ORIGINAL ARTICLE

Fibrinogen and von Willebrand factor-independent platelet aggregation *in vitro* and *in vivo*

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Summary. *Background:* Fibrinogen (Fg) has been considered essential for platelet aggregation. However, we recently demonstrated formation of occlusive thrombi in Fg-deficient mice and in mice doubly deficient for Fg and von Willebrand factor (Fg/VWF$^{-/-}$). *Methods and results:* Here we studied Fg/VWF-independent platelet aggregation *in vitro* and found no aggregation in citrated platelet-rich plasma of Fg/VWF$^{-/-}$ mice. Surprisingly, in Fg/VWF$^{-/-}$ plasma without anticoagulant, adenosine diphosphate induced robust aggregation of Fg/VWF$^{-/-}$ platelets but not of β_3-integrin-deficient ($\beta_3^{-/-}$) platelets. In addition, $\beta_3^{-/-}$ platelets did not significantly incorporate into thrombi in Fg/VWF$^{-/-}$ mice. This Fg/VWF-independent aggregation was blocked by thrombin inhibitors (heparin, hirudin, PPACK), and thrombin or thrombin receptor activation peptide (AYPGKF-NH$_2$) induced aggregation of gel-filtered Fg/VWF$^{-/-}$ platelets in 1 mM Ca^{2+} PIPES buffer. Notably, aggregation in PIPES buffer was only 50–60% of that observed in Fg/VWF$^{-/-}$ plasma. Consistent with the requirement for thrombin *in vitro*, hirudin completely inhibited thrombus formation in Fg/VWF$^{-/-}$ mice. These data define a novel pathway of platelet aggregation independent of both Fg and VWF. Although this pathway was not detected in the presence of anticoagulants, it was observed under physiological conditions *in vivo* and in the presence of Ca^{2+} *in vitro*. *Conclusions:* β_3 integrin, thrombin, and Ca^{2+} play critical roles in this Fg/VWF-independent aggregation, and both plasma and platelet granule proteins contribute to this process.

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Introduction

Platelet adhesion and subsequent aggregation at sites of vascular injury are key events required to arrest bleeding. However, the same hemostatic processes may also contribute to the generation of inopportune thrombi within atherosclerotic arteries. It has been demonstrated that von Willebrand factor (VWF) and fibrinogen (Fg) are two essential molecules that mediate platelet adhesion and aggregation [1]. Deficiencies in either of these molecules are associated with bleeding disorders [i.e. von Willebrand disease (VWD) and afibrinogenemia]. Although no Fg/VWF double-deficient patients have been reported, our data have shown that Fg/VWF$^{-/-}$ mice are viable and form occlusive thrombi after vascular injury [2]. The molecular basis for thrombosis and hemostasis independent of VWF and Fg is, however, largely unknown.

The molecules involved in platelet adhesion and aggregation have been intensively studied. It has been demonstrated that the platelet membrane glycoprotein (GP) Ib complex and its ligand, VWF, initiate platelet adhesion, particularly at high shear [1,3–5]. Subsequent stable adhesion is mediated by several platelet integrin receptors and their ligands (e.g. integrin $\alpha_2\beta_1$/collagen, integrin $\alpha_{IIb}\beta_3$/Fg and VWF) as well as interaction between GPVI and collagen [1,4,6]. Following initial adhesion, additional platelets aggregate on the layer of adherent platelets to form a hemostatic plug. This is probably mediated by the synergistic action of several platelet receptors and their ligands, including $\alpha_{IIb}\beta_3$/Fg [7,8], $\alpha_{IIb}\beta_3$/VWF, GPIb complex/VWF [1,9,10], P-selectin/sulfatides [11], and β_1 integrin/fibronectin (Fn) [4,12]. In addition, glycolipids, heparans, proteoglycans, and SLAM family receptors may also be involved in this process [13,14].

It has been well-demonstrated *in vitro* that Fg is required for platelet aggregation both in afibrinogenemic patients and

in gene-deficient animals [15,16]. This suggests that bridging ligands other than Fg may play either a minor role or a secondary role following Fg engagement during platelet aggregation. It is intriguing, therefore, that platelet-rich thrombi are able to form in mice lacking Fg and, even more surprisingly, in mice lacking both Fg and VWF [2]. In addition, although it has been reported that $\alpha_{IIb}\beta_3$ integrin plays a central role in platelet aggregation [8,17], platelets from Glanzmann thrombasthenic patients lacking $\alpha_{IIb}\beta_3$ proteins are still able to aggregate, suggesting that β_3 integrin-independent platelet aggregation exists [13,18]. Furthermore, thrombus formation occurs in $\beta_3^{-/-}$ mice [19], although it is not clear whether platelet aggregation is involved in this process. Thus, it is of interest to investigate whether β_3 integrin is required for Fg/VWF-independent platelet aggregation.

In this study, we investigated the mechanism of Fg/VWF-independent platelet aggregation by performing *in vitro* aggregation assays and *in vivo* intravital microscopy. We first observed robust adenosine diphosphate (ADP)-induced Fg/VWF-independent platelet aggregation in non-anticoagulated plasma *in vitro*. Further experiments using $\beta_3^{-/-}$ platelets and different thrombin inhibitors demonstrated that both β_3 integrin and thrombin were critical for this aggregation. Moreover, both plasma and platelet granule-released proteins contributed synergistically to this novel platelet aggregation pathway.

