Characterization of the Role of Integrin α5β1 in Platelet Function, Hemostasis, and Experimental Thrombosis

Emily Janus-Bell1,∗ Alexandra Yakusheva1,2,∗ Cyril Scandola1 Nicolas Receveur1 Usman Muhammad Ahmed1 Clarisse Mouriaux1 Catherine Bourdon1 Cécile Loubière1 Anita Eckly1 Yotis A. Senis1 Mikhail A. Panteleev2 Christian Gachet1 Pierre H. Mangin1

1Université de Strasbourg, INSERM, EFS Grand-Est, BPPS UMR-S1255, FMTS, Strasbourg, France
2Center for Theoretical Problems of Physicochemical Pharmacology, Cellular Hemostasis Lab, Moscow, Russia

Address for correspondence Pierre H. Mangin, PhD, Université de Strasbourg, INSERM, EFS Grand-Est, BPPS UMR-S1255, FMTS, 10 rue Spielmann, F-67065 Strasbourg, France (e-mail: pierre.mangin@efs.sante.fr).

Abstract

Objective Integrins are key regulators of various platelet functions. The pathophysiological importance of most platelet integrins has been investigated, with the exception of α5β1, a receptor for fibronectin. The aim of this study was to characterize the role of α5β1 in megakaryopoiesis, platelet function, and to determine its importance in hemostasis and arterial thrombosis.

Approach and Results We generated a mouse strain deficient for integrin α5β1 on megakaryocytes and platelets (PF4Cre-α5−/−). PF4Cre-α5−/− mice were viable, fertile, and presented no apparent signs of abnormality. Megakaryopoiesis appears unaltered as evidence by a normal megakaryocyte morphology and development, which is in agreement with a normal platelet count. Expression of the main platelet receptors and the response of PF4Cre-α5−/− platelets to a series of agonists were all completely normal. Adhesion and aggregation of PF4Cre-α5−/− platelets under shear flow on fibrinogen, laminin, or von Willebrand factor were unimpaired. In contrast, PF4Cre-α5−/− platelets displayed a marked decrease in adhesion, activation, and aggregation on fibrillar cellular fibronectin and collagen. PF4Cre-α5−/− mice presented no defect in a tail-bleeding time assay and no increase in inflammatory bleeding in a reverse passive Arthus model and a lipopolysaccharide pulmonary inflammation model. Finally, no defects were observed in three distinct experimental models of arterial thrombosis based on ferric chloride-induced injury of the carotid artery, mechanical injury of the abdominal aorta, or laser-induced injury of mesenteric vessels.

Conclusion In summary, this study shows that platelet integrin α5β1 is a key receptor for fibrillar cellular fibronectin but is dispensable in hemostasis and arterial thrombosis.

Keywords► platelets► arterial thrombosis► integrin α5β1► fibronectin

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Introduction

Platelets adhere, become activated, and aggregate at a site of vessel injury to form a hemostatic plug which stops bleeding. They are also involved in maintaining vascular integrity and in the arrest of inflammatory bleeding in various organs. On the other hand, platelets play an instrumental role in arterial thrombosis by inducing the formation of an occlusive thrombus in a diseased artery, which results in life-threatening ischemic pathologies such as myocardial infarction or ischemic stroke. The molecular mechanisms involved in the interactions of platelets with an injured vessel wall have been extensively investigated. The initial step of attachment of circulating platelets is ensured by binding of the glycoprotein (GP) Ib–IX complex to subendothelial von Willebrand factor (vWF) at elevated shear rates. When the flow is slower, β1 and β3 integrins assist the GPIb–IX complex to allow further platelet recruitment as well as stable adhesion to various extracellular matrix proteins. This enables the interaction of GPVI with its ligands, including collagen, which initiates platelet activation. Aggregation results through interactions of integrin αIIbβ3 with plasma fibrinogen, forming a plug that seals the breach in healthy vessels or a pathological thrombus in diseased arteries.

