Materials and Methods

The Institutional Animal Care and Use Committee of University of California San Francisco approved these studies.

Materials

Par3 and *Par4* knockout mice[1,2] and *Gp6* knockout mice[3] were bred at least 7 generations into a C57BL6 background. Tail bleeding studies were performed on littermate offspring from crosses capable of generating offspring of different genotypes before genotyping was performed, and thrombosis assays were performed blind to genotype. Anti-CD62 (P-selectin) (Wug.E9 clone) and anti-$\alpha_{IIb}\beta_3$ (JON/A clone) antibodies utilized in platelet activation studies were from Emfret analytics (Eibelstadt, Germany). The concentrations of ferric chloride ($FeCl_3$) solutions are specified by both molarity and % (weight per volume of anhydrous $FeCl_3$) for convenience in making comparisons across studies that utilize both nomenclatures.

Methods

Preparation of washed mouse platelets. Murine platelets were isolated as described previously[4]. Briefly, mice were anesthetized with ketamine (75 mg/kg), xylazine (15 mg/kg), and acepromazine (2.5 mg/kg) and blood from the inferior vena cava was withdrawn into a syringe containing 150 µL acid citrate dextrose (ACD) and mixed gently. Blood samples were further mixed with 500 µL PIPES (20 mM)-buffered saline (pH 6.5), and centrifuged at 100×g for 20 minutes at 37°C without braking. The platelet-rich supernatant was removed and added to 500 µl low pH platelet wash buffer [140 mM NaCl, 10 mM $NaHCO_3$, 2.5 mM KCl, 0.5 mM Na_2HPO_4, 1.0mM $MgCl_2$, 6.46 mM trisodium citrate, 0.1% weight/volume (w/v) dextrose, 0.35% w/v bovine serum albumin (BSA; Fraction V), pH 6.5] supplemented with 5 U/mL Apyrase (Grade III). Platelets were pelleted by centrifugation at 500×g for 15 minutes with no braking. The platelets were resuspended in 400 µL calcium-free Tyrode's HEPES (CFTH) buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 Na_2HPO_4, 1.0 mM $MgCl_2$, 10 mM HEPES, 0.9% w/v dextrose, 0.35% w/v BSA, pH 7.4).

Ex vivo platelet activation studies. Platelet activation was assessed by measuring agonist-induced P-selectin exposure and integrin $\alpha_{IIb}\beta_3$ activation using flow cytometry[4]. Briefly, 23 µL of washed platelets at 40,000 platelets/µL concentration suspended in CFTH recalcified with 2 mM $CaCl_2$ was used in assay. 1µL of Thrombin to a final concentration of 1pM to 1 µM together with 1µL each of FITC-conjugated anti-CD62 (P-selectin) antibody and PE-conjugated anti-$\alpha_{IIb}\beta_3$ antibody was added to platelets. 15 minutes later, samples were diluted by adding 400 µL PBS and platelet-bound antibody was measured using C6 Accuri flow cytometer. 20,000 events with forward scatter channel threshold of ≤ 32,000 were analyzed for each sample. Mean fluorescence intensity for each antibody was plotted as a function of agonist concentration and EC_{50} values were calculated from those dose-response curves. Platelets from individual mice

were prepared and analyzed separately. Results reflect an average of 3 or more mice per genotype.

In vivo tail bleeding assay. Hemostasis in mice was assessed by measuring the amount of blood loss after transection of the tail tip[4]. Briefly, 25-31 day-old mice were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture and maintained on heating pads. The tail was transected 2 mm from the tip and immediately immersed in 37 °C saline in a clear test tube for 10 minutes. Tails still bleeding at this time were cauterized and mice were allowed to recover from anesthesia. Blood cells collected in above test tubes were pelleted by centrifugation at 250 ×g for 15 min and the re-suspended in 3 mL erythrocyte lysis buffer (8.3 g/L NH4Cl, 1.0 g/L KHCO3, and 0.037 g/L EDTA in water). Blood loss was determined by measuring Optical Density at 575 nM using a SPECTRAmax190 (Molecular Devices) and is expressed as arbitrary units (AU). In a standard curve constructed by spiking collection tubes with a known volume of blood, OD varied linearly with the volume of blood added over the range of the assay. The maximum blood loss observed in the assay resulted in an OD of 3.8 AU corresponded to 215 µL of whole blood or about 15% of blood volume. Tail tips were used for genotyping.

In vivo carotid thrombosis assay. Carotid thrombosis studies were performed as described[4]. Briefly, 8- to 14-wk-old mice were anesthetized with ketamine (75 mg/kg), xylazine (15 mg/kg), and acepromazine (2.5 mg/kg). The left carotid artery was isolated and blood flow was continuously monitored using a perivascular flow probe (MA0.5PSB; Transonic Systems Inc.) connected to a TS420 flow meter (Transonic Systems Inc.) connected to an Adinstruments Powerlab 4/30 and Chart software. The surgical field was blotted dry and two 1 mm × 2 mm pieces of Whatman #3 filter paper soaked in 0.5 M (8%) or 1.25 M (20%) $FeCl_3$ were applied to the artery (one above and one below) for 3 min or 5 min respectively. Filter paper was then removed and the surgical field was rinsed twice with saline to remove remaining $FeCl_3$ solution. Flow was measured for the next 20 minutes. Time from removal of the $FeCl_3$ paper to stable occlusion (defined as no flow for > 2 minutes) was recorded for each artery. Results are expressed as percent of arteries with continued blood flow as a function of time. For the purpose of calculating median time to occlusion, arteries that did not occlude after application of 0.5 M (8%) or 1.25 M (20%) $FeCl_3$ injury were assigned occlusion times of 1200 seconds, the specified end of the protocol.

Statistical analysis. Data from carotid occlusion assay in Figures 3 and 5 were analyzed using log-rank test; the p-value for significance of follow-on individual comparisons was adjusted using Bonferroni's method. All other data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. The two group variables used were *Par3* and *Par4* genotype for the data in Figures 1 and 2, and *Par4* and *Gp6* genotype for the data in Figure 4. Figure 1 used a 2x2 group table (*Par3*+/+ and *Par3*-/- on one axis and *Par4*+/+ and *Par4*+/- on the other). Figure 2 used a 3x3 group table with *Par3*+/+, *Par3*+/-, and *Par3*-/- on one axis and *Par4*+/+, *Par4*+/-, and *Par4*-/- on the other. Figure 4 used a 3x3 group table with *Par4*+/+, *Par4*+/-, and *Par4*-/- on one axis and *Gp6*+/+, *Gp6*+/-, and *Gp6*-/- on the other.

* indicates P<0.05, ** indicates P<0.01, ***indicates P<0.001, ****indicates P<0.0001.

References

1. Sambrano GR, Weiss EJ, Zheng YW, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature*. 2001;413:74-78.

2. Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV Jr, Tam C, Coughlin SR. A dual thrombin receptor system for platelet activation. *Nature*. 1998;394:690-694.

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;102:1701-1707.

4. Cornelissen I, Palmer D, David T, Wilsbacher L, Concengco C, Conley P, Pandey A, Coughlin SR. Roles and interactions among protease-activated receptors and P2ry12 in hemostasis and thrombosis. *Proc Natl Acad Sci USA*. 2010;107:18605-18610.