Material and methods

Experimental animals

Fg/VWF$^{-/-}$ mice and $\beta_3^{-/-}$ mice have been previously described [2,20]. $\beta_3^{-/-}$ mice were backcrossed onto the BALB/c background at the Massachusetts Institute of Technology. Syngeneic BALB/c and C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and used as controls. Genotypes of all experimental animals were confirmed by polymerase chain reaction analysis. All animal care and experimental procedures were approved by the Animal Care Committee at St Michael's Hospital.

Platelet and plasma preparation

Mice (6–8 weeks old) were anesthetized and bled from the retro-orbital plexus using heparin-coated glass capillary tubes. The blood was collected into a tube containing 3% acid citrate dextrose (ACD; 1/9, v/v) or 3.8% sodium citrate (1/9, v/v). Platelet-rich plasma (PRP) was obtained by centrifugation at $300 \times g$ for 7 min. Platelets were isolated from the PRP (ACD used as an anticoagulant) using a Sepharose 2B column in PIPES buffer (PIPES 5 mM, NaCl 1.37 mM, KCl 4 mM, glucose 0.1%, pH 7.0) [2,21]. Platelet-poor plasma (PPP) was prepared from Fg/VWF$^{-/-}$ mouse whole blood with or without anticoagulant, as previously described [21].

Platelet aggregation

Platelet aggregation was performed at 37 °C using a computerized Chrono-log aggregometer (Chrono-Log Corporation, Havertown, PA, USA). The method of platelet aggregation in anticoagulated PRP has been previously described [21]. For platelet aggregation in the non-anticoagulated PPP, equal amounts of gel-filtered Fg/VWF$^{-/-}$ platelets or $\beta_3^{-/-}$ platelets in PIPES buffer were mixed with non-anticoagulated PPP (final platelet concentration of 2.5×10^8 mL^{-1}). Subsequently, 20 μM ADP was added to induce platelet aggregation. Similarly, Fg/VWF$^{-/-}$ platelet aggregation was also induced by ADP, or PAR-4 thrombin receptor activation peptide (TRAP, AYPGKF-NH$_2$; Sigma-Aldrich, St Louis, MO, USA), or other agonists [100 μM phorbol myristyl acetate (PMA; Sigma-Aldrich)], 10 μM A23187 (Calbiochem, Darmstadt, Germany), 2 and 20 μg mL^{-1} collagen (Nycomed Pharma, Ismaning, Germany), 100 μM epinephrine (Chrono-Log, Havertown, PA, USA), in the presence of thrombin inhibitors, heparin (250 IU mL^{-1}), r-hirudin (50 μg mL^{-1}), or PPACK (120 μM) (Sigma-Aldrich) in Fg/VWF$^{-/-}$ plasma. In another set of experiments, Fg/VWF$^{-/-}$ mouse platelets in PIPES buffer were treated with 1–4 U mL^{-1} thrombin (Sigma-Aldrich), 0.5–1 mM TRAP, 100 μM PMA, 20 μg mL^{-1} collagen, and 10 μM A23187 in the presence of 1 mM Ca^{2+}. Platelet aggregates were observed under a Zeiss Axiovert 135-inverted microscope (32X, 0.4NA; Zeiss, Oberkochen, Germany) and pictures were taken with a digital camera (DP70, Olympus, Japan).

Detection of GPIbα, β1 integrin and P-selectin on the platelet surface

Resting gel-filtered platelets (10^6) from wild-type (BALB/c) and $\beta_3^{-/-}$ mice were incubated for 30 min with rat anti-mouse GPIbα antibody p0p5 (10 μg mL^{-1}, provided by B. Nieswandt, University of Wurzburg, Germany) in 50 μL of PIPES buffer. The expression of GPIbα was detected with fluorescein isothiocyanate (FITC)-conjugated anti-rat polyvalent immunoglobulin G (1:100; Sigma-Aldrich). Similar samples were incubated with FITC-conjugated hamster anti-rat CD29 (anti-β$_1$ integrin) that crossreacts with mouse β$_1$ integrin (1:100; BD Pharmingen, San Jose, CA, USA) to detect the expression of β$_1$ integrin. In order to examine P-selectin expression on platelet surfaces, resting or activated (1 U mL^{-1} thrombin or 20 μM ADP) platelets were incubated with FITC-conjugated rat anti-mouse CD62P (P-selectin, BD Pharmingen). All samples were analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

In vivo *intravital microscopy thrombosis model*

Intravital microscopy was performed as previously described [2,21]. Briefly, platelets were isolated from donor Fg/VWF$^{-/-}$ mice and fluorescently labeled with calcein acetoxymethylester (1 μg mL^{-1}; Molecular Probes, Eugene, OR, USA). Experi-

mental Fg/VWF$^{-/-}$ mice were injected with the fluorescently labeled platelets (5×10^6 g^{-1}) through the lateral tail vein. The mice were anesthetized and FeCl$_3$ was used to induce a mesenteric arteriolar injury. The sites of injured vessels were visualized and recorded with an intravital microscopy system. In the group undergoing thrombin inhibition, r-hirudin (3 mg kg^{-1}) (kindly provided by Dr Fareed, Loyola University, Chicago, IL, USA) was mixed with fluorescently labeled platelets prior to i.v. injection.

