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ORIGINAL ARTICLE

Compromised ITAM-based platelet receptor function in a patient with immune thrombocytopenic purpura

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Summary. *Background:* Receptors on platelets that contain immunoreceptor tyrosine-based activation motifs (ITAMs) include collagen receptor glycoprotein (GP) VI, and FcγRIIa, a low affinity receptor for immunoglobulin (Ig) G. *Objectives:* We examined the function of GPVI and FcγRIIa in a patient diagnosed with immune thrombocytopenic purpura (ITP) who had unexplained pathological bruising despite normalization of the platelet count with treatment. *Methods and Results:* Patient platelets aggregated normally in response to ADP, arachadonic acid and epinephrine, but not to GPVI agonists, collagen or collagen-related peptide, or to FcγRII-activating monoclonal antibody (mAb) 8.26, suggesting ITAM receptor dysfunction. Plasma contained an anti-GPVI antibody by MAIPA and aggregated normal platelets. Aggregating activity was partially (~60%) blocked by FcγRIIa-blocking antibody, IV.3, and completely blocked by soluble GPVI ectodomain. Full-length GPVI on the patient platelet surface was reduced to ~10% of normal levels, and a ~10-kDa GPVI cytoplasmic tail remnant and cleaved FcγRIIa were detectable by western blot, indicating platelet receptor proteolysis. Plasma from the patient contained ~150 ng mL^{-1} soluble GPVI by ELISA (normal plasma, ~15 ng mL^{-1}) and IgG purified from patient plasma caused FcγRIIa-mediated, EDTA-sensitive cleavage of both GPVI and FcγRIIa on normal platelets. *Conclusions:* In ITP patients, platelet autoantibodies can curtail platelet receptor function. Platelet ITAM receptor dysfunction may contribute to the increased bleeding phenotype observed in some patients with ITP.

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Introduction

Glycoprotein (GP) VI is a member of the Ig receptor family, and contains two Ig domains in the extracellular region, a mucin-like domain, a transmembrane domain and a cytoplasmic tail [1,2]. GPVI agonists include collagen, collagen-related peptide (CRP) and snake toxins [3,4]. GPVI is homologous to immune receptors and is expressed in a non-covalent complex with the Fc receptor γ chain (FcRγ). The cytoplasmic tail of GPVI assembles Src family kinase members, Fyn or Lyn, *via* a proline-rich sequence in the cytoplasmic tail, and also binds calmodulin through a membrane-proximal sequence upstream of the Fyn/Lyn-binding site [3,5]. The binding of ligand to GPVI causes disulfide-dependent receptor dimerization [6] and phosphorylation of an immunoreceptor tyrosine-based activation motif (ITAM) domain within the cytoplasmic tail of FcRγ, and assemblage of Syk tyrosine kinase. Activation of Syk initiates a cascade of tyrosine phosphorylation of adapter proteins, including LAT, SLP-76 and phospholipase Cγ2 (PLCγ2) [3]. Engagement of GPVI leads to shape change and eventual activation of the platelet integrin, $\alpha_{IIb}\beta_3$, which binds von Willebrand factor (VWF) or fibrinogen and mediates platelet aggregation. GPVI signaling events also lead to dissociation of calmodulin from the cytoplasmic tail of GPVI, and shedding of a soluble ~55-kDa ectodomain fragment of GPVI [7–9]. Shedding of GPVI has also been observed in human platelets treated with thrombin [10] and in mouse platelets treated with the mitochondrial targeting reagent, carbonyl cyanide m-chlorophenyl-hydrazone [11] or anti-mouse GPVI antibodies [12,13].

Recently, we showed that ligand-dependent activation of GPVI or FcγRIIa, a second ITAM-containing receptor on platelets, using either GPVI or FcγRIIa ligands also activated metalloproteinase-dependent shedding of GPVI as well as proteolytic cleavage within the cytoplasmic tail of FcγRIIa [14]. Calpain inhibitors prevented this ITAM receptor-mediated

cleavage of FcγRIIa, and purified calpain could cleave a recombinant cytoplasmic tail of FcγRIIa in at least two sites upstream of the ITAM sequence. IgG prepared from plasma of patients with heparin-induced thrombocytopenia (HIT), where patients have antibodies that recognize a heparin/platelet-activating factor complex and induce platelet activation through FcγRIIa [15], also induced cleavage of both GPVI and FcγRIIa.

