Platelet glycoprotein VI in the regulation of thrombus growth

Summary

Glycoprotein VI (GPVI) is the major signalling receptor for collagen. Mouse studies have shown that GPVI is critical for arterial thrombus formation but has a limited contribution to haemostasis. Individuals with an inherited deficiency in GPVI also have only a mild bleeding diathesis. Together, these observations suggest that targeting GPVI may suppress arterial thrombosis while preserving haemostasis. The recent discovery that GPVI is also a receptor for fibrinogen and fibrin suggests that its role extends beyond the onset of thrombus formation. In addition, it raised question about the functional significance of these new interactions. **Chapter 1** provides a general introduction to the work performed with emphasis on the roles of platelets in thrombosis and haemostasis, relevant platelet receptors and ligands, and the architecture of arterial thrombi. As an overarching hypothesis, I proposed that GPVI acts as a relevant signalling receptor for fibrin and fibrinogen in thrombus propagation and stability. Therefore, I investigated the relative contribution of collagen, fibrinogen and fibrin to thrombus growth and stability using inhibitors of GPVI and downstream tyrosine kinases. In **Chapter 2**, I examined the reasons for conflicting results in the literature on whether fibrin and fibrinogen bind to monomeric or dimeric GPVI. I analysed the differences between the various (dimeric and monomeric) GPVI constructs used by the groups involved in the contradictory results, the binding assays employed, and the methods used for fibrin generation. This analysis highlighted the importance of knowing the precise molecular structure of the various GPVI constructs and the role of charge interactions in the ligand binding. As some of the GPVI domains are highly charged, it was concluded that the intramolecular charges gave rise to diverse orientations of the various GPVI constructs, thereby accounting for the discrepant results. Hence, the different forms of GPVI constructs, along with a different use of reagents and techniques could explain the discrepant results.

In **Chapter 3**, I explored the role of platelet GPVI in flow-dependent thrombus formation on collagen and non-collagen surfaces in the

presence or absence of coagulation. For the studies, I used blood from four homozygous and three heterozygous individuals, from three unrelated families, with an inherited mutation in the *GP6* gene, which prevents GPVI expression on platelets. The results show that GPVI is critical for flow-dependent platelet aggregation and phosphatidylserine exposure on both collagen and non-collagen surfaces, whereas it is not required for platelet adhesion. In addition, I confirmed that GPVI can be activated through charge interactions as spreading of platelets on collagen, VWF and negatively charged glass was reduced or abolished with platelets lacking expression of GPVI. Given the unique presentation of GPVI-deficient individuals in the Chilean population, we sequenced the *GP6* exon 6 in 1212 DNA samples, representative of the Chilean population, and calculated that about 4079 individuals carrying the *GP6* mutation may be living in Chile without genetic diagnosis or recognised bleeding phenotype.

In **Chapter 4**, I studied the role of GPVI in supporting platelet adhesion, activation, and aggregation under flow conditions on a range of fibrin and fibrinogen surfaces, in comparison to a collagen surface. In the study platelet responses elicited by GPVI were compared to those relying on integrin αIIbβ3 and GPIb-V-IX. Using Fab 9O12 (blocking GPVI), PRT-060318 (inhibiting Syk tyrosine kinase) and blood from Glanzmann patients lacking integrin αIIbβ3, I concluded that αIIbβ3 is key for platelet adhesion to fibrin and fibrinogen and that it synergises with GPVI in provoking adhesion-dependent platelet activation. Furthermore, I showed that thrombi formed on fibrin and fibrinogen are relatively small when compared to collagen, with platelets showing P-selectin expression, transient cytosolic Ca^{2+} rises and a low phosphatidylserine exposure. In **Chapter 5** I investigated the effect of two small molecules, losartan and honokiol, reported as GPVI antagonists, on platelet responses. Both compounds indeed decreased thrombus formation on collagen, but they were not specific for GPVI, as they also impaired platelet activation induced by CLEC-2 agonists.

Chapter 6 observes whether the tyrosine kinase cascade downstream GPVI and integrin αIIbβ3 is involved in the process of thrombus stabilization, and whether this can be targeted to cause disaggregation of platelets. To do that, flow studies were performed on immobilised collagen and human plaque material under non-coagulating conditions, thus preventing the formation of fibrin. Aggregate stability was challenged by post-perfusion of Syk, Src or Btk inhibitors. In addition, the effects of these inhibitors were compared to antagonism of the secondary mediators of platelet activation, ADP and thromboxane A_2 (TxA_2). We found that inhibition of Syk and Src increased platelet disaggregation of preformed thrombi, both on collagen and plaque material, to a similar extent as blockage of ADP and TxA_2. No additive effect was seen when tyrosine kinase inhibitors were combined with ADP and TxA_2 blockers, suggesting that the pathways act in synergy to maintain thrombus stability. In contrast, selective inhibition of Btk did not significantly impair the stability of the preformed aggregates. We concluded that targeting Syk might be promising to disrupt the thrombus shell, a region of the thrombus which is devoid of fibrin and consists of loosely aggregated platelets.

The review of **Chapter 7** provides an inventory of the literature evidence supporting a role of platelet GPVI in venous thromboembolism, based on findings with humans and mice. This concerned studies that related GPVI to experimental thrombosis, pulmonary thromboembolism and thrombo-inflammation. In humans, a common variant rs1613662 in the *GP6* gene is linked to venous thromboembolism. Overall, the literature shows emerging evidence for platelet GPVI acting in the venous part of the circulation, including leukocyte-dependent thrombo-inflammation, venous thromboembolism, pulmonary thromboembolism and cancer metastasis. We critically evaluated whether fibrin might be the responsible GPVI agonist under venous thrombosis conditions. A general conclusion was that platelet GPVI may be a suitable co-target in the prevention of venous thrombosis due to its role in thrombus

consolidation and platelet-leukocyte complex formation. **Chapter 8** discusses the findings of this thesis, noting the importance of having an antagonist specific for GPVI interaction with fibrin and fibrinogen given the prospective of targeting GPVI and its signalling in thrombotic complications.