Examination of β$_3$$^{-/-}$ platelet incorporation into thrombi in Fg/VWF$^{-/-}$ mice by confocal intravital microscopy

Male Fg/VWF$^{-/-}$ mice were injected i.v. with fluorescently labeled Fg/VWF$^{-/-}$, β$_3$$^{-/-}$ or β$_3$$^{+/+}$ platelets (2.5×10^6 g^{-1}), as described above. Thrombus formation was induced with FeCl$_3$ and pictures were taken with the digital camera DP70 combined with a confocal cell imaging module (BD Biosciences, Rockville, MD, USA) under the intravital fluorescence microscope. We monitored several independent arterioles and venules within the same mouse as well as in comparison with different mice.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was assessed by unpaired Student's *t*-test.

Results

Fg/VWF$^{-/-}$ platelets aggregated in vitro in non-anticoagulated Fg/VWF$^{-/-}$ plasma

To elucidate the pathway of thrombus formation in Fg/VWF$^{-/-}$ mice [2], we examined platelet aggregation *in vitro*. Using a routine method for PRP preparation with 3% ACD, ADP was capable of inducing platelet aggregation in wild-type and VWF$^{-/-}$ PRP, but not in Fg/VWF$^{-/-}$ or Fg$^{-/-}$ PRP (Fig. 1A). Similar results were obtained using either 3.8% sodium citrate as an anticoagulant (Fig. 1B) or citrated PRP after the pH was adjusted to 7.3–7.5 with NaOH (data not shown). As there is no fibrin formation in Fg/VWF$^{-/-}$ mice, we next prepared Fg/VWF$^{-/-}$ plasma without adding exogenous anticoagulant. When this non-anticoagulated plasma was mixed with gel-filtered Fg/VWF$^{-/-}$ platelets, robust aggregation occurred following ADP stimulation (Fig. 2A).

β3$^{-/-}$ platelets did not aggregate in vitro in non-anticoagulated Fg/VWF$^{-/-}$ plasma

Because it was reported that platelet aggregation occurs with platelets from patients lacking α$_{IIb}$β$_3$ proteins [13,18], we reasoned that Fg/VWF$^{-/-}$ platelet aggregation may occur in either a β$_3$ integrin-dependent or -independent manner. Therefore, we examined whether Fg/VWF-independent platelet aggregation *in vitro* was mediated by β$_3$ integrin. As shown
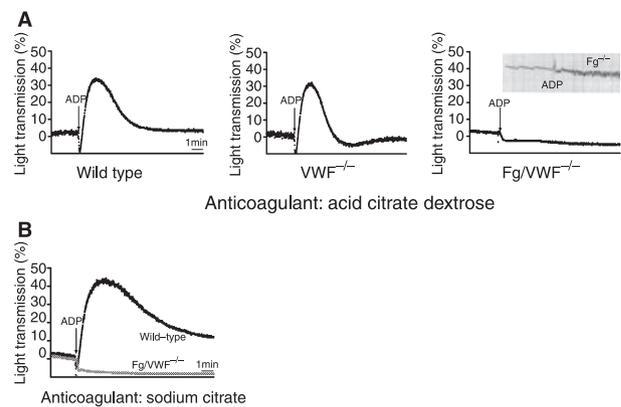

Fig. 1. No platelet aggregation occurred in anticoagulant-treated fibrinogen/von Willebrand factor (Fg/VWF)$^{-/-}$ platelet-rich plasma (PRP) *in vitro*. Adenosine diphosphate (ADP)-induced platelet aggregation in acid citrate dextrose (A) and sodium citrate (B) anticoagulated PRP. PRP from wild-type and different gene deficient mice are indicated. $n=3$ in three independent experiments.
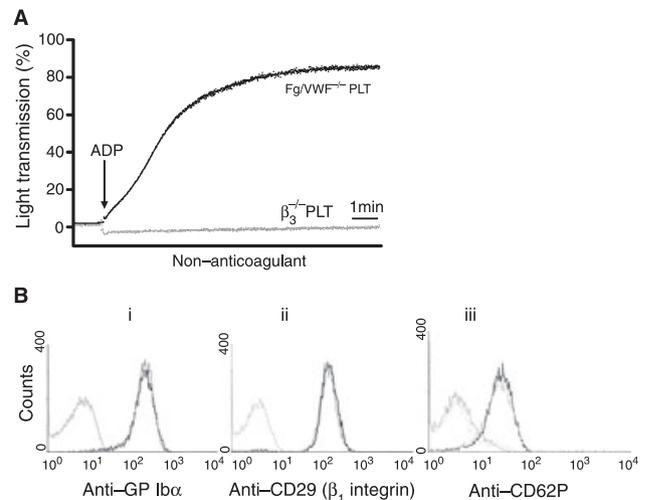

Fig. 2. Fibrinogen/von Willebrand factor (Fg/VWF)$^{-/-}$, but not β$_3$$^{-/-}$, platelets aggregated in non-anticoagulated Fg/VWF$^{-/-}$ plasma after ADP treatment. (A) ADP-induced robust aggregation of Fg/VWF$^{-/-}$ platelets in non-anticoagulant-treated Fg/VWF$^{-/-}$ plasma (black line). No aggregation of β$_3$$^{-/-}$ platelets was observed in the same plasma (gray line). PLT, platelets; $n = 3$. (B) No differences in (i) glycoprotein Ibα, (ii) β$_1$ integrin and (iii) P-selectin expression levels were observed between β$_3$$^{-/-}$ platelets and wild-type platelets. (i), (ii) Secondary antibody alone (negative control, light gray line), wild-type platelets (black line), β$_3$$^{-/-}$ platelets (dark gray line). (iii) Resting wild-type platelets (black dashed line), thrombin-activated wild-type platelets (black line), resting β$_3$$^{-/-}$ platelets (gray dashed line), thrombin-activated β$_3$$^{-/-}$ platelets (gray line). $n = 3$.

