Targeting Glycoprotein VI and the Immunoreceptor Tyrosine-Based Activation Motif Signaling Pathway

David Stegner, Elizabeth J. Haining, Bernhard Nieswandt

Abstract—Coronary artery thrombosis and ischemic stroke are often initiated by the disruption of an atherosclerotic plaque and consequent intravascular platelet activation. Thus, antiplatelet drugs are central in the treatment and prevention of the initial, and subsequent, vascular events. However, novel pharmacological targets for platelet inhibition remain an important goal of cardiovascular research because of the negative effect of existing antiplatelet drugs on primary hemostasis. One promising target is the platelet collagen receptor glycoprotein VI. Blockade or antibody-mediated depletion of this receptor in circulating platelets is beneficial in experimental models of thrombosis and thrombo-inflammatory diseases, such as stroke, without impairing hemostasis. In this review, we summarize the importance of glycoprotein VI and (hem)immunoreceptor tyrosine-based activation motif signaling in hemostasis, thrombosis, and thrombo-inflammatory processes and discuss the targeting strategies currently under development for inhibiting glycoprotein VI and its signaling. (Arterioscler-Thromb-Vasc-Biol. 2014;34:00-00.)

Key Words: antiplatelet agents ■ immunoreceptor tyrosine-based activation motif ■ platelet inhibitors

Hemostasis and thrombosis are processes balanced by a complex interplay between the cells and soluble factors of the blood and vasculature. As the effectors of this balancing act, platelets have a central role in the outcomes of vascular damage and disease. Circulating along the margins of the blood vessels, platelets are captured and activated by ligands exposed in damaged blood vessels. This activation of platelets leads to thrombus formation at sites of damage via the action of the major platelet integrin (αIIb)3 and the provision of a procoagulant surface for the activation of the coagulation cascade. In healthy individuals, this process is confined and controlled such that blood flow is maintained in the arteries and arterioles, whereas the thrombus protects and supports the repair of sites of damage.2 In the context of vascular disease and atherosclerosis, this process can result in the total blockade of a vessel by a thrombus, causing ischemic damage to downstream areas. The heart attacks and strokes that commonly arise from thrombosis are the world’s leading cause of death, and because of the central role of platelets in this process, antiplatelet therapies are commonly used to combat this world health burden.3 The cyclooxygenase inhibitor aspirin, often in combination with a thienopyridine drug targeting the P2Y12 ADP receptor in platelets, such as clopidogrel, is the standard treatment for patients at risk of a vascular event. These drugs attenuate the action of the platelet secondary mediators thromboxane A2 and ADP, thus preventing the amplification of platelet activation during thrombus formation. Although this therapy reduces the recurrence of vascular events, the increased risk of bleeding because of platelet inhibition is a particular concern for patients who have experienced stroke, and a further subset of patients remain refractory to either or both antiplatelet approaches.4 Therefore, improved antiplatelet therapies that target alternative pathways of platelet activation and carry a decreased risk of bleeding are required.

Several major platelet surface receptors have been suggested as new targets for antiplatelet drugs; among these, the platelet collagen receptor glycoprotein VI (GPVI) possesses several unique attributes, which make it a particularly attractive target. First, GPVI is only expressed in platelets and megakaryocytes, allowing specific targeting of the receptor. Second, it has been known since its discovery that loss of GPVI from platelets is not associated with severe bleeding complications in vivo.5 Third, the interaction between GPVI and collagen is an early event in platelet activation.4 Therefore, targeting this would interrupt thrombosis earlier than current therapeutics and may offer a more effective reduction of platelet reactivity and procoagulant activity. Finally, GPVI has been implicated in the platelet-mediated damage that occurs in ischemically injured cerebral vasculature and also in the progression of atherosclerosis. This suggests that targeting GPVI may have beneficial effects beyond the treatment and prevention of acute and secondary vascular events.6 This brief review will focus on discussing the current methods of targeting GPVI, which are under development for use as antithrombotics.

