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ORIGINAL ARTICLE

Dimers of the platelet collagen receptor glycoprotein VI bind specifically to fibrin fibers during clot formation, but not to intact fibrinogen

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Abstract

Objective: The platelet collagen receptor glycoprotein VI (GPVI) has an independent role as a receptor for fibrin produced via the coagulation cascade. However, various reports of GPVI binding to immobilized fibrin(ogen) are not consistent. As a collagen receptor, GPVI-dimer is the functional form, but whether GPVI dimers or monomers bind to fibrin remains controversial. To resolve this, we analyzed GPVI binding to nascent fibrin clots, which more closely approximate physiological conditions.

Methods and results: ELISA using biotinyl-fibrinogen immobilized on streptavidin-coated wells indicated that GPVI dimers do not bind intact fibrinogen. Clots were formed by adding thrombin to a mixture of near-plasma level of fibrinogen and recombinant GPVI ectodomain: GPVI dimer (GPVI-Fc_2 or Revacept) or monomer (GPVI-His: single chain of Revacept GPVI domain, with His tag). Clot-bound proteins were analyzed by SDS-PAGE/immunoblotting. GPVI-dimer bound to noncrosslinked fibrin clots with classical one-site binding kinetics, with μM-level K_D, and to crosslinked clots with higher affinity. Anti-GPVI-dimer (mFab-F) inhibited the binding. However, GPVI-His binding to either type of clot was nonsaturable and nearly linear, indicating very low affinity or nonspecific binding. In clots formed in the presence of platelets, clot-bound platelet-derived proteins were integrin αIIbβ3, present at high levels, and GPVI.

Conclusions: We conclude that dimeric GPVI is the receptor for fibrin, exhibiting a similar K_D to those obtained for its binding to fibrinogen D-fragment and D-dimer, suggesting that fibrin(ogen)'s GPVI-binding site becomes exposed after fibrin formation or cleavage to fragment D. Analysis of platelets bound to fibrin clots indicates that platelet GPVI binds to fibrin fibers comprising the clot.

KEYWORDS
collagen, fibrin clot, glycoprotein VI, platelets, thrombosis

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1 | INTRODUCTION

To maintain hemostasis, thrombus formation is initiated when flowing blood contacts exposed subendothelial tissues of injured blood vessels, activating two reaction pathways. One pathway involves binding of the platelet-specific collagen receptor glycoprotein VI (GPVI) to subendothelial collagen exposed in injured vessels, initiating a signaling cascade leading to platelet activation, aggregation, and thrombus formation.[1-4] Constitutively present GPVI dimers comprise 29% of the total GPVI (monomers and dimers) in resting platelets. The dimers bind with high affinity to collagen fibers, but not soluble monomeric collagen,[2] and are the functional form of this receptor.[5,6] Platelet activation increases the number and clustering of dimers, bringing signaling molecules into closer proximity to enhance platelet activation.[7]

The second pathway occurs simultaneously, driven by tissue factor release from damaged sub-endothelium that produces active thrombin through the coagulation cascade; thrombin converts fibrinogen in blood to fibrin monomers, which then polymerize to form a gel-like fibrin clot. Clots are stabilized by activated factor XIII, factor XIIIa, which catalyzes fibrin crosslinking. Thrombin also activates nearby platelets, leading to phosphatidylserine expression and formation of a prothrombinase complex with coagulation factors V and VIII, enhancing thrombin formation, further driving fibrin formation and platelet activation. Many blood cells, including platelets are trapped within the fibrin clot and numerous fibrinolysis- and coagulation-related proteins are bound to the fibrin fibrils.[8] Many of these proteins, such as α2-plasmin inhibitor, plasminogen, von Willebrand factor (VWF), fibronectin, and albumin,[9-11] are covalently crosslinked to fibrin by factor XIII and affect fibrin clot stability. Intravital microscopy has detected fibrin fibers at the site of injury, and thrombi with tightly packed and activated platelet cores were formed in the intravascular space over the injured site.[12,13] Loosely adherent platelets formed a shell-like layer outside the platelet core.[12]

Recently, GPVI was reported to bind both fibrin and fibrinogen, independent of its function as a collagen receptor, leading to platelet activation.[14,15] Thus, thrombus formation is a process combining platelet activation and blood coagulation, with GPVI involved in both systems. Details about this role of GPVI in coagulation, whether there is direct binding to both fibrin and fibrinogen, and whether GPVI-monomer or -dimer can serve as the receptor remain controversial. Watson's group reported that GPVI monomer binds to fibrin and fibrinogen, whereas the dimer does not, using assays with immobilized fibrin or fibrinogen.[16,17] However, in our hands, these results could not be reproduced by a similar ELISA method using a fully glycosylated dimeric recombinant Fc-fusion protein of the GPVI-extracellular domain (GPVI-Fc_2) and indeed we found the opposite results. We found that GPVI-Fc_2 scarcely bound to fibrin, and not to fibrinogen at all, but did bind to a part of the fibrinogen molecule, the D-fragment, and to the crosslinked fibrin degradation product D-dimer, both with one-site binding kinetics.[18] We found that the corresponding GPVI-monomer did not bind well to any of these fibrinogen substrates in ELISA assays,[18] which was later confirmed by Zhang et al.[10] Using a different analysis system, Ebrahim et al[19] reported that GPVI-dimer does not bind to fibrin under flow conditions.

Essentials

- Glycoprotein VI (GPVI) was reported to bind to fibrin (ogen) but its mechanism is controversial.
- Fibrin clots formed with GPVI, fibrinogen, and thrombin were used to quantitate GPVI-binding.
- Only GPVI-dimer binds to the clots like a classical receptor; the monomer does not.
- Platelet surface GPVI also binds to the clots, but integrin αIIbβ3 is the major fibrin receptor.

In the present studies, we developed a method to quantitate the binding of GPVI-dimer and -monomer to fibrin clots formed from fibrinogen by thrombin in the presence of GPVI. Our system avoids the shortcomings of ELISA assays that rely on immobilization of a low concentration of soluble fibrin. Interaction of GPVI with such immobilized fibrin or fibrinogen would be very different from the interaction with fibrin clots formed with near-physiological concentrations of fibrinogen; such clots comprise larger fibrin fibers that have a characteristic three-dimensional structure and form a complex network. Whilst retaining the simplicity of a biochemically defined system, by studying the GPVI–fibrin interaction during clot formation, our assay method is closer to pathophysiology.

Fibrin protofibrils are formed by the polymerization of fibrin monomers, produced by thrombin cleavage of the fibrinopeptide A from the E-domain of fibrinogen. The fibrin E-domain can now interact with the D-domain of another fibrin(ogen) molecule, inducing a conformational change and fibrin-specific structure in the D-domain, which is the binding site for plasminogen and tissue plasminogen activator.[20,21] Fibrin-bound factor XIII is also activated by thrombin and catalyzes both fibrin–fibrin and also plasma protein–fibrin crosslinks. Fiber formation is a very complex process, making it imperative that we understand the basic interaction between fibrin and each specific ligand, in this case GPVI, in a defined system as the first step in the absence of other proteins.

Our results indicate that GPVI-dimer binds specifically to fibrin clots but GPVI-monomer binds either nonspecifically or with very low affinity to the fibrin clots. We also suggest that GPVI contributes significantly to the binding of resting platelets to fibrin. We demonstrate here that the collagen-binding GPVI-dimer is the form that also binds specifically to fibrin clots.

