METHODS

Reagents

Anti-factor XI antibodies 1A6, 14E11 and 10C9 were generated as described.[1–3] Plasmin and corn trypsin inhibitor (CTI) were from Enzyme Research Laboratories, Inc., hirudin from Hypen Biomed, Phe-Pro-Arg-chloromethylketone (PPACK) from Santa Cruz, fibrillar collagen from Chrono-Log Corp, lipidated tissue factor (TF; Innovin® PT reagent) from Siemens, and ellagic acid (aPTT reagent) from Pacific Haemostasis. Rabbit anti-fibrinogen antibody was from Cappel MP Biomedicals, LLC, anti-rabbit-AF350 from Life technologies, anti-CD41-PE and anti-CD62P-APC were from BD Pharmingen, CD31-eFluor450 was from eBioscience. Anti-thrombin API (H-85) antibody was from Santa Cruz Biotech and anti-integrin αIIb from Abnova. FXI- and FXII-depleted plasmas were from Haematalogic Technologies Inc. All other reagents were from Sigma-Aldrich, Inc. or previously named sources.[4]

Blood collection, preparation of plasmas and clotting times

Human venous blood was drawn by venipuncture from healthy adult volunteers into sodium citrate in accordance with the OHSU Institutional Review Board. Platelet-poor plasma (PPP) was prepared by centrifugation of citrated whole blood (in 0.32% w/v sodium citrate) from three separate donors at 2150×*g* for 10 min. Further centrifugation of the plasma fractions at 2150×*g* for 10 min yielded PPP, which was then pooled and stored at −80°C until use.

Clotting times of human whole blood or PPP were measured with a KC4 Coagulation Analyzer (Trinity Biotech PLC, Bray, Ireland). Samples were pretreated at room temperature (RT) for 3 min with blocking FXI antibodies 1A6 (50 μg/mL), 14E11 (50 μg/mL), 10C9 (50 μg/mL), FXII inhibitor CTI (40 μg/mL), or thrombin inhibitor hirudin (25 μg/mL) followed by incubation with activated partial thromboplastin time (aPTT) or prothrombin time (PT) reagent at 37°C, and clotting time was recorded. For aPTT assays, coagulation was initiated by the addition of Ca^{2+} (16.6 mM final). In select experiments, FXI- or FXII-depleted plasmas were used.

Ex vivo flow experiments

Glass capillary tubes/chambers (0.2×2×200 mm; VitroCom) were coated as described previously.[5] Surfaces were blocked with 5 mg/mL denatured bovine serum albumin (BSA) for 1 h prior to assembly into a flow system as shown in Fig.2A. Sodium citrate (0.38% w/v) anticoagulated whole blood was recalcified and perfused through the chamber for 10 min at an initial wall shear rate (300 or 1000 s^{-1}). Downstream samples were collected directly into 100 μM PPACK and 1.5% w/v sodium citrate (1 tube/min of perfusion) to final 50% dilution and evaluated using fluorescence-activated cell sorting (FACS).

Microscopy

After blood perfusion, glass capillaries were washed with phosphate-buffered saline (PBS) and Hepes/Tyrode buffer (136 mM NaCl, 2.7 mM KCl, 10 mM Hepes, 2 mM $MgCl_2$, 0.1% BSA; pH7.4) containing 1.5% sodium citrate and 100 μM PPACK, followed by incubation with blocking buffer (1% BSA, 1% FCS in Hepes/Tyrode buffer) for 30 min. Glass capillaries were incubated with rabbit anti-serum against human fibrinogen (1:100) for 10 min and washed with PBS followed by an incubation with CD41-PE (1:50), CD62-FITC (1:50), and goat anti-rabbit Alexa Fluor 350 (1:500) in dark for 10 min. Glass capillaries were washed with PBS, fixed with

paraformaldehyde (PFA 4%), and sealed with mounting media. Samples were analyzed on a Zeiss Axio Imager 2 microscope 6 (Carl Zeiss MicroImaging GmbH, Germany)

Western blots

Thrombi formed on collagen/TF were lysed for 5 min with 1× lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% NP-40, 2 mM PMSF and 10 U DNAse I) at 4°C, followed by treatment with 1 µM plasmin for 40 min at RT. Local fibrin deposition was evaluated by separating combined eluate samples on non-reducing SDS–PAGE gels, transferring to PVDF membrane and immunoblotting with rabbit anti-fibrinogen followed by anti-rabbit-HRP. Local platelet deposition and thrombin content was similarly evaluated by separating combined eluate samples on separate reducing SDS–PAGE gels and immunoblotting for CD41 or thrombin. Proteins were detected using ECL.