in Fig. 2A, ADP did not induce β$_3$$^{-/-}$ platelet aggregation in the same non-anticoagulated Fg/VWF$^{-/-}$ plasma. This absence of aggregation was not due to the reduction of other potential platelet receptors such as GPIbα and β$_1$ integrins in β$_3$$^{-/-}$ mice, as their expression levels were unchanged as measured by flow cytometry (Fig. 2B). We also observed a similar P-selectin expression on wild-type BALB/c platelets and β$_3$$^{-/-}$ platelets

before and after thrombin (1 or 10 U mL^{-1}) stimulation (Fig. 2B). There was also no reduction of these receptors on wild-type BALB/c platelets as compared with Fg/VWF$^{-/-}$ platelets (C57BL/6J/129 background, data not shown). These results excluded the possibility that GPIbα, β_1 integrins or P-selectin deficiency was responsible for the inability of $\beta_3^{-/-}$ platelets to aggregate.

$\beta_3^{-/-}$ platelets did not significantly incorporate into the thrombi formed in Fg/VWF$^{-/-}$ mice

To elucidate the role of β_3 integrin in Fg/VWF-independent platelet aggregation *in vivo*, we injected fluorescently labeled $\beta_3^{-/-}$ platelets into Fg/VWF$^{-/-}$ mice to determine whether $\beta_3^{-/-}$ platelets could incorporate into thrombi formed in Fg/VWF$^{-/-}$ mice. Fluorescently labeled Fg/VWF$^{-/-}$ platelets or wild-type ($\beta_3^{+/+}$) platelets were used as controls. We found that injected wild-type platelets (data not shown) and Fg/VWF$^{-/-}$ platelets formed bright thrombi at the site of vessel injury (Fig. 3, lower panel). In contrast, when $\beta_3^{-/-}$ platelets were injected, only weakly fluorescent thrombi formed (Fig. 3, upper panel). Confocal imaging of the thrombi further demonstrated that the fluorescently labeled $\beta_3^{-/-}$ platelets did not significantly incorporate into the dark thrombi, which were formed by endogenous, unlabeled Fg/VWF$^{-/-}$ platelets (Fig. 3, upper panel).

Fg/VWF$^{-/-}$ platelet aggregation *in vitro* was inhibited by high doses of thrombin inhibitors

As shown in Fig. 2A, ADP-induced Fg/VWF$^{-/-}$ platelet aggregates did not exhibit significant deaggregation, which
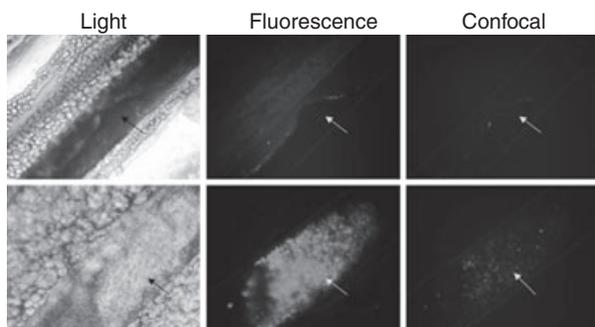

Fig. 3. In contrast to fibrinogen/von Willebrand factor (Fg/VWF)$^{-/-}$ platelets, $\beta_3^{-/-}$ platelets did not significantly incorporate into thrombi in Fg/VWF$^{-/-}$ mice. Upper panel: fluorescently labeled $\beta_3^{-/-}$ platelets were injected into Fg/VWF$^{-/-}$ mice, and weakly fluorescent thrombi were observed at the site of injury under intravital microscopy. Confocal imaging of the thrombi showed that only dark thrombi (unlabeled) were formed and that a few fluorescently labeled $\beta_3^{-/-}$ platelets were temporarily trapped on the surface of the dark thrombi. Lower panel: after injection of fluorescently labeled Fg/VWF$^{-/-}$ platelets, bright thrombi formed at the site of injury. Confocal imaging of these thrombi showed that fluorescently labeled Fg/VWF$^{-/-}$ platelets were evenly distributed within the thrombi. The blood vessel is indicated by the dashed lines and the site of thrombus formation is indicated by the arrows. Representative blood vessels are shown. $n = 3$–5 in three separate experiments.

suggested that platelet granule release probably occurred [22]. As we did not add exogenous anticoagulant during preparation of the plasma in these experiments, trace amounts of thrombin may have remained in the plasma, which may promote ADP-induced thrombin generation on the platelet surface [23]. Therefore, we hypothesized that thrombin may play a role in Fg/VWF$^{-/-}$ platelet granule release and aggregation. To test this hypothesis, we preincubated the Fg/VWF$^{-/-}$ plasma with thrombin inhibitors [heparin (250 IU mL^{-1}), hirudin (50 μg mL^{-1}), or PPACK (120 μM)]. Interestingly, these three different (i.e. indirect, direct, and allosteric) thrombin inhibitors completely inhibited ADP-induced Fg/VWF$^{-/-}$ platelet aggregation (Fig. 4A). Experiments with wild-type PRP have shown that none of these thrombin inhibitors affects ADP-induced platelet aggregation (data not shown). These data suggest that ADP-induced Fg/VWF$^{-/-}$ platelet aggregation probably requires thrombin generation and thrombin signaling.