Platelets express at their surface five different integrins of the β1 and β3 families, namely α2β1, α5β1, α6β1, αvβ3, and αIIbβ3 whose main ligands are collagen, fibronectin, laminins, vitronectin, and fibrinogen, respectively. αIIbβ3, the most abundant integrin at the platelet surface, enables platelet adhesion and aggregation through its binding to fibrinogen. This receptor plays a major role in hemostasis as evidenced by the hemorrhagic disorder known as Glanzmann’s thrombasthenia, in which αIIbβ3 is absent or nonfunctional. It is also the target of a class of potent antiplatelet agents, illustrating its key involvement in arterial thrombosis. The role of the other integrins, notably the β1 integrins, appears to be limited to the initial step of platelet adhesion and activation through interactions with extracellular matrix proteins. Concerning their importance, it has been shown that the absence of either α2β1 or α6β1 has no major impact on the tail-bleeding time in mice, but reduces thrombosis in several experimental models. In contrast, the importance of α5β1 in hemostasis and arterial thrombosis has never been studied.

Integrin α5β1 is a well-known receptor for fibronectin, which is broadly expressed on various cell types and plays an important role in migration and differentiation, especially during fetal development. As a consequence, knocking out the α5 gene results in death at the embryonic stage due to a defect in the mesoderm. Concerning platelets, it has been shown that α5β1 together with αIIbβ3 plays a central role in platelet adhesion to fibronectin under shear flow. Plasma fibronectin is very weak in supporting platelet adhesion and activation when compared to cellular fibronectin, which is probably explained by the presence of additional binding domains in the latter. In addition, both forms of fibronectin increase markedly their reactivity after polymerization and fiber formation, especially for cellular fibronectin. Howev-
mice. Platelet count and volume were analyzed in an automatic cell counter (Scil Animal Care Company, Altorf, France) and surface GP expression was determined by flow cytometry. Expression of the α5 subunit was quantified in platelet lysates using automated capillary-based immunoassay (ProteinSimple Wes, San Jose, United States), as previously described.19

Platelet-Rich Plasma Aggregations
Mouse platelet-rich plasma (PRP) was prepared by centrifugation of blood collected on hirudin (100 U/mL) and adjusted to 300,000 platelets/μL with platelet-poor plasma from the same mouse. Platelet aggregation was measured as reported previously.20

Preparation and Properties of Washed Mouse Platelets
Washed mouse platelets were prepared as reported previously.20 Agonist-induced binding of soluble fibronectin and exposure of P-selectin were determined as previously described,11 while phosphatidylserine exposure was quantified by Alexa Fluor 488-annexin V binding.

In Vitro Flow-Based Adhesion Assay
PDMS flow chambers (0.1 × 1 mm) were coated with vWF-binding protein (DDR2, 100 μg/mL), fibronectin (100 μg/mL), laminins (100 μg/mL), collagen (200 μg/mL), or soluble cellular fibronectin (300 μg/mL) overnight at 4°C. Mechanical stretching of soluble cellular fibronectin was performed to form fibrillar cellular fibronectin as described previously.17 To prevent nonspecific adhesion, the channels were blocked with phosphate-buffered saline containing human serum albumin (10 mg/mL) for 30 minutes at room temperature. Hirudinated (100 U/mL) whole blood was drawn from the abdominal aorta of anesthetized mice. The hirudinated blood was perfused through the chambers at the indicated wall shear rates and platelet adhesion was observed in real time and analyzed as detailed elsewhere.11 Thrombus formation on fibrillar cellular fibronectin was monitored as previously reported.17

In Vivo Thrombosis Models
Platelets were labeled by administering 3,3’-dihexyloxacarbocyanine iodide to anesthetized mice. Ferric chloride (FeCl3)-mediated thrombosis was induced by applying a 3 × 3 mm Whatman filter paper saturated with 7.5% FeCl3 laterally to the carotid artery for 2.5 minutes. Thrombosis was initiated mechanically by pinching the abdominal aorta with forceps for 15 seconds. Thrombus formation was monitored in real time by bright field and fluorescence microscopy (Leica DM IRB) using a CMOS ORCA Flash V2 camera (Hamamatsu Photonics, Massy, France).

