Focusing on plasma glycoprotein VI

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Summary

New methods for analysing both platelet and plasma forms of the platelet-specific collagen receptor, glycoprotein VI (GPVI) in experimental models or human clinical samples, and the development of the first therapeutic compounds based on dimeric soluble GPVI-Fc or anti-GPVI antibody-based constructs, coincide with increased understanding of the potential pathophysiological role of GPVI ligand binding and shedding. Platelet GPVI not only mediates platelet activation at the site of vascular injury where collagen is exposed, but is also implicated in the pathogenesis of other diseases, such as atherosclerosis and coagulopathy, rheumatoid arthritis and tumour metastasis. Here, we describe some of the critical mechanisms for generating soluble GPVI from platelets, and future avenues for exploiting this unique platelet-specific receptor for diagnosis and/or disease prevention.

Keywords

Glycoprotein VI, shedding, platelets, thrombosis, thrombocytopenia

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Introduction

The analysis in 2011 of the human platelet sheddome, after excluding plasma proteins and microparticles, identified 69 soluble membrane proteins derived from activated platelets (1). While the pathophysiological importance of some of these shedding events remains to be determined, one of the identified receptors shed in response to platelet activation by the protein kinase C (PKC)-activator phorbol myristate acetate (PMA), is the collagen receptor, glycoprotein (GP)VI. Immunoblotting human platelet lysates with an antibody against the cytoplasmic domain of GPVI that recognises both intact receptor and the membrane-associated fragment, shows that platelet GPVI from healthy individuals is essentially all intact with no cleaved remnant fragment detectable, but that treatment of platelets *in vitro* with PMA, artificial activators of shedding such as calmodulin inhibitors or *N*-ethylmaleimide (NEM), coagulation factors, or GPVI ligands including collagen, collagen-related peptide (CRP) or GPVI-targeting snake toxins results in time-dependent loss of intact GPVI (~62 kDa) and the appearance of an ~10-kDa remnant fragment (2, 3). A ~55-kDa ectodomain fragment of soluble GPVI (sGPVI) is released into the supernatant, and can be detected in human plasma by ELISA (4–6). Recent studies show that compared to healthy individuals, sGPVI is significantly elevated in plasma from patients with atherothrombotic, immune-related or other diseases, providing evidence for a pathological or protective role for GPVI shedding *in vivo*, and/or supporting the value of measuring proteolytic fragments of GPVI as platelet-specific markers. As discussed below, these findings also raise the possibility of a functional role for the shed monomeric GPVI fragment through interaction with collagen or other proteins, while a recombinant dimeric form of sGPVI (expressed as a fusion protein with human Fc) inhibits GPVI-dependent thrombus formation in experimental models, and has been administered to healthy human subjects for the first time in 2011 (7).

Platelet GPVI

GPVI is a type I transmembrane glycoprotein of the immunoreceptor family, with two extracellular immunoglobulin domains, and a mucin domain, transmembrane domain, and cytoplasmic tail of ~60 residues that regulates signalling/shedding (8). On platelets, GPVI is physically and functionally linked to the GPIb-IX-V complex, *via* a direct interaction of the extracellular domains of GPVI and GPIbα, the ligand-binding subunit of GPIb-IX-V (9). GPIbα interacts with von Willebrand factor (VWF) and thrombospondin, coagulation factors (thrombin, kininogen, factor XII and XI) and receptors on activated endothelial cells and platelets (P-selectin) or activated leukocytes ($\alpha_M\beta_2$; Mac-1) (reviewed in [10]). Interaction of platelet GPVI/GPIb-IX-V with extracellular matrix components – VWF, thrombospondin, collagen or laminin – induces rapid platelet activation and signalling leading to activation of platelet integrins, including $\alpha_{IIb}\beta_3$ which binds VWF and fibrinogen, $\alpha_2\beta_1$ which binds collagen and $\alpha_6\beta_1$ which binds laminin (10–14). Further, both collagen and laminin are able to signal *via* GPVI associated with the Fc receptor γ-chain (GPVI/FcRγ). The cytoplasmic tail of GPVI constitutively binds activated Src ki-