Immune thrombocytopenic purpura (ITP) is a common autoimmune mucocutaneous bleeding disorder, characterized by isolated thrombocytopenia in which anti-platelet autoantibodies, primarily against $\alpha_{IIb}\beta_3$ and GPIb-IX-V cause mononuclear macrophage-mediated platelet destruction, chiefly in the spleen [16,17]. Recent studies indicate megakaryocyte maturation and thrombopoiesis can also be affected by anti-platelet autoantibodies [18]. Classically, ITP is treated with corticosteriods and/or intravenous infusion of immunoglobulin (Ig) which may reduce platelet loss *via* Fcγ receptor (FcγR)-mediated inhibition of dendritic cells [19]. Newer therapies are aimed either at slowing platelet clearance, by targeted modulation of FcγRs or stimulating megakaryocyte platelet production using recombinant thrombopoietin-like agents [17,20]. Whether anti-platelet antibodies also directly affect platelet activation status and function is not known.

The occurrence of anti-GPVI autoantibodies in ITP has been previously reported [21–24]; however, the molecular mechanism linking occurrence of anti-GPVI autoantibodies with thrombocytopenia and accelerated clearance of platelets from the circulation remains to be defined. Boylan and colleagues (2004) demonstrated that the autoantibody bound to platelet GPVI, and hypothesized that this binding caused the observed depletion of GPVI from the platelet surface and associated mild thrombocytopenia [24]. A similar effect has been reported in mice [12,13,25] or monkeys [26] treated with anti-GPVI antibodies, where a transient thrombocytopenia and removal of platelet surface GPVI was observed. The mechanism of removal of GPVI in these reports involved receptor shedding and/or receptor internalization [12,13,26]; mouse platelets lack FcγRIIa.

Here we report evidence of an exaggerated bleeding phenotype in a patient with ITP caused by ITAM receptor dysfunction associated with accentuated cleavage of platelet GPVI and FcγRIIa, elevated levels of soluble GPVI and an autoantibody against GPVI. Our data suggests that, at least in patients with 'anti-GPVI-mediated ITP' [24], platelet autoantibodies can curtail platelet receptor function, and thus contribute to a more pronounced bleeding phenotype than expected based on platelet count.

Materials and methods

Reagents

The protease inhibitor cocktail, Complete^{TM}, was from Roche Diagnostic (Mannheim, Germany). The GPVI-specific agonist, collagen-related peptide (CRP) with amino acid sequence $GCO(GPO)_{10}GCOG\text{-}NH_2$ (O, hydroxyproline), was prepared by cross-linking cysteinyl residues at the N- and C-termini using (3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide (Sigma, St Louis, MO, USA) as described elsewhere [27].

Antibodies

MAb VM58 against GPIV (CD36), which activates platelets *via* interaction of its Fc portion with platelet FcγRIIa, has been characterized previously [28]. Anti-GPVI hybridoma medium 6B12 from Dr Mark Kahn (PA, USA); anti-GPVI mAbs 1G5 [8], 4B8 and 1A12 were all raised against the extracellular domain of human platelet GPVI and all recognize a single platelet protein of 62-kDa by western blot. A rabbit polyclonal antibody was raised by injection of a recombinant extracellular fragment of human GPVI, made as described previously [29] and affinity purified on a column of the same injected protein coupled to Affigel 10–15. This antibody recognized a 62-kDa protein in Western blots of platelet lysates eluted on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. Purified polyclonal antibodies against the cytoplasmic tails of GPVI and GPIbα have been described previously [8]. The anti-FcγRIIa blocking mAb IV.3 was purified from hybridoma medium on protein A-Sepharose (Amersham, Buckinghamshire, UK). A platelet-activating mAb, 8.26, raised against human FcγRII [30], was affinity purified from mouse ascites on a column of protein A-Sepharose; 8.26 and antiserum raised in rabbits against the recombinant cytoplasmic tail of human FcγRIIa were from Professor Mark Hogarth (Melbourne, Australia).

Platelet and plasma preparation

Platelets were prepared from blood of patients or normal donors as described previously [31] once informed consent was provided according to the Declaration of Helsinki. Collection of blood was carried out using procedures approved by the Human Ethics Committee of Royal Perth Hospital or Monash University. To obtain platelet-rich plasma (PRP), blood was collected into (final concentration) 0.32% (w/v) trisodium citrate, and centrifuged for 20 min at $160 \times g$ at RT (room temperature). Platelet-poor plasma (PPP) was obtained by centrifuging PRP at $1000 \times g$ for 10 min at RT. For experiments requiring washed platelets, blood was collected into acid–citrate–dextrose and centrifuged at $160 \times g$ for 20 min to obtain PRP. Platelets were washed three times in CGS (37.5 mM sodium citrate, 5 mM glucose, 0.15 M NaCl, pH 7.0) then resuspended in Tyrode's or flow cytometry buffers [14].

