Differential Role of Glycoprotein VI in Mouse and Human Thrombus Progression and Stability

Emily Janus-Bell[1,*] Muhammad Usman Ahmed[1,*] Nicolas Receveur[1] Clarisse Mouriaux[1] Bernhard Nieswandt[2] Elizabeth E. Gardiner[3] Christian Gachet[1] Martine Jandrot-Perrus[4,5] Pierre H. Mangin[1]

[1] Université de Strasbourg, INSERM, EFS Grand-Est, BPPS UMR-S1255, FMTS, F-67065 Strasbourg, France
[2] Institute of Experimental Biomedicine, University Hospital and Rudolf Virchow Center, University of Würzburg, Würzburg, Germany
[3] ACRF Department of Cancer Biology and Therapeutics, The John Curtin School of Medical Research, The Australian National University, Canberra, Australia
[4] Université de Paris INSERM, Hôpital Bichat, Paris, France
[5] Acticor Biotech, Paris, France

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

Thromb Haemost 2021;121:543–546.

Introduction

Platelet glycoprotein (GP) VI is a promising, safe, antithrombotic target as its absence or blockade prevents in vitro thrombus formation and experimental thrombosis in various animal models without impacting the tail-bleeding time.[1] In addition, patients with a mutation in the GPVI gene exhibit only a mild bleeding diathesis,[2] further suggesting that GPVI does not play a critical role in hemostasis. GPVI is the main platelet activation receptor for collagen and viewed as being important in the initiation of thrombus formation.[1] In addition to collagen, GPVI interacts physically or functionally with other adhesive proteins including laminins, fibrin, and fibrinogen.[3–7] We have shown that human GPVI activates platelets on immobilized fibrinogen and that this process is key for the progression and stability of human thrombi.[7,8] In sharp contrast, we observed that mouse GPVI does not promote such an activation, as platelets deposited on fibrinogen do not fully spread.[7] In this study, we investigated the consequence of absence of GPVI/fibrinogen-mediated platelet activation in mice on the regulation of thrombosis in comparison to the human system. It is important to appreciate species difference between humans and mice as the latter represent the most broadly used animal model to study experimental thrombosis and that a significant part of our current understanding of the molecular mechanism of thrombosis relies on experiments performed with these animals.

Results and Discussion

To investigate the role of GPVI in mouse thrombus build-up, beyond its role as a collagen receptor, we used a flow-based assay. We preformed thrombi by perfusing hirudinated whole blood of wild-type (WT) mice over immobilized type-I fibrillar collagen for 1 minute at 300 s^{-1} and stained platelet aggregates with $DiOC_6$ (green). We next perfused hirudinated blood from WT or $GPVI^{-/-}$ mice for 6 minutes at 300 s^{-1} which were stained with the anti-GPIbβ antibody RAM.1-A647 (red) to visualize thrombus progression. Three-dimensional reconstructed confocal microscopy images showed that the second population of red platelets formed thrombi on the top of the first population of green aggregates similarly with $GPVI^{-/-}$ and WT blood (►**Fig. 1A**). This was confirmed by measuring thrombus volume, which indicated that the aggregates formed with $GPVI^{-/-}$ mouse blood presented a similar volume to WT thrombi (WT: $5.7 \pm 0.9\,\mu m^3/\mu m^2$; $GPVI^{-/-}$: $5.1 \pm 0.6\,\mu m^3/\mu m^2$, $p > 0.05$; ►**Fig. 1B**). This result demonstrates that mouse GPVI does not play a critical role in thrombus build-up beyond its role as an initial collagen receptor and is in sharp contrast with the key role played by human GPVI.[7] Moreover, this result also implies that murine thrombus progression in the absence of thrombin in mice primarily occurs via GPVI-independent activation pathways, notably those relying on soluble agonists such as thromboxane A2 and adenosine diphosphate (ADP; ►**Fig. 1C**). These in vitro observations are consistent with in

* *These authors contributed equally to this work.*

published online
October 29, 2020

DOI https://doi.org/10.1055/s-0040-1718737.
ISSN 0340-6245.