Received on: April 11, 2014; final version accepted on: May 28, 2014.
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Arterioscler-Thromb-Vasc-Biol is available at http://atvb.ahajournals.org DOI: 10.1161/ATVBAHA.114.303408
Structure and Signaling of GPVI

GPVI is a platelet- and megakaryocyte-specific immunoglobulin superfamily protein with copy numbers reported between 4000 and 6000 per platelet. It is a 58-kDa type I transmembrane protein that is constitutively associated with the Fc receptor γ (FcRγ) chain. The FcRγ chain is essential for GPVI function because it is not only required for GPVI expression in platelets but also contains the immunoreceptor tyrosine-based activation motif (ITAM) that forms the basis of GPVI signal transduction (Figure 1). The structure of GPVI and the molecular basis for its interaction with its major ligand collagen are discussed in detail in 2 recent reviews.9,10 In addition to collagen, GPVI also interacts with 2 other components of the extracellular matrix, laminin11 and fibronectin.12 GPVI signals via a tyrosine phosphorylation cascade, which is similar to that of other immune receptors. Ligand-induced cross-linking of GPVI initiates the phosphorylation of the FcRγ chain in the ITAM motif by the Src kinases Fyn and Lyn.9,13 This phosphorylation event induces the recruitment and subsequent activation of the tandem SH2 domain–containing protein tyrosine phosphatase (spleen tyrosine kinase Syk), which in turn initiates a downstream signaling cascade that involves a large number of kinases, adaptor, and effector molecules, which form the GPVI signalosome (Figure 1). A central effector protein of the signalosome is phospholipase Cγ2, and the action of this protein leads to platelet activation via protein kinase C activation and store-operated calcium entry. The function of GPVI in hemostasis and thrombosis has been investigated using several mouse models of GPVI deficiency14–17 and by the assessment of patients who lack GPVI through either genetic defects18,19 or autoimmunity.20,21 All studies have demonstrated the requirement of GPVI for the recruitment and activation of platelets by collagen, in addition to functional hemostasis despite GPVI deficiency. However, in animal models of thrombosis, the evidence on the necessity of GPVI for arterial thrombosis is less compelling.22,23 The variability of these results may be attributed to differences in the nature and extent of vessel injury in each model. In particular, under conditions of strongly activated coagulation, the relevancy of GPVI seems to be reduced. It is thought that the high levels of thrombin generated in these cases may provide a compensatory mechanism in triggering integrin activation.22,24,25 What does seem clear is that in instances where subendothelial matrix mediates platelet activation, GPVI is essential for stable thrombus formation.4 A major ligand for GPVI, fibrillar collagen type I, is enriched in the fibrous cap of atherosclerotic plaques, and studies in which experimental plaque rupture has been used to trigger thrombus formation have clearly revealed a central role for GPVI under these conditions.26,27 GPVI is therefore thought to be an essential receptor in platelet responses to vascular disease, demonstrating the relevancy of GPVI as anti-thrombotic target.

**Fcγ Receptor IIA**

In addition to GPVI, the Fc receptor FcγRIIA also signals through an ITAM which it bears in its own cytoplasmic tail (Figure 1). Interestingly, this receptor is expressed on human but not mouse platelets. FcγRIIA mediates the activation of human platelets exposed to immune complexes, tumor cells, and bacteria and is critically involved in the thrombocytopenia and thrombosis caused by these agents.28–32 This receptor also enhances platelet integrin outside-in signaling, thereby contributing to general thrombus stabilization.33,34

**C-Type Lectin-Like Receptor 2**

The third ITAM-related receptor on platelets is the recently discovered C-type lectin-like receptor 2 (CLEC-2). This receptor, like FcγRIIA, is not associated with the FcRγ chain but contains half an ITAM (a YXXL sequence with an upstream stretch of acidic amino acids) in its cytoplasmic tail and so is termed a (hem)ITAM receptor (Figure 1). CLEC-2 is the target of the snake venom toxin rhodocytin35 and is the receptor for the sialoglycoprotein podoplanin36 but paradoxically has no known ligand expressed in the vasculature. In the case of CLEC-2, Src family kinases seem to be downstream of Syk in the ITAM cascade, which is in contrast to the classical understanding of ITAM signaling.37 CLEC-2 has been proposed as a target for antithrombotic agents because, like GPVI, CLEC-2 deficiency has only a minor impact on hemostasis but prevents formation of stable vessel occluding thrombi.38,39 However, CLEC-2 is also involved in several