Bleeding Time
The bleeding time and volume of blood lost were determined by transversally severing a 3-mm segment from mouse tails, as reported previously.21

Cutaneous and Pulmonary Inflammation Models
An rpA reaction was elicited in anesthetized mice by intradermal injection of an antiovine serum albumin antibody (60 μg/spot), followed by retro-orbital injection of bovine serum albumin (75 mg/kg), as previously described.1

A lung inflammation model was induced in anesthetized mice by intranasal inoculation of Pseudomonas aeruginosa LPS (1 μg/mouse) in 60 μL of saline, as previously described.1

Statistical Analyses
Statistical analyses were performed with GraphPad Prism software (see figure legends).

Results
Characterization and Megakaryopoiesis of PF4Cre-α5-Deficient Mice
To study the role of platelet integrin α5β1, we crossed a mouse strain floxed for the α5 gene with a strain expressing Cre recombinase under control of the PF4 promoter (PF4Cre-α5+/−). We used a quantitative biochemical approach to provide evidence that platelets from these mice expressed almost no integrin α5 anymore as compared to PF4-Cre mice (∗Fig. 1A, B). No obvious abnormalities were detected in PF4Cre-α5+/− mice that were bred and developed normally. These mice have no increase in embryonic lethality, nor variation of the litter size and they present no abnormality in survival. Physical appearance and behavior are also unchanged.

PF4Cre-α5+/− mice presented a normal maturation and morphology of their megakaryocytes as assessed by their observation of ultrastructure on TEM images (∗Fig. 1C). While the number of megakaryocytes in the bone marrow appears slightly increased in PF4Cre-α5+/− mice, the distribution of the different maturation stages was unchanged in PF4Cre-α5+/− as compared to control mice, suggesting no major impact of integrin α5 in megakaryopoiesis (∗Fig. 1D, E). In agreement, we observed that deletion of α5 had no impact on the platelet count or volume (∗Table 1). Thus, PF4Cre-α5-deficient mice appeared to be normal and PF4Cre-α5-deficient megakaryocytes displayed no difference in development and maturation.

Characterization of PF4Cre-α5-Deficient Platelets
The surface expression of the major GPs was normal (∗Table 1), except for that of integrin α5 (∗Fig. 1A, B). Using light-transmission aggregometry, we found that platelets

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from PF4Cre-α5−/− mice aggregated normally in response to a series of agonists including adenosine diphosphate (ADP) (5 μmol/L), collagen (2.5 μg/mL), and U46619 (2 μmol/L) in PRP, presenting the advantage to contain plasma fibronectin (Fig. 2A, B). Similar results were obtained with washed platelet aggregation to various agonists (Supplementary Fig. S1, available in the online version). In addition, no differences in P-selectin exposure, fibronogen binding or annexin V binding in response to ADP, thrombin, proteinase-activated receptor 4 (PAR-4) peptide or convulxin were observed by flow cytometry in PF4Cre-α5−/− platelets as compared to controls (Fig. 2C–E). This confirmed that platelets deficient in integrin α5 respond normally to a GPVI ligand and a series of soluble agonists. These results indicate that PF4Cre-α5-deficient platelets presented no defect in response to soluble agonists.

Characterization of PF4Cre-α5-Deficient Platelet Adhesion under Shear Flow

We next evaluated the ability of α5β1 to support platelet adhesion to various surfaces under flow by perfusing hirudinated whole blood over immobilized proteins. We observed normal adhesion and rolling of PF4Cre-α5−/− platelets recruited to vWF bound to DDR2 at 1,500 s⁻¹ (Fig. 3A) and normal adhesion of these platelets to fibronogen and laminin at 300 s⁻¹ (Fig. 3B). In contrast, adhesion of PF4Cre-α5−/− platelets to immobilized fibrillar cellular fibronectin displayed a major defect as compared to controls, with a 78% reduction in the number of adherent platelets at 8 minutes (Ctrl: 7.1 ± 0.7 × 10³/mm²; PF4Cre-α5−/−: 1.6 ± 0.05 × 10³/mm²) (Fig. 3C, D). A detailed analysis indicated that α5β1 was important to establish the initial bond with fibronectin, as the recruitment of PF4Cre-α5−/− platelets to the surface was decreased by 55% as compared to controls (Fig. 3E). Moreover, study of the adhesive behavior