2 | MATERIALS AND METHODS

2.1 | Materials

We used a fibrinogen that is a plasminogen-, VWF-, and fibronectin-depleted human fibrinogen preparation (fibrinogen-3; Enzyme Research Laboratories). The fibrinogen was biotinylated using sulfosuccinimidyl-2-[biotinamido] ethyl-1,3-dithiopropionate (EZ-Link

sulfo-NHS-SS-biotin; Thermo Scientific) according to the manufacturer's instructions. Biotin incorporation was 0.712 mol biotin per mol fibrinogen, and the derivative's clotability was the same as the unlabeled fibrinogen. Fibrinogen was also labeled with AlexaFluor-555 using a labeling kit (Invitrogen); 0.65 mol AlexaFluor-555 was incorporated per mol fibrinogen. Dimeric forms of GPVI, GPVI-Fc_2 and Revacept, were previously reported. GPVI-Fc_2 contains the extracellular domain of GPVI (amino acids 21-234) fused with human IgG Fc, with C-terminal Myc and His tags.[1] Revacept comprises amino acids 21-269 fused with IgG Fc with a C-terminal His tag.[22] Both are fully glycosylated. Single-chain GPVI-His consists of amino acids 21-269 of GPVI, with a C-terminal His-tag. Revacept and GPVI-His were kindly supplied by AdvanceCore (Martinsried).

The following antibodies were used in western blotting: anti-GPVI (1G5; binds to monomeric and dimeric GPVI; Biocytex), anti-GPIb (clone 486805; R&D Systems), anti-CD61 (clone VIPL2; Novus Biologicals), anti-human albumin (15C7; Abcam), and rabbit anti-tubulin (EPR16774, Abcam). Anti-integrin αIIbβ3 antibody M148 (Abcam) was labeled with an AlexaFluor-488 labeling kit (Invitrogen). Fluorescently labeled secondary antibodies were from Li-Cor Biosciences. The inhibitory GPVI dimer–specific antibody mFab-F has been reported previously.[3]

2.2 | ELISA analysis of GPVI binding to biotinylated fibrinogen

ELISA-plate (Nunc MaxiSorp flat-bottomed; Thermo Fisher Scientific) wells were coated with streptavidin (10 μg/ml; Sigma); blocked with 0.5% bovine serum albumin (BSA); reacted with biotin-fibrinogen (10 μg/ml); and blocked with 10 μM biotin. After the final wash, wells were incubated with GPVI-Fc_2 followed by 1G5/IR Dye 800CW anti-mouse antibody, and binding was quantitated by the Li-Cor Odyssey CLx Imaging System. Fibrinogen preparations were also directly immobilized and GPVI-Fc_2 binding was similarly measured.

2.3 | Washed platelet preparation

Citrate-anticoagulated blood from healthy volunteers was obtained with informed consent in accordance with the Treaty of Helsinki. Washed platelets were prepared as described previously[23] and resuspended in modified HEPES-Tyrode's buffer (HT: 134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 10 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 5.5 mm glucose, pH 7.3).

2.4 | Quantitative analyses of GPVI binding to fibrin clots

The reaction mixture consisted of fibrinogen (1 mg/ml, final concentration) and equal concentrations of GPVI-Fc_2 and albumin (control protein). Thrombin (1 U/ml) was added, and the mixture was then incubated for 30 min at 37°C. Formed clots were pelleted by centrifugation at 14 000 rpm for 15 min in a microcentrifuge. The clots were washed twice with 0.5 ml of phosphate-buffered saline and dissolved in 0.1 ml of 1% SDS/8M urea solution. Each sample was mixed with 1/3 volume of SDS-sample buffer and 10 μl of each subjected to nonreduced SDS-PAGE and immunoblotting. The blots were stained with a mixture of 1G5 (mouse anti-pan GPVI) and anti-mouse albumin as primary antibodies and IRDye 800CW anti-mouse antibody (LI-COR) as secondary antibody; GPVI-Fc_2 and albumin bands were quantitated by the Odyssey image analyzer. Because GPVI-Fc_2 and the control protein were stained using different primary antibodies, we normalized the GPVI and control protein bands to a normalization standard (applied to the same gel), which was a sample containing 25 μg/ml each of GPVI-Fc_2 and control protein, as well as fibrinogen, with no added thrombin, which was then subjected to nonreduced SDS-PAGE/Western blotting along with the clot samples. The amounts of GPVI-Fc_2 or control protein in the fibrin clots were calculated as a percent of the respective bands in the normalization standard, and the specific binding is deduced from the difference in the percent of the GPVI-Fc_2 band and that of the control band at each GPVI-Fc_2 concentration.

To quantitate GPVI-Fc_2 binding to crosslinked fibrin clots, the reaction mixture contained fibrinogen (1 mg/ml final concentration), equal concentrations of GPVI-Fc_2 and human Fc (control protein), Ca^{2+} (2 mM) and FXIII (2 μg/ml). Clot formation was induced by adding thrombin as described previously. The obtained crosslinked clots were each washed as described above and then dissolved with SDS/urea containing 2% 2-mercaptoethanol and subjected to reduced SDS-PAGE/western blotting. The blots were stained with IRDye 800CW anti-human antibody (LI-COR). GPVI-Fc and human Fc were quantitated as described previously and expressed as a percent of the normalization standard (25 μg/ml each of GPVI-Fc_2 and human Fc).

For quantitation of GPVI-His in either noncrosslinked or crosslinked clots, the western blots were stained with mouse anti-His antibody (Abcam, primary antibody) and IRDye 800 CW anti-mouse antibody (secondary antibody).

2.5 | Effects of inhibitors on GPVI-Fc_2 binding to fibrin clot

We determined how other GPVI-binding proteins: fibrinogen D-fragment, D-dimer, CRP-XL (crosslinked collagen-related peptide, a GPVI-specific agonist; synthesized by the Farndale lab), and mFab (anti-GPVI dimer) affected GPVI-Fc_2 binding to the clots. Each mixture of GPVI-Fc_2, fibrinogen, and test proteins was preincubated for 10 min and then clotting initiated by adding thrombin. The clots were then processed as described above and analyzed for fibrin-bound GPVI-Fc_2 by SDS-PAGE/immunoblotting.

2.6 | Analysis of platelet receptor binding to fibrin clots

Washed platelets (5 × 10^8/ml in HT) were mixed with fibrinogen (1 mg/ml) in the absence or presence of human IgG Fab fragment (200 μg/ml; Jackson ImmunoResearch Laboratories), mFab-F [2,3] (200 μg/ml), or Integrilin (10 μM, Sigma); clots were formed by adding thrombin (1 U/ml), as described previously. To reduce extensive fibrin crosslinking, all the reactions contained 20 μM ZED1301 (FXIII inhibitor; Zedira). Each clot was mixed with an equal volume of lysis buffer (2% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.4, containing 1/100 volume of protease inhibitors cocktail and phosphatase inhibitors cocktail [Merck]) and incubated for 30 min on ice. Lysates were slowly rotated overnight at 4°C. The fibrin clots were pelleted by centrifugation, washed twice with 1% NP-40 lysis buffer, dissolved in SDS/urea solution, and analyzed by SDS-PAGE/immunoblotting. We also determined if other major platelet surface receptors (GPIb, integrin β3) and tubulin were associated with the clots. Because some proteins are precipitated in the cytoskeletal fraction under similar conditions, platelets in the absence of fibrinogen were also treated with lysis buffer at a 2-min incubation time, and the relative amount of the platelet proteins were calculated. The original sample containing platelets and fibrinogen is dissolved directly with SDS sample buffer and used as control for calculating the relative amounts of protein in each condition. The amount of clot-bound protein is expressed as relative amount to the corresponding band in the control sample.