Microparticle-free plasma was isolated from citrated PRP by centrifugation at $1000 \times g$ followed by centrifugation of the supernatant at $100\,000 \times g$ for 60 min and assayed for levels of soluble GPVI by ELISA. GPVI-depleted plasma was used as a negative control for ELISAs (described under Measurement of soluble GPVI in plasma) and was prepared by batch adsorption of 10 mL of microparticle-free plasma with anti-GPVI mAb 1G5 coupled to Affigel 10–15. The plasma-1G5-Affigel suspen-

sion was rocked overnight at 4 °C then centrifuged at 8000 × *g* for 20 min to obtain GPVI-depleted plasma and stored in aliquots at −70 °C until required.

Platelet aggregation

Aggregometry was performed using human citrated PRP, using a Chronolog 680 aggregometer (Chronolog Corporation, Havertown, PA, USA) as previously described [6,32]. Aggregation was induced by addition of Type II collagen or arachadonic acid (Helena Laboratories; Beaumont, TX, USA), CRP, epinephrine (AstraZeneca, North Ryde, Australia) and ADP (Sigma, MO, USA). To evaluate functional ITAM-dependent signaling, platelet aggregation was assessed in response to platelet-activating mAb 8.26, against human FcγRII [30]. MAb 8.26 causes rapid aggregation of citrated PRP from healthy donors, which could be blocked by pre-incubation of PRP with 5 μg mL^{-1} FcγRIIa-blocking mAb, IV.3. Aggregating activity of patient PPP was assessed by mixing 50 μL of PPP isolated from either the patient or a healthy donor, with PRP isolated from a healthy donor. In some samples, PRP was pre-incubated for 5 min with 10 μg mL^{-1} IV.3 or 80 μg mL^{-1} of an affinity-purified soluble GPVI ectodomain protein [29] prior to commencement of aggregation.

Flow cytometry

Washed platelets were resuspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.4, containing 0.1% (w/v) bovine serum albumin and 10 mM EDTA to inhibit platelet metalloproteinase activity. Levels of platelet receptors were estimated by incubating 5×10^7 platelets with saturating concentrations of mAbs directed against GPIbα (AK2), GPVI (1A12, 4B8 or 1G5), or an isotype-matched irrelevant control, re-washed, and bound antibody was detected using secondary FITC-conjugated anti-mouse antibody in a FACSCalibur (Becton Dickinson, San Jose, CA, USA) as previously described [14].

Purification of Ig from human serum

Serum from the ITP patient or a healthy donor was fractionated by 0–40% ammonium sulfate precipitation, resuspended in 20 mM KH_2PO_4, 50 mM NaCl, pH 8.0, and dialyzed into the same buffer at 4 °C [14]. To remove albumin, dialysed antibody was loaded onto a 10 × 1-cm column of DEAE-Affigel Blue (BioRad, Hercules, USA) and unbound fractions pooled and dialyzed into TS buffer (0.01 M Tris-HCl, 0.15 M NaCl, pH 7.4).

Shedding of GPVI from the platelet surface

Ligand-induced shedding of GPVI from platelets was performed essentially as described previously [7,14]. Either control or patient platelets (~5×10^8 mL^{-1}) in Tyrode's buffer were mixed in the absence or presence of (final concentration) 100 μg mL^{-1} control Ig or Ig isolated from patient plasma, or 1.0 μg mL^{-1} convulxin. After 0–240 min at RT, samples were made 10 mM in EDTA, and centrifuged to separate platelets from supernatants. Samples were analyzed by SDS-PAGE, and Western blotting with anti-GPVI mAb 6B-12, or affinity-purified anti-FcγRIIa cytoplasmic tail antibody. Blots were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL (Amersham, Buckinghamshire, UK).

Measurement of soluble GPVI in plasma

A sandwich enzyme-linked immunosorbent assay (ELISA) was established to measure protein levels of soluble GPVI in aliquots of human plasma from healthy donors, similar to an assay previously described to measure soluble P-selectin [33]. Conditions of the assay were optimized using recombinant soluble GPVIex diluted in GPVI-depleted plasma or platelet-poor plasma (data not shown). Briefly, wells of 96-well microtitre plates (Nunc, Roskilde, Denmark) were coated with 1 μg mL^{-1} affinity purified polyclonal anti-GPVIex in buffer containing 15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6, overnight at RT, then washed with PBS containing 0.2% (v/v) Tween-20 (PBS-T). Wells were blocked with 1% (w/v) bovine serum albumin (BSA) (Sigma, MO, USA) in PBS for 1 h at RT, rinsed with PBS-T then triplicate aliquots of microparticle-free human plasma, or known amounts of recombinant GPVIex diluted in 10% (v/v) GPVI-depleted plasma/PBS were added to wells for 1 h at RT. Wells were washed then incubated for 1 h at RT with 1 μg mL^{-1} anti-GPVI mAb 1A12 or an isotype-matched control mAb, washed and incubated with HRP-rabbit anti-mouse IgG that had been adsorbed against human and bovine IgG (Pierce, Rockford, IL, USA). Wells were washed again and the amount of HRP conjugated antibody bound to the wells was detected by addition of 100 μL superSignal ELISA pico chemiluminescence substrate (Pierce) and measurement of emitted light using a Wallac-Victor2 luminescence plate reader (PerkinElmer, Rowville, Australia).

