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Non-redundant roles of platelet glycoprotein VI and integrin αIIbβ3 in fibrin-mediated microthrombus formation

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Running title: GPVI and αIIbβ3 in thrombus formation on fibrin

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Abstract

Objective- Fibrin is considered to strengthen thrombus formation via integrin αIIbβ3, but recent findings indicate that fibrin can also act as ligand for platelet glycoprotein VI.

Approach and Results- To investigate the thrombus-forming potential of fibrin and the roles of platelet receptors herein, we generated a range of immobilised fibrin surfaces, some of which were cross-linked with factor XIIIa and contained von Willebrand factor-binding peptide (VWF-BP). Multicolour microfluidics assays with whole-blood flowed at high shear rate (1000 s^{-1}) indicated that the fibrin surfaces, regardless of the presence of factor XIIIa or VWF-BP, supported platelet adhesion and activation (P-selectin expression), but only microthrombi were formed consisting of bilayers of platelets. Fibrinogen surfaces produced similar microthrombi. Markedly, tiggering of coagulation with tissue factor or blocking of thrombin no more than moderately affected the fibrin-induced microthrombus formation. Absence of αIIbβ3 in Glanzmann's thrombasthenia annulled platelet adhesion. Blocking of glycoprotein VI with Fab 9O12 substantially, but incompletely reduced platelet secretion, Ca^{2+} signalling and aggregation, while inhibition of Syk further reduced these responses. In platelet suspension, glycoprotein VI blockage or Syk inhibition prevented fibrin-induced platelet aggregation. Microthrombi on fibrin surfaces triggered only minimal thrombin generation, in spite of thrombin binding to the fibrin fibres.

Conclusions- Together, these results indicate that fibrin fibres, regardless of their way of formation, act as a consolidating surface in microthrombus formation via non-redundant roles of platelet glycoprotein VI and integrin αIIbβ3 through signalling via Syk and low-level Ca^{2+} rises.

Abbreviations: AF647, Alexa Fluor 647; FITC, fluorescein isothiocyanate; GPIb-V-IX, glycoprotein Ib-V-IX; GPVI, glycoprotein VI; VWF, von Willebrand factor; FXIIIa, factor XIIIa; OG488, Oregon Green 488; PPACK, D-Phe-Pro-Arg chloromethyl ketone; PRP, platelet-rich plasma; PS, phosphatidylserine.

Introduction

Glycoprotein (GP) VI is a platelet immunoglobulin (Ig) receptor, expressed at 3,000-4,000 copies per platelet, and known to be involved in the onset of thrombus formation.[1,2] Common concept is that GPVI mediates the initial activation of platelets in contact with exposed collagen in the vasculature, assisted by platelet integrins and by GPIb-V-IX which interacts with collagen-bound von Willebrand factor (VWF).[3-5] In the platelet membrane, GPVI is constitutively associated with the Fc receptor γ-chain, containing an intracellular immunoreceptor tyrosine-based activation motif (ITAM). Ligand binding induces clustering of GPVI and ensuing phosphorylation of the ITAM motif via Src family kinases.[6] This leads to activation of the tyrosine kinase Syk through its tandem SH2 domain, culminating in activated phospholipase Cγ2 and ensuing Ca^{2+} mobilisation.[2,6]

Since 2015, it has been recognised that GPVI can also act as a receptor for fibrin and fibrinogen.[7-9] Relevance of this finding comes from the earlier observation that fibrin formation can be both an initial and propagating process in vaso-occlusive thrombus formation upon vascular damage.[10-12] In this setting, the role of GPVI as a functional receptor for fibrin implies a crucial contribution of this receptor interaction in thrombus growth and in the propagation of coagulation. This idea is supported by the observations that: *(i)* GPVI binding to fibrin can trigger platelet procoagulant activity and ensuing thrombin generation;[8] and *(ii)* fibrin binds to procoagulant platelets via the cross-linking transglutaminase factor XIIIa (FXIIIa).[13] However, some authors have questioned the role of GPVI as a fibrin receptor in blood.[14]

Integrin αIIbβ3 is known as the conventional platelet receptor for fibrinogen and fibrin, being expressed at 50,000 to 80,000 copies per platelet.[15-17] Similarly to GPVI, integrin αIIbβ3 promotes platelet adhesion and activation via outside-in through Src-family and Syk protein tyrosine kinases.[6,18] Questions then arising are: *(i)* what are the roles of GPVI and αIIbβ3 in fibrin-dependent platelet activation; *(ii)* how do these receptor interactions contribute to thrombus formation; *(iii)* how can they prevent endless growth of the platelet-fibrin thrombus; and *(iv)* which is the role of blood flow and shear in this process.

In the present paper, we aimed to answer these questions and resolving the dispute on the role of GPVI. We studied the relative contribution of GPVI and integrin αIIbβ3 in fibrin-dependent platelet activation and thrombus formation under defined flow conditions. Since fibrin is known to bind VWF,[19] we also explored the contribution herein of the VWF receptor, GPIb-V-IX. Our results show that GPVI provides a weakly activating signal that relies on αIIbβ3-dependent platelet adhesion and Syk activation to form small-sized thrombi. Markedly, our data also indicate that the platelet-activating role of thrombin is dampened on fibrin surfaces.