2.7 | Confocal imaging of platelet binding to fibrin clots

Each clotting mixture contained 80% unlabeled fibrinogen and 20% Alexa Fluor 555-labeled fibrinogen (total fibrinogen concentration of 1 mg/ml). After adding thrombin (0.2 U/ml) to the mixture, it was immediately pipetted into a well of an 8-chamber Nunc Lab-Tek II Chamber slide (Thermo Scientific). The formed clots on the slides were stored at 4°C overnight and then treated with a 1/100 dilution of Protease Inhibitor Cocktail, washed twice with PBS, and blocked with 1% BSA/PBS for 1 h before use.

Washed platelets in HT buffer (100 μl of 3-5 × 10^7 platelets/ml), added with hFab (human Fab, 100 μg/ml), mFab-F (100 μg/ml), or Dasatinib (50 nM), were aliquoted over the clot on the slide. After 30 min at 37°C, the unadhered platelets were discarded; clots washed four times with PBS; 200 μl of formalin was added and incubated for 10 min to fix the samples, followed by thorough washing with PBS. Samples were stained with AlexaFluor-488-labeled M148 (anti-αIIbβ3, 4 μg/ml in 0.5% BSA/PBS). Vectashield (nonhardening formula; Vector Laboratories) was dropped over the sample and a coverslip placed over it. The samples (coverslip-side down) were imaged with an FV300 IX81 laser-scanning confocal microscope, with a ×60 oil immersion objective (Olympus UK).

2.8 | Statistical analyses

The paired *t*-test was applied to compare the data using Prism version 8 software (GraphPad).

3 | RESULTS

3.1 | GPVI-Fc_2 does not bind to fibrinogen

We previously reported,[18] assessed by ELISA, that GPVI-Fc_2 bound strongly to fibrinogen D-fragment, but only very weakly to fibrinogen. Because others[17] reported that GPVI monomers bound to fibrinogen, we verified that the lack of binding to fibrinogen was not due to steric hinderance, conformational change, or limited exposure of its binding site because of directly immobilizing it on polystyrene wells or other surface.[24] We examined GPVI-Fc_2 binding to fibrinogen by using biotinylated fibrinogen indirectly immobilized to streptavidin-coated wells; less than one biotin molecule was incorporated per fibrinogen molecule, so most of the fibrinogen would be immobilized by a single biotin-streptavidin bond. GPVI-Fc_2 bound to neither indirectly immobilized nor directly immobilized fibrinogen, but showed strong, saturable binding to fibrinogen fragment D (Figure 1), verifying that it does not bind to intact fibrinogen.

3.2 | GPVI-Fc_2 binding to fibrin clots

We developed an assay to measure GPVI-Fc_2 binding to fibrin clots. In this assay, the bound proteins on fibrin clots are separated from the unbound form in solution by centrifugation to form pellets and by thoroughly washing the pellets with buffer. The fibrin pellets are then dissolved in SDS/urea solution and the amounts of GPVI-Fc_2 are analyzed by immunoblotting.

The validity of our assay was shown by the following results: (1) very little albumin or human Fc, used to determine nonspecific binding, was bound to the clots, so the procedures were sufficient to remove the unbound proteins trapped in the gel. (2) In our analyses, the normalization control sample containing 25 μg/ml GPVI-Fc_2, and 187.5 ng of protein is applied to SDS-PAGE after mixing with sample buffer. Figures 2 and 3 show that most of the GPVI bands are less than 80% of the control band, which corresponds to 150 ng at most. There is an almost linear correlation between the amount of GPVI-Fc_2 applied to SDS-PAGE and protein band strength up to about 150 ng (Figure 4), validating our method for quantitative measurement.

GPVI-Fc_2 bound specifically to the noncrosslinked fibrin clots with typical saturation kinetics, consistent with a classical one-site receptor; K_D values ranged from 111.4 (Figure 2B graph) to 248.4 μg/ml (mean ± SD = 1.19 ± 0.26 μM, n = 4).

GPVI-Fc_2 binding to crosslinked clots was examined by including Ca^{2+} (2 mM) and FXIII (2 μg/ml) in the clotting mixtures; these more-resistant clots were dissolved in 1% SDS/8M urea/2%

mercaptoethanol and detected as GPVI-Fc (single chain) by anti-human IgG antibody because 1G5 does not react with reduced GPVI. GPVI-Fc_2 also binds specifically to the crosslinked clots, but its affinity was increased by about 2.4-fold; K_D = 81.66 μg/ml for the Figure 3 curve (0.496 ± 0.166 μM, *n* = 4).

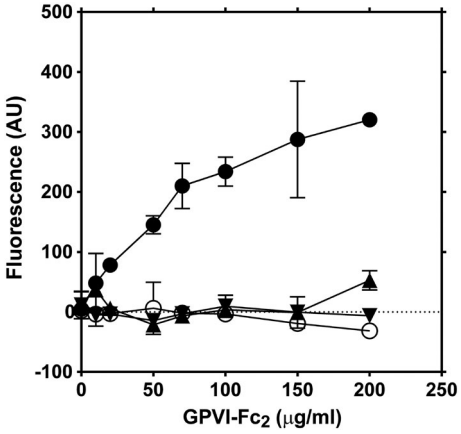

FIGURE 1 ELISA analysis of GPVI-Fc_2 binding to immobilized fibrinogens. Biotinylated fibrinogen was immobilized to streptavidin-coated wells and fibrinogen, biotin-fibrinogen, and fibrinogen D fragment were directedly immobilized on the wells of the ELISA plate. After washing, fibrinogen-coated wells were reacted with different concentrations of GPVI-Fc_2 for 1 h and the bound GPVI-Fc_2 was detected by 1G5 (anti-GPVI primary antibody, 5 μg/ml)/IR 800CW anti-mouse IgG (secondary antibody). ○, biotin-fibrinogen immobilized on streptavidin-coated surface; ▲, biotin-fibrinogen directly immobilized on surface; ▲, nontreated fibrinogen directly immobilized; ●, fibrinogen fragment D directly immobilized. GPVI-Fc_2 bound to fibrinogen fragment D in a dose-dependent and saturable manner. However, GPVI-Fc_2 did not bind to any of the immobilized intact fibrinogens, regardless of if they were directly or indirectly immobilized on the surface. GPVI, glycoprotein VI; GPVI-Fc_2, GPVI-extracellular domain; IgG, immunoglobulin G

3.3 | Analysis of GPVI monomer binding to fibrin clot

We compared the binding of dimeric and monomeric GPVI to fibrin clots by using fully glycosylated proteins having the same amino acid sequence with respect to the GPVI portion: Revacept (GPVI-dimer), a recombinant dimeric protein comprising two chains of the extracellular domain of GPVI, and GPVI-His, which contains a single chain of the same GPVI extracellular domain, and hence serves as GPVI monomer in these experiments. Binding curves for Revacept were similar to those obtained for GPVI-Fc_2 binding to noncrosslinked and crosslinked clots, with K_D values of 1.18 ± 1.32 μM (*n* = 2) and 0.62 ± 0.17 μM (*n* = 2), respectively (Figure 5B). These are similar to the corresponding values determined for GPVI-Fc_2, and crosslinking increases Revacept affinity by about twofold, as it did for GPVI-Fc_2.