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**Figure 1.** (hem)Immunoreceptor tyrosine-based activation motif (ITAM) receptors in platelets. Glycoprotein VI (GPVI) is a type I transmembrane protein that is constitutively associated with the Fc receptor γ (FcRγ) chain, which bears an ITAM. Receptor clustering by ligands initiates phosphorylation of the FcRγ chain in the ITAM motif by the Src family kinases (SFK), which induce the recruitment and subsequent activation of spleen tyrosine kinase (Syk), which in turn initiates a downstream signaling cascade that involves a large number of kinases, adaptors, and effectors. This cascade leads to phospholipase Cγ2 (PLCγ2) activation, which results in the formation of a membrane raft, integrin outside-in signaling, and calcium mobilization. In platelets, this leads to platelet activation and the recruitment of G-protein–coupled receptors (GPCRs). GPVI mediates the activation of human platelets exposed to immune complexes, tumor cells, and bacteria and is critically involved in the thrombocytopenia and thrombosis caused by these agents.28–32 This receptor also enhances platelet integrin outside-in signaling, thereby contributing to general thrombus stabilization.
processes beyond thrombosis and hemostasis, including the separation of lymphatics from the blood vasculature, the maintenance of high endothelial venule barrier function, and the formation of lymph nodes. This, in combination with its enigmatic role in hemostasis, suggests that CLEC-2 currently is not a serious antithrombotic target. Importantly for GPVI targeting, GPVI and CLEC-2 have some redundant functions because combined loss of both receptors results in a more pronounced antithrombotic effect in vivo than the loss of either protein individually. However, this increased protection is accompanied by a profound bleeding phenotype.

**GPVI in Thromboinflammation**

In recent years, the term thromboinflammation has been introduced to highlight the interdependence of thrombotic and inflammatory processes under certain pathological conditions. This is best established in the setting of reperfusion injury (eg, in reperfused brain tissue after stroke) where a significant contribution of platelet receptors to inflammatory neuronal damage has been revealed. GPVI, in particular, has been recognized as a key platelet receptor under these conditions. In the transient middle cerebral artery occlusion mouse model of transient ischemic stroke, GPVI deficiency reduced the damage to the brain after transient ischemia. Indirect evidence also supporting a role for GPVI in ischemic stroke comes from a study showing that transient ischemic attack and stroke correlate with elevated plasma levels of soluble GPVI in patients with acute ischemic stroke, it is hypothesized that increased GPVI activation occurs in this setting. More direct evidence comes from recent studies in mice that have hyperactive GPVI signaling because of a deficiency in the hematopoietic adapter molecules Src-like adapter protein and Src-like adapter protein 2. These studies demonstrated that increased GPVI signaling results in increased damage to the brain in the transient middle cerebral artery occlusion model (Cherpokova et al, unpublished data). Although the mechanisms behind these observations are incompletely understood, GPVI-mediated platelet activation clearly plays a role in the pathophysiology of thrombo-inflammatory diseases. In addition to thromboinflammation in reperfusion injuries, GPVI has also been recognized as a key platelet receptor in more classical inflammatory disease settings. One elegant study has demonstrated that GPVI contributes to inflammation via microparticle formation, independently of thrombus formation, in a model of rheumatoid arthritis, and a requirement for GPVI and CLEC-2 has been demonstrated in the maintenance of vascular integrity under inflammatory conditions.

**Blocking the Collagen–GPVI Interaction**

Blocking the interactions between receptors and ligands is a common approach in therapy. In the case of GPVI, blocking its interaction with collagen could significantly reduce platelet activation in diseased vessels, providing powerful prevention of thrombosis. One approach currently under development is the competitive inhibition of GPVI binding sites on collagen by a soluble dimeric GPVI-Fc fusion protein (Figure 2). In mouse models of thrombosis, pretreatment with this protein, called Revacept, was reported to prevent occlusive thrombus formation and reduce infarction size after ischemic stroke. These results were in agreement with those seen in GPVI-deficient mouse models, albeit less pronounced, indicating the potential efficacy of this approach. Revacept is now under phase II trial (clinicaltrials.gov: NCT01645306) after phase I indicated that it is safe in healthy subjects. Interestingly, the Revacept fusion protein seems to be in some way unique because GPVI-Fc fusion proteins generated by others failed to produce a detectable antithrombotic effect in several in vivo thrombosis models in mice. As a small aside, GPVI fusion proteins have also been suggested as useful tools in localizing sites of exposed collagen, and therefore damage in the vasculature, and are currently under investigation as tracers for fibrosis in patients.