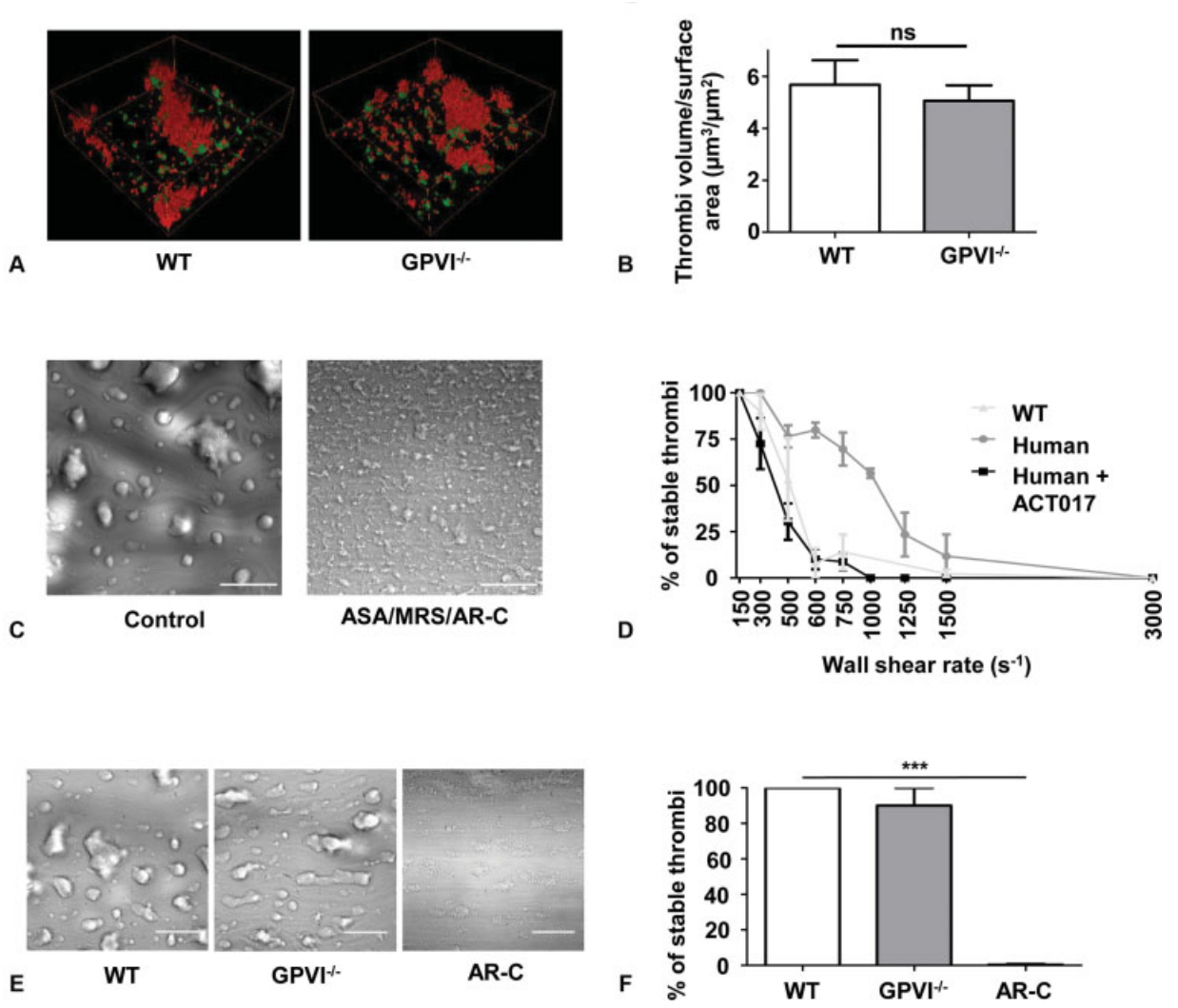

Fig. 1 GPVI plays a key role in human but not in mouse thrombus progression and stability. (**A**) Representative 3D reconstructed confocal images of wild-type (WT) and GPVI-deficient ($GPVI^{-/-}$) mouse thrombi obtained after perfusing hirudinated whole blood stained with RAM.1-A647 2 µg/mL (in *red*) over WT aggregates for 6 minutes at 300 s^{-1}. The preformed thrombi were obtained by perfusing WT mice hirudinated whole blood stained with $DiOC_6$ 1 µmol/L (in *green*) over fibrillar collagen for 1 minute at 300 s^{-1}. (**B**) Quantification of WT ($n = 6$) and $GPVI^{-/-}$ ($n = 7$) thrombus volume by confocal microscopy. (**C**) Representative DIC images obtained after perfusing WT mice hirudinated whole blood for 5 minutes at 300 s^{-1} and washed with PBS for 3 minutes at 300 s^{-1}. For the ASA/MRS/ARC69931MX (AR-C) condition, WT whole blood was treated with 1 mM of aspirin, 10 µM of MRS 2500, and 10 µM of AR-C, 10 minutes before perfusion. Scale bar represents 50 µm. (**D**) The curves represent the percentage of stable thrombi obtained 8 minutes after perfusion of PBS over WT mouse ($n = 4$) and human ($n = 3$) aggregates at indicated wall shear rates. Thrombi were preformed by perfusing human or WT mouse hirudinated whole blood over collagen. For the ACT017 condition, 50 µg/mL of ACT017 was perfused over human thrombi at indicated wall shear rates. (**E**) Representative DIC images obtained 8 minutes after perfusion of PBS at 300 s^{-1} over preformed aggregates. Thrombi were preformed by perfusing WT mice hirudinated whole blood over collagen, followed by the perfusion of WT or $GPVI^{-/-}$ whole blood. For the ARC69931MX (AR-C) condition, 10 µM of AR-C in PBS was perfused over preformed WT aggregates. Scale bar represents 50 