In marked contrast, binding curves of GPVI-His are almost linear and nonsaturating (Figure 5A), suggesting that the binding of GPVI monomer to the fibrin clots is nonspecific or of very low affinity. Crosslinking fibrin did not affect GPVI-His binding.

3.4 | The effects of inhibitors on GPVI-Fc_2 binding to fibrin clots

We examined how proteins that interfere with the interaction between GPVI-Fc_2 and fibrinogen fragment-D or D-dimer affect dimer binding to the clot (Figure 6). The inhibitory GPVI-dimer-specific antibody mFab-F partially but significantly inhibited the GPVI-Fc_2 binding to the clot (100 μg/ml, *p* = .070; 150 μg/ml, *p* = .0014; 200 μg/ml, *p* = .0019 *n* = 6), indicating a GPVI-dimer-specific interaction. Fragment-D and D-dimer weakly inhibited (*p* = .0129 and *p* = .0235, respectively), supporting the conclusion that GPVI-Fc_2 binds to these fragments.[18] Although the GPVI-specific agonist

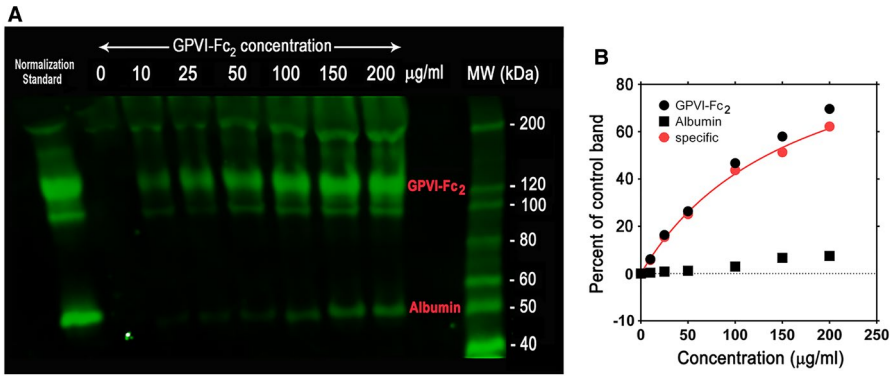

FIGURE 2 GPVI-Fc_2 binding to noncrosslinked fibrin clot. Details of the preparation of the fibrin clot and quantitation of the fibrin-bound GPVI are described in the Methods. (A) A typical gel pattern for analyzing fibrin-bound GPVI-Fc_2 by our method. (B) The quantitative relationship between the amount of GPVI-Fc_2 and band strength; total clot-bound GPVI-Fc_2 and albumin (nonspecific binding) are expressed as a percent of the normalization standard (clotting mixture containing 25 μg/ml each of GPVI-Fc_2 and albumin, but no added thrombin). Specific binding of GPVI-Fc_2 (%, red circles) is calculated by subtracting nonspecific binding (albumin, %, black squares) from the total binding of GPVI-Fc_2 (%, black circles) and fit to a one-site binding model (red curve). The GPVI-Fc_2 binding to the clot is dose-dependent and saturable, exhibiting classical receptor binding kinetics. GPVI, glycoprotein VI; GPVI-Fc_2, GPVI-extracellular domain

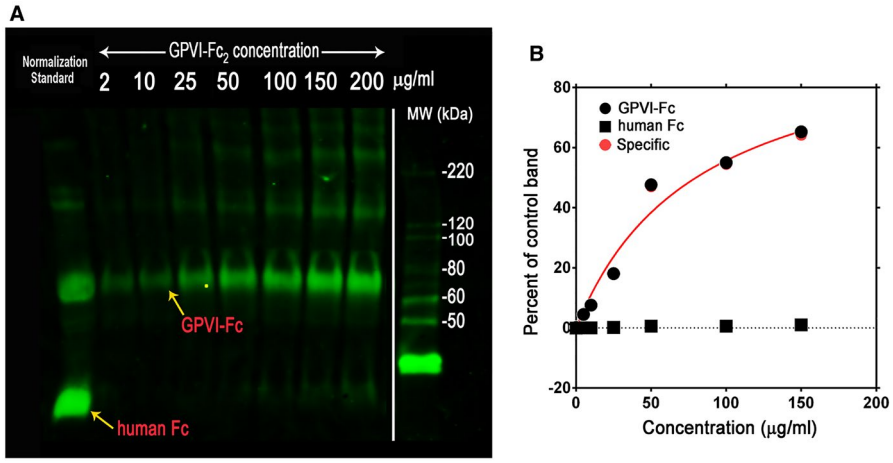

FIGURE 3 GPVI-Fc_2 binding to crosslinked fibrin clot. GPVI-Fc_2 binding to crosslinked fibrin clot (formed in the presence of Ca^{2+} and factor XIII) was analyzed as described in Figure 2 and the Methods. (A) Typical gel pattern for analyzing the GPVI-Fc_2 binding to crosslinked fibrin clot. (B) Quantitative results. GPVI-Fc_2 was reduced, and thus detected as GPVI-Fc with anti-human IgG antibody that detected the Fc portion of GPVI-Fc and the control protein was human Fc. After normalization with the standard, total binding of GPVI-Fc_2 (black circles, %) was subtracted by the value for human Fc (black squares, %) to obtain specific binding of GPVI-Fc_2 (%, red circles); specific binding was fitted to a one-site model. GPVI-Fc_2 binding to the crosslinked clot is dose-dependent and saturable, but had higher affinity than its binding to the noncrosslinked clots. GPVI, glycoprotein VI; GPVI-Fc_2, GPVI-extracellular domain

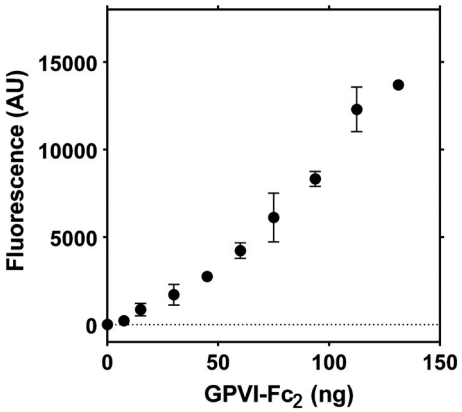

FIGURE 4 Relationship between band strength and applied amount of GPVI-Fc_2. Amount of GPVI-Fc_2 as detected by our SDS-PAGE/Western blotting system nearly linearly increases with the amount of GPVI-Fc_2 applied to SDS-PAGE. This validates that our system for analyzing GPVI-Fc_2 binding to clots is quantitative in the concentration of GPVI-Fc_2 that we used. GPVI, glycoprotein VI; GPVI-Fc_2, GPVI-extracellular domain

CRP-XL strongly inhibited GPVI-Fc_2 binding to fragment-D and D-dimer,[18] it did not affect GPVI-Fc_2 binding to fibrin clots.

3.5 | Analysis of platelet receptor binding to fibrin clot

These data indicate that purified GPVI-Fc_2 binds to fibrin clots, so we formed clots from a mixture of platelets and fibrinogen to examine how the platelet-surface receptors, GPVI, GPIb, and CD61 (integrin β3 subunit), contribute to the interaction between platelets and fibrin in the clot.