A second approach to interrupt the GPVI–collagen interaction is the use of monoclonal anti-GPVI Fab fragments that block the collagen binding site of the receptor (Figure 2). This strategy may have improved efficacy over that of GPVI-Fc fusion proteins, because anti-GPVI Fab fragments would bind irreversibly to platelets and can easily saturate their target. Ex vivo models of collagen-induced thrombus formation in rats and cynomolgus monkeys and an in vivo arterial thrombosis...
A2 signaling (targeted by aspirin), may result in severely impaired hemostasis. Despite this, the GPVI targeting approach of targeted GPVI downregulation seems unaccompanied by a severe bleeding risk and could even be more efficient than blocking the receptor or receptor binding. However, the irreversible nature of this approach is perhaps worth a note of caution. Furthermore, it is essential to advance the understanding of anti-GPVI antibody effects before these powerful observations can be fully harnessed for therapy.

**Targeting (hem)ITAM Signaling**

Blocking GPVI function may also be achieved by targeting the tyrosine kinases and adaptor proteins downstream of GPVI. Inhibitors of the kinase Syk, which are already under development for the treatment of some cancers, may also be useful for the treatment and prevention of thrombosis. Examples include the inhibitor fostamatinib, currently in clinical trials for the treatment of chronic lymphocytic leukemia, rheumatoid arthritis, and asthma and PRT318, which, in addition to being studied in the context of chronic lymphocytic leukemia, has also been shown to reduce heparin-induced thrombocytopenia and immune complex–mediated thrombosis in mice.

The major drawback of targeting signaling molecules downstream of GPVI is a lack of specificity because Syk and many other components of the GPVI signalosome are important in immune cell signal transduction. Thus, it has to be thoroughly evaluated whether the side effects on the immune system are outweighed by the desired antithrombotic potential of Syk inhibitors. Furthermore, disruption of GPVI receptor signaling cannot be considered without also understanding the effect that this could have on the 2 other ITAM signaling receptors expressed in platelets and also the outside-in signaling of the aIIbβ3 and β1 integrins. In particular, clarification is needed as to whether Syk, and therefore (hem)ITAM, inhibition will reproduce the severe defect in hemostasis caused by the combined deficiency of GPVI and CLEC-2. Importantly, early studies using bone marrow chimeric Syk-deficient mice or pharmacological inhibition of Syk in vivo reported grossly normal hemostasis. However, a direct comparison of Syk-deficient mice with mice lacking CLEC-2 and GPVI in hemostasis models would be helpful in judging the relative safety of both antithrombotic approaches.

**Conclusions**

GPVI is a promising pharmacological target for effective and safe treatment of thrombotic diseases and, as indicated by several recent studies, inflammatory diseases. The appeal of GPVI is a result of its restricted expression and the possibility of its easy targeting by experimental blockade or immunodepletion. The latter may represent a feasible long-term prophylactic treatment, because patients with anti-GPVI autoantibodies demonstrate that GPVI immunodepletion cannot be considered without also understanding the effect that this could have on the 2 other ITAM signaling receptors expressed in platelets and also the outside-in signaling of the aIIbβ3 and β1 integrins. In particular, clarification is needed as to whether Syk, and therefore (hem)ITAM, inhibition will reproduce the severe defect in hemostasis caused by the combined deficiency of GPVI and CLEC-2. Importantly, early studies using bone marrow chimeric Syk-deficient mice or pharmacological inhibition of Syk in vivo reported grossly normal hemostasis. However, a direct comparison of Syk-deficient mice with mice lacking CLEC-2 and GPVI in hemostasis models would be helpful in judging the relative safety of both antithrombotic approaches.
strategies discussed here have the potential to contribute to, and improve, the treatment of vascular disease and thrombotic disorders in the future.

Acknowledgments

We thank the current and past members of our group who have contributed to the work in improving the understanding of (hem) immunoreceptor tyrosine-based activation motif receptor function in platelets.

Sources of Funding

Work of the authors was supported by the Deutsche Forschungsgemeinschaft (SFB 688 to D. Stegner and B. Nieswandt).

Disclosures

None.

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Arterioscler Thromb Vasc Biol. published online June 12, 2014;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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