Proline-rich tyrosine kinase Pyk2 regulates deep vein thrombosis

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SUPPLEMENTARY APPENDIX

METHODS

DVT analysis.

Partial inferior vena cava (IVC) ligation was performed as previously described.[1] Briefly, mice were anesthetized with xylazine (5mg/kg) and ketamine (50mg/kg). After laparotomy, intestines were exteriorized, and warm sterile saline (37°C) was applied during the whole procedure to prevent drying. After gentle separation from the aorta, IVC was ligated by a 7.0 polypropylene suture immediately below the renal veins (toward the tail) over a 30-gauge needle placed outside the vessel so that piercing or any other injury to the IVC wall was completely avoided and then the needle was removed to obtain a partial flow restriction. The side and back branches of IVC were ligated. After surgery, peritoneum and skin were closed by monofilament absorbable suture and 6.0 silk, respectively. Mice were euthanized after 24 or 48 hours, and thrombi formed in the IVC distally to the suture were collected for analysis. Thrombus weight and thrombus length were measured. A total of 17 WT and 31 Pyk2-KO mice were used for these experiments.

For cross-transfusion experiments, platelets were isolated by centrifugation at 150g for 10 minutes from whole blood of donor mice anticoagulated with acid citrate dextrose (1:6, vol/vol). The obtained PRP was pooled and infused in recipient mice that had been previously rendered deeply thrombocytopenic by one single i.v. injection of a saturating amount (100µl) of an anti-mouse anti-platelet serum (APAS), prepared as previously described[2], that induce platelet count decrease by ≥95% within 2 minutes. APAS was injected one hour before platelet transfusion. Approximately 1.0×10^9 platelets were reinfused into each animal, leading to a restoration of the platelet count to about 80% of basal.[2] Deep venous thrombosis induced by partial ligation of vena cava was performed 1 h after platelet transfusion as above described. Platelets obtained from Pyk2-KO mice were reinfused in recipient thrombocytopenic C57BL6 mice and, vice versa, platelets taken from C57BL6 mice were reinfused in thrombocytopenic Pyk2-KO mice. A total of 4 mice/group were used.

Flow cytometry.

HUVECs were cultured in M199 medium supplemented with 20% FBS, 50µg/ml endothelial cell growth supplement, 100µg/ml streptomycin/penicillin, 100µg/ml heparin, 2mM l-glutamine until 90–100% confluence as described,[3] and used from passage II to V. Serum starved cells were preincubated with 10µM PF-4594755 for 30 minutes and stimulated with 1ng/ml IL-1β for 4 hours, detached by mild trypsinization, centrifuged for 5 minutes at 200g, resuspended in PBS, and incubated with PE-conjugated anti-VCAM-1 mAb, FITC-conjugated anti-P-selectin mAb or anti-TF mAb combined with FITC anti-mouse IgG for 30

minutes. Samples were analyzed by flow cytometry using a Cytoflex Instrument, Beckman Coulter.[4] THP-1 cells, a human monocytic cell line,[5] were grown in RPMI medium supplemented with 10% FBS, 2mM L-glutamine, 100µg/ml streptomycin/penicillin. 1×10^6 THP-1 cells were pre-treated with 10µM PF-4594755 for 30 minutes at 37°C. Samples were stimulated with 10µg/ml LPS for 3 hours in a humidified 5% CO_2 atmosphere at 37°C, followed by incubation for 30 minutes in the dark with anti-TF mAb combined with FITC anti-mouse IgG, and then analyzed by flow cytometry. For the analysis of platelet-leukocyte aggregates, 50µL of whole blood in 4% sodium citrate were stimulated with 0.1U/ml thrombin for 10 minutes, fixed with 1% PFA and incubated for 30 minutes with FITC-labeled anti-CD41/CD61 and PerCP-conjugated anti-mouse CD45.[6] For leukocytes subtype identification, anti-Ly6G or anti-CD115 antibodies were used. Leukocytes were identified based on their morphology and positivity for CD45. Platelet-leukocyte aggregates were quantified as percent of leukocytes positive for the platelet antigen CD41/CD61 on the leukocyte surface.[2] Phosphatidylserine (PS) exposure on platelet surface was assessed by measuring Annexin V binding using a commercial kit. Mouse PRP was stimulated for 10 minutes with 0.5U/mL thrombin and 250ng/mL convulxin or with 1U/mL thrombin and 500ng/mL convulxin and stained for Annexin V, according to the manufacturer's instructions

Real Time PCR.