Figure 7 shows representative gel patterns for the clot-bound (n = 13) and cytoskeleton-associated (n = 6) platelet-derived proteins. GPIb is associated with both the fibrin clot and cytoskeleton, with similar strong staining observed in both. GPVI and CD61 were only in the clot. CD61 staining is significantly inhibited by the αIIbβ3 inhibitor Integrilin (p < .0001, n = 13) and enhanced by mFab-F (p = .0034, n = 13). Clot-bound GPVI is increased in the presence of Integrilin (p = .0415, n = 13). However, we could not detect any effect of mFab-F on GPVI binding. The low intensity of the GPVI band combined with the weak inhibitory effect of mFab-F would account for lack of observed significant effect. Only a small amount of tubulin is bound to fibrin and not affected by any of the inhibitors.

3.6 | Confocal imaging of platelets bound to crosslinked and noncrosslinked clots

Fluorescently labelled fibrin clots were formed with or without crosslinking, and then washed platelets allowed to adhere to the clots, followed by staining for αIIbβ3. In the noncrosslinked clots, almost all the control platelets were round in shape with no protrusions, suggesting that they are not activated, with only a few well-spread platelets observed. Particularly evident was the "halo" of fibrin, colocalizing with integrin αIIbβ3 on the platelet periphery. Dasatinib (tyrosine kinase inhibitor, which inhibits Src kinases) prevented all spreading and decreased association with fibrin, but the platelets still adhered. mFab-F did not affect the platelet binding in four of six preparations, but there is apparently less fibrin bound around the platelet perimeter.

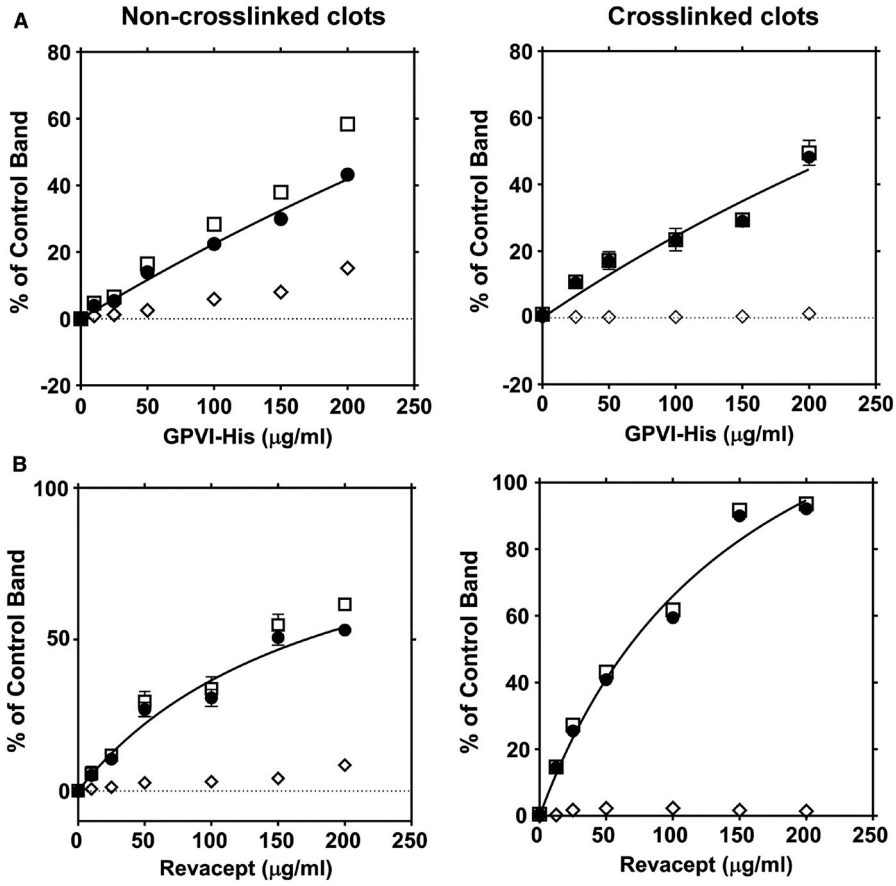

FIGURE 5 Analysis of Revacept and GPVI-His binding to fibrin clots. Revacept, which contains 2 chains of the extracellular domain of GPVI, was used as GPVI-dimer and GPVI-His, comprised of one chain of the same GPVI-extracellular domain as Revacept, was used as GPVI-monomer. Binding was measured by the same methods as described in Figures 2 and 3. Revacept and GPVI-His were detected by 1G5 or anti-human IgG and anti-His antibodies, respectively. (A) Typical curves for the binding of GPVI-His to noncrosslinked (left) and crosslinked fibrin (right) clots. The binding in the concentration range of 50 to 200 μg/ml is nearly linear, consistent with it being of very low affinity or nonspecific. Crosslinking the clots had no effect on GPVI-His binding. (B) Typical curves for the binding of Revacept to noncrosslinked fibrin (left) and to crosslinked fibrin (right). Revacept binding is dose-dependent and saturable and Revacept has a higher affinity for the crosslinked fibrin clot than the noncrosslinked one. □, total binding of Revacept or GPVI-His; ●, specific binding; ◊, binding of control proteins

Platelets adhered to crosslinked clots had notably different morphology. The control platelets were well-spread on crosslinked fibrin, and mFab-F did not affect this. Dasatinib appeared to decrease the number of well-spread platelets and the platelets had a spikey appearance. No sample had evident fibrin colocalized on the platelet periphery. These results suggest that resting platelets would be slightly activated on the fibrin fibers and the crosslinked fibrin would more strongly activate them, resulting in spreading.

4 | DISCUSSION

Thrombus formation is a process combining platelet activation and blood coagulation, with platelet GPVI involved in both systems by functioning independently as a collagen receptor and fibrin receptor, respectively. The collagen-binding functional form of GPVI has been established to be a dimer composed of two GPVI molecules, but there are ongoing controversies about which form of GPVI, the monomer or dimer, is the functional fibrin receptor and about the GPVI binding to fibrinogen or fibrin. Our present study aimed to resolve these issues.

The discrepant observations by different laboratories may arise from using ELISA assays with immobilized fibrinogen or fibrin, a system far from the physiological environment of this interaction. ELISA cannot address the GPVI-fibrin-fiber interactions under physiological conditions, where platelets would interact with clots formed from a three-dimensional network of fibrin fibers that have been crosslinked via the transglutaminase activity of FXIIIa. Physiologically, fibrin forms a gel-like clot at higher fibrinogen concentrations by thrombin. Fibrin has specific affinity for numerous plasma proteins, including plasminogen, factor XIII, fibronectin, VWF, and prothrombin[25]; and plasminogen binding to fibrin depends on fibrin structure.[21] Thus, the artificially formed and immobilized fibrin used in ELISA assays, typically formed with fibrinogen concentrations <100 μg/ml, is in the soluble form (monomeric or partially polymerized fibrin) and as such has properties