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nase, Lyn, which phosphorylates an immune receptor-based activation motif (ITAM) in FcRγ, leading to activation of Syk, phosphatidylinositol 3-kinase (PI3K), and downstream effectors such as PKC and phospholipase Cγ (PLCγ) (11, 13, 15). GPIb-IX-V is also co-associated with the platelet Fc receptor, FcγRIIa, a "single-chain analogue" of GPVI/FcRγ, consisting of two extracellular immunoglobulin domains, a transmembrane domain and an ITAM-containing cytoplasmic tail, and can utilise FcRγ signalling pathways within a GPVI/GPIb-IX-V adheso-signalling complex (9, 16–18). Engagement of GPVI/GPIb-IX-V also leads to activation of intracellular complexes involving the receptor-associated protein, TRAF4, and its binding partner $p47^{phox}$ of the NADPH oxidase complex (Nox2) which generates intracellular reactive oxygen species (ROS) in activated platelets (19, 20). GPVI/GPIb-IX-V signalling pathways not only activate integrins including $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, but also divergent pathways leading to activation of sheddases that mediate proteolysis of GPVI, GPIbα and GPV.

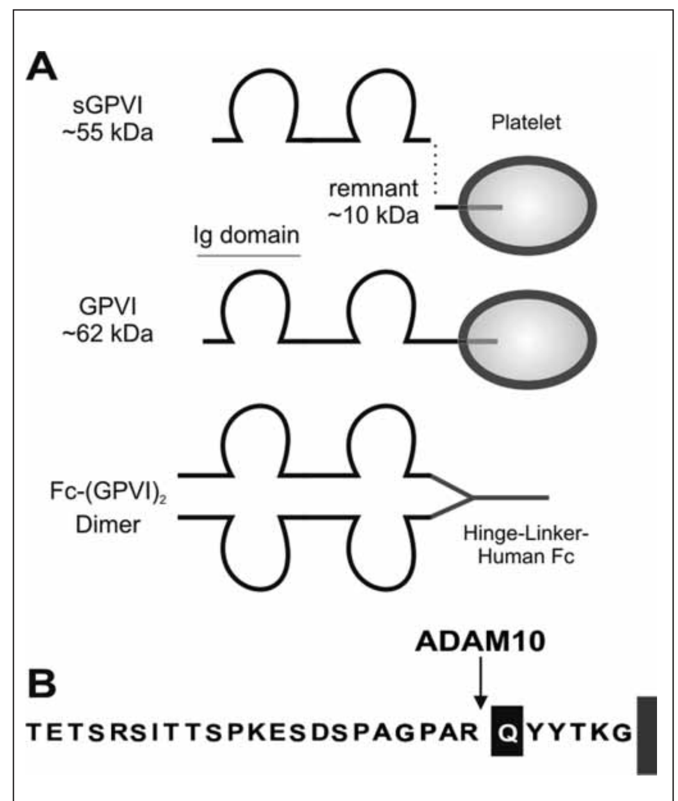

Figure 1: Platelet and soluble GPVI. A) Schematic of intact GPVI on platelets cleaved by endogenous platelet sheddase(s) to generate a ~55-kDa soluble fragment (sGPVI) and a ~10-kDa membrane-associated remnant fragment. Fc-$(GPVI)_2$ is a recombinant dimeric form of sGPVI conjugated via a hinge-linker sequence to human Fc. Ig, immunoglobulin (disulfide bonds are not shown). B) The membrane-proximal extracellular sequence of human GPVI corresponding to a synthetic peptide cleaved by recombinant ADAM10. The highlighted Gln (Q) residue mutated to Lys prevents shedding of recombinant GPVI.