MATERIALS AND METHODS

The authors declare that materials and data are available upon reasonable request from the authors. An extended version of Materials and Methods is available in the online-only Data Supplement.

Major Resources

Please see the Major Resources Table in the online-only Data Supplement.

Blood Withdrawal and Platelet Preparation

Blood was obtained by venepuncture from healthy volunteers (male and female), who had

not received anti-platelet or anticoagulant medication for at least two weeks. Informed consent was obtained according in compliance with the ethical principles of the Declaration of Helsinki, and studies were approved by the local Medical Ethics Committee (METC 10-30-023, Maastricht University). Blood samples were collected into 3.2% trisodium citrate (Vacuette tubes, Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Blood samples were also obtained from two patients with diagnosed Glanzmann's thrombasthenia, *i.e.* one homozygous patient lacking expression of integrin αIIbβ3 on platelets, and one heterozygous patient with 50% of normal αIIbβ3 expression.

Platelet-rich plasma (PRP) was obtained from the citrated blood by centrifugation at 870 g for 10 minutes.[20] After addition of 1:10 vol./vol. acid-citrate-dextrose (80 mM trisodium citrate, 183 mM glucose, 52 mM citric acid), the PRP was centrifuged at 2360 g for 2 minutes. Platelet pellets were resuspended into Hepes buffer pH 6.6 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose and 0.1 % bovine serum albumin). After addition of apyrase (1 U/mL) and 1:15 vol./vol. acid-citrate-dextrose, another centrifugation step was performed to obtain washed platelets. The platelet pellet was resuspended into Hepes buffer pH 7.45 (10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 5.5 mM glucose and 0.1 % bovine serum albumin).[20]

Microfluidic Flow Experiments

Glass coverslips were coated for 1 hour with two 1.5-mm diameter spots, each 3-mm apart, which contained the indicated type of fibrin or fibrinogen (upstream) and, when indicated, collagen type III (50 µg/mL) as a reference spot (downstream), and collagen type I (50 µg/mL). This microspot coating procedure eliminates cross-talk of thrombus formation between the adjacent surfaces.[21] For fibrin spots, fibrinogen (1 mg/mL, 0.5 µL) was applied for 30 minutes, after which α-thrombin (20 nM, 1 µL) was supplemented for additional 30 minutes. Where indicated, mixtures (1 µL) of α-thrombin (20 nM), FXIIIa (0.7 µg/mL) and $CaCl_2$ (10 mM) were applied on top of the fibrinogen. Residual FXIIIa activity of the fibrinogen preparation used was determined as 6% in comparison to plasma. Indicated spots were post-coated with the peptide VWF-BP (100 µg/mL, 1 µL). After the completion of coating, coverslips were blocked with 1% BSA in Hepes buffer pH 7.45 for 30 minutes.

As a standard, whole blood was flowed over the coated spots using a microfluidics chamber under conditions allowing coagulation.[12] In brief, 1.0 mL samples of citrated blood were co-perfused with recalcification medium using two pulse-free micro-pumps (Model 11 Plus, 70-2212, Harvard Apparatus), and a y-shaped mixing tubing. The recalcification medium (in a second 1 mL syringe) consisted of 32 mM $MgCl_2$ and 63 mM $CaCl_2$ in Hepes buffer pH 7.45. Complete mixing was achieved at a volume ratio of 10 (blood) to 1 (recalcification medium).[22] Flow rates were adjusted to give a total wall-shear rate of 1000 s^{-1} or 100 s^{-1}.

Fluorescent labels added per blood sample were $DiOC_6$ (platelet staining), AF568-annexin A5 (phosphatidylserine, PS exposure), and AF647-anti-CD62P mAb (P-selectin expression), as described.[23] When appropriate, samples were preincubated for 10 minutes with vehicle, inhibitor PRT-060318 (10 µM, in 0.4 µg/mL pluronic plus 0.5% DMSO) and Fab 9012 (50 µg/mL, in saline). Inhibition of GPVI was achieved with Fab 9O12 (50 µg/mL), which has previously been shown to interfere in the interaction of GPVI and fibrin.[8] Brightfield and multicolour fluorescence images were recorded per spot over time.[24] Per donor, all control and intervention conditions were repeated at least in duplicates. Collected time series of brightfield and fluorescence microscopic images were analysed by using pre-defined

scripts,[25] formatted in the open source package Fiji.[26]

Scanning Electron Microscopy

For electron microscopy, fibrin-coated spots were prepared as for flow studies and coated on a Sefar matrix (sieve mesh, pores: 170 μm) using 96-wells plates.[27] Samples were fixed with 4% paraformaldehyde for 1 hour. After wash with phosphate-buffered saline, the samples were dehydrated by a 5-step gradient of ethanol (30-100%), and then dried by 10-minutes treatment with hexamethyl disilizane/ethanol (1:1) and 1 hour exposure to air. Dried samples were mounted onto aluminium pin studs with 12 mm carbon conductive tabs (Ted Pella, Redding, CA, USA), were sputter coated with gold (Quorum Technologies, Ashford, UK; vacuum pump: Edwards, Crawley, UK) on carbon tabs, and imaged. Table-top electron microscopy was performed, as before.[27]

Fibrin Suspension Preparation

Fibrinogen (1 mg/mL) was mixed with $CaCl_2$ (10 mM), FXIIIa (0.7 μg/mL) and thrombin (1 U/mL), and was left to polymerise for 1 hour. Subsequently, PPACK (20 μM) was added to inactivate the thrombin, the clot mixture was agitated until it turned liquid, and it was left for 15 minutes. The gel solution was then ultrasonicated at 20 kHz and amplitude of 80-100 μm until clear; this was followed by a centrifugation step at 1000 g for 5 minutes. The obtained pellet was homogeneously resuspended into Hepes buffer pH 7.45.