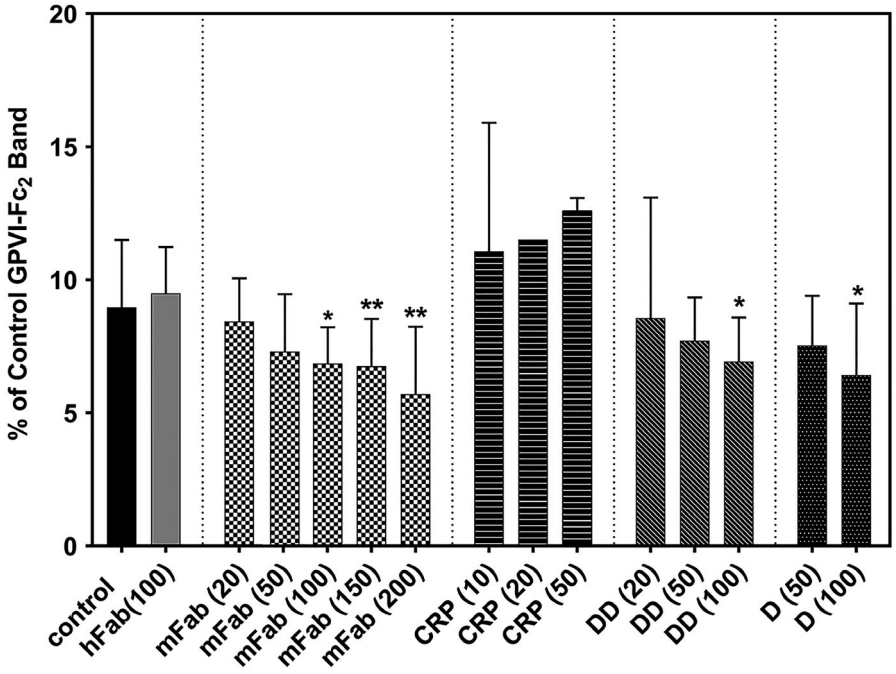

FIGURE 6 Effects of inhibitors on GPVI-Fc_2 binding to fibrin clots. The effects of the anti-GPVI dimer-specific antibody mFab-F; collagen mimetic CRP-XL; fibrinogen fragment D, and D-dimer on GPVI-Fc_2 binding to noncrosslinked fibrin were analyzed. The concentrations of each antagonist in μg/ml are given in parentheses. mFab-F significantly inhibits the binding at concentrations greater than 100 μg/ml and fragment D and D-dimer weakly inhibited the binding at the highest concentration of 100 μg/ml. CRP-XL had no effect. $^*p < .05$, $^{**}p < .01$, compared with control (no added inhibitor). GPVI, glycoprotein VI; GPVI-Fc_2, GPVI-extracellular domain

not fully reflecting those of fibrin fibers in physiological clots. The inhibition of GPVI-dependent platelet activation by GPRP, an inhibitor of fibrin clot formation,[15-17] supports our hypothesis that GPVI binds only to fibrin fibers. Here we developed a system to quantitatively determine GPVI dimer and monomer binding to fibrin fibers in clots to reconcile the different findings. We determined GPVI binding to fibrin clots formed from thrombin cleavage of near-serum concentrations of fibrinogen, which mimics the clots formed from a three-dimensional network of fibrin fibers as seen in vivo. Furthermore, we have used fully glycosylated recombinant proteins of the GPVI extracellular domain (GPVI-Fc_2 and Revacept and GPVI-His, the one-chain form containing the same GPVI-domain as Revacept along with a His tag) to assess this interaction, also removing further uncertainty caused by using truncated or incompletely glycosylated recombinant proteins of the GPVI extracellular domain.

We verified that neither directly immobilized fibrinogen nor indirectly immobilized biotin-fibrinogen bound to GPVI-Fc_2, but observed binding of fibrinogen D-fragment that was concentration-dependent and saturable (Figure 1), as reported previously. This suggests that the GPVI-dimer binding site in the D-domain of fibrinogen is not exposed to the surface in an intact fibrinogen molecule and its processing by the coagulation cascade to fibrin is required to make these sites available.

In our clot assay, GPVI-Fc_2 shows a typical one-site binding curve with a K_D of 1.19 ± 0.26 μM for noncrosslinked fibrin and 0.50 ± 0.17 μM for crosslinked fibrin, but this was not a significant difference ($p = .065$). These K_D values are similar to the K_D (0.3-0.4 μM) that we reported for GPVI-Fc_2 binding to immobilized D fragment or D-dimer[18] by ELISA and similar to its K_D (0.576 μM) for collagen as measured by surface plasmon resonance.[1] GPVI dimer, and not GPVI monomer, was indicated to bind to collagen fibers that are prepared from collagen gel but GPVI dimer does not bind to soluble (mostly monomeric) collagen.[23] The GPVI-dimer binding to fibrin clots is inhibited by the GPVI-dimer-specific antibody mFab-F (Figure 6), and this is weakly inhibited by fragment D and D-dimer, a consistent set of observations. The modest effect of fragment D or D-dimer is reasonable because each is competing with a high concentration of fibrin. CRP-XL, however, was not inhibitory, although it strongly inhibited GPVI-Fc_2 binding to fragment-D or D-dimer,[18] suggesting that

GPVI-Fc_2 binding sites for fibrin and fragment D would be slightly different and CRP-XL would bind closely to the latter site. The binding site of fibrinogen for GPVI dimer would be exposed after fibrin clot formation or proteolytic degradation by plasmin to fragment D. The reported conformational change in fibrinogen upon its conversion to fibrin[20,21] may enable GPVI dimer to access its binding site in the D-domain.

Revacept, whose structure is similar to GPVI-Fc_2, specifically bound to noncrosslinked (K_D = 1.18 ± 1.32 μM) and crosslinked fibrin clots (K_D = 0.617 ± 0.172 μM; Figure 5B). In marked contrast, monomeric GPVI-His exhibited nonsaturating binding to either type of clot, consistent with either very low affinity or nonspecific binding to the clots (Figure 5A). The low affinity of GPVI-His could be deduced from its monovalency compared with bivalent Revacept. Onselaer et al[16] and Mangin et al[17] reported that GPVI-monomer bound to both fibrin and fibrinogen, with a K_D of about 0.3 μM, but detected no GPVI-dimer binding. GPVI-His binding to fibrin in our experiments is nonsaturable, and we cannot explain why this nonsaturable binding is so high because the nonspecific bindings of the control proteins (albumin and IgG Fc protein) are much lower. GPVI-His might have a weak affinity for the fibrin clot and our assay method, which is more complex because the clot is a gel, may enhance the overall binding of GPVI-His. Previous papers[16,17] did not