Results and discussion

Patient

A 55-year-old Caucasian female presented with epistaxis and lower limb purpura and was found to have severe isolated thrombocytopenia with a platelet count of 2×10^9 L^{-1} (normal range 150–400 × 10^9 L^{-1}). Bone marrow examination showed plentiful megakaryocytes without other abnormality and normal cytogenetics confirming the diagnosis of ITP. Treatment was initiated with prednisolone, 1 mg kg^{-1} day^{-1}, with rapid improvement of her platelet level. However, even when her platelet count was 167×10^9 L^{-1}, she still experienced extensive spontaneous pathological bruising on her trunk and limbs. There was no past or family history of a bleeding diathesis, and she was on no medications that could exacerbate a bleeding tendency. Initial investigations confirmed an isolated defect in collagen-induced platelet aggregation and a prolonged

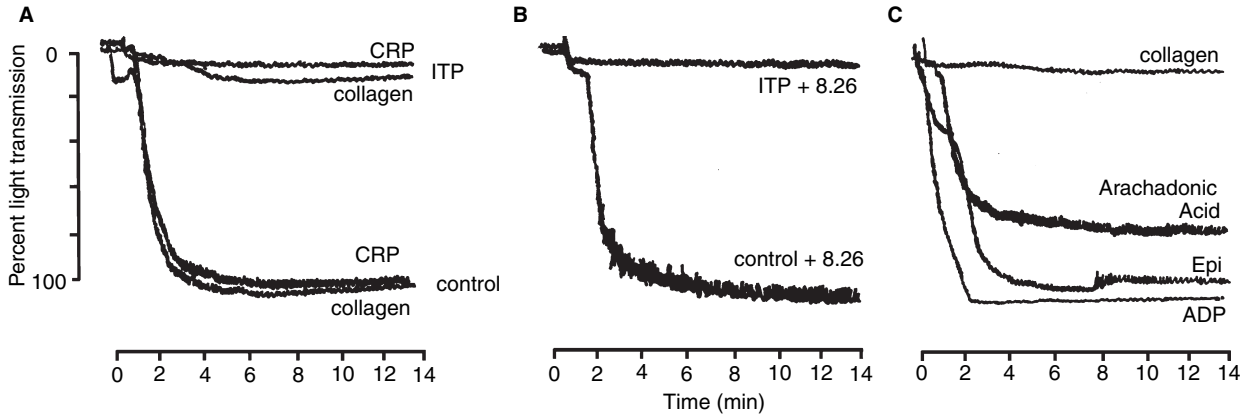

Fig. 1. An immune thrombocytopenic purpura (ITP) patient exhibited abnormal platelet aggregation in response to glycoprotein (GP) VI- and FcγRII-specific agonists. (A) Aggregation of citrated platelet-rich plasma (PRP) isolated from a healthy donor control or the patient diagnosed with ITP in response to 5 μg mL^{-1} collagen or 4.5 μg mL^{-1} collagen-related peptide (CRP). (B) Platelets isolated from the ITP patient do not aggregate in response to 2 μg mL^{-1} anti-FcγRII activating mAb 8.26. (C) Platelets from the ITP patient exhibited normal aggregation in response to arachadonic acid (1 mM), ADP (4 μM) and epinephrine (7 nM), but not collagen (1 μg mL^{-1}). Results are representative of three identical experiments performed with platelets isolated on three separate days.

collagen-epinephrine PFA-100 closure time of more than 300 s (normal, 75–145 s) and a collagen-ADP closure time of 105 s (normal 65–105 s). Prothrombin time, activated partial thromboplastin time and thrombin time were all within the normal ranges, as were plasma fibrinogen and VWF levels. Platelet-associated IgG was elevated and serum from the patient also contained a circulating autoantibody against GPVI detected by a mAb-specific immobilization of platelet antigen (MAIPA) [34] assay (data not shown).