Platelet Aggregometry

Platelet aggregation was monitored by light transmission aggregometry using an automated Chronolog aggregometer (Havertown PA, USA) at 37°C with stirring at 1200 rpm. Platelet suspensions (2 x 10^8/mL) were incubated at 37°C for 2 minutes, antagonists were added for 10 minutes, followed by agonists sonicated fibrin, collagen-I (5 μg/mL), or α-thrombin (0.1 U/mL).

Cytosolic Ca^{2+} Measurements

Washed human platelets (2 x 10^8/mL) were loaded with Fluo-4 acetoxy methyl ester (8 μM) and pluronic (0.4 mg/mL) by a 40 minutes incubation in the presence of apyrase (1 U/mL).[28] After centrifugation step in the presence of acid-citrate-dextrose, the Fluo-4-loaded platelets were resuspended into Hepes buffer pH 7.45. Blood samples were supplemented with 10% of autologous Fluo-4-loaded platelets; inhibitors were added after 5 minutes. Changes in cytosolic $[Ca^{2+}]_i$ during flow-dependent adhesion of labelled platelets were recorded for 5 minutes, using a Zeiss LSM 510 confocal microscope, essentially as described before.[29] Time series of fluorescence images were analysed for changes in fluorescence intensity and for platelet adhesion, using Fiji/image J software.

Data Handling and Statistics

Data are represented as means ± SD. Statistical analysis was performed using GraphPad Prism v8 software (San Diego, CA, USA). Significance was determined using a two-way ANOVA (Dunnett and Sidak's multiple comparison test) or a one-way ANOVA (Dunnett's multiple comparison test); differences with $P<0.05$ were considered as significant.

Heatmaps were generated with the program R. For the heatmap representation, all parameters were univariate scaled to 0-10.[21] According to earlier procedures,[30] thrombus values of duplicate or triplicate flow runs from one blood donor were averaged to obtain

one parameter set per spot. Mean values of control and inhibitor runs were then compared per blood sample. For subtraction heatmaps, a conventional filter of $P<0.05$ (one-way ANOVA) was applied to determine relevant effects, as described before.[25,30]

RESULTS

Fibrin microstructure of coated spots

In order to assess the suitability of fibrin-coatings for flow chamber studies, we prepared a series of spotted fibrinogen surfaces which were treated with a thrombin mixture in the presence or absence of the cross-linking transglutaminase, FXIIIa, and/or a peptide (VWF-BP) capable to capture free VWF from blood.[31] Ultrastructural observation of the different preparations by scanning electron microscopy showed that in all conditions multiple layers of fibrin were formed, which presented as microstructures with both thicker and thinner fibres (Figure 1, arrows). The addition of VWF-BP did not alter the overall fibre structure. However, addition of FXIIIa resulted in fibrin fibres that appeared to be less densely packed with an overall thicker size. The latter observation may be due to the local high transglutaminase concentration upon the fibrin formation. For comparison, also fibrinogen-only spots were examined with or without VWF-BP. Electron microscopy did not reveal any fibrous structures in this case (data not shown).

Formation of only small-sized microthrombi on fibrin surfaces under flow

We examined how the different types of fibrin(ogen) spots formed with or without FXIIIa and/or VWF-BP (coded as S1-S6, see Table 1) were able to support thrombus formation under flow in microfluidics chambers. Therefore, citrated whole blood (labelled with $DiOC_6$, AF568-annexin A5, and AF647-anti-CD62P mAb) was flowed over sets of two spots at defined conditions, in coagulating condition (absence of PPACK).[22] After 10 minutes of flow at arterial shear rate (1000 s^{-1}), on all fibrin(ogen) surfaces, platelets adhered and formed small aggregates, not extending two or three cell layers, which we characterised as bilayer aggregates or microthrombi (Figure 2A; representative images of all six spots in Suppl. Figure I). In contrast, simultaneous flow experiments using collagen-I spots produced larger thrombi composed of multi-layered platelet aggregates (see below), such as reported before.[32] No platelets adhered to coverslip areas in between the coated spots.[21]

Capturing of multicolour fluorescence microscopic images at time points of 2, 4, 6, 8 and 10 minutes allowed to assess the kinetics of the process, in terms of six parameters. These were: $DiOC_6$ platelet adhesion (P1), thrombus morphological score (P2), thrombus contraction score (P3), bi- or multilayer score (P4), bi- or multilayer size (P5), P-selectin expression (P6), and PS exposure (P7).[25] End-stage brightfield and triple-coloured microscopic images from fibrin-FXIIIa spots (S3) are shown in Figure 2A. To compare the time-dependent parameter increases per type of fibrin spots (S1-4) and fibrinogen spots (S5-6), we scaled all values per parameter across surfaces (scale 0-10), and represented the results in a practical heatmap format (Figure 2B).