Regulation of GPVI shedding

GPVI function, and platelet responsiveness to collagen, may be regulated by i) GPVI expression levels (FcRγ is required for GPVI surface expression) that affect the propensity for cross-linking and activation of ITAM signalling pathways (21–23), ii) inhibitory receptors such as PECAM-1 or CEACAM-1 which contain an immune receptor-based inhibition motif (ITIM) that can recruit phosphatases and attenuate GPVI signalling (24–26), iii) internalisation, a process which potentially decreases GPVI surface density or possibly redistribution of GPVI to the open canalicular system (27–29) as previously proposed for GPIb-IX-V and other receptors in activated platelets; and iv) metalloproteinase-mediated ectodomain shedding. While the physiological importance of all of these potential pathways is not known in detail, GPVI shedding is a key mechanism for controlling GPVI function on platelets (▶Fig. 1), because it is rapid, irreversible and proteolysis near the outer surface of the membrane generates a ~10-kDa membrane-associated remnant (consisting of the transmembrane and cytoplasmic domains) and a ~55-kDa *N*-terminal soluble fragment (consisting of the two immunoglobulin domains plus mucin) which can be measured as a quantifiable marker of the extent of shedding induced experimentally or *in vivo* (30, 31). Unlike the other regulatory mechanisms, there is also increasing evidence that ectodomain shedding occurs *in vivo*, and is increased in human disease.

Shedding of GPVI from human platelets is induced in activated platelets, by activation-independent pathways and by anti-platelet antibodies: that is, GPVI shedding may be triggered by mechanisms that are likely to be relevant in physiology/pathology (ligand-induced, dependent upon platelet activation or coagulopathy, or due to autoantibodies) or by artificial means that can be used *in vitro* or may be relevant to certain drugs (calmodulin inhibitors that may be related to psychoactive drugs, thiol modifiers such as NEM, or apoptotic drugs used for anticancer treatments) (▶Table 1). Examining some of these causes of GPVI shedding in detail provides information on mechanisms involved in shedding, and how these may be relevant to future therapeutics or use of proteolysed GPVI as a biomarker:

GPVI ligands and platelet activation

Analysis of platelet GPVI shedding from human platelets showed that the GPVI ligand, collagen, and other GPVI ligands (CRP, convulxin) induced shedding whereas platelet agonists against GPIbα (von Willebrand factor) or thrombin receptor (thrombin, TRAP) were relatively ineffective (32). Shedding was metalloproteinase-dependent, and was blocked by GM6001, TAPI or EDTA (32, 33). Shedding was also blocked by inhibitors of Src/Lyn (PP1, PP2), Syk (piceatannol), and PI3K (wortmannin) involved in GPVI/FcRγ-dependent pathways (32). Integrin engagement and platelet aggregation is not required, however, since platelets from healthy donors treated with $\alpha_{IIb}\beta_3$ inhibitor (RGD) or platelets from an individual with absent GPVI-dependent platelet aggregation, were still able to

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shed GPVI to near-normal levels (32, 34). The PKC-activator, PMA, known to activate ADAM-family sheddases in other cells, also induced shedding of GPVI (1, 2). Differential shedding of GPVI due to GPVI ligands or other platelet agonists suggested that separate mechanisms controlled regulation of GPVI, GPIbα and other platelet receptors. Contemporary studies also showed an important role for ADAM17 (tumour necrosis factor-α-converting enzyme, TACE) in GPIbα shedding, using mice expressing catalytically-inactive ADAM17 or inhibitors (35, 36). In addition, recombinant ADAM10, but not ADAM17, cleaved a synthetic peptide spanning the sheddase-cleavage site in GPVI (►Fig. 1), whereas only ADAM17 cleaved a peptide based on the corresponding sequence of GPIbα (2). Circulating human platelets express essentially all intact GPVI, with no membrane-associated remnant fragment, whereas both intact and cleaved GPIbα is detectable on the same platelets (2). Finally, GPVI shedding from human platelets may be partially blocked by the ADAM10-selective inhibitor, GI254023 (37). Interestingly, however, on mouse platelets, deficiency of active ADAM10 does not prevent GPVI shedding induced by activation-independent pathways, suggesting redundancy in any purported ADAM10-mediated shedding pathway (38). Together, these findings suggest that GPVI shedding is more tightly regulated than GPIbα shedding, and that platelet activation alone is not sufficient to induce GPVI shedding.