Fg/VWF$^{-/-}$ platelet aggregation *in vitro* was induced by TRAP

In order to confirm the role of thrombin in Fg/VWF-independent platelet aggregation, we used TRAP (AY-PGKF-NH$_2$) to directly induce Fg/VWF$^{-/-}$ platelet aggregation. As shown in Fig. 4B, TRAP induced Fg/VWF$^{-/-}$ platelet aggregation in Fg/VWF$^{-/-}$ plasma that was preincubated with heparin, hirudin or PPACK. These data confirm that inhibition of aggregation by heparin, hirudin or PPACK was thrombin-specific and that thrombin receptor activation induced Fg/VWF-independent platelet aggregation.

Hirudin inhibited Fg/VWF$^{-/-}$ thrombus formation *in vivo*

We then used intravital microscopy to investigate the effect of hirudin (3 mg kg^{-1}) on platelet aggregation and thrombus formation in Fg/VWF$^{-/-}$ mice (Fig. 4C). The number of early single platelets deposited at the site of injury was not significantly different between hirudin-treated and untreated Fg/VWF$^{-/-}$ mice. Approximately 5–10 min after injury, platelets adhered more stably to the vessel wall and started to form visible platelet aggregates in untreated Fg/VWF$^{-/-}$ mice. However, we did not observe any apparent platelet aggregates in hirudin-injected mice during the 40 minute experiments. These data differ from those in hirudin-injected wild-type mice, in which platelet aggregation and thrombus formation occur [24]. Thus, thrombin is required for Fg/VWF-independent platelet aggregation *in vivo*.

Both platelet granule and plasma protein(s) contributed to Fg/VWF$^{-/-}$ platelet aggregation

Because Fg/VWF$^{-/-}$ platelet aggregation was dependent on thrombin signaling, it is possible that granule release may play an important role in this aggregation pathway. To test this possibility, we induced platelet aggregation with additional strong and weak agonists. As shown in Fig. 5, high-dose (20 μg mL^{-1}) collagen, PMA (100 μm) and calcium ionophore
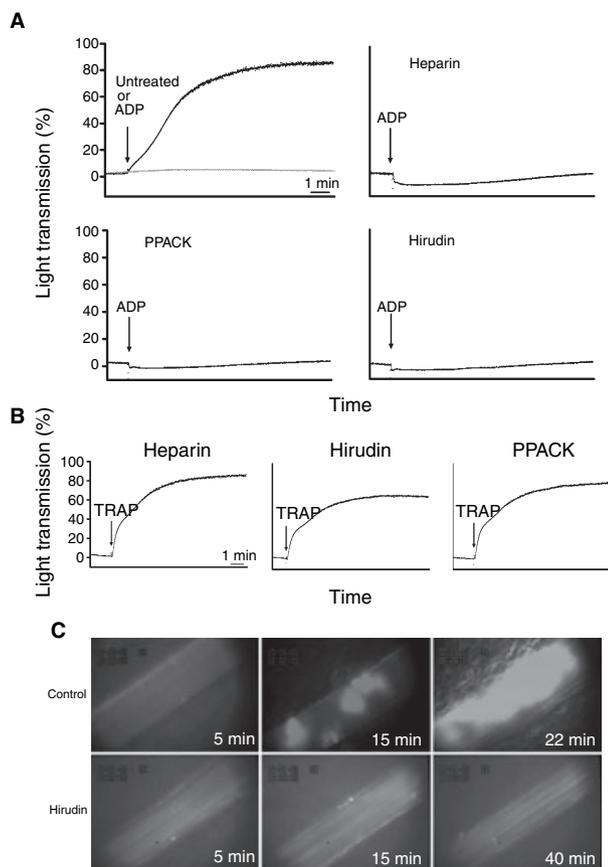
Fig. 4. Thrombin was critical for fibrinogen/von Willebrand factor (Fg/VWF)-independent platelet aggregation *in vitro* and *in vivo*. (A) Thrombin inhibitors inhibited ADP-induced Fg/VWF$^{-/-}$ platelet aggregation in Fg/VWF$^{-/-}$ plasma. Upper left panel: control: Fg/VWF$^{-/-}$ platelets were mixed with non-anticoagulant-treated Fg/VWF$^{-/-}$ plasma (gray line) and treated with ADP (black line). Other panels: Fg/VWF$^{-/-}$ platelets were mixed with Fg/VWF$^{-/-}$ plasma, which was preincubated with the indicated thrombin inhibitors before ADP treatment. (B) In contrast to ADP, thrombin receptor activation peptide induced robust Fg/VWF$^{-/-}$ platelet aggregation in Fg/VWF$^{-/-}$ plasma in the presence of thrombin inhibitors. (C) Hirudin inhibited platelet aggregation in Fg/VWF$^{-/-}$ arterioles in an intravital microscopy thrombosis model. Time after FeCl$_3$ injury is indicated in the lower right corner. Blood flow was from right to left. Upper panel: in Fg/VWF$^{-/-}$ mice, single fluorescently labeled platelets adhered to the arterioles 5 min after injury. Delayed thrombus formation occurred with frequent embolization (15 min), and the majority of the Fg/VWF$^{-/-}$ mouse arterioles eventually occluded. Lower panel: in the hirudin-injected group, no significant difference was found in the platelet–vessel wall interaction during the first 3–5 min when compared with the non-hirudin-injected group. Platelet aggregation and thrombus formation were completely inhibited in hirudin-treated arterioles.