Overall, the heatmap analysis pointed to similar parameter increases over time for all types of fibrin spots (S1-4), although rates of platelet adhesion (P1) were slightly higher in spots containing VWF-BP (S2, S4). The latter observation pointed to a moderate enhancement of platelet adhesion but not to bilayer microthrombus formation, by plasma-bound VWF. On all fibrin spots, the adhered platelets gradually increased in P-selectin expression (P6), but remained low in PS exposure (P7), thus indicating an only moderate

platelet activation state.[2] Addition of FXIIIa during fibrin formation (S3, S4) slightly increased the formation of platelet aggregates, when compared to no added FXIIIa (S1, S23). Furthermore, the two spots with fibrinogen (S5, S6) were even less active in supporting platelet adhesion, bilayer aggregate formation and platelet activation (P-selectin expression) in comparison to the fibrin surfaces (Figure 2A-B).

Given the presence of 6% residual FXIIIa in the fibrinogen preparation used for the coating, we checked if transglutaminase contributed to platelet-activating effects by generating spots of fibrin (S1) and fibrin-FXIIIa (S3) in the presence of the FXIIIa inhibitor T101. However, after 10 minutes of whole-blood flow, both end-stage images (Suppl. Figure II, panel A) and scaled time-dependent parameters P1,5-7 did not show significant effects of this inhibitor (Suppl. Figure II, panels B-C). Together, these results pointed to a no more than modest role of FXIIIa-induced cross-linking in fibrin-dependent microthrombus formation.

Major role of Syk kinase in microthrombus formation on fibrin independently of coagulant strength

In order to assess tyrosine kinase-dependent signalling, blood samples were preincubated with the selective Syk kinase inhibitor PRT-060318, known to completely abolish the thrombus formation on collagen-like surfaces.[30] This inhibitor lowered platelet adhesion and abolished the bilayer aggregate formation and P-selectin expression on all prepared fibrin and fibrinogen spots (Figure 2A-B). Subtraction heatmaps of the scaled treatment effects over time showed for essentially all parameters a relevant reduction with PRT-060318, but not with DMSO vehicle (Figure 2C). For instance, on fibrin-FXIIIa spots, PRT-060318 gradually suppressed platelet adhesion parameters P1-2 (Figure 2Di-ii) and even tended to suppress the low PS exposure (Figure 2Diii).

Subsequent flow experiments were carried out with fibrin-FXIIIa (S3) spots, which were used as a standard. We first examined how the formation of microthrombi relied on the extent of coagulation, *i.e.* thrombin generation. Therefore, blood samples were incubated either with the thrombin inactivator PPACK, or with 5 or 10 pM tissue factor to trigger the extrinsic coagulation pathway. During 10 minutes of flow at 1000 s^{-1}, microscopic images again were captured and analysed for $DiOC_6$ platelet adhesion (P1), multilayer score (P5), P-selectin expression (P6) and PS exposure (P7). Markedly, the presence of either PPACK and tissue factor did not significantly change platelet adhesion, size of microthrombi or the P-selectin expression at fibrin-FXIIIa spots, when compared to control runs (Suppl. Figure III, panel A). In addition, Syk inhibition with PRT-060318 had a similar lowering effect on all parameters, regardless of the presence of PPACK or tissue factor (Suppl. Figure III, panel B).

A marked finding was that, for all variable coagulation conditions, the extent of PS exposure remained low for fibrin-FXIIIa spots (0.1-0.4 SAC%). On the other hand, parallel flow experiments on collagen-I spots caused formation of large-sized platelet thrombi, with high staining for $DiOC_6$ and high PS exposure, with or without tissue factor (Suppl. Figure IV). Hence, the low level of PS exposure observed on fibrin-FXIIIa spots was not due to limitations of the flow set-up, in agreement with earlier findings.[22]

To further assess the apparently limited role of thrombin in the microthrombus formation on fibrin spots, we compared to the effects of the thrombin receptor antagonist atopaxar and the thrombin inhibitor refludan. When added to the blood, neither end-stage images nor (subtraction) heatmaps of scaled parameters P1,5-7 indicated any effect of these interventions (Suppl. Figure V, panels A-B). In addition, we measured the ability of thrombi

formed on fibrin-FXIIIa and collagen-I spots to support (PS-dependent) thrombin generation, using an earlier described procedure based on the thrombin-induced cleavage of substrate Z-GGR-ACM.[33] The observed no more than minimal thrombin generation on fibrin-FXIIIa spots supported the conclusion that the procoagulant activity of the fibrin surface is low in comparison to the collagen-I surface (Suppl. Figure V, panels C-D). Together, these results indicated that the low thrombogenic effect of fibrin surfaces relies on Syk kinase signalling, which is relatively independent of coagulation triggering.

