SUPPLEMENTAL DATA

Shear-induced integrin signaling in platelet phosphatidylserine exposure, microvesicle release and coagulation

Short title: Gα_{13} and integrin signaling facilitates clotting

Aiming Pang[*1,6], Yujie Cui[*1,7], Yunfeng Chen[2,4,#], Ni Cheng[1], M. Keegan Delaney[1,5], Minyi Gu[5], Aleksandra Stojanovic-Terpo[1], Cheng Zhu[2,3,4] and Xiaoping Du[1]

[1]Department of Pharmacology, University of Illinois at Chicago. Chicago, IL 60612, [2]Woodruff School of Mechanical Engineering, [3]Coulter Department of Biomedical Engineering, and [4]Petit Institute for Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, GA 30332, [5]Dupage Medical Tehcnology, Inc. Willowbrook, IL 60527, [6]Hematopoietic Stem Cell Transplantation Center, Institute of Hematology and Blood Diseases Hospital, Peking Union Medical College and Chinese Academy of Medical Sciences, Tianjin, China 300020,[7]School of Medical Laboratory, Tianjin Medical University, Tianjin, China 300203

* Equal contribution first authors

[#]Current address: Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037, USA

Supplemental Methods

Preparation of mouse platelets

Eight- to 12-week-old mice were anesthetized with isofluorane, fresh blood was drawn from mouse inferior vena cava using ACD (85mM trisodium citrate, 83mM dextrose, and 21mM citric acid) as anticoagulant[1,2]. Blood from 5-6 mice of either genotype was pooled. After adding final concentrations of 1 U/mL apyrase and 0.1 μg/mL PGE1 to blood. Platelets were isolated and washed as described previously[3], and resuspended in modified Tyrode's buffer[4] (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 0.42 mM NaH_2PO_4, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4, containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$), and allowed to rest for at least 1 hour at room temperature before use.

Peptide micellar formulation.

As previously described[5], PEG2000-DSPE (Avanti Polar Lipids Inc. Alabaster, AL), L-α-phosphatidylcholine (egg PC, Type XI-E, Sigma-Aldrich, St. Louis, MO), and peptides were mixed in methanol, and allowed to form a dry thin film using a rotary evaporator (Buchi, Rotavapor R-215). The dry thin film was rehydrated to form micellar nanoparticles in 0.15M NaCl as previously described[5].

Preparation for Dual biomembrane force probe assay

Following an Institutional Review Board approved protocol at Georgia Tech, 8-10 µL of human blood was collected from finger prick and centrifuged to isolate RBCs. The RBCs were biotinylated by incubating with Biotin-PEG3500-NHS (JenKem) solution[6-8], and swollen by incubating with nystatin (Sigma-Aldrich)[6,8]. Glass beads (Distrilab Particle Technology) were thiolated and incubated with Streptavidin−Maleimide (SA-MAL) overnight, washed with and resuspended in phosphate buffer (27.6 g/L $NaH_2PO_4 \cdot H_2O$, 28.4 g/L Na_2HPO_4) to make SA beads. To make FN or Annexin V beads, the SA beads were further incubated with soluble biotinylated FN (a gift from Dr. Andres Garcia, Georgia Tech) or Annexin V (BD Biosciences, CA) for 3 hours, washed with and resuspended in phosphate buffer.

Bone marrow transplantation

As described in our previous publications[5,9], bone marrow stem cells were isolated from femur and tibias of 8-week-old Integrin $\beta_3^{-/-}$ mice using the MACS lineage cell depletion kit (Miltenyi Biotec, Germany). Stem cells were subsequently infected twice with concentrated lenti-virus containing shRNA or cDNA constructs to be transfected with wild-type or AAA mutant β_3 as described previously[5]. The recipient Integrin $\beta_3^{-/-}$ mice were irradiated for 5Gy. The transfected stem cells were retro-orbitally injected with 27 1/2G needle's syringe into recipient mice (1×10^6 cells per recipient mouse) 24 hours after irradiation. Retro-orbital injection provides similar haematopoietic reconstitution as compared to IV injection[10].

Flow cytometric analysis of platelet-derived MV.

Platelet MV release and PS exposure were analyzed by flow cytometry as described previously[11]. Washed human platelets with or without thrombin stimulation were subjected to shear. The platelets were then labeled with phycoerythrin (PE)-conjugated IgG control or anti-integrin α_{IIb} antibody and analyzed via flow cytometry to determine whether the MV were derived from platelets. The MV and platelets were distinguished according to their light scattering pattern as previously described[12,13].