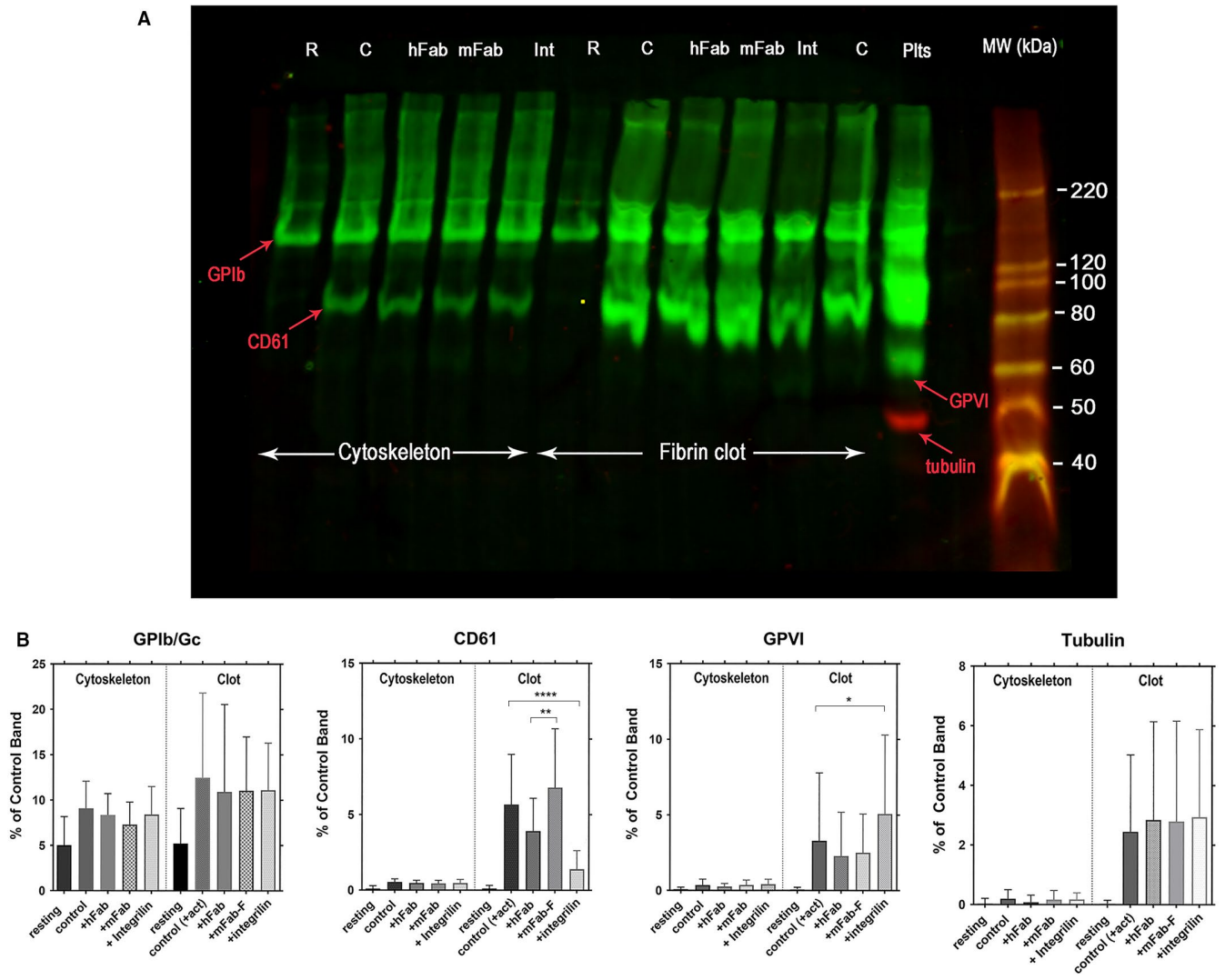

FIGURE 7 Analysis of the contribution of GPVI on platelet binding to fibrin clot. Fibrin clots were formed in the presence of platelets and the following clot-bound proteins originating from platelets were analyzed: GPIb/Gc (glycocalicin), CD61 (integrin β3), GPVI, and tubulin were measured. The platelet cytoskeleton of thrombin-activated platelets was also precipitated in separate experiments to compare the difference in associated proteins. We determined the effect of inhibitors on the amount of these associated proteins. (A) An example of the gel pattern used to analyze the clot and cytoskeleton-associated proteins from platelets. The immunoblots were stained with appropriate primary antibodies followed by fluorescently labeled secondary (see Methods) and quantitated by a Licor Odyssey scanner. R (negative control; no thrombin treatment), C (control, no added inhibitor), hFab (human Fab only control), mFab (mFab-F, anti-GPVI-dimer), Int (Integrilin, antagonist of integrin αIIbβ3), Plts (platelets; original sample, no treatment). (B) Summary of the quantitation of platelet proteins associated with the clot and platelet cytoskeleton. The amounts of protein bands were calculated as percent of the original number of corresponding bands in platelets shown in plts in the figure. GPIb/Gc is found in both the cytoskeleton and clot fractions. CD61 is only associated with the clot and is significantly inhibited by integrilin (****$p < .0001$, compared with the control) and increased by mFab-F (**$p \leq .005$, compared to the control Fab [hFab]). GPVI is only associated with the clot fraction and integrilin that inhibits αIIbβ3 increases the amount of clot-associated GPVI (*$p \leq .05$). GPVI, glycoprotein VI; GPVI-Fc_2, GPVI-extracellular domain

report the concentration dependence of GPVI monomer binding to fibrin but nonetheless concluded that it binds to fibrin, despite saturability of binding being ignored.

Interaction between fibrin and platelet-surface receptors αIIbβ3, GPVI, and GPIb were examined by our clot system. Fibrin clots were formed in the presence of platelets, the platelets were dissolved with platelet lysis buffer, and then proteins bound to fibrin were analyzed by immunoblotting. The very low amount of β-tubulin (used as the negative control) detected in fibrin (Figure 7) indicated that our lysis condition successfully dissolves platelets and there is no retention of nonspecifically bound proteins in the fibrin fraction. Some platelet proteins other than GPVI and integrin β3 may also precipitate with the cytoskeleton under our lysis conditions, particularly GPIb.[26,27] To exclude this possibility, we analyzed the proteins precipitated in the absence of fibrinogen. Similar amounts of GPIb were found in the cytoskeletal precipitate and in the fibrin clot, so