Shear-dependent contribution of integrin αIIbβ3 in microthrombus formation on fibrin

Considering that also integrin αIIbβ3 interaction with fibrin(ogen) can trigger Syk activation,[6,34] we went on to determine the role of this integrin in the microthrombus formation. Therefore, blood samples from two patients with Glanzmann's thrombasthenia were obtained, and flowed over fibrin-FXIIIa spots (S3) at arterial (1000 s^{-1}) or venous (100 s^{-1}) shear rate. Markedly, with blood from the homozygous patient, completely lacking platelet surface expression of αIIbβ3, the platelets failed to adhere to fibrin, regardless of the shear rate (Figure 3A-B). Control experiments indicated that VWF was present on the S3 (Fibrin FXIIIa) surfaces (see below). With blood from the heterozygous patient, presenting with reduced platelet αIIbβ3 expression, the platelets again did not adhere at the high shear rate. However, there was substantial platelet adhesion (P1) at the low shear rate. In the latter case, the normal P-selectin expression (P6) pointed to residual platelet activation. Taken together, these results pointed to a crucial, shear-dependent role of the αIIbβ3 integrin in the flow-dependent platelet interaction with fibrin.

Complementary roles of GPVI, αIIbβ3 and GPIb-V-IX in microthrombus formation on fibrin

Subsequent flow experiments were carried out with spots of fibrin-FXIIIa (S3) in combination with downstream collagen-III (S7), which was considered as a reference platelet-activating surface. The comparative analysis of fibrin-FXIII and collagen-III surfaces showed that the microthrombi on fibrin were less contracted and activated than those on the collagen III (Figure 4A-B). Considering that both GPVI and integrin αIIbβ3 can activate Syk kinase, in this setting we established the role of GPVI in the thrombus-forming process at both surfaces. Whole blood samples were treated with anti-GPVI Fab 9012, and flowed over the spots for 10 minutes at high shear rate of 1000 s^{-1}. Microscopic images, captured from these surfaces over time were analysed for the same parameters as before (P1,5-7). The results showed that, for fibrin-FXIIIa (S3), GPVI inhibition caused significant decreases of platelet adhesion, bi-layered aggregation and P-selectin expression (Figure 4A-B). The effects of Fab 9O12 on platelet adhesion and activation were similar for fibrin-FXIIIa and collagen-III spots. However, these were lower in comparison to Syk inhibition (compare Figures 2C and 4C). This notion agrees with a complementary activation pathway of Syk kinase, involving integrin αIIbβ3.

Fibrin has previously been shown to bind to VWF.[19] For the fibrin-FXIIIa spots exposed to flowing blood, we could confirm the binding of plasma-derived VWF by staining with an FITC-labelled anti-VWF antibody (Figure 5A). The presence of VWF was yet lower than on the reference spot, collagen-III. To determine contribution of the VWF-GPIb-V-IX axis to the microthrombus, we used an established blocking anti-GPIbα antibody, RAG35.[35] For both spots S3 (fibrin-FXIIIa) and S7 (collagen-III), the addition of RAG antibody resulted in a marked reduction in platelet adhesion, bilayer formation and P-selectin expression, already observable from the first minutes of flow (Figure 5B-C). Subtraction heatmaps however

pointed to larger effect for collagen-III than for fibrin-FXIIIa spots (Figure 5D).

Role of Syk and GPVI in fibrin-induced platelet aggregation

To further confirm the moderate signalling via Syk kinase and GPVI in fibrin-induced platelet activation, we examined the aggregation response of platelets upon stimulation with a sonicated, homogeneous fibrin suspension. This suspension was treated with PPACK to remove thrombin traces. Similar to the results of microthrombus formation, pre-treatment of platelets with Syk inhibitor PRT-060318 abrogated the fibrin-induced aggregation (Figure 6A-B). Similarly, the GPVI blocking Fab 9O12 suppressed fibrin-induced aggregation, but a residual shape change and aggregation remained. In comparison, both inhibitors also antagonised the platelet aggregation induced by collagen-I, but not by thrombin (Figure 6). As expected, treatment with the integrin antagonist tirofiban blocked the fibrin-induced aggregation response by >80% (n = 3, data not shown).

Fibrin-induced platelet Ca^{2+} signalling under flow

In collagen-induced platelet activation, GPVI adhesion under flow is known to induce a prolonged and high Ca^{2+} signal, leading to massive P-selectin expression and PS exposure.[21,36] To investigate the Ca^{2+} signal of platelets flowed over fibrin-FXIIIa, blood samples were supplemented with autologous Fluo-4-loaded platelets, and fluorescent $[Ca^{2+}]_i$ rises were measured in real time by confocal microscopy. The results show a consistent, moderate increase in Fluo-4 fluorescence in fibrin-adhered platelets, which was suppressed by Fab 9O12 (Figure 7A). A near complete major reduction in fluorescence increase was observed upon Syk inhibition with PRT-060318. The effects on Fluo-4 fluorescence increases paralleled effects on platelet adhesion (Figure 7B). However, detailed analysis of traces from single adhered platelets confirmed suppression of transient, spiking Ca^{2+} signal generation in the presence of Fab 9O12 or PRT-060318 (Suppl. Figure VI, panels A-C). Of note, no platelet adhesion could be observed in the presence of integrin inhibitor, tirofiban. Taken together, these results confirm that fibrin interaction activates platelets via Syk kinase and GPVI.