Total RNA was isolated using 1mL TRIzol® Reagent (Ambion by Life technology), from HUVEC resting, stimulated with 1ng/ml IL-1β in the presence or absence of 10µM Pyk2 inhibitor PF-4594755. RNA was quantified and 1µg of total RNA served as template for single-strand cDNA synthesis. Reverse transcription was performed using iScript Reverse Transcription Supermix. Primers for Tissue Factor (TF¬_For: AACACCATGGCACCTTTTGC; TF_Rev: GTAACCAGGTCGTCCAAGCA) were added to 4.5µl of cDNAs and the RT-PCR reaction was run by adding PowerUpTM SYBERTM Green Master Mix (Applied Biosystem by ThermoFisher Scentific). Results, normalized to GAPDH and reported as relative expression, were calculated by the Agilent aria software (Agilent).

Neutrophil isolation and NET formation.

Neutrophils were isolated essentially as described.[7] Mice were euthanized, and bone marrow cells were flushed in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin. The cell suspension was filtered with 70µm cell strainer and then centrifuged (250×g for 7 minutes). The pellet was resuspended in hypotonic buffer for erythrocytes lysis, and then centrifuged again at 250×g for 7 minutes. Cells were resuspended in 1mL Ca^{2+}-Mg^{2+}-free HBSS and centrifuged on 62.5% Percoll for 30 minutes at 1000×g at room temperature. Typically, $1-2 \times 10^6$ neutrophils were harvested per mouse. Neutrophils (1×10^5 cells) were left untreated or stimulated by addition of 1×10^8 platelets pre-activated with 0.1U/ml thrombin for 30 minutes, before being plated onto glass coverslips in 12-well plates. The plates were

citospun, and adherent cells were fixed with 2% paraformaldehyde and permeabilized with ice-cold 0.25% Triton-X-100. For fluorescent labeling of NETs, cells were stained with Hoechst 33342 (1:1000 dilution).

VWF secretion measurement.

VWF:Ag was measured in HUVECs supernatant by Asserachrom VWF:Ag Kit was from Diagnostica Stago. HUVEC suspended in serum-free medium were seeded in 24-well plates at a concentration of 1.5×10^5 cells/well, preincubated with 10 µM PF-4594755 for 30 minutes and then stimulated with 100µM histamine for 4 hours in a humidified atmosphere with 5% CO_2 at 37°C. After incubation the supernatant was collected, centrifuged at 10,000xg for 3 minutes and frozen at - 80°C for later assay.

Platelet preparation and adhesion assay.

Murine platelets were prepared from blood collected from abdominal vena cava and anticoagulated with ACD/3.8% Na-citrate (2:1), as described.[8] Washed platelets were finally resuspended in HEPES buffer (10mM HEPES, 137mM NaCl, 2.9mM KCl, 12mM $NaHCO_3$, 5.5mM glucose pH 7.4) at a concentration of 3×10^8 cells/ml. Evaluation of platelet adhesion and spreading was performed using a fluorescence microscopy-based method, upon plating 3×10^7 platelets treated with 0.5mg/ml botrocetin onto glass coverslips coated with 10µg/ml VWF in PBS. After 60 minutes, non-adherent cells were removed. Adherent platelets were fixed, permeabilized, stained by TRITC-conjugated phalloidin and analyzed under a fluorescence microscope, Olympus BX51. The number of adherent cells was determined using the Image J software. For each experiment, five different fields were analyzed by two independent observers.

Neutrophil and platelet rolling over HUVECs.

Confluent HUVECs (>80%) were let to adhere overnight in complete medium on a channel slide µ-Slide VI (IBIDI GMBH) (37°C, 5% CO2, EGM-2 + 10% FBS) and were stimulated with IL-1β (1ng/ml, 20 minutes, 37°C). Isolated neutrophils (2×10^6/ml) in RPMI were perfused for 10 minutes (shear rate 200 sec^{-1}) and images of neutrophils rolling over activated HUVECs (identified as bright spots by contrast phase microscopy) were acquired (20X) live, 1 frame per second (FPS) using a Zeiss Observer A1 microscope equipped with a Axiocam MRm digital camera, and analyzed off-line. Washed murine platelets were loaded with the fluorescent vital probe CellTracker™ Red CMTPX Dye (1µM 30 minutes, 37°C in the dark) and whole blood was then reconstituted by mixing loaded platelets with the pellet resulting from the washing procedure to a hematocrit of 45% and stored in the dark until use. Platelets, in reconstituted blood, were then perfused (shear rate 250sec^{-1}) over confluent HUVECs stimulated with IL-1β as above described. Images of platelet adhesion was acquired (20X) live (1FPS) for 5 minutes and analyzed off-line with ImageJ.

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Statistical analysis.

The reported figures are representative of at least 4 different experiments. Statistical analysis was performed using Prism Version 8 software. Categorical data were analyzed with the Fisher exact test (DVT incidence), and continuous data were compared with unpaired t-test or with one-way ANOVA multiple comparison, with Sidak's post-test as appropriate. Neutrophil and platelet adhesion data were analyzed with non-linear regression (Boltzmann sigmoidal least square fit) and maximum surface coverage and EC_{50} were calculated (GraphPad Prism v 8.2) Data are expressed as mean ± SD.

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