Coagulation

While characterising our in-house ELISA for measuring shed sGPVI, we found that while plasma levels were not significantly affected by common anticoagulants (trisodium citrate, acid-citrate-dextrose, or EDTA), serum obtained from blood collected into a clotting tube contained vastly elevated levels of sGPVI, calculated to be close to complete loss of platelet GPVI (4). Further studies revealed that coagulation was a potent inducer of GPVI shedding, and that generation of activated factor X (FXa) of the common (intrinsic or extrinsic) coagulation pathway played an important role (37). Shedding was metalloproteinase-dependent (inhibited by GM6001 and GI254023), as well as by FXa inhibitors. The mechanism for coagulation-induced shedding of GPVI remains to be determined, however, shedding is independent of platelet activation (that is, not blocked by PP1/PP2, piceatannol or wortmannin), possibly implying a direct effect on a sheddase(s).

Artificial triggers and drugs

While collagen exposure (ligand-induced) and/or coagulation (FXa-induced) GPVI shedding could be related to physiological/pathological prothrombotic/procoagulant conditions *in vivo* (see next section), analysis of non-physiological triggers are nonetheless relevant to platelet GPVI expression/shedding. NEM has been reported to induce activation of sheddases on other cells (references in [2, 30]). NEM activates both ADAM-family and matrix metalloproteinase (MMP)-family metalloproteinases. One mechanism involves modification by NEM of a free thiol (cysteine) within the pro-domain of ADAM10; however, the role of the pro-domain in the function of platelet ADAM10/ADAM17 is unclear. Whilst immature ADAMs can be detected on the surface of cultured cells, in other vascular cell types, only mature ADAMs protein (lacking a prodomain) are present on the surface of circulating primary cells (39, 40). Calmodulin inhibitors such as W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide) or trifluoperazine (an antipsychotic used to treat schizophrenia or other disorders) can dissociate calmodulin from the cytoplasmic domains of sheddase substrates, and/or of sheddases, and induce activation-independent shedding in other cells. Although the cytoplasmic domain of GPVI binds calmodulin, and the dissociation occurs in activated platelets or following treatment with W7 (32), whether this alone regulates shedding is uncertain. Off-target effects of calmodulin inhibitors could also be involved in inducing platelet receptor shedding. Another pathway for increasing shedding of GPVI, GPIbα and other receptors is the mitochondrial poison, CCCP (carbonyl cyanide M-chlorophenylhydrazone), with cellular stress coincident with increased surface receptor shedding (2, 27, 35). The pro-apoptotic Bcl-x(L)-inhibitory BH3 mimetics (Abt-737, and the anticancer drug Abt-263) also induce both thrombocytopenia and thrombocytopathy associated with increased time-dependent shedding of platelet GPVI and GPIbα (41).

Antibodies

Anti-GPVI antibodies have been shown to induce depletion of platelet surface GPVI both *in vitro* and *in vivo* (3, 29, 42–46). In these examples, depletion may involve either metalloproteinase-mediated shedding or internalisation, and may be independent of activation. Other antibodies against GPVI or other platelet antigens cause activation-dependent shedding by engagement of platelet FcγRIIa (blocked by the anti-FcγRIIa monoclonal antibody, IV.3) (47). These different pathways are important in terms of potential therapeutic depletion of GPVI, as well as monitoring or treatment of autoimmune disease.

In rare individuals, anti-GPVI antibodies do not result in profound thrombocytopenia from immune-clearance or aberrant platelet activation (*via* GPVI or FcγRIIa), but there is loss of platelet surface GPVI and a selective defect to collagen or other GPVI ligands (reviewed in [48]). Similarly in mice, injection of a rat anti-GPVI monoclonal antibody JAQ1 results in selective platelet GPVI depletion *in vivo* without substantial thrombocytopenia, and protection from arterial thrombosis (44). In another study, human platelets were injected into mice, and could be depleted of GPVI by injection of anti-human GPVI antibodies (49). Also injecting monkeys with anti-GPVI antibodies or Fab fragments resulted in depletion *in vivo* of circulating platelet GPVI, and loss of GPVI-dependent aggregation (29). These inhibitory agents were generated

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Table 1: Shedding of platelet GPVI. See the text for details.

