Platelet glycoprotein VI in the regulation of thrombus growth

Impact

The role of the haemostatic system is to prevent excessive blood loss in case of vessel injury. Platelets are the main regulators of haemostasis alongside with the coagulation system. The endothelium lining the blood vessels prevents platelet activation by separating platelets from thrombogenic factors present in the subendothelium. Damage to the endothelium exposes extracellular matrix proteins such as collagen fibres, which triggers platelet activation, plug formation and vascular occlusion of the site of bleeding. On the other hand, the same platelet and coagulation activation processes, at a site of rupture of an atherosclerotic plaque can lead to formation of occlusive thrombi, and life-threatening complications such as heart attack and stroke. Patients at risk of arterial thrombosis are treated prophylactically with anti-platelet agents, such as aspirin and $P2Y_{12}$ ADP receptor antagonists, but such medications have the risk of life-threatening bleeding. There is hence an urgent need for novel anti-platelet agents that selectively target arterial thrombosis over haemostasis. GPVI is the principal collagen signalling receptor on platelets. The fact that GPVI-deficient individuals have an only mild bleeding diathesis suggests that this receptor is not crucial in haemostasis. Further, the recent observation that fibrinogen and fibrin also bind to GPVI indicates a role for this receptor in collagen-independent thrombus formation, alongside the roles of ADP and TxA_2. In this thesis, I investigated the role of GPVI in platelet activation, thrombus growth and stability with the aim to further elucidate the benefits of targeting this receptor in patients with arterial cardiovascular disease.

In 2015, the finding that fibrin and fibrinogen are ligands for GPVI, raised questions about the functional significance of these two interactions. At that time, one of the most debated questions was whether GPVI binds to fibrin or fibrinogen as monomer or dimer. Moreover, the observation that fibrin and fibrinogen bind to monomeric GPVI was surprising, as in contrast with the accepted theory at the time that GPVI dimerises following collagen binding. In order to understand the reasons accounting for the published discrepancies, we critically analysed the GPVI constructs

used by the various groups, alongside other key reagents and the techniques employed (***Chapter 2***). This analysis highlighted the importance of the structures of the GPVI constructs and the role of charge interactions in ligand binding. As GPVI contains domains that are highly charged, it was concluded that the charges may give rise to diverse orientations of the various GPVI constructs, thereby accounting for the discrepancies. In line with this observation, I also showed that GPVI can be crosslinked by charge interactions, as platelet spreading on VWF or glass surface, is abolished in GPVI-deficient platelets (***Chapter 3***). Solving the whole structures of GPVI with collagen and fibrin(ogen) will have important implications in understanding the role of these interactions in various disease and in designing therapeutics that effectively target GPVI interaction with one ligand or another.

As a follow-up, I investigated the mechanism of fibrin- and fibrinogen-induced platelet activation via GPVI under arterial shear rate (***Chapter 4***). As integrin αIIbβ3 is a well-known fibrin and fibrinogen receptor, I asked how the two receptors contribute to platelet activation. I concluded that platelet activation via GPVI in response to either fibrin or fibrinogen is supported by integrin αIIbβ3 outside-in signalling. Furthermore, I showed that binding of fibrinogen to GPVI is important for stability of platelet aggregates. Considering these observations, antagonists specific for fibrinogen- and fibrin-GPVI interaction, could be effective in the primary and secondary prevention of cardiovascular events as they may contribute to inhibit the formation of occlusive thrombi during the first stages of thrombus formation. Moreover, inhibition of GPVI may help to increase permeability of thrombi rich in fibrin in combination with anti-fibrinolytic agents.

