Online data supplement

Flow studies on human GPVI-deficient blood under coagulating and non-coagulating conditions

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Supplementary materials and methods

Reagents

D-Phe-Pro-Arg chloromethyl ketone (PPACK), anti-Syk (sc-1240) monoclonal antibody (mAb), anti-PLCγ2 mAb (sc-5283), anti-GPIbα mAb (sc-59052) and anti-Rho A mAb (sc-418) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Collagen type I was from Nycomed Pharma (Munich, Germany). D-dimer was from HYPHEN BioMed (Neuville-sur-Oise, France). Von Willebrand factor (VWF), anti-P-LAT (Y200) (ab68139) mAb and anti-Btk antibody (ab189434) came from Abcam (Cambridge, UK). Rhodocytin was purified as described[1]. Recombinant tissue factor (rTF; Innovin) was purchased from Siemens (Erlanger, Germany). The antibody to the GPVI tail has been described[2]. Annexin-A5-Alexa Fluor-647 was from Invitrogen (Bleiswijk, The Netherlands). Other reagents including bovine serum albumin (BSA: fatty acid free, ≥96%), formalin, anti-α-tubulin mouse mAb (T6199), anti-FcRγ antibody (06-727), anti-P-Tyrosine mAb (4G10) (05-321) and anti-LAT antibody (06-807) were by Sigma-Aldrich (St. Louis, MO, USA). Anti-P-Syk (Y525/526) mAb (2710), anti-P-PLCγ2 (Y1217) antibody (3871) and GPIIb mAb (13807) were from Cell Signaling (Massachusetts, USA). Anti-CLEC-2 antibody (AF1718) were from R&D Systems (Minneapolis, USA). Z-Gly-Gly-Arg AMC.HCl substrate was from Bachem (Torrance, CA, USA). BigDye Terminator sequencing kit v3.1 and NuPAGETM 4-12% Bis-Tris gel were purchased from Thermo Fisher Scientific (Massachusetts, USA). mAb 6F1 was a kind gift from Barry Coller (Rockefeller, USA).

Mice

Experiments were performed in accordance with UK laws (Animal [Scientific Procedures] Act 1986) with approval of local ethics committee and UK Home Office approval under PPL P0E98D513. The following mice were used: the ITAM-deficient strains. The GPVI-null mice were the kind gift of Jerry Ware[3] and were bred as homozygous mice with wild type mice purchased. All mice were on a C57Bl/6 background.

Blood and platelet preparation

Washed platelets were obtained by centrifugation using prostacyclin (56 nM) and resuspended in Tyrode (137 mM NaCl, 0.36 mM Na_2HPO_4, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 0.05 g glucose, 2 mM $MgCl_2$; pH6.2). They were pelleted by centrifugation at 1500 g for 9 min and resuspended at $2x10^7$/mL for static adhesion or $4x10^8$/mL for aggregation and phosphorylation measurements.

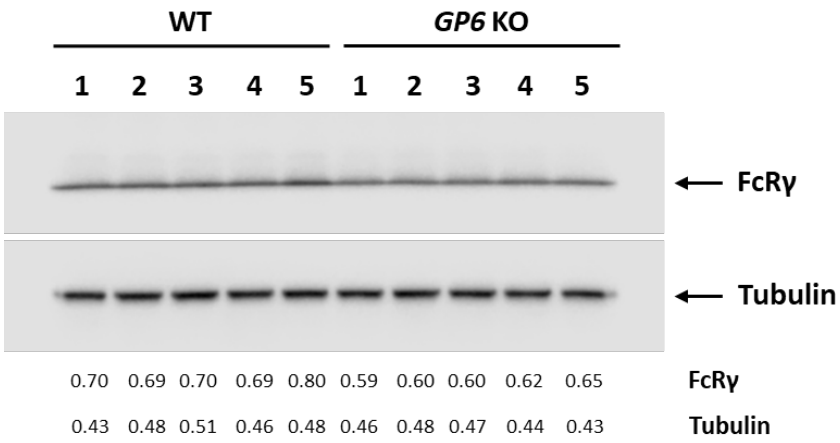
Western blotting

Immunoblotting was performed with rabbit anti-human GPVI cytoplasmic tail antibody (1µg/ml), anti-FcRγ (1:500), anti-Syk (1:200), anti-α-tubulin (1:1000), anti-phosphotyrosine (1:1000), anti-P-PLCγ2 (Y1217) (1:250), anti-P-LAT (Y200) (1:500), anti-P-Syk (Y525/526) (1:500), anti-GPIbα (1:500), anti-PLCγ2 (1:200), anti-Btk (1:500), anti-CLEC-2 (1:500), anti-LAT (1:500), anti-Rho A (1:200) and anti-GPIIb (1:500) overnight at 4°C.