Defective ITAM receptor-dependent platelet aggregation in patient platelets

At concentrations of agonist inducing robust aggregation of control PRP, aggregation of PRP isolated from the patient diagnosed with ITP was abnormal in response to GPVI agonists, collagen and CRP (Fig. 1A), and purified FcγRII-activating mAb, 8.26 (Fig. 1B). Responses to epinephrine, arachadonic acid and ADP were normal indicating normal functional expression of $\alpha_{IIb}\beta_3$ (Fig. 1C) and diminished aggregation was achieved with higher doses (>10 μg mL^{-1}) of collagen (data not shown). Aliquots of the patient's plasma rapidly induced aggregation of PRP isolated from a healthy donor and this aggregating activity could be blocked by inclusion of human recombinant GPVI encoding the two Ig domains of GPVI [29], or partially blocked (40% of maximal aggregation) by FcγRIIa-blocking antibody, IV.3 (Fig. 2). These results indicate that plasma containing the anti-GPVI autoantibody activates platelets directly through GPVI, with only partial dependence on FcγRIIa, as the anti-FcγRIIa antagonist IV.3 incompletely inhibited aggregating activity.

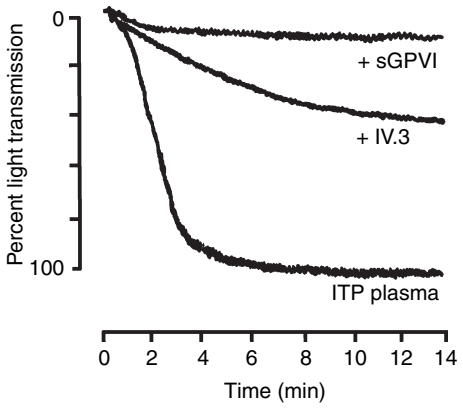

Fig. 2. Plasma isolated from the patient contains aggregating activity. Aggregation of citrated platelet-rich plasma (PRP) from a healthy donor treated with platelet-poor plasma from the patient with immune thrombocytopenic purpura (ITP). Inclusion of 80 μg mL^{-1} recombinant, purified glycoprotein (GP) VIex or 10 μg mL^{-1} FcγRIIa-blocking antibody IV.3 to PRP 5 min prior to commencement of aggregation interfered with the plasma aggregatory activity. Results are representative of three identical experiments performed with platelets isolated on three separate days.

Levels of GPVI are reduced on patient platelets

The patient's platelets were analyzed by flow cytometry to investigate whether defective GPVI-dependent platelet aggregation was as a result of deficiency of GPVI. While GPIbα was expressed within normal ranges on control and patient platelets [35], levels of GPVI on the ITP patient platelets were approximately 10–20% of levels on control platelets as detected using any of three anti-GPVI mAbs (Fig. 3).

A 55-kDa GPVI ectodomain fragment can be cleaved from platelets treated with GPVI agonists or FcγRIIa-activating antibodies, leaving an ~10-kDa remnant associated with the platelet membrane [7,14]. Further, activation of ITAM receptor signaling pathways initiated intracellular calpain-dependent cleavage of FcγRIIa, generating a ~30-kDa platelet-associated fragment containing the extracellular and transmembrane domains but lacking most of the cytoplasmic tail of FcγRIIa [14]. In washed, resting platelets from healthy donors, levels of full-length GPVI are relatively