µm. (**F**) Quantification of the percentage of stable thrombi of WT ($n = 5$), $GPVI^{-/-}$ ($n = 5$) ,and WT + AR-C ($n = 5$) after perfusing PBS with or without AR-C over preformed aggregates for 8 minutes at 300 s^{-1}. All the experiments were done with PDMS flow chambers coated with type I fibrillar collagen at 200 µg/mL. Results are presented as mean ± standard error of the mean (SEM). Ns $p > 0.05$, $^{***}p < 0.001$. Data were compared using Mann–Whitney and chi-square test. 3D, three-dimensional; DIC, differential interference contrast; GPVI, glycoprotein VI; PBS, phosphate-buffered saline.

vivo results showing that following $FeCl_3$ injury of the carotid artery, which does not expose subendothelial proteins such as collagen, thrombus build-up is largely independent of GPVI.[9]

We next studied the role of GPVI in thrombus stability and compared this process in humans and mice. Therefore, we preformed aggregates in a similar way to that described for ►**Fig. 1(A, B)**, before testing the thrombus stability by perfusing PBS over the surface. We observed that mouse platelet aggregates were much less stable when compared to human thrombi (►**Fig. 1D**). Indeed, a reduction in thrombus stability in mouse versus human platelet thrombi was already observed at wall shear rates of 300 s^{-1}. This difference became very obvious at 600 s^{-1} where almost all mouse thrombi disaggregated while more than 80% of human platelet aggregates were still stable (►**Fig. 1D**). These results indicate that mouse platelet aggregates are much less stable than their human counterpart in such an experimental setting. In parallel, we observed that the blocking anti-GPVI agent ACT017 reduced the stability of human platelet aggregates to the level of mouse thrombi (►**Fig. 1D**). This result confirms that GPVI-fibrinogen