Table 1 Platelet counts, volumes, and expression of major surface glycoproteins in Ctrl and PF4Cre-α5−/− mice

|                      | Ctrl             | PF4Cre-α5−/−     | Number of mice |
|----------------------|------------------|------------------|----------------|
| Platelet count       | 1.095 ± 0.50 × 10³/μL | 1.119 ± 0.29 × 10³/μL | 13             |
| Mean platelet volume | 4.98 ± 0.07 μm³  | 4.68 ± 0.02 μm³  | 13             |
| αIIbβ3               | 5.28 ± 0.98      | 4.87 ± 1.20      | 6              |
| α2                   | 10.50 ± 0.25     | 11.03 ± 0.19     | 6              |
| α5                   | 1.23 ± 0.04      | 0.32 ± 0.04      | 9              |
| α6                   | 13.57 ± 0.22     | 13.27 ± 0.13     | 6              |
| β1                   | 4.69 ± 0.92      | 4.55 ± 1.01      | 6              |
| GPIbα                | 5.01 ± 0.95      | 5.04 ± 0.15      | 6              |
| GPV                  | 0.99 ± 0.06      | 0.97 ± 0.03      | 6              |
| GPIX                 | 2.04 ± 0.12      | 2.06 ± 0.03      | 6              |
| GPVII                | 1.66 ± 0.05      | 1.46 ± 0.04      | 6              |

Abbreviation: GP, glycoprotein.

Note: Platelet counts and volumes in Ctrl and PF4Cre-α5−/− mice were analyzed with an automatic cell counter; values represent the mean ± standard error of the mean (SEM). The surface expression of various glycoproteins on platelets in whole blood from Ctrl and PF4Cre-α5−/− mice was evaluated using selective antibodies and flow cytometry; results are expressed as the mean fluorescence intensity (MFI) ± SEM.
of the recruited platelets showed a marked increase in numbers of PF4Cre-α5−/− platelets detaching from the surface and a clear decrease in stationary adhesion, highlighting the importance of α5β1 in stabilizing the bonds between platelets and fibronectin (Fig. 3F). These results indicated that platelet integrin α5β1 is a major receptor for fibronectin supporting platelet attachment and maintaining the bonds to ensure stable adhesion.
A Major Defect of PF4Cre-α5−/− Platelet Activation and Aggregation on Fibrillar Cellular Fibronectin and Collagen

Platelet activation is a key step in thrombus formation. Microscopic fluorescence images showed a clear JonA-PE signal for control platelets accumulating on fibrillar cellular fibronectin under flow at 300 s⁻¹, while only a weak signal was detected for PF4Cre-α5−/− platelets, indicating that α5β1 is important to promote αIIbβ3 activation on fibronectin (Ctrl: 26.9 ± 8.8 × 10⁴ AU; PF4Cre-α5−/−: 3.5 ± 2.7 × 10⁴ AU) (→ Fig. 4A, B). We observed that thrombi formed in control blood but not in PF4Cre-α5−/− blood, highlighting a key role of α5β1 in platelet aggregation on fibrillar cellular fibronectin (→ Fig. 4C, D). Surprisingly, a defect in thrombus formation was also observed when PF4Cre-α5−/− blood was perfused over fibrillar collagen, a surface which does not directly activate α5β1, suggesting that this integrin participates in thrombus build-up, probably through interactions with plasma fibronectin (→ Fig. 4E, F). This role of mouse α5β1 in thrombus growth over fibrillar collagen was however not observed when human blood was perfused with a blocking anti-α5 antibody, suggesting a species difference (data not shown). Altogether, these findings indicated that integrin α5β1 plays an important role in thrombus growth on fibronectin.