A. Pathways for inducing GPVI shedding *in vitro*	Signalling dependent	Independent of platelet activation	Refs	
GPVI ligands				
Collagen	Yes		(27, 32, 33)	
C-reactive protein	Yes		(2, 32)	
Snake toxins	Yes		(2, 32, 33)	
Coagulation (FXa)		Yes	(1, 2)	
Artificial triggers and drugs				
PMA (PKC activator)	Yes		(37)	
NEM		Yes	(2, 4, 9)	
Calmodulin inhibitors (W7)		Yes	(2, 32)	
CCCP		Yes	(2, 27)	
Abt-737/Abt-263		Yes	(41)	
Antibodies				
Anti-GPVI antibodies		Yes	(42–45, 49)	
Anti-GPVI/anti-platelet abs acting via FcγRIIa	Yes	Yes	(3, 47)	
B. Plasma sGPVI in human disease[a]	**Control sGPVI, ng/ml[b]**	**Patient sGPVI, ng/ml[b]**	**P**	**Refs**
Acute ischaemic stroke (n=159)[#]	19.7 ± 8.1	21.5 ± 9.1	0.0168	(51)
Disseminated intravascular coagulation (DIC) (n=29)[#]	12.5 (9.0–17.3) [c]	53.9 (39.9–72.8) [c]	<0.001	(37)
Immune thrombocytopenia purpura (ITP) (n=1)[#]	~15	~150	-	(43)
Lupus nephritis (n=1)[##]	9.2 ± 3.3	~11	-	(46)
Atrial fibrillation (n=78)[###]	1.4 ± 0.8	1.9 ± 1.1	0.038	(60)
Alzheimer's disease (n=30)[###]	0.75 ± 0.43	0.55 ± 0.18	0.033	(6)
Stable angina pectoris (n=1,371)[§]	8.4 ± 3.6	9.8 ± 4.8	0.002	(5)

[a]sGPVI measurements from four separate laboratories using different ELISA reagents and detected using enhanced chemiluminescence [#]ref. (4), optical density [##] ref (46) and [###]ref. (6), and bead-based sandwich immunoassay [§]ref (5). [b]mean ± SD. [c]mean (95%confidence intervals).

from monoclonal antibodies that recognise an epitope on GPVI for a human anti-GPVI autoantibody that caused GPVI depletion in a patient. Human anti-platelet autoantibodies (heparin/platelet factor 4-dependent) or mouse monoclonal antibodies (14A2 against CD151, VM58 against CD36) induce metalloproteinase-mediated GPVI shedding from healthy platelets, dependent on engagement of FcγRIIa. FcγRIIa ligands and GPVI ligands both activate dual pathways not only leading to GPVI shedding, but also proteolytic inactivation of FcγRIIa by intracellular cleavage and deletion of the ITAM required for FcγRIIa signalling (3).

sGPVI in human plasma

sGPVI presents as an excellent candidate plasma marker of platelet activation because (i) it is a platelet-specific receptor; (ii) there is no detectable GPVI remnant fragment on resting platelets, indicating that levels of the receptor on normal circulating platelets are stable; (iii) preliminary data indicates plasma levels of sGPVI are not influenced by age, gender, or smoking; and (iv) preliminary data demonstrates that GPVI shedding is a measurable consequence of treatment of human platelets with ITAM receptor agonists, including anti-platelet autoantibodies as found in ITP patients and in thrombotic diseases including stroke and stable angina pectoris. Together, these studies suggested that plasma sGPVI levels could provide a quantitative platelet-specific marker for multiple human diseases or could serve as a negative-predictor to rule out abnormal platelet activation

sGPVI can be measured in fresh or stored frozen human plasma by ELISA, and normal reference ranges in healthy donors have been established, albeit that absolute values vary between assays in different laboratories using different capture and detection reagents and methods of variable sensitivity (▶Table 1). In our studies, plasma sGPVI is 19 ng/ml (sd, 7.1, range 4–40, *n*=10). This would represent ~10% of total blood GPVI at a platelet count of 200 x 10^9/l (~6,000 copies/platelet). In contrast, plasma levels of soluble GPIbα (glycocalicin) are 1–3 μg/ml, representing about two-thirds of total blood GPIbα (~35,000 copies/platelet), suggesting that sGPVI might be a more sensitive platelet-specific readout of sheddase activation by various causes. This concept is supported by several lines of new evidence including analysis of sGPVI in clinical samples (▶Table 1).