Table 1 Comparision of mouse and human system with focus on GPVI

	Human GPVI	Mouse GPVI
Platelet count	300 ± 150 ($\times 10^3$) platelets/μL	956 ± 38 ($\times 10^3$) platelets/μL
Copy number per platelet	3,000–4,000	4,500–5,500
Structure	• Human and mouse GPVI share a 64% homology • Mouse GPVI cytoplasmic tail lacks the 24 C-terminal residues of human GPVI	
Physiological ligands	Fibrillar collagen Fibrinogen Fibrin Laminin	Fibrillar collagen Fibrin Laminin
Nonphysiological ligands	Convulxin CRP	Convulxin CRP (slightly less reactive)
GPVI shedding	ADAMs family members (ADAMs 10 and 17), physiological and nonphysiological ligands	ADAMs family members and nonphysiological ligands
Signaling pathway	No major differences identified to date (FcRγ chain, Src kinases, Syk, PI3 kinases, LAT, SPLP76, and calmodulin all involved in human and mouse GPVI function)	
Dependency on soluble mediators	Human and mouse GPVI-initiated platelet activation relies on soluble agonists ADP and TxA2 for full activation especially with low collagen concentrations	
GPVI/collagen interaction	Human and mouse GPVI activate platelets through collagen which promotes thrombus formation	
GPVI/fibrinogen interplay	Human GPVI interaction with fibrinogen supports thrombus growth and stability	Mouse GPVI does not interact with fibrinogen
Arterial thrombosis	Under evaluation in phase II studies	• Reduced experimental thrombosis in several models when GPVI is absent/blocked • Protection in an experimental stroke model in GPVI-immunodepleted mice
Hemostasis	Patients with GPVI deficiencies (acquired or genetic) have a modest/no bleeding diathesis. No spontaneous bleeding and no prolonged bleeding time in healthy volunteers treated with anti-GPVI agents (phase I studies)	GPVI- or FcγR-deficient mice • No spontaneous bleeding • No bleeding during surgery • No prolonged tail-bleeding time and normal volume of blood loss when GPVI is absent or blocked

Abbreviations: ADP, adenosine diphosphate; CRP, C-reactive protein; GPVI, glycoprotein VI.

mediated platelet activation in humans markedly increases thrombus stability. In contrast, when we perfused GPVI$^{-/-}$ or WT blood over aggregates formed with WT blood as described for the data in ►**Fig. 1**(**A, B**), we observed that GPVI$^{-/-}$ platelet aggregates were as stable as WT platelet thrombi (►**Fig. 1E**). Quantification confirmed no difference in the number of stable platelet aggregates between GPVI$^{-/-}$ and WT thrombi (WT: $100 \pm 0\%$; GPVI$^{-/-}$: $90 \pm 10\%$; ►**Fig. 1F**). As a positive control, we used ARC69931MX (10 μM), an antagonist of the ADP receptor $P2Y_{12}$, which efficiently destabilized mouse platelet aggregates (►**Fig. 1E, F**). Together, these results indicate that mouse platelet thrombi are less stable than human thrombi and that their stability at low shear does not critically rely on GPVI, whereas GPVI is critical for the stability of human platelet thrombi. We propose that this species difference does limit the relevance of using standard mouse models to study the thrombus stabilizing action of GPVI and limits extrapolation of data obtained on the progression and stability of thrombi in mice to the human system with regard to GPVI.

In conclusion, this study highlights a significant species difference between the human and mouse hemostatic systems with regard to the contribution of GPVI in thrombus progression and stability beyond GPVI's role as a collagen receptor (►**Table 1**). The reason why mouse GPVI does not contribute to thrombus build-up and stability most likely results from an inability to promote platelet activation on fibrinogen. This adhesive protein, found in every layer of a growing thrombus, is very well known to support platelet aggregation through its interaction with integrin αIIbβ3, but also through its interplay with human GPVI to promote and maintain platelet activation. This species difference is critical when judging the importance of GPVI in thrombosis as most of our knowledge is acquired from in vivo experiments performed in mice. This study suggests that the importance of GPVI in cardiovascular events in humans might be more important than previously anticipated based on experiments in mice. This is particularly important to highlight since anti-GPVI agents are currently under evaluation in phase II studies in the setting of acute coronary syndromes and ischemic stroke.

Authors' Contributions

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

Funding
This work was supported by ARMESA and by a grant from the Concours mondial de l'innovation: TherAVC 2.0 (BPI France). M.U.A. was supported by a PhD fellowship from HEC Pakistan.

Conflict of Interest
M.J.-P.: founder of Acticor Biotech. All other authors have declared that they have no conflict of interest.

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