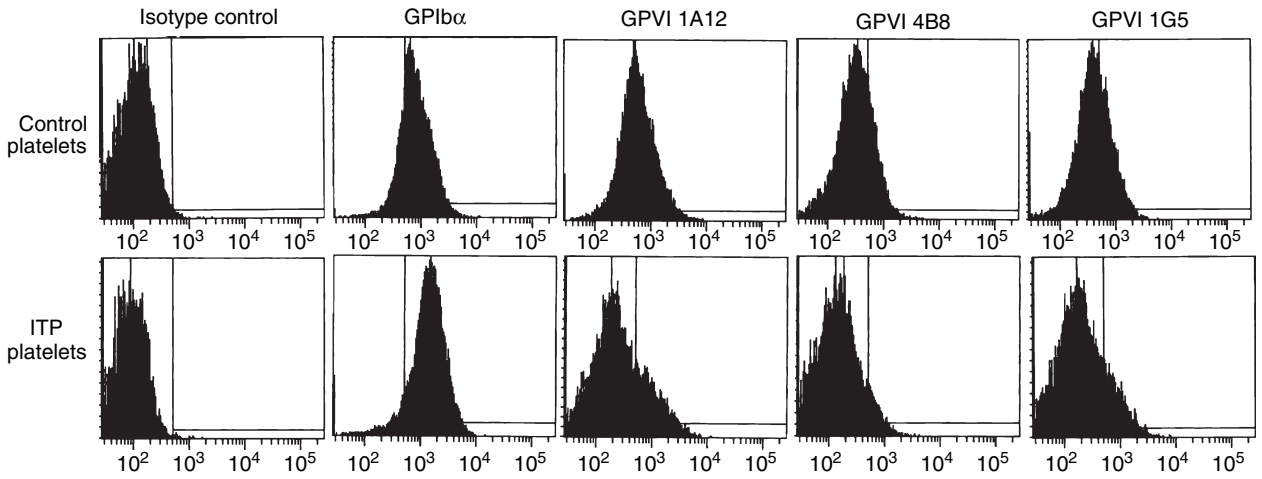

Fig. 3. Surface levels of glycoprotein (GP) VI on platelets from the patient were abnormal. Flow cytometry of resting washed platelets from a healthy donor (upper panel) or the immune thrombocytopenic purpura (ITP) patient (lower panel) using an isotype-matched control antibody or mAbs against GPIbα (AK2) or GPVI (1A12, 4B8 and 1G5) as described in Methods. Bound antibody was detected with a FITC-conjugated anti-mouse secondary antibody.

stable for up to 4 h and the 10-kDa remnant fragment of GPVI is below detectable levels [7–9]. Washed platelets isolated from the ITP patient or a healthy donor were lysed and separated by SDS-PAGE and levels of platelet receptors were examined by Western blot. Reduced levels of intact GPVI and FcγRIIa in patient platelet lysates, relative to levels found in healthy donor platelets, was confirmed by Western blotting with antibodies raised against the cytoplasmic tails of GPVI or FcγRIIa (Fig. 4) consistent with the observed lack of aggregation in response to GPVI- and FcγRIIa-dependent agonists (Fig. 1B) by patient platelets. The remnant 10-kDa membrane-associated fragment of GPVI was evident in lysed platelets from the ITP patient (Fig. 4), as was the 30-kDa FcγRIIa cleavage fragment [14] on prolonged exposure (data not shown), indicating that proteolytic shedding pathways had been activated in these platelets and that GPVI and FcγRIIa had both been partially cleaved. Proteolysis was specific for these ITAM receptors, as levels of full length and cleaved forms of GPIbα in ITP patients were similar to those seen in lysed platelets from healthy individuals, and within reported ranges for expression of GPIbα [35]. However, as GPIbα function was not specifically examined, we cannot exclude the possibility that levels of GPIbα also undergo some degree of modulation. These data suggest either that there was insufficient intact receptor to allow activating antibody-mediated cross-linking to occur or that intracellularly-cleaved FcγRIIa behaved as a dominant-negative receptor, preventing functional dimerization of FcγRIIa and activation of intracellular signaling events. Taken together, these data strongly suggest that circulating platelets from the ITP patient had been activated and GPVI had been shed from the surface. It is likely the GPVI receptor shedding and internal cleavage of FcγRIIa explains the persistent bleeding phenotype in this patient despite a normalized platelet count.

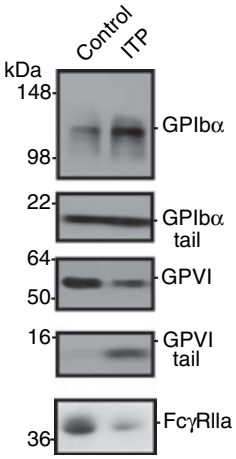

Fig. 4. Circulating platelets isolated from an immune thrombocytopenic purpura (ITP) patient show evidence of glycoprotein (GP) VI shedding and cleavage of FcγRIIa. Aliquots of washed platelets isolated from either a control or the ITP patient were lysed in sodium dodecylsulfate (SDS)-containing buffer and examined by SDS-polyacrylamide gel electrophoresis (PAGE) (10^7 platelets per lane) and Western blot using antibodies directed against the cytoplasmic tail of GPIbα, GPVI or FcγRIIa which detect both full-length and cleaved forms of each receptor. Bound antibody was detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody and enhanced chemiluminesence. All lanes within each figure came from the same experiment, and the same gel/Western blot. Data are representative of two identical experiments performed on separate days.

Purified Ig fraction from the serum of a patient with ITP induces FcγRIIa-dependent shedding of platelet GPVI and proteolysis of FcγRIIa