First, in a 59-year old woman with immune thrombocytopenic purpura (ITP), an anti-GPVI autoantibody induced metalloproteinase-mediated shedding of GPVI from healthy donor platelets, and increased levels of plasma sGPVI in the patient (5–10–fold higher than normal levels) (42, 43). This was also one of the first clear demonstrations of elevated plasma sGPVI in human disease, and this is likely to be the case in other immune diseases with anti-

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platelet antibodies (acting *via* FcγRIIa) (3, 42, 43). If shedding of GPVI is elevated, this may be indicative of thrombocytopenia caused by increased immune clearance, rather than low platelet count due to decreased production, particularly in the absence of other known or potential causes of shedding (coagulopathy, atherothrombosis, drugs). In this regard, others recently analysed both reticulated platelet fraction (increased staining of mRNA in young vs. old platelets) and plasma glycocalicin relative to platelet count (glycocalicin index) as a diagnostic tool in ITP (50). Second, we showed that there was a small but significant increase in sGPVI in plasma from 159 patients with acute ischaemic stroke (<7 days) compared with age/sex-matched community-based controls (p=0.0168) (51). In separate studies, platelet GPVI was elevated in stroke patients (52), and inhibiting GPVI protects against ischaemic brain injury in experimental models of stroke (53, 54). Further, patient sGPVI was significantly elevated in the stroke subtype associated with large artery disease, but not in cardioembolic stroke (51). In 59 cases, sGPVI levels significantly decreased several months after anti-platelet treatment. In this study, sGPVI also correlated with plasma soluble P-selectin (p=0.0007), which may be derived from platelets and/or endothelial cell, suggesting that sGPVI is at least as useful a marker of elevated platelet activity under pathological conditions as plasma P-selectin, with advantages over other activation markers in terms of platelet specificity, sensitivity of detection, as an early indicator and/or age-dependence (5, 31, 51, 55). Elevated sGPVI could be explained by increased thrombosis due to atherosclerosis and collagen exposure in acute stroke patients. In one of the stroke studies, it was also shown that elevated sGPVI correlated with a Gln317 polymorphism of GPVI compared with heterozygote or Leu317 (p=0.0241). GPVI*a* and *b* haplotypes involve seven polymorphisms, with five causing substitutions at Ser219Pro, Lys237Glu, Thr249Ala, Gln317Leu, His322Asn. GPVI*a* (associated with Gln317) and GPVI*b* exhibit comparable ligand-binding, but differ in intracellular interactions and signalling which could be related to differential susceptibility to shedding (23).

Further studies have shown that increased coagulation in 29 individuals with disseminated intravascular coagulation (DIC) was also associated with significantly elevated plasma sGPVI, compared to healthy donor plasma or plasma from patients with thrombocytopenia due to chemotherapy (37). Differences between DIC and control remained significant after correction for platelet count, age, sex and coagulation markers, and sGPVI in the DIC group correlated with coagulation markers. GPVI is prothrombotic, by promoting collagen-induced or tissue factor-dependent thrombin generation, thrombus formation, or pulmonary embolism, or by GPVI-dependent phosphatidyl serine exposure on activated platelets or generation of microparticles (56–59). Shedding of GPVI in clotting blood might also be a mechanism to limit coagulation. Bigalke et al. have also shown that plasma sGPVI is significantly increased in acute coronary syndrome and atrial fibrillation, and also showed significantly increased platelet GPVI (31, 60). This group also used a bead-based ELISA method to show significantly elevated sGPVI in plasma from stable angina pectoris compared with myocardial infarct patients (5).