A23187 (10 µm) were also able to induce Fg/VWF$^{-/-}$ platelet aggregation in Fg/VWF$^{-/-}$ plasma (hirudin-anticoagulated). The same results were obtained when PPACK was used as the anticoagulant. However, weak agonists such as epinephrine and low-dose (2 µg mL^{-1}) collagen did not induce this aggregation (data not shown).

To distinguish between the contribution of platelet granule proteins and plasma proteins, we analyzed gel-filtered Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer. No Fg/VWF$^{-/-}$
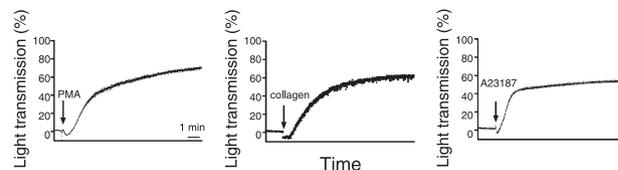

Fig. 5. Fibrinogen/von Willebrand factor (Fg/VWF)$^{-/-}$ platelet aggregation induced with phorbol myristyl acetate, collagen or A23187 induced robust Fg/VWF$^{-/-}$ platelet aggregation in Fg/VWF$^{-/-}$ plasma in the presence of hirudin.

platelet aggregation was induced by ADP in this buffer. There was also no significant aggregation after either thrombin (1 U mL^{-1}) or TRAP (500 µm) treatment. However, after adding 1 mm CaCl$_2$ to the PIPES buffer, both thrombin and TRAP, but not ADP, induced Fg/VWF$^{-/-}$ platelet aggregation (Fig. 6A,B). Concomitantly, we found that P-selectin, a marker for granule release, was expressed on the Fg/VWF$^{-/-}$ platelet surface after either 1 U mL^{-1} thrombin (Fig. 6C, left panel) or 500 µm TRAP treatment, but was not expressed after ADP (20 µm) treatment in 1 mm Ca^{2+} PIPES buffer. Similar results were also observed in micro-aggregates from thrombin-induced platelet aggregation (Fig. 6C, right panel). It is notable that ADP was also not able to induce P-selectin expression on wild-type platelets in the same PIPES buffer (data not shown). Together, these data suggest that platelet granule protein(s) contributed to Fg/VWF-independent platelet aggregation. It appears that this process requires millimolar levels of Ca^{2+} and the moderate levels of platelet-released Ca^{2+} are insufficient to support this aggregation in PIPES buffer. Notably, there was no significant enhancement of aggregation after adding higher doses of Ca^{2+} and/or Mg^{2+} (data not shown).