Fibrin in consolidating microthrombus formation

We explored why the fibrin-adhered platelet were insensitive to thrombin, for instance in flow with tissue factor. As a first approach, we adapted an earlier protocol, where single immobilised platelets in a flow chamber were triggered to generate star-like fibrin fibres.[37] These fibrin-forming platelets were post-perfused for up to 10 minutes with blood samples, again containing labels for platelets ($DiOC_6$), and P-selectin and PS exposure. Recording of brightfield and tri-colour fluorescence images from the same microscopic fields after blood flow showed considerable overlap between the staining (Suppl. Figure VII, panel A). Detailed analysis showed that the stainings concentrated around the immobilised platelets, rather than at the extending fibrin fibres (Supp. Figure VII, panel B). Thus, the $DiOC_6$-labelled microaggregates showed high overlap with the P-selectin exposing prior immobilised platelets, which was confirmed by measuring the overlap coefficients R (Suppl. Figure VII, panel C). This overlap was further increased by blood flow in the presence of integrin $\alpha IIb\beta 3$ antagonist tirofiban.

In an earlier study, we observed high binding of OG488-labelled thrombin to fibrin-containing thrombi.[11] This could be confirmed using the present coagulant flow conditions for fibrin-FXIIIa and collagen-I spots (Suppl. Figure VIII, panels A-B). As a control, blocking of

the coagulation process with PPACK suppressed cleavage of the OG488-labelled prothrombin probe, with as a consequence binding to PS-exposing platelets only (see figure and Ref. [11]). Taking together, these data suggest that fibrin fibres provide a relatively poor surface for newly-adhering platelets, but can trap locally cleaved prothrombin.

DISCUSSION

While GPVI has been identified as a receptor for fibrin and also fibrinogen,[7-9] the relative strength of the platelet-activating effect of fibrin via GPVI has not been examined in detail. It has been established that blood flow over immobilised collagens or collagen-related peptides via GPVI causes strong platelets activation responses, *i.e.* a prolonged Ca^{2+} signal, high integrin activation, P-selectin expression, PS exposure, and massive thrombus formation.[21,38-40] The present data, using a variety of immobilised fibrin surfaces, point to a weaker GPVI-dependent platelet-activating effect of blood flow over fibrin, in that fewer platelets adhered showing transient Ca^{2+} signals, residual P-selectin expression and limited PS exposure, altogether resulting in the formation of only small-sized microthrombi.

The microthrombus formation under flow appeared to be hardly influenced by producing the fibrin with or without added VWF-BP or FXIIIa. However, this does not rule out a role of VWF or FXIIIa in the thrombus-forming process, because fibrin can capture VWF from the blood plasma,[19] and FXIIIa can also be produced in coagulating plasma and released from activated platelets.[13] Evidence for a relative weakness of fibrin (in comparison to collagen) as platelet-activating surfaces was further corroborated by experiments showing that fibrin fibres extending from immobilised platelets are relatively ineffective in trapping newly perfused platelets, when compared to the immobilised platelets themselves.

Comparative analysis of the microthrombi on fibrin-FXIIIa and collagen-III spots indicated that the platelets on fibrin had a lower activation state than those on collagen. Nevertheless, on both surfaces, inhibition of Syk (PRT-060318) or blockade of GPVI (Fab 9O12) suppressed the flow-dependent platelet adhesion, aggregate formation and activation (P-selectin expression). In agreement with these findings, also light transmission aggregation studies using stirred platelet suspensions showed that the fibrin-induced aggregation process is abolished by both PRT-060318 and Fab 9O12.

Complete or partial defects in expression of integrin αIIbβ3 (Glanzmann's patients) resulted in an annulled platelet adhesion to fibrin under flow, which in case of partial deficiency was limited to the high shear rate condition. In addition, we could establish a role of GPIb-V-IX by using the blocking anti-GPIbα antibody RAG35.[35] This antibody substantially but not completely decreased platelet adhesion to both fibrin-FXIIIa and collagen-III spots, while the remaining adhered platelets still displayed P-selectin expression. Together, these findings point to complementary and non-redundant roles of GPVI, αIIbβ3 and GPIb-V-IX in the microthrombus formation on fibrin surfaces. Since the tyrosine kinase Syk is known to be phosphorylated and activated downstream of both GPVI and αIIbβ3,[6,30,41] our results suggest that concomitant activation via both receptors is required for formation of the microthrombi. This idea is supported by a previous study showing that Syk phosphorylation is a continuous process in murine thrombus growth, and that secondary Syk inhibition can annul platelet adhesion even on pre-formed thrombi under flow.[42] A non-redundant contribution of GPVI and αIIbβ3 can also be derived from the observation that perfusion of blood from patients with GPVI deficiency over fibrin spots resulted in an abolished aggregate formation, although individual platelets still adhered (unpublished data, but see

Ref. [43]).