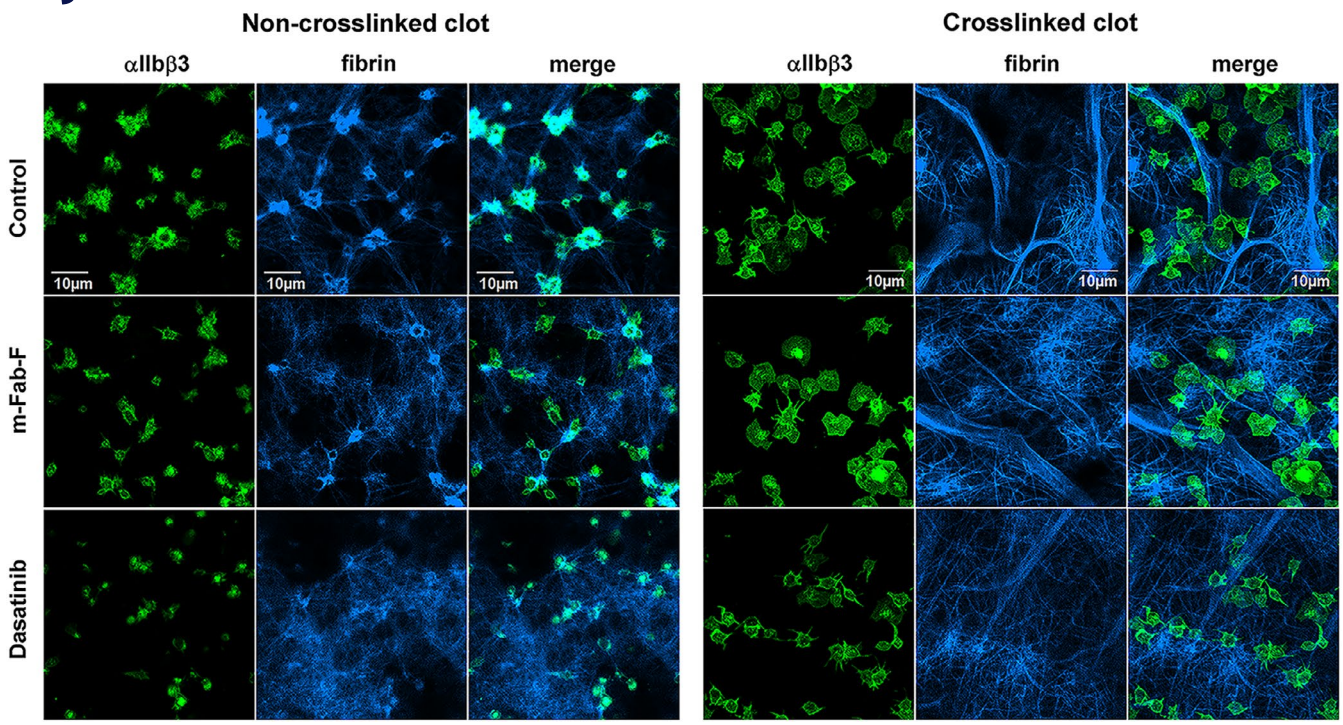

FIGURE 8 Confocal images comparing platelets bound to noncrosslinked and crosslinked clots. Clots were formed with a mixture of unlabeled and Alexa Fluor 555-labeled fibrinogen (1 mg/ml final concentration) without (noncrosslinked clots) and with Ca^{2+} and factor XIII (crosslinked clots) by adding thrombin; details are described in the Methods. Platelets (control: no inhibitor, m-Fab-F: 100 μg/ml, Dasatinib: 50 nM) were allowed to bind to the clots for 30 min, unadhered platelets removed from the clots and then the clots were fixed for each condition. Noncrosslinked clots: only a few platelets were spread on the noncrosslinked clots, the rest were round in shape, with evident fibrin colocalizing with integrin αIIbβ3 on the periphery of the platelets in the control. Dasatinib-treated prevented all spreading and decreased association with fibrin, compared with the controls, but the platelets still adhered. mFab-F did not affect the platelet binding, but in four of six preparations, there is apparently less fibrin bound around the platelet perimeter. Crosslinked clots: Control platelets were well spread on the crosslinked fibrin, and mFab-F did not affect this. Dasatinib appeared to decrease the number of well-spread platelets. None of the samples had evident fibrin colocalized on the platelet periphery, compared with those in the noncrosslinked clot

the GPIb associated with the fibrin clot can mainly be ascribed to its association with the cytoskeleton. GPIb was reported to bind to VWF in platelet adhesion to fibrin under flow conditions,[28] but because our fibrinogen preparation is VWF-free, this interaction is not relevant to our assay. Both platelet-derived GPVI and integrin β3 were detected significantly in the clots, indicating that the fibrin clot contains the platelet proteins specifically binding to fibrin. Integrilin markedly decreased clot-bound β3, indicating that active αIIbβ3 contributes to platelet binding to fibrin. However, Integrilin increased the amount of clot-bound GPVI, suggesting that GPVI in platelets can bind to fibrin, in lieu of active αIIbβ3. This is consistent with our observation that αIIbβ3-deficient platelets from a Glanzmann's thrombasthenia patient retained low-level but significant binding to fibrin and fragment-D as assessed by both static and flow adhesion methods.[18] Zhang et al[10] analyzed the effect under flow conditions of an anti-GPVI antibody on platelet adhesion to fibrin made from purified fibrinogen and showed it to be inhibited by the antibody, supporting our results. However, the antibody did not inhibit platelet adhesion to fibrin fibers formed on immobilized tissue factor, and, accepting the latter result over the former, they concluded that GPVI was not a functional platelet receptor for fibrin. The study by Zhang et al suggests that a different mechanism may be involved in platelet adhesion under flow.

Mammadova-Bach et al[14] and Alshehri et al[15] reported that fibrin enhances thrombin-induced platelet activation. Alshehri et al show that Syk and FcR-gamma phosphorylations were induced mainly through GPVI-dependent activation, but they were strongly enhanced in the presence of fibrin and thrombin. Onselaer et al[16] later reported that the addition of fibrin induced weak platelet aggregation and platelet spreading on a fibrin surface, indicating that fibrin activated the platelets. Because the clot assay requires the addition of thrombin, it would be difficult to resolve activation by fibrin from thrombin-induced activation. Thus, it would be reasonable to admit that most of the platelets would be activated by thrombin. Only activated platelets bind to fibrin,[29] so they would bind to fibrin mainly through activated αIIbβ3. Our results thus suggest that GPVI may significantly contribute to the binding of resting platelets to fibrin.

To analyze the interaction of resting platelets with fibrin clots, we allowed resting platelets to adhere to the fibrin clot and observed the morphology of the adhered platelets and fibrin (Figure 8). Control platelets bound to noncrosslinked fibrin showed that some platelets are spread, whereas spreading is slightly decreased by

mFab-F and strongly decreased by dasatinib, a tyrosine kinase inhibitor that strongly inhibits GPVI-induced platelet activation but only weakly inhibits thrombin and ADP-induced platelet activation through the inhibition of outside-in activation by integrin αIIbβ3.[30,31] Furthermore, control platelets show strong colocalization with fibrin(ogen) and integrin αIIbβ3 and the colocalization is decreased by mFab-F and dasatinib. These results suggest that platelets are weakly activated on fibrin and the activation is decreased by inhibition of GPVI binding to fibrin and its activation pathway. Platelet activation by fibrin via the GPVI pathway has been reported in previous studies.[14-16] In contrast, platelet binding to crosslinked fibrin is very different. Most of the control platelets are spread on the fibrin, and mFab-F and dasatinib had little or no apparent effect, although dasatinib-treated platelets show filopodia and less spreading. In these crosslinked clots, we could not detect colocalization of integrin αIIbβ3 and fibrin (ogen), which might be because crosslinked fibrin forms tightly packed fibrin fibers and fibrin is unable to move to the platelet surface. These results suggest stronger activation on crosslinked fibrin than on noncrosslinked fibrin, a phenomenon that should be explored in future studies.

This study resolves the question of which form of GPVI acts as the fibrin receptor. The dimer is the form of GPVI specifically interacting with fibrin as a receptor and this is consistent with the expected higher avidity of dimeric receptors. From the analogy between collagen binding and fibrin binding by GPVI, we can make the following hypothesis: GPVI dimers bind to both collagen and fibrin fibers, but not to their monomeric forms (soluble collagen and fibrinogen, respectively). This means that a binding site specifically formed in the dimer comprising two GPVI monomers recognizes a complementary binding site in the tertiary structure of either type of fiber. It follows that GPVI monomers, which cannot form such a binding site, is unable to bind to either collagen or fibrin fibers.

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CONFLICT OF INTEREST

None of the authors (Drs. Moroi, Induruwa, Farndale, and Jung) have any conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Masaaki Moroi designed and performed experiments, analyzed the data, wrote the paper, made figures, and provided antibodies; Stephanie M. Jung designed and performed experiments, performed the confocal imaging, wrote the paper, made figures, provided antibodies, and obtained funding; Isuru Induruwa performed some of the experiments; and Richard W. Farndale obtained funding, discussed the data with us, and critically read the paper.

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