Nevertheless, Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer was significantly less robust than that in Fg/VWF$^{-/-}$ PPP (Figures 2A and 6). As shown in Fig. 6, thrombin-induced Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer ($41.3 \pm 5.9\%, n = 3$) was less than that in Fg/VWF$^{-/-}$ plasma ($73.0\% \pm 7.2\%, n = 3; P < 0.05$); TRAP-induced Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer ($44.7\% \pm 2.9\%, n = 3$) was also less than that in hirudin-pretreated Fg/VWF$^{-/-}$ plasma ($73.3\% \pm 5.9\%, n = 3; P < 0.05$). In addition, thrombin- or TRAP-induced Fg/VWF$^{-/-}$ platelet aggregation in 1 mm CaCl$_2$ PIPES buffer was also less than ADP-induced aggregation in Fg/VWF$^{-/-}$ PRP (Figures 2A and 6A,B). Furthermore, platelet aggregates that formed following thrombin or TRAP stimulation in PIPES buffer were considerably smaller than those in Fg/VWF$^{-/-}$ plasma (Fig. 6A,B). In fact, the extent of Fg/VWF$^{-/-}$ platelet aggregation and the morphology of these aggregates in Fg/VWF$^{-/-}$ plasma (Fig. 6A,B) were indistinguishable from their wild-type controls (data not shown). Dose–response experiments showed that the 1 U mL^{-1} thrombin or 500 µm TRAP used in the gel-filtered platelet aggregation in PIPES buffer achieved a platelet aggregation plateau, and no further enhancement of aggregation was observed with higher con-
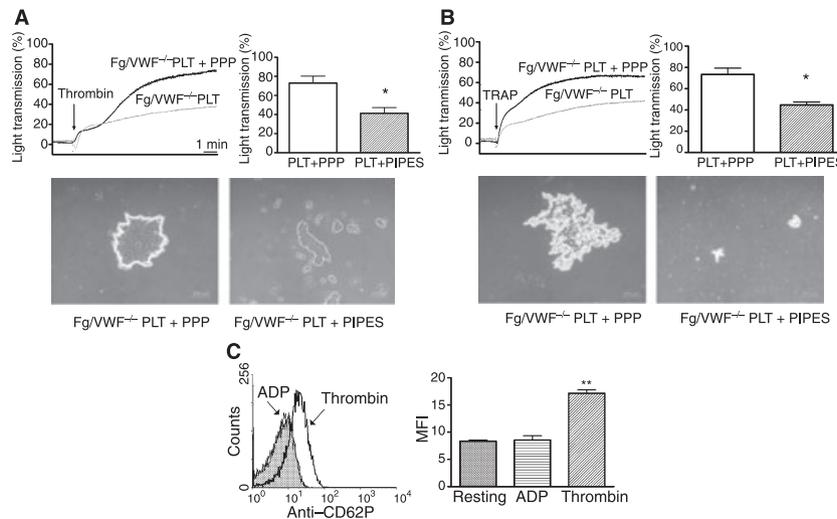
Fig. 6. Both plasma and platelet granule protein(s) contributed to the fibrinogen/von Willebrand factor (Fg/VWF)-independent platelet aggregation *in vitro*. (A) Thrombin induced Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer and in Fg/VWF$^{-/-}$ plasma. Upper left panel: gel-filtered Fg/VWF$^{-/-}$ platelets, suspended in either 1 mM CaCl2 PIPES buffer or non-anticoagulated Fg/VWF$^{-/-}$ plasma, were treated with thrombin. Platelet aggregation in PIPES buffer (gray line) is approximately 50–60% of that observed in Fg/VWF$^{-/-}$ plasma (black line). Upper right panel: Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer is significantly lower than that observed in non-anticoagulated Fg/VWF$^{-/-}$ plasma. *$P < 0.05$, $n = 3$. Lower panel: Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer formed smaller aggregates than in Fg/VWF$^{-/-}$ plasma, as visualized by light microscopy. (B) Thrombin receptor activation peptide (TRAP) induced Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer and in Fg/VWF$^{-/-}$ plasma. Upper left panel: gel-filtered Fg/VWF$^{-/-}$ platelets, suspended in either PIPES buffer (1 mM CaCl2, 50 μg mL^{-1} hirudin) or hirudin preincubated Fg/VWF$^{-/-}$ plasma, were treated with TRAP. Platelet aggregation in PIPES buffer (gray line) is 50–60% of that in Fg/VWF$^{-/-}$ plasma (black line). Upper right panel: Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer is lower than that seen in non-anticoagulated Fg/VWF$^{-/-}$ plasma. *$P < 0.05$, $n = 3$. Lower panel: Fg/VWF$^{-/-}$ platelet aggregation in PIPES buffer formed smaller aggregates than in Fg/VWF$^{-/-}$ plasma. (C) P-selectin expression on activated Fg/VWF$^{-/-}$ platelets in PIPES buffer was examined by flow cytometry. After treatment with thrombin under static conditions for 15 min, there was significant P-selectin expression on the platelet surface (black line, left panel) as compared with resting platelets (filled area), while ADP (arrow indicated) failed to induce P-selectin expression (gray line). Similar results were observed from platelet aggregation assays. Small micro-aggregates formed after treatment with thrombin for 5 min; this is when P-selectin was detected on platelets by flow cytometry (right panel). **$P < 0.01$, $n = 3$ in three independent experiments.

centrations of thrombin (2 and 4 U mL^{-1}) or TRAP (1 mM). These data suggest that both plasma and platelet granule proteins contributed to the Fg/VWF-independent platelet aggregation.

Discussion

Fibrinogen is considered an essential bridging molecule for platelet aggregation [8,15,16,25]. In patients with afibrinogenemia, residual platelet aggregation is thought to be mediated mainly by VWF [26]. However, we demonstrated here that under more physiological conditions (i.e. non-anticoagulated plasma), robust platelet aggregation occurs in the absence of both Fg and VWF *in vitro*. We further demonstrated that Fg/VWF-independent platelet aggregation is dependent on β$_3$ integrin, thrombin, platelet secretion and divalent Ca^{2+} cations. Both plasma and platelet granule proteins contribute to this novel pathway of platelet aggregation.

The critical role of Fg in platelet aggregation was established by studying specimens from patients with afibrinogenemia (< 200 μg mL^{-1} Fg in their plasma) [15,27]. However, because significant residual Fg exists in most afibrinogenemic blood, exogenous anticoagulant reagents, such as sodium citrate, are routinely used to prevent coagulation during blood preparation. Under these circumstances, afibrinogenemic PRP

responded poorly to ADP-induced aggregation. De Marco *et al.* [26] reported that the monoclonal antibody LJP5, which specifically blocks VWF/α$_{IIb}$β$_3$ interaction but not Fg/α$_{IIb}$β$_3$ interaction, was able to abolish residual platelet aggregation in afibrinogenemic citrated-PRP. As some residual Fg was present in both the plasma and platelets in these cases, it is thus not clear whether residual Fg is required to initiate this VWF-mediated platelet aggregation. It remains unclear, therefore, whether Fg-independent aggregation indeed occurs in citrated blood, and whether Fg/VWF-independent aggregation can be induced in non-anticoagulated blood.

Our Fg/VWF-double deficient mice (in which Fg and VWF are completely deleted) provided an opportunity to study Fg- and VWF-independent platelet aggregation, particularly under more physiological conditions where the effects of anticoagulant reagents were minimized. The data presented in this study differ from the aggregation data seen in the afibrinogenemic patients who have residual Fg. We demonstrated that Fg, at least in murine blood, is probably an indispensable bridging molecule for platelet aggregation in anticoagulated blood (Figs 1 and 4A). However, under more physiological conditions (i.e. non-anticoagulated blood), robust platelet aggregation can be induced in the absence of both Fg and VWF (Fig. 2).