Novel related observations were: *(i)* the low PS exposure of platelets on fibrin (although still dependent on low level GPVI), *(ii)* the relative inability of thrombin to alter fibrin-dependent microthrombus formation, and *(iii)* a low-level thrombin generation of platelets on fibrin in comparison to collagen. An explanation for these observations is the finding, supported by earlier studies,[11] that fibrin captures (fluorescent-labelled) thrombin, apparently without ability to cleave its substrate Z-GGR-ACM. This agrees with the earlier notion of irreversible thrombin binding to a fibrin network.[44] A suggestion then is that under the present microfluidic conditions fibrin-bound thrombin is unable to activate platelets. Clearly, more research needs to be done to better understand this phenomenon. Hence, our present findings lead to the concept that on fibrin a low platelet GPVI activation and an inactivation of thrombin induces only weak support of thrombus formation; or in other words, that platelet interaction with fibrin in particular consolidates the process of thrombus formation. However, we cannot rule out that under certain (patho)physiological static or flow conditions the role of fibrin is enlarged.[45]

The overall observation of fibrin-induced microthrombus formation suggests that fibrin fibres act as consolidating elements of the thrombus shield, such in contrast to vascular collagens which trigger the formation of larger size thrombi. Given that thrombus growth is regulated by secondary mediators, such as ADP and thromboxane A_2, which activate platelets in the thrombus core, it is not evident that the fibrin-GPVI interaction substitutes the high GPVI activation induced by collagens. A local inactivation of thrombin by fibrin may further contribute to this dampening process.

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Disclosures

JWMH is founder and co-owner at FlowChamber b.v. The other authors declare that no competing interests exist.

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Highlights

- Platelet adhesion to a fibrin layer under flow elicits only moderate glycoprotein VI activation.
- The interaction with glycoprotein GPVI interaction relies on $\alpha IIb\beta 3$ and forms small, bilayered thrombi.
- This consolidated thrombus formation is restricted by thrombin binding to fibrin.

Tables

Table 1. Coding of spots (*S*) and parameters (*P*) in whole-blood thrombus formation. Coding of variables in the order of appearance in heatmaps. Assumed relevant platelet-adhesive receptors are indicated per spot. Parameters were evaluated from microscopic images over time periods as indicated in figures. Abbreviation: SAC, surface-area-coverage.

S	Microspot coating	Adhesive receptors
S1	Fibrin	GPVI, αIIbβ3
S2	Fibrin + VWF-BP	GPIb, VI, αIIbβ3
S3	Fibrin FXIIIa	GPVI, αIIbβ3
S4	Fibrin FXIIIa + VWF	GPIb, VI, αIIbβ3
S5	Fibrinogen	GPVI, αIIbβ3
S6	Fibrinogen + VWF-BP	GPIb, VI, αIIbβ3
S7	Collagen III	GPIb, VI, α2β1

P	Image type	Description	Unit or scaling
Platelet parameters			
P1	$DiOC_6$	platelet adhesion	%SAC
P6	AF647 α-P-selectin	platelet activation	%SAC
P7	AF568 annexin A5	platelet PS exposure	%SAC
Thrombus parameters			
P2	brightfield	thrombus morphology score	1-5
P3	brightfield	thrombus aggregation score	1-3
P4	brightfield	thrombus contraction score	1-3
P5	brightfield	thrombus multilayer coverage	%SAC

Figure Legends

Figure 1. Fibrillar microstructure of immobilised fibrin surfaces. Representative scanning electron microscopy (SEM) images of immobilised fibrin spots, produced from fibrinogen coatings with or without FXIIIa and VWF-BP. Mixtures were allowed to generate fibrin fibres on a SEFAR filter for 30 minutes. Representative images are shown; scale bar 2 μm. Arrow A points to a thin fibre, arrow B indicates a thick fibre.

Figure 2. Major role of Syk kinase in microthrombus formation on fibrin or fibrinogen surfaces. Blood samples pre-incubated with vehicle (Ctrl) or Syk inhibitor PRT-060318 (PRT, 10 μM), labelled and flowed at 1000 s^{-1} over spots S1-6 (Table 1). Surfaces were imaged (brightfield and fluorescence) at time points t = 0, 2, 4, 6, 8, 10 minutes to obtain parameters P1 ($DiOC_6$ platelet adhesion), P2 (thrombus morphological score), P3 (contraction score), P4 (bi- or multilayer score), P5 (bi- or multilayer size), P6 (P-selectin expression), and P7 (PS exposure). Effects of PRT were assessed per blood sample, surface and parameter. **A**, Representative brightfield and fluorescence images of microthrombi on fibrin-FXIIIa spots (S3) after 10 minutes. Scale bar, 50 μm. **B**, Mean parameter values from 3-4 blood samples over time, univariate scaled to 0-10 per parameter. Heatmap of the scaled parameters, demonstrating effects of PRT. Rainbow colour code indicates scaled values from 0 (blue) to 10 (red). **C**, Subtraction heatmap representing effects of PRT, filtered for relevant differences (n = 3-4, $P<0.05$, two-way ANOVA). Colour code showing relevant decrease (green) or increase (red) in comparison to control runs. **Di**, Time-dependent increase in platelet adhesion (P1) in the presence or absence of PRT (**Di**). Mean effects of PRT on platelet adhesion (P1) and platelet activation (P6) after 10 minutes (**Dii**); mean effects of PRT on platelet PS exposure (P7) after 10 minutes (**Diii**). Data are means ± SD (n = 3-4), *$P<0.01$, **$P<0.001$ (two-way ANOVA).