Finally, one of the sheddases implicated in GPVI shedding, ADAM10 (an α-secretase), is involved in protective processing of amyloid precursor protein (APP) (61, 62). APP cleavage by β-secretases leads to pathological APP fragments associated with Alzheimer's disease or dementia. Human platelets express APP, and systemic processing could be relevant to pathology. Initial analysis has shown that sGPVI levels are decreased in 30 individuals with Alzheimer's disease compared with controls, consistent with decreased activity of ADAM10 (or other α-secretase) (6). Further studies may investigate the potential of sGPVI as a surrogate marker or GPVI expression/shedding as protective/pathological in neurological disorders. Approaches for upregulation of ADAM10 activity for Alzheimer's or other diseases potentially applicable to platelets (not involving gene expression) include interventions such as statins which modify membrane lipid fluidity and enhance ADAM10-mediated cleavage of receptors on other cells (62, 63). The potential role of shed sGPVI in regulating the activity of other cell types is also of current interest, because of the identification of new GPVI-binding receptors on other cell types, and the development of recombinant forms of sGPVI for therapeutic use.

Clinical intervention at GPVI/collagen

Experimental and clinical studies on the role of platelet GPVI and regulation of its expression and shedding has coincided with new approaches for clinical intervention or diagnosis: First, competitive inhibitors of collagen-GPVI interaction have potential to block GPVI-dependent thrombotic events, based on the effects of GPVI blockade or deficiency on arterial thrombosis and ischaemic stroke in animal models (29, 53, 54, 64–67). Particularities related to GPVI-knockout mice strains, antibody blockade and thrombosis models are discussed elsewhere (66). GPVI defects in human or other animals are generally considered to have minimal impact on skin bleeding times, suggesting anti-platelet strategies targeting GPVI/collagen could be more selective for pathological thrombosis rather than compromising haemostasis (7, 11, 13, 29, 44, 48, 67). This remains to be proven, and some individuals with GPVI defects have more than mild bleeding episodes which could partially depend on other contributing factors, or on the type of insult (48). Approaches for targeting GPVI-collagen include inhibitors based on anti-GPVI antibodies (single chain or humanised forms) (68, 69), or recombinant monomeric sGPVI or dimeric sGPVI expressed as an Fc-$(GPVI)_2$ fusion protein (▶Fig. 1). In *ex vivo* or animal studies to date, the dimeric form is significantly more efficacious than monomer as an antithrombotic (7, 64, 65, 70–72). For collagen binding, Fc-$(GPVI)_2$ conjugated to a detectable probe has been tried for intravascular or *ex vivo* imaging of exposed collagen (31, 73). The capacity of blocking GPVI-collagen to inhibit haemostasis is also demonstrated by the binding of plasma kallikrein (PK) to collagen under hyperglycaemic conditions to inhibit collagen-induced platelet aggregation and enhance cerebral haematoma expansion in rodents (74, 75). This suggests both alternative inhibitory approaches and potential safety issues associated with

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blockade of GPVI-collagen by any approach. An Fc-$(GPVI)_2$ construct, Revacept, administered to healthy individuals dose-dependently inhibited collagen responsiveness and importantly, showed no early signs of aberrant bleeding (7). As shown using anti-GPVI monoclonal antibodies or derivatives, it is also possible to deplete platelet GPVI *in vivo*, without immune clearance or platelet activation, and attenuate platelet collagen responsiveness (29, 44). These methods are likely to be highly selective for platelet GPVI due to its limited cellular distribution and specificity of antibodies. The achievable plasma concentrations, half-time and other pharmacokinetic properties of both Fc-$(GPVI)_2$ in healthy human subjects and humanised anti-GPVI antibodies in monkeys enable prolonged inhibition of *ex vivo* collagen responsiveness for up to one week or more (7, 29). Although these types of agents are not likely to be orally available, and might not be suitable for long-term anti-platelet clinical use, the solving of the crystal structure of the GPVI ectodomain, the likelihood that GPVI forms a functional homodimer on the platelet surface *via* an interaction between the ectodomains, and identification of the interactive site between the GPVI ectodomain and the negatively-charged sulphated tyrosine-containing sequence of GPIbα, provide opportunities for screening/developing new small molecule competitive or allosteric inhibitors (9, 70, 76, 77), particularly if the efficacy of existing inhibitors fulfils current expectations (7).

