SUPPLEMENTARY APPENDIX

Rate-limiting roles of the tenase complex of factors VIII and IX in platelet procoagulant activity and formation of platelet-fibrin thrombi under flow

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Methods

Animals

Experiments were approved by the local animal experimental and care committee. *F8*$^{-/-}$ and *F9*$^{-/-}$ mice and matched wildtype mice came from the Jackson Laboratory and Charles River, respectively. All mice were of C57Bl/6 background. Blood was obtained by aortic puncture under anesthesia (ketamine/xylazine; Eurovet) and collected into 1/10 volume of 129 mM trisodium citrate, as described.[1]

Human blood preparation

Blood from healthy volunteers, a patient with hemophilia A (4% FVIII) and a patient with hemophilia B (5% FIX) was taken after full informed consent, in accordance with the Declaration of Helsinki. Blood samples were collected by puncture of the median cubital vein into 1/10 volume of 129 mM trisodium citrate.[2] For both patients, other coagulation factors were in the normal range.

Labels and antibodies

Annexin A5 labeled with fluorescein isothiocyanate (FITC) or Alexa Fluor (AF)647 was from PharmaTarget; fibrinogen labeled with Oregon Green (OG)488, AF546 or AF647 came from Invitrogen. Fluorogenic thrombin substrate peptide, Z-Gly-Gly-Arg aminomethyl coumarin (Z-GGR-AMC), was from Bachem, and FXa substrate peptide Pefafluor from Pentapharm. Polyclonal AF647-labeled antibodies against human/mouse FVIII(a) (ABIN895941) or FIX(a) (ABIN1403082) and AF647-labeled irrelevant IgG (A21240) were obtained from Antibodies Online. FITC-labeled antibody against human/mouse von Willebrand factor (VWF) came from Affinity Biologicals (GAVWF-APFTC). Human FXa was purified and labeled (active-site with N^a-[(acetylthio)acetyl]-*D*-Phe-Pro-Arg chloromethyl ketone, followed by mild treatment with NH_2OH and reaction of the thiol generated with AF647-5-iodoacetamide, as described.[2, 3] It was confirmed that the active-site labeled factor lacked protease activity, but retained normal binding properties.

Whole blood flow chamber experiments

Flow experiments were performed with a parallel-plate transparent flow chamber (3 mm width, 50 µm depth), containing a coverslip coated with spots of type I Horm collagen (Nycomed Pharma), blocked with Hepes buffer pH 7.45 (137 mM NaCl, 5 mM Hepes, 2.7 mM KCl, 2 mM $MgCl_2$, and 0.1% glucose), containing 1% bovine serum albumin (BSA).[4] Samples of citrate-anticoagulated blood (400-1500 µL) were flowed at defined wall-shear rate, as described for mouse[5] and human[2] blood. Coagulation was introduced by co-perfusion of recalcification buffer using a dual syringe pump system (1:1 mouse: 110 mM NaCl, 13.3 mM $CaCl_2$, 6.7 mM $MgCl_2$, 0.1% glucose and 0.1% BSA; 1:10 human: 10 mM Hepes, 136 mM NaCl, 2.7 mM KCl, 6.3 mM $CaCl_2$, 3.15 mM $MgCl_2$, 0.1% glucose and 0.1% BSA). Where indicated, blood samples were supplemented with tissue factor (Innovin, 0.1-100 pM; Dade Behring), recombinant human FVIII (2 U/mL), recombinant human FIX (2 U/mL) or rivaroxaban (0.4 or 4 µg/mL, all f.c.). Fluorescent probes, OG488-fibrinogen or AF647-fibrinogen (16 µg/mL, f.c.), were added to the blood prior to perfusion. To detect phosphatidylserine exposure, thrombi on coverslips were post-stained with 0.5 µg/mL FITC-annexin A5. Coagulation factors were detected by post-staining with AF647-labeled antibodies against FVIII(a) or FIX(a) (1:20); control staining was performed with irrelevant AF647-labeled IgG at the same dilution. Where indicated, VWF was stained with FITC-labeled anti-VWF antibody (20 µg/mL), and FXa binding sites were identified with AF647-labeled FXa (16.5 µg/mL).

Brightfield phase-contrast and fluorescence images were captured with a non-confocal microscopic system.[4] Where indicated, differential interference contrast and confocal fluorescence images were taken with a Live7 Zeiss system, as described.[4] In double or triple labeling experiments, laser/filter settings were such that cross-channel fluorescence was essentially absent. Randomly captured images were analyzed for surface area coverage of thrombi and of fluorescent labels using Metamorph software (Molecular Devices).

Real-time measurement of thrombin and FXa generation in flow chambers

To measure serine protease activities, blood samples were preincubated with the fluorogenic thrombin substrate Z-GGR-AMC or FXa substrate Pefafluor, each 0.5 mM. Thrombi were generated as above on a collagen surface (4 minutes perfusion at 1000 s^{-1}), after which fluorescence accumulation from cleaved substrate was recorded under stasis, by capturing microscopic images every 30 seconds.[2] Regions of interest with/out thrombi images were analyzed for fluorescence intensity changes with ImageJ software (open source).

Statistical analysis

Significance of difference between groups was determined with the independent samples t-test, using the statistical package for social sciences (SPSS 11.0). Data are expressed as mean ± SEM. Statistical significance was set at $P < 0.05$.

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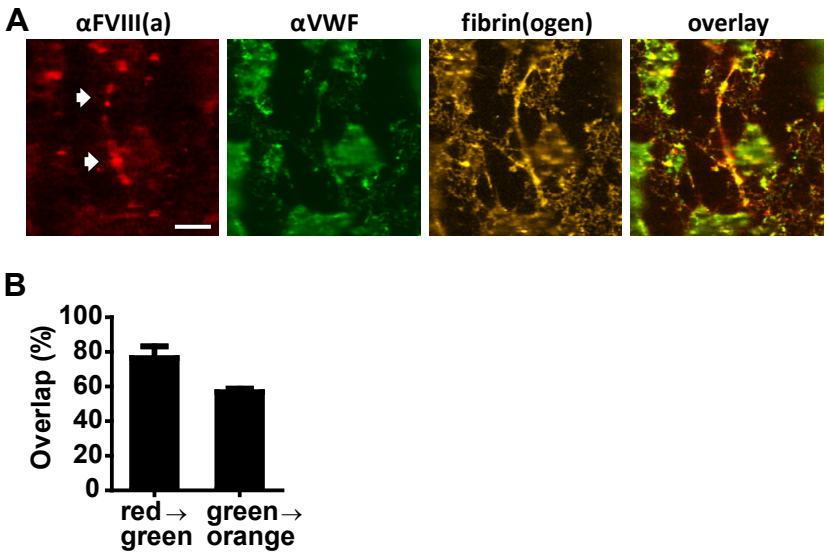

Supplemental Figure 1. Localization of FVIII(a), VWF and fibrin(ogen) on murine platelet-fibrin thrombi. Blood from wildtype mice was perfused over collagen at 1000 s^{-1} under coagulant conditions, as for Figure 1. Thrombi were post-stained with AF647-labeled antibody against FVIII(a) (red), FITC-labeled antibody against VWF (green), and AF546-labeled fibrinogen (orange), as indicated. (A) Representative confocal fluorescence images with overlay (arrow = collagen, bars 20 μm). (B) % Overlap analysis of red (α-FVIII) → green (α-VWF), and green (α-VWF) → orange [fibrin(ogen] fluorescence (pixels thresholded above background). Mean ± SEM; n = 3.