Recalcification time

Human platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were isolated from blood anti-coagulated with 1/10 volume of 3.8% citrate[11]. Recalcification time of citrated PRP was the time from addition of 20 mM $CaCl_2$ to the formation of a fibrin clot, which was monitored either in a cone-and-plate rheometer (Thermo Scientific Haake) or a turbidometric lumi-aggregometer (Chrono-Log) as described previously[11]. For the cone-and-plate rheometer method, 150 µL of mice platelet suspension in human PRP was pipetted onto a 60 x 15mm polystyrene dish and 6000 s^{-1} shear was applied at 37˚C. Coagulation was indicated by a sharp increase in viscosity. The test was stopped for samples that failed to coagulate after 1 hour. For the turbidometric aggregometer method, PRP was used as the baseline reference (100% light transmittance). Clotting

was detected by turbidity change following addition of 20 mM $CaCl_2$ at 37˚C with stirring (1200 rpm).

Laser-induced mouse arteriolar thrombosis model and fluorescence intravital microscopy

As described previously[14], Male C57BL/6 mice (6–8 weeks old) were anesthetized with intraperitoneal injection of ketamine and xylazine, and placed on a thermo-controlled blanket (37˚C). The mouse cremaster muscle was exteriorized and superfused with thermo-controlled (37˚C) bicarbonate-buffered saline for the duration of experiments. After infusion of DyLight 649-labeled anti-mouse GPIbβ and Alexa Fluor (AF) 488–labeled monoclonal anti-fibrin antibody (clone 59D8, provided by Dr. Hartmut Weiler, The Blood Research Institute, Milwaukee, WI) into mice, platelet and fibrin accumulation around the site of injury was visualized following laser-induced injury to cremaster arterioles[15].

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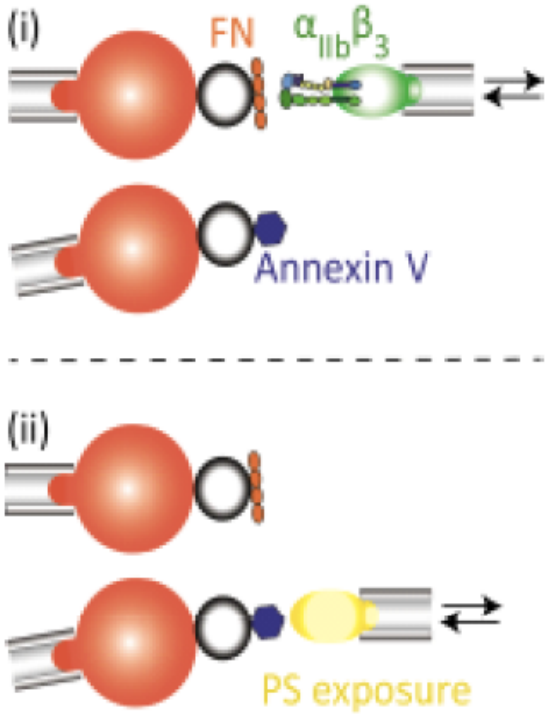
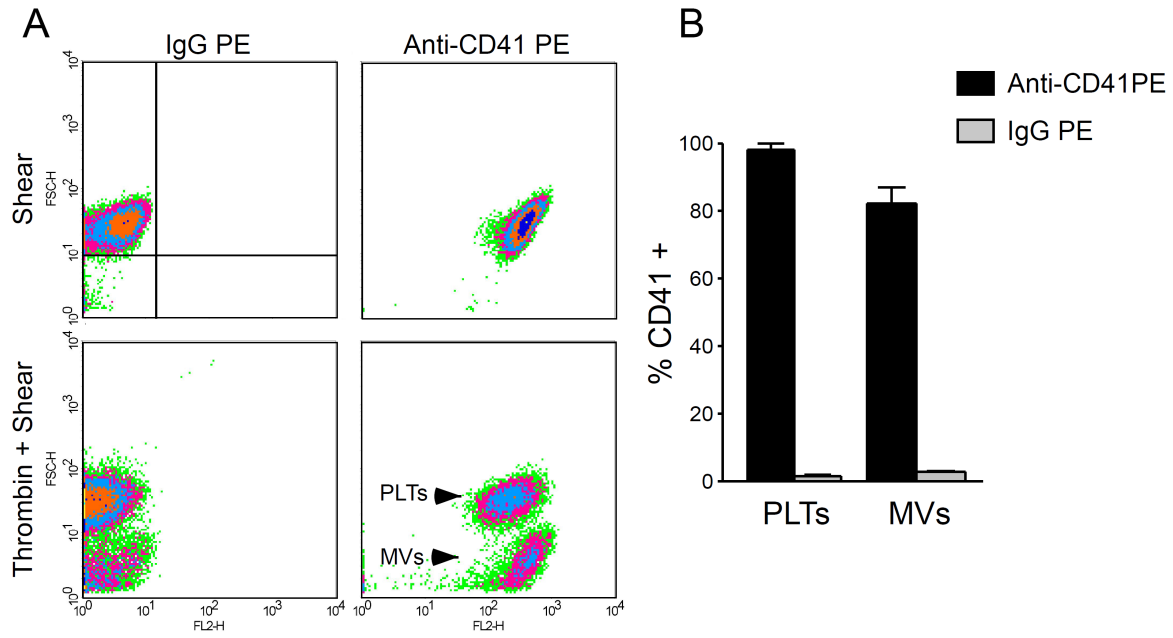
Supplemental Figures