The evidence from animal experiments that GPVI can be targeted to suppress arterial thrombosis with limited effects on haemostasis has raised wide interest towards this receptor but, there remained concern over whether anti-GPVI agents would cause excessive bleeding. One way

to explore this aspect is by studying the clinical history and functional response of platelets from patients with the genetic loss of GPVI (***Chapter 3***). By using the Maastricht flow chamber, I showed that GPVI signalling regulates platelet phosphatidylserine exposure, and that it supports platelet aggregation on collagen and non-collagen surfaces. Considering the mild bleeding diathesis of individuals with GPVI deficiency, this finding suggests that, in GPVI-deficient individuals, a compensatory mechanism provides for the lack of GPVI. The mild bleeding phenotype is further supported by the calculation that about 4079 individuals, homozygous for the mutation, may be living in Chile without known bleeding disorder. In light of these observations and the finding that GPVI is key in thrombus formation on atherosclerotic plaques,[1, 2] these results strengthen the idea that anti-GPVI agents can selectively target arterial thrombosis over haemostasis. As of today, glenzocimab is the only one anti-GPVI antibody undergoing phase II trial in patients with acute ischemic stroke (NCT03803007) and resulted are awaited. In the previous phase I clinical trial, it was shown to be well tolerated, with inhibition lasting up to 24 h for the highest dose of 2,000 mg.[3] Moreover, inhibition of GPVI might be beneficial in different scenarios where the use of classic anti-platelet medications can be either ineffective or unsafe. For instance, in patients undergoing percutaneous injury interventions whereby the use of inhibitors of the integrin αIIbβ3 carries the risk of excessive bleeding. Interestingly, these patients experience high incidence of microvascular obstruction despite receiving dual antiplatelet therapy consisting of inhibitors of secondary mediators,[4, 5] therefore indicating that novel alternative therapies that target different signalling pathways are needed to reduce this risk.

Another therapeutic approach for targeting GPVI is represented by small-molecule inhibitors either directly affecting GPVI or interferring with its downstream signalling. The advantages of these compounds are the oral bioavailability and low production costs. Several small-molecule GPVI

inhibitors have been reported, and we investigated the effects of two of these, namely losartan and honokiol (***Chapter 5***). Although both compounds decreased thrombus formation on collagen, they showed a lack of specificity for GPVI, as they also impaired platelet activation induced by CLEC-2. Further, a sub-nanomolar potency of honokiol, reported in the literature, could not be reproduced. A sub-class of small-molecule inhibitors is represented by tyrosine kinase inhibitors. Advantages of several of these compounds are that they have already reached the clinics, are well tolerated and are orally available. Furthermore, there is evidence of a decreased risk of thrombosis in some patients on anti-tyrosine kinase treatment, however bleeding complications appear in these patients.

In light of these observations and the finding that platelet activation by fibrin(ogen) is driven by the interplay of integrin αIIbβ3 and GPVI, we investigated whether the inhibition of Syk, which signals dowstream of the two receptors, could affect the formation of an occlusive thrombus (***Chapter 6***). I concluded that fibrinogen regulates the stability of the thrombus shell region by binding to GPVI and by maintaining the integrin αIIbβ3 in an active state. I found that blockage of Syk increases instability of the platelet aggregates at arterial shear rate and that this caused a similar extent of disaggregation, as seen with inhibitors of the secondary mediators, ADP and TxA_2. Overall, this work indicates that targeting GPVI and the downstream signalling pathway could be used to prevent thrombus growth or to induce instability on a newly formed aggregate. The risk of bleeding related to signalling inhibitors should be further examined, however encouraging data comes from fostamitinib, an approved anti-Syk inhibitor currently used for chronic immune thrombocytopenia which has shown low bleeding risk.[6] Finally, we explored whether binding of fibrin to GPVI could contribute to venous thrombosis and discussed evidence supporting an inflammatory role of the receptor at venous shear conditions (***Chapter 7***). Despite these

observations, limited studies have been performed in investigating GPVI as a possible anti-thrombotic target in the venous setup. To better understand the role of GPVI in venous thromboembolism, new studies need to be performed in comparison with the currently used antithrombotic regiment.

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