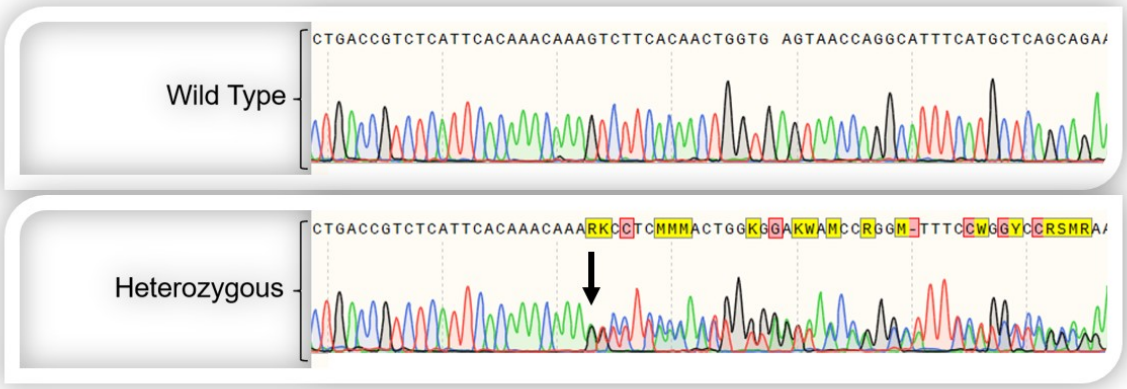
References

1. Hooley E, Papagrigoriou E, Navdaev A, et al. The crystal structure of the platelet activator aggretin reveals a novel (alphabeta)2 dimeric structure. *Biochemistry*. 2008;47(30):7831-7837.
2. Gardiner EE, Karunakaran D, Shen Y, Arthur JF, Andrews RK, Berndt MC. Controlled shedding of platelet glycoprotein (GP)VI and GPIb-IX-V by ADAM family metalloproteinases. *J Thromb Haemost*. 2007;5(7):1530-1537.
3. Kato K, Kanaji T, Russell S, et al. The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. *Blood*. 2003;102(5):1701-1707.

Supplementary figures



Supplementary Figure 1. Western blot of the FcR γ-chain (FcRγ) in five wild type and five *GP6* KO mouse platelets. The densitometry levels are shown. The same amount of platelet protein was loaded in each lane as shown by reprobing for tubulin.



Supplementary Figure 2. Representative gene sequence traces of a wild type sample and *GP6*^{het} sample. Overlapping sequences represent two separate alleles, starting at the insert site of the c.711_712insA mutation (arrowed).

Supplementary Table 1: Bleeding Assessment Tool (BAT) score of GPVI homozygous and heterozygous individuals.

A bleeding assessment tool (BAT) developed by one of the authors [1] was used to generate a score for *GP6*^{hom} individuals. The BAT score of six *GP6*^{het} individuals (5 female, 1 male) was between 0 – 2; one *GP6*^{het} individual had a BAT score of 5.5. The asterix illustrate the individuals who donated blood in this study.

	BAT score	Bleeding symptoms
***GP6*^{hom}**		
Female, age 33*	19	Bleeding after tooth extraction and surgery, heavy menstrual bleeding, abnormal bruising, superficial hematomas, hemoptysis (once).
Female, age 9*	10	Abnormal bruising.
Female, age 22	18	Heavy menstrual bleeding leading to acute anaemia, required multiple blood transfusions, abnormal bruising, epitaxis, excessive bleeding after minor injury.
Male, age 20*	6.5	Ecchymosis in unusual sites, prolonged bleeding after mild injuries, superficial hematomas.
Female, age 12*	1	Easy bruising; no surgery or menarche.
Female, age 22	6	Heavy menstrual bleeding, easy bruising, epistaxis.
Female, age 39	11	Heavy bruising, heavy menstrual bleeding, bleeding after minor injuries; no bleeding during cesarean section.
Male, age 10*	8	Epitaxis (3 cautaries), bruising in unusual sites, prolonged bleeding after mild injuries.

References

1. Quiroga T, Goycoolea M, Panes O et al. High prevalence of bleeders of unknown cause among patients with inherited mucocutaneous bleeding. A prospective study of 280 patients and 299 controls. *Haematologica* 2007; 92:357-365