Patient anti-platelet antibodies potentially cause platelet activation either directly through binding to platelet surface antigens, or indirectly *via* Fc engagement of FcγRIIa. Mouse anti-human GPVI mAbs against different epitopes can simi-

larly activate platelets by FcγRIIa-dependent or independent mechanisms [36]. For example, the antibody, OM1, induces GPVI-dependent platelet aggregation without a requirement for FcγRIIa (that is, aggregation is not blocked by IV.3), presumably by receptor cross-linking as OM1 Fab fragments did not activate platelets. In contrast, another antibody, OM2, activated platelets in an FcγRIIa-dependent (IV.3 inhibitable) manner [36]. In previous reports, we demonstrated that Ig preparations isolated from serum of patients with HIT, who have circulating anti-platelet antibodies, also caused cleavage of GPVI and FcγRIIa [14]. Both of these cleavage events are likely to impair receptor function and this dysfunction may contribute to the platelet abnormalities observed in HIT. We examined whether the anti-GPVI autoantibody IgG isolated from serum prepared from ITP patient blood could also cause cleavage of ITAM-containing receptors on washed platelets from healthy donors. Platelets were incubated with Ig prepared from control or ITP patient serum and levels of GPVI and FcγRIIa in platelet lysates or the shed fragment of GPVI in supernatants were assessed by Western blot. GPVI (Fig. 5A) and FcγRIIa (Fig. 5C) levels in platelet lysates remained stable for up to 4 h in the presence or absence of IV.3 or control Ig. However, levels of both receptors were reduced in platelets incubated with ITP Ig. The cleavage of GPVI from the platelet surface and appearance of the 55-kDa GPVI fragment in the supernatant was blocked by inclusion of either IV.3 or EDTA (Fig. 5A,B) indicating that cleavage of GPVI was mediated by metalloproteinases. Similar to previous observations [14], cleavage of FcγRIIa was also blocked by inclusion of IV.3 (Fig. 5C, upper panel) or EDTA (Fig. 5C, lower panel). The level of potency of ITP total Ig after a 4-h incubation is less than observed with mAb VM58, a purified FcγRIIa-activating mAb; however, chronically-exposed patient platelets are non-responsive to FcγRIIa agonists and even partial proteolysis