Another approach for targeting platelet GPVI is inhibition of signalling pathways. In one individual where GPVI is surface-expressed and binds ligand, there is absent GPVI-dependent platelet aggregation, whereas platelets aggregate in response to other agonists (34). Although the defect is not identified, this demonstrates the potential to selectively block GPVI without interfering with other platelet activation pathways. Signalling by GPVI/FcRγ and FcγRIIa is ITAM-mediated, involving a cytoplasmic ITAM in the Fc receptor chains, which activate Syk (11, 13, 78). Although Syk-independent pathways may be involved (19, 78), new generation orally-available Syk inhibitors presently used clinically for treating immunological defects in leukocytes have also been shown to inhibit platelet ITAM signalling (79, 80). The potential use of these therapeutics as anti-platelet drugs, or off-target effects on platelet reactivity when prescribed for other reasons, requires evaluation.

Finally, physiological or pathological shedding of GPVI *in vivo*, or a potential therapeutic induction of GPVI shedding to generate a monomeric soluble ectodomain, or the use of dimeric Fc-$(GPVI)_2$ as a drug or for collagen imaging, raises the question of whether monomeric and/or dimeric forms of sGPVI are bioactive towards other cells. Apart from collagen and laminin, a recently-identified novel GPVI binding partner is the extracellular matrix metalloproteinase (MMP) inducer, EMMPRIN (CD147) (81). Like GPVI, EMMPRIN is a glycoprotein of the immunoglobulin superfamily expressed on the surface of tumour cells (where it up-regulates expression of MMPs and promotes angiogenesis) and is also expressed on platelets, human leukocytes and monocytes and mediates platelet-monocyte interactions (82, 83). EMMPRIN expression on vascular cells positively correlates with platelet activation markers and is increased in patients with coronary artery disease (82). Blocking GPVI activity by Fc-$(GPVI)_2$, antibodies or other means could have potential anti-inflammatory effects, and/or stimulate leukocytes through direct interaction with EMMPRIN (or other receptors). It would be worthwhile investigating expression of EMMPRIN on other vascular cells. Although experimental studies involving GPVI-targeting agents show minimal effects on platelet count or turnover *in vivo*, there is also potential for these agents to target megakaryocytes in bone marrow, a possible action of some anti-GPVI autoantibodies (42).

Conclusion

Since human GPVI was identified by cloning in 1999 (8), initial laboratory studies on structure-activity, cell expression and signalling, have progressed through experimental models of thrombosis and stroke, to initial pre-clinical and clinical studies of GPVI-targeting therapeutics (7). Regulated ectodomain shedding of GPVI from human platelets has implications for platelet reactivity, generation of proteolytic fragments as potential markers or mediators, and therapeutic approaches for selective targeting of GPVI. The expression of GPVI-binding EMMPRIN on tumour cells also highlights the potential role of platelets/GPVI in tumour metastasis. The interaction between tumour cells and platelets has been found to increase the metastatic potential in the circulation, and a role for GPVI has been suggested from experimental models where GPVI deficiency is associated with decreased metastasis of injected tumourogenic cells (84, 85). Similarly, hepatitis C virus (HCV) interacts with GPVI, possibly regulating passage of the virus through the liver (86, 87), and the glycan-based interaction between platelet GPVI/GPIbα and *Staphylococcal* superantigen-like protein 5 (SSL5) suggests a role in platelet activation in *S. aureus* infection (88). GPVI also has potential roles in platelet-related inflammatory responses in arthritis or glomerulonephritis (59, 89). Therefore, studies of platelet GPVI might be directly relevant in thrombosis, bleeding, coagulopathy, inflammation, infectious diseases and cancer.

Conflicts of interest
None declared.

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