quantification of the endocytosed CypHer5E. The second part was incubated for 30 minutes on ice with saturating amounts of a PE-conjugated anti-GPVI mAb, which showed no competition with an anti-GPVI mAb present in the sample or an isotype-matched negative control Ab, diluted, and analyzed in a flow cytometer for quantification of platelet surface GPVI. Quantification of platelet CD62P was performed with a PE-conjugated anti-human CD62P Ab (Beckman Coulter) under similar conditions as described above. In some experiments, fluorescence intensities were expressed as mean fluorescence intensity (MFI), from which the value of MFI of control was subtracted. All data were analyzed with FlowJo software (Tree Star Inc.).

Statistics. Comparisons between 2 groups and also those among more than 2 groups were made with a Student's unpaired or paired t test and with a Dunnett test, respectively. When analyzing the data, including the values below the limit of sensitivity of the assay, the appropriate nonparametric (Wilcoxon, Mann-Whitney) test was performed. A value of $P < 0.05$ was considered statistically significant. The analyses were performed with the StatLight statistical software (Yukms Co. Ltd.).

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