Figure 3. Abolished microthrombus formation on fibrin in Glanzmann's thrombasthenia. A, Representative brightfield and fluorescence images from microthrombi after flow of blood from control subjects or two Glanzmann patients over spots of fibrin-FXIIIa (10 minutes). Flow perfusion was at arterial shear rate of 1000 s^{-1} (**Ai**) or at venous shear rate of 100 s^{-1} (**Aii**). Scale bars 50 μm. **B**, Time-dependent increases in platelet activation (P-selectin expression, P6) and platelet adhesion ($DiOC_6$, P1) for controls (Ctrl, n = 4), heterozygous and homozygous patients at time points t = 0, 2, 4, 6, 8 and 10 minutes. Graphs at shear rates of 1000 s^{-1} (**Bi**) and 100 s^{-1} (**Bii**). Means ± SD (n = 4 for controls), **$P<0.001$, ***$P< 0.0001$ (two-way ANOVA).

Figure 4. Effect of GPVI inhibition on parameters of microthrombus formation on immobilised fibrin-FXIIIa or collagen III. Blood samples pre-incubated with vehicle (Ctrl) or GPVI blocking agent (9012 Fab, 50 μg/mL) were flowed over spots S3 (fibrin-FXIIIa, upstream) and S7 (collagen-III, downstream) for 10 minutes at 1000 s^{-1}. Microthrombi formed were imaged to obtain parameters P1 ($DiOC_6$ platelet adhesion), P5 (bi- or multilayer size), P6 (P-selectin expression), and P7 (PS exposure). **A**, Representative brightfield and fluorescence images from fibrin-FXIIIa (**Ai**) and collagen-III spots (**Aii**) at 10 minutes. **B**, Heatmap of scaled parameters, demonstrating mean effects of GPVI inhibition on thrombus formation per spot. Effects of GPVI inhibition were assessed per blood sample,

surface and parameter. Mean values from individual blood samples were univariate scaled to 0-10 per parameter. Rainbow colour code indicates scaled values between 0 (blue) and 10 (red). **C**, Subtraction heatmaps representing scaled effects of GPVI inhibition, filtered for relevant changes (P<0.05, two-way ANOVA per surface and parameter). Colour code represents decrease (green) or increase (red) in comparison to control runs. Scale bar 50 μm. **D**, Graphs representing PS exposure (P7) for fibrin-FXIIIa (**Di**) and collagen-III (**Dii**) at 10 minutes. Data are means ± SD (n = 7), *P<0.05 (two-way ANOVA).

Figure 5. Roles of VWF and GPIb-V-IX in microthrombus formation on immobilised fibrin-FXIIIa or on collagen III. Blood samples pre-incubated with vehicle (Ctrl) or GPIb blocking antibody RAG35 (20 μg/mL) were flowed at 1000 s^{-1} over spots S3 (fibrin-FXIIIa, upstream) and S7 (collagen-III, downstream). After 10 minutes, microthrombi formed were imaged to obtain parameters P1 ($DiOC_6$ platelet adhesion), P5 (bi- or multilayer size), P6 (P-selectin expression), P7 (PS exposure). **A**, Staining of microthrombi formed on S3 and S7 spots after 10 minutes for VWF using FITC-labelled anti-VWF antibody (green) or irrelevant control antibody. **B-E**, Effects of RAG35 antibody on thrombus parameters were assessed over time per blood sample for S3 and S7 surfaces. Scale bar 50 μm. Shown are representative end stage brightfield and fluorescence images for fibrin-FXIIIa (**Bi**) and collagen-III (**Bii**). Furthermore, heatmap of univariate scaled parameters (0-10), indicating increased build-up of microthrombi over time in the absence or presence of RAG35 antibody (**C**). Rainbow colour code shows scaled values between 0 (blue) and 10 (red). In addition, subtraction heatmap representing scaled effects of GPIb blocking (**D**). Filtering was applied for relevant changes (n = 6, P<0.05, two-way ANOVA per surface and parameter). Colour code represents decrease (green) or increase (red) in comparison to control runs. Means ± SD (n = 6), P<0.05 (two-way ANOVA).

Figure 6. Comparative effects of GPVI or Syk inhibition on fibrin-mediated platelet aggregation. Platelets in suspension were treated with Syk inhibitor PRT-060318 (10 μg/mL) or GPVI-inhibitor 9O12 (50 μg/mL) for 10 minutes, before agonist addition. Platelet aggregation was monitored by conventional light transmission aggregometry. **A**, Quantitation of maximal aggregation upon stimulation with fibrin, collagen or thrombin. **B**, Representative aggregation traces upon stimulation with indicated agonist. Mean ± SD (n = 5), ****P<0.0001 (one-way ANOVA).

Figure 7. Effects of GPVI or Syk inhibition on Ca^{2+} signalling in fibrin-adhered platelets under flow. Blood samples containing autologous Fluo-4-loaded platelets were pre-incubated with vehicle (Ctrl), GPVI blocking agent 9O12 Fab (50 μg/mL) or Syk inhibitor PRT-060318 (10 μM), and flowed over S3 spots (fibrin-FXIIIa), as for Figure 2. Fluorescence changes in cytosolic $[Ca^{2+}]_i$ of adhered platelets were recorded by confocal microscopy for 5 minutes. Time series of fluorescence images were analysed for threshold increases in fluorescence intensity representing platelet activation (**Ai-ii**), and for fluorescence coverage as a measure of platelet adhesion (**Bi-ii**). Panels present data from parallel flow runs; dots represent values from analysed images. Bars are means (n = 3 experiments), **P< 0.001, ****P<0.0001 (one-way ANOVA).

Supplemental Material

See accompanying pdf