α5β1 Does Not Act as a Major Platelet Receptor in Experimental Thrombosis

Mice deficient in platelet integrin α5β1 were studied in three distinct models of localized vascular injury to expose the subendothelium matrix, which is known to contain fibrillar cellular fibronectin. When the common carotid artery was injured with a 7.5% solution of FeCl₃, PF4Cre-α5−/− mice (n = 9) presented a similar profile of thrombus formation and disaggregation as compared to controls (n = 6) (area under the curve: Ctrl: 6 ± 2 × 10⁸ µm²; PF4Cre-α5−/−: 10 ± 2 × 10⁸ µm²; p > 0.05; → Fig. 5A, B; → Supplementary Fig. S2A, available in the online version). A comparable result was obtained after mechanical injury of the abdominal aorta with forceps, the thrombus area in PF4Cre-α5−/− mice (n = 8) being not significantly different from that in control mice (n = 8) (area under the curve, Ctrl: 12 ± 4 × 10⁷ µm²; PF4Cre-α5−/−: 17 ± 4 × 10⁷ µm²; p > 0.05; → Fig. 5C, D; → Supplementary Fig. S2B, available in the online version). Finally, following laser-induced injury of mesenteric arteries, PF4Cre-α5−/− (n = 5 vessels on three mice) also formed thrombi in a similar manner to control mice (n = 6 vessels in three mice) (area under the curve, Ctrl: 7 ± 1 × 10⁷ µm²; PF4Cre-α5−/−: 7 ± 1 × 10⁸ µm²; p > 0.05) (→ Fig. 5E, F; → Supplementary Fig. S2C, available in the online version). Hence mouse platelet α5β1 did not appear to be a major receptor for arterial thrombosis.

Platelets from PF4Cre-α5−/− Mice Display No Important Hemostatic Defect

PF4Cre-α5−/− mice did not present any spontaneous bleeding. In addition, PF4Cre-α5−/− mice showed no signs of excessive bleeding during surgery as compared to control animals, suggesting that the lack of this integrin on platelets does not critically affect hemostasis. This was further supported by a normal bleeding time (n = 6) (Ctrl: 302 ± 119 s; PF4Cre-α5−/−: 121 ± 17 s) and normal volume of blood lost (n = 6) (Ctrl: 259 ± 130 µL; PF4Cre-α5−/−: 130 ± 84 µL) in a tail-bleeding time assay (→ Fig. 6A, B). Finally, we did not observe any effect on inflammatory bleeding in PF4Cre-α5−/− mice as compared to control mice in an rpA model of skin inflammation (→ Fig. 6C, D) and in a LPS pulmonary inflammation model (→ Fig. 6E, F). These results do not favor a major role of integrin α5β1 in the murine hemostatic system.

Discussion

Platelets express numerous adhesion receptors, notably integrins, to sense the proteins exposed on the
subendothelium of an injured vessel and to ensure their efficient recruitment to the site of injury. We show in this study, using tissue-specific knock-out combined with in vitro flow-based assays, that integrin α5β1 is a functionally key receptor for efficient platelet adhesion, activation, and aggregation on immobilized fibrillar cellular fibronectin, which are found in the vessel wall. Interestingly, in the absence of α5β1, the other main platelet receptor for fibronectin, αIIbβ3, was inefficient in promoting normal platelet adhesion, activation, and aggregation, highlighting the importance of α5β1 in these processes. We also identified an unexpected role of α5β1 in platelet aggregation on collagen. Despite these important functional roles identified in vitro, we found that platelet α5β1 did not play an essential role in the arrest of bleeding after trauma or under inflammatory conditions. Moreover, three distinct experimental models of thrombosis revealed no impact on thrombus formation in α5β1-deficient mice as compared to controls, suggesting that this receptor is unlikely to represent a major factor in arterial thrombosis.

We observed that α5β1-deficient platelets aggregated normally in response to a series of soluble agonists and to collagen. In addition, fibrinogen binding and P-selectin and annexin V exposure in response to soluble agonists were completely normal, indicating no role of α5β1 in the amplification step of platelet activation driven by these agonists. Adhesion under flow of α5β1-deficient platelets to many adhesive surfaces including fibrinogen, laminins, and vWF...
was likewise unchanged. In contrast, we observed a marked impairment of the adhesion of these platelets to immobilized fibrillar cellular fibronectin and collagen. These results suggest that α5β1 is a highly specialized platelet receptor for probably only one adhesive protein, fibronectin. This is not a unique case, as platelets express many receptors to sense a single adhesive protein, notably the GPIb–IX complex and integrins α2β1, α6β1, and αvβ3 which bind respectively to vWF, collagen, laminins, and vitronectin. The reason why platelets have maintained distinct specific receptors is probably to ensure efficient adhesion to a wide range of matrices which can be exposed by different types of vessels and at different depths of injury.