FACS analysis

Pre- and post-chamber blood samples were collected into 100 µM PPACK and 1.5% w/v Na-citrate (1:1, vol/vol) and incubated with 1:50 dilution antibodies for 30 min at RT. Reactions were fixed by diluting 1:10 with RT 12.5% Cytofix[BD]. 10,000 single platelets were determined by a PE-conjugated platelet marker CD41a and the characteristic forward and side-scatter scatter patterns via FACS (Canto II; BD Biosciences, Heidelberg, Germany). Platelet CD62P expression levels and single platelet consumption were determined as previously described.[6] Microaggregate formation was determined by the upshift in fluorescence intensity in CD31/CD41a double-positive events.

In vivo thrombosis model

Nonterminal studies were performed using male baboons (*Papio anubis*, 9-11 kg). All studies were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University. Thrombosis experiments were conducted as previously described.[7,8] Briefly, prosthetic vascular graft segments (4 mm ID × 20 mm, expanded-polytetrafluoroethylene; W. L. Gore & Associates, Flagstaff, AZ) coated with collagen were acutely introduced into chronic femoral arteriovenous shunts as shown in Fig.6A, and exposed to blood flow. In parallel, control experiments were performed with tubing alone. The flow rate through the graft was restricted to 100 mL/min (measured by the Transonics Systems flow meter, Ithaca, NY) by clamping the proximal shunt segment, thereby producing initial mean wall-shear rates of 265 s^{-1}. Local thrombus formation was assessed in real time with quantitative gamma camera imaging of radiolabeled platelet accumulation within the graft segment.

In vivo assessment of distal platelet consumption

Samples were collected distal to the graft from the bloodstream adjacent to the vessel wall (intraluminal boundary layer) into 3.8% w/v Na-citrate (1:9, vol/vol) before graft deployment (0 min), and then at 10 and 20 mins. Blood was collected at a rate of 100 µL/min during 10-min intervals through a 0.64-mm id port located 10 mm distal to the graft as shown in Fig.6A. To maintain patency of the sampling port, PPACK (1 mM), which inhibits thrombin and other coagulation proteases,[9] was infused at a rate of 20 µL/min into a second 0.64-mm id port located 3 mm proximal to and in line with the collection port. Anticoagulant infusion and local blood sampling were regulated using syringe pumps (Harvard Apparatus, Holliston, MA).

Platelet counts were determined using a micro-60 automated cell counter (Horiba ABX Diagnostics, Montpellier, France).

Data analysis

Data are shown as mean±SEM. Statistical significance of differences between means was determined by ANOVA. If means were shown to be significantly different, multiple comparisons were performed by the Tukey test. Probability values of $P < 0.05$ were selected to be statistically significant.

References:

1. Kravtsov DV, Matafonov A, Tucker EI, et al. Factor XI contributes to thrombin generation in the absence of factor XII. *Blood*. 2009;114(2):452-458.

2. Cheng Q, Tucker EI, Pine MS, et al. A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo. *Blood*. 2010;116(19):3981-3989.

3. Puy C, Tucker EI, Wong ZC, et al. Factor XII promotes blood coagulation independent of factor XI in the presence of long-chain polyphosphates. *J Thromb Haemost*. 2013;11(7):1341-1352.

4. White-Adams TC, Berny MA, Tucker EI, et al. Identification of coagulation factor XI as a ligand for platelet apolipoprotein E receptor 2 (ApoER2). *Arterioscler Thromb Vasc Biol*. 2009;29(10):1602-1607.

5. Baker SM, Phillips KG, McCarty OJT. Development of a Label-free Imaging Technique for the Quantification of Thrombus Formation. *Cell Mol Bioeng*. 2012;5(4):488-492.

6. McCarty OJT, Abulencia JP, Mousa SA, Konstantopoulos K. Evaluation of Platelet Antagonists in In Vitro Flow Models of Thrombosis. In: *Anticoagulants, Antiplatelets, and Thrombolytics*. Vol 93. New Jersey: Humana Press; 2003:21-34.

7. Hanson S, Griffin J, Harker L, Kelly A, Esmon C, Gruber A. Antithrombotic Effects of Thrombin-Induced Activation of Endogenous Protein-C in Primates. *J Clin Invest*. 1993;92(4):2003-2012.

8. Gruber A, Hanson SR. Factor XI-dependence of surface- and tissue factor-initiated thrombus propagation in primates. *Blood*. 2003;102(3):953-955.

9. Angliker H, Wikström P, Rauber P, Stone S, Shaw E. Synthesis and properties of peptidyl derivatives of arginylfluoromethanes. *Biochem J*. 1988;256(2):481-486.