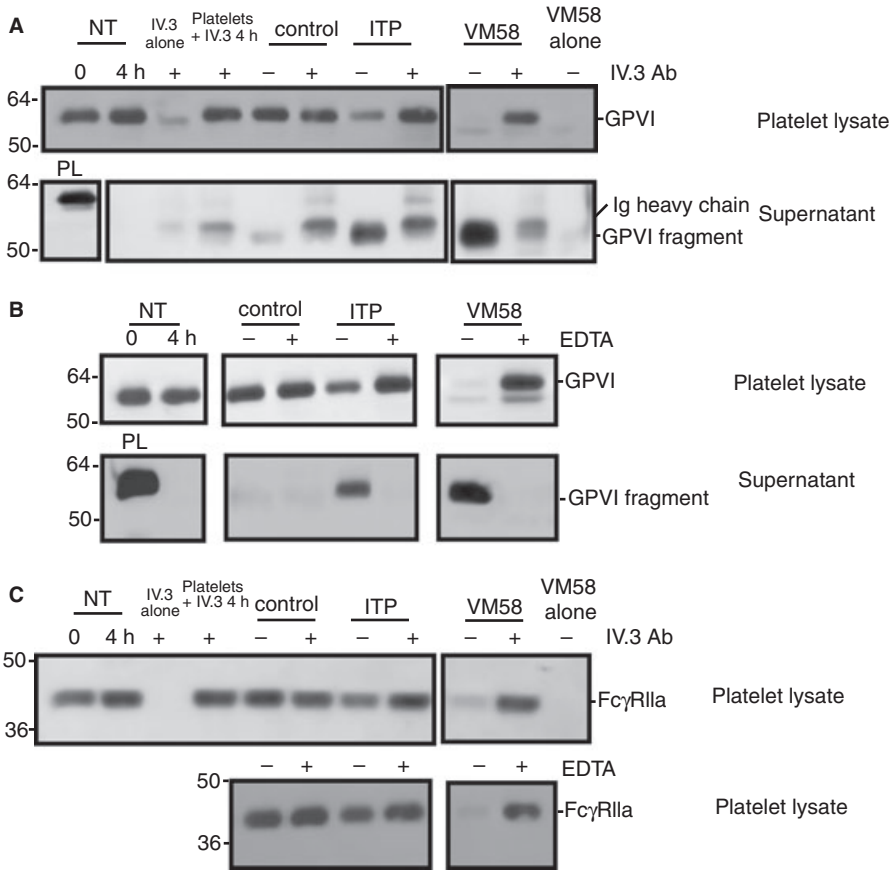

Fig. 5. Cleavage of either glycoprotein (GP) VI or FcγRIIa induced by Ig from an immune thrombocytopenic purpura (ITP) patient. Washed platelets were incubated with either 2 μg mL^{-1} VM58 for 1 h, or 100 μg mL^{-1} Ig prepared from control or ITP patient serum for 4 h. Some samples also contained 10 μg mL^{-1} blocking anti-FcγRIIa mAb, IV.3, or 10 mM EDTA. Equivalent amounts of VM58 and IV.3 alone, or mixed with platelets in the absence of agonist, were run in parallel lanes to distinguish bands corresponding to Ig heavy chains. Panels (A) indicate GPVI on platelets (upper panel) and GPVI fragment in supernatant (lower panel) in the presence or absence of IV.3; Western blot with 6B12 Panels (B) indicate GPVI on platelets (upper panel) and GPVI fragment in supernatant (lower panel) in the presence or absence of EDTA; western blot with 6B-12. (C) FcγRIIa levels on platelets were assessed by Western blotting with anti-FcγRIIa tail antiserum in the presence or absence of IV.3 (upper panel) or EDTA (lower panel). PL = platelet lysate, containing full-length GPVI for reference. NT, no treatment. Vertical lines have been inserted to indicate a repositioned gel lane. All lanes within each figure came from the same experiment, and the same gel/Western blot. Lanes marked NT, IV.3 alone, Platelets + IV.3 4h, control Ig, VM58 and VM58 alone have been previously published in a separate report examining cleavage of receptors by Ig isolated from two patients with heparin-induced thrombocytopenia (HIT) [14].

may impair function as discussed above. Taken together, these data imply that treatment of platelets with ITP Ig triggers FcγRIIa-dependent signaling pathways leading to activation of both GPVI and FcγRIIa proteolytic events. Under conditions of chronic exposure, loss of authentic GPVI and FcγRIIa can trigger ablation of receptor function (even if not all FcγRIIa is proteolysed).

Measurement of soluble GPVI in plasma by ELISA

Plasma soluble cell adhesion molecule levels may reflect cardiovascular and atherosclerotic disease [37,38] and directly impact inflammatory responses [39]. In these cases, soluble forms of immunoglobulin superfamily members, intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), as well as E-selectin and L-selectin, originate from a variety of cells including endothelial and smooth muscle cells, fibroblasts and leukocytes. On platelets, platelet (P)-selectin is a marker of platelet activation and soluble P-selectin in serum (either released from the platelet surface or exported as an alternatively-spliced soluble form) correlates with cardiovascular disease and is used clinically to identify people at risk of coronary heart disease [40]. Soluble GPVI has recently been affinity purified from plasma of normal individuals [24]. In order to quantitate soluble GPVI in plasma, we developed a sensitive sandwich ELISA, using an immobilized polyclonal anti-GPVI ectodomain antibody to capture soluble GPVI from diluted, ultracentrifuged plasma. Addition of known amounts of recombinant soluble GPVI to GPVI-depleted plasma or normal plasma indicated that the assay was additive and had a sensitivity of 0.5–500 ng mL^{-1} (E.E.G., *et al*., unpublished data). Using this assay, levels of (mean of triplicate samples ± SD) 148 ± 0.4 ng mL^{-1} soluble GPVI (~2.7 nM) were detected in plasma from the ITP patient. GPVI in plasma from six healthy individuals, including a control collected on the same day as the patient, was 15 ± 7 ng mL^{-1} (range 8–23 ng mL^{-1}, 0.14–0.42 nM) and consistent with levels of 0.018–0.114 nM in a preliminary report by others [41]. By comparison, ICAM-1, which has been shown to be shed from cell surfaces by ADAM17 under experimental conditions reflecting inflammation [42] increased 2-fold in plasma from patients with unstable angina over control individuals (373 ± 18 vs. 208 ± 13 ng mL^{-1}) [43,44]. The observed tenfold increase in soluble GPVI in ITP patient plasma together with the low levels of platelet GPVI by FACS and detection of the 10-kDa GPVI cleavage remnant is likely to reflect irregular platelet metalloproteinase activity and aberrant platelet activation in the patient with ITP; these observations are consistent with our previous studies indicating that activation through either GPVI or FcγRIIa leads to ITAM-dependent proteolysis of both receptors [14]. It remains to be determined how accurately soluble GPVI levels reflect platelet activity in plasma, but at least in patients with anti-GPVI antibody activity, it appears soluble GPVI levels in plasma reflect platelet ITAM receptor engagement and may well indicate ITAM receptor dysfunction. Whether GPVI shedding is a frequent characteristic in immune thrombocytopenic purpura, and whether soluble levels of GPVI in plasma can be used as a clinical marker of platelet activation represent new avenues of enquiry.

Addendum

E. E. Gardiner, M. Al-Tamimi, R. K. Andrews, M. C. Berndt and R. I. Baker. designed the research, analyzed data and co-wrote the manuscript. M. Moroi contributed vital reagents. E. E. Gardiner, M. Al-Tamimi, F.-T. Mu, D. Karunakaran and J. Y. Thom carried out the experiments.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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