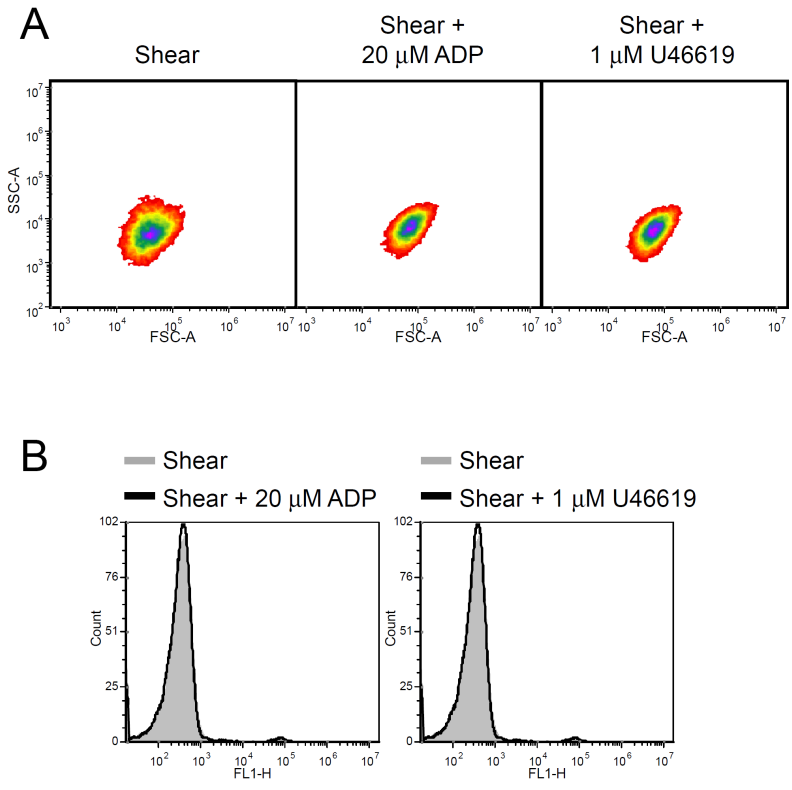
Supplemental Figure 1. An illustration of the biomembrane dual force probe (BFP) analysis of integrin- and outside-in signaling-dependent transduction of mechanical force leading to PS exposure.



Supplemental Figure 2. Staining platelets and platelet-derived MVs with a platelet-specific marker. Washed human platelets with or without thrombin stimulation were subjected to shear. The platelets were then labeled with phycoerythrin (PE) conjugated IgG control or anti- integrin α_{IIb} antibody and analyzed via flow cytometry for MV release and expression of α_{IIb}. (A) Cytograms showing size vs fluorescence. (B) Quantification of the percent of platelets or platelet-derived MVs that were positive for α_{IIb} staining.



Supplemental Figure 3. ADP or TXA2 alone does not induce PS exposure or MV release in the presence of shear. Washed mouse platelets were subjected to shear alone, or stimulated with 20 μM ADP or 1 μM U46619 and subjected to shear (3000/sec). The platelets were then labeled with Annexin V and analyzed via flow cytometry for MV release and PS exposure. (A) SSC vs FSC cytograms. (B) Fluorescence histograms of Annexin V binding.



Supplemental videos for Figure 6:

Supplemental video 1: Saline control

Supplemental video 2: Scambled peptide

Supplemental video 3: mP6

Supplemental videos for Figure 7:

Supplemental video 4: $G\alpha13^{fl/fl}$ control mice

Supplemental video 5: $G\alpha13^{fl/fl\text{-}PF4cre}$ (platelet specific $G\alpha13$ knockout mice)