It is well established that platelets express two main receptors for fibronectin, α5β1 and αIIbβ3. Our results support a major role for α5β1 as a receptor for fibronectin, since in its absence, we observed a dramatic reduction in platelet adhesion and activation on fibrillar cellular fibronectin with almost no aggregation at all. As a consequence, our observations also indicate that αIIbβ3 alone is not sufficient to compensate for the absence of α5β1 in the case of adhesion to a fibronectin surface. We further found that α5β1, in addition to its role in initiating platelet adhesion and activation on fibronectin, contributed to thrombus growth on collagen, a process known to be mainly ensured by αIIbβ3. However, the impairment we observed when perfusing PF4Cre-α5β1/− mice did not result in an increased tail-bleeding time, which might not maintain vascular integrity and stop inflammatory bleeding. The molecular mechanism relies on the immunoreceptor tyrosine-based activation motif, GPVI, and C-type lectin-like receptor II, which promote platelet activation. An unresolved question is which receptors support platelet adhesion at sites of inflammatory bleeding, since the process appears to be independent of two major adhesion receptors, the GPIb–IX complex and integrin αIIbβ3. We have recently identified a role of β1 integrins in this process (Janus-Bell et al, submitted manuscript). However, our results exclude a key role of α5β1, since no bleeding phenotype was observed in PF4Cre-α5β1/− mice using a cutaneous rPA model and a LPS pulmonary inflammation model. Further studies will be required to identify the adhesion receptors important to stop inflammatory bleeding, it is tempting to speculate that compensatory mechanisms between such receptors could explain why no major bleeding occurs when only one of them is absent.

Using three distinct models of arterial thrombosis, in different vessels and under distinct rheological conditions, we did not observe any significant difference in any experimental model of thrombosis. These results indicate that α5β1 alone does not play a central role in experimental thrombosis. Moreover, α5β1 appears to be less important than the other two β1 integrins, α2β1 and α6β1, which have both been shown to contribute to experimental thrombosis in at least one of the models used in this study. Additionally, the apparent discrepancy we observed between an important role of α5β1 in platelet aggregation on fibronectin in vitro and the absence of a role in experimental models of thrombosis could be linked to the nature of the surface exposed to the flowing blood in vivo, which might not contain enough fibrillar cellular fibronectin. Another explanation could be the ability of other platelet adhesion receptors to compensate for the lack of α5β1 on platelets. As it has been reported that an evolved atherosclerotic plaque is particularly rich in fibrillar cellular fibronectin, it might be interesting to test the impact of α5β1 blockade in apolipoprotein E-deficient models of atherosclerotic plaque rupture. To date, there is no information available about the role of human α5β1 integrin in arterial thrombosis in humans.

In conclusion, in vitro experiments performed with α5β1-deficient mouse platelets indicated that this integrin plays a very important role in supporting platelet adhesion, activation, and aggregation on fibrillar fibronectin and participates in thrombus growth. However, integrin α5β1 appears to be dispensable for hemostasis under normal and inflammatory conditions and is also not essential in in vivo models of experimental thrombosis.
What is known about this topic?

- Platelets express β1 and β3 integrins to allow adhesion, activation, and aggregation.
- α5β1 and αIIbβ3 are the main platelet receptors for fibronectin supporting platelet adhesion to fibronectin under flow condition.

What does this paper add?

- This study highlights the important role of α5β1 in supporting platelet adhesion, activation, and aggregation on fibriellar cellular fibronectin using PF4Cre-α5-deficient mice.
- We provide evidence that in the absence of α5β1, the other platelet receptor for fibronectin, αIIbβ3, is unable to support normal platelet adhesion to immobilized fibronectin with no platelet aggregation at all.
- Platelet α5β1 is dispensable for hemostasis under normal and inflammatory conditions and does not play a key role in experimental thrombosis.

Author Contributions

A.Y., C.S., E.J.B., and N.R. acquired, analyzed, and interpreted the data and wrote the manuscript; C.B., C.L., C.M., and U.M.A. acquired and analyzed the data; A.E., M.A.P., and Y.A.S. designed the research, interpreted the data, and wrote the manuscript; C.G. contributed to writing of the manuscript; P.H.M. conceived and designed the research, interpreted the data, wrote the manuscript, and handled funding and supervision.

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Conflict of Interest

None declared.

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