Is β$_3$ integrin required for this Fg/VWF-independent platelet aggregation? It has been reported that platelet aggregation

occurs in patients lacking $\alpha_{IIb}\beta_3$ integrin (type I Glanzmann thrombasthenia) [13,18]. We also found a very low level of platelet aggregation (< 10% of normal level) in gel-filtered $\beta_3^{-/-}$ platelets in PIPES buffer after stimulation with thrombin (unpublished data). These observations are consistent with the concept that multiple platelet receptors may be involved in platelet aggregation [4,10–14]. However, in the present study, using $\beta_3^{-/-}$ mice as well as our newly developed anti-mouse β_3 integrin blocking antibody (data not shown), we found that β_3 integrin is the essential receptor that mediates this Fg/VWF-independent platelet aggregation (Figs 2 and 3).

What are the ligand(s) mediating Fg/VWF-independent platelet aggregation? Fn [28], thrombospondin-1 (TSP-1) [29], vitronectin (Vn) [21], and CD40L [30] may be candidates. We recently demonstrated that plasma Fn promotes thrombus growth and stability [12]. It is as yet unclear, however, whether this results from Fn supporting platelet aggregation, stabilizing the fibrin clot, or self-assembling [31,32]. We are also not certain whether this results from plasma Fn or platelet granule Fn. The latter comes mainly from plasma via β_3 integrin internalization [33]. In the present study, removal of plasma Fn from Fg/VWF$^{-/-}$ plasma with a Sepharose 4B-gelatin column did not significantly affect Fg/VWF$^{-/-}$ platelet aggregation (data not shown), suggesting that plasma Fn is not the likely ligand. We also demonstrated that plasma vitronectin and CD40L may not be the ligands. Fg/VWF$^{-/-}$ platelet aggregation occurred to a similar extent in vitronectin$^{-/-}$ and CD40L$^{-/-}$ plasma as in their wild-type controls after Fg and VWF were removed by a Centricon membrane (100 kD cut-off, data not shown). Furthermore, the plasma concentration of TSP-1 is negligible. Thus, there may be other ligand(s) in plasma that enhance platelet aggregation.

Platelet granule Fn, vitronectin, TSP-1, and CD40L may, however, play a role in this Fg/VWF-independent aggregation. We found that gel-filtered Fg/VWF$^{-/-}$ platelets were able to aggregate in 1 mM Ca^{2+} PIPES after thrombin treatment (Fig. 6) or treatment with strong agonists such as PMA, high dose collagen, or A23187 (data not shown). This suggested that platelet-released β_3 integrin ligands were the major bridging molecules. Our earlier work demonstrated that platelet Fn content increased more than 3-fold in Fg$^{-/-}$ and Fg/VWF$^{-/-}$ mice [2,33]. We recently found that released platelet vitronectin significantly supported platelet aggregation, although plasma vitronectin inhibited this process [21]. Thus, platelet Fn, vitronectin and TSP-1, as well as other ligands such as CD40L, may synergistically contribute to this Fg/VWF-independent platelet aggregation after treatment with a potent agonist.

We found that thrombin is critical for Fg/VWF-independent platelet aggregation *in vitro* and *in vivo* (Fig. 4). Interestingly, although thrombin inhibitors do not affect ADP-induced $\alpha_{IIb}\beta_3$ activation and subsequent ligand binding [34], they did inhibit ADP-induced Fg/VWF$^{-/-}$ platelet aggregation in plasma (Fig. 4A). This suggested that ADP might play a key role, e.g. via interaction with its receptor P2Y$_{12}$ [35], in Fg/VWF$^{-/-}$ platelet aggregation, by inducing thrombin generation.

We also found that physiological (mM) levels of Ca^{2+} ions were required for Fg/VWF$^{-/-}$ platelet aggregation and that small amounts of platelet-released Ca^{2+} were insufficient to support this aggregation. This is intuitive because β_3 integrin is required for this new pathway of platelet aggregation and both the β-propeller and MIDAS domains of integrins require Ca^{2+} in order to maintain structural integrity. Although the Fg/$\alpha_{IIb}\beta_3$ and VWF/$\alpha_{IIb}\beta_3$ interactions may be less dependent on divalent cations and are able to bridge platelets in citrated plasma, other ligands, such as Fn, bind to β_3 integrin in a divalent cation-dependent manner [36]. It is very likely that physiological levels of Ca^{2+} (but not the residual levels present in citrated plasma) may be required to maintain the appropriate conformation of β_3 integrin for recognition of other ligands (i.e. ligands other than Fg and VWF). Furthermore, in the processes of ADP-induced thrombin generation and platelet degranulation, Ca^{2+} may also be required. Thus, citrated blood, as used in earlier studies, may mask the interaction between β_3 integrin and other plasma and platelet granule ligands.

In summary, the current theory that Fg and VWF are essential for platelet aggregation was established based on studies using anticoagulated blood *in vitro*, which may differ from pathophysiological conditions *in vivo*. At the site of injury, including the rupture of an atherosclerotic plaque, thrombin, divalent cations, and other platelet agonists are present, which may allow β_3 integrin to bind ligands other than Fg and VWF. These ligands may play an important role in supporting hemostasis in VWD and afibrinogenemia, and may also contribute to the thrombotic process in normal individuals. Identification and characterization of these ligands should be of great interest for future studies.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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