MATERIALS AND METHODS

Materials

Paraformaldehyde (PFA) and glutaraldehyde were from Electron Microscopy Sciences (Hatfield, PA). Human VWF was isolated from factor VIII concentrates (EFS-Alsace, Strasbourg, France) according to a previously described method.\(^1\) Botrocetin was purified from snake venom (Latoxan, Valence, France) as described previously.\(^2\) Acid citrate dextrose (ACD) solution was from Bioluz (St-Jean-de-Luz, France) and hirudin from Transgene (Illkirch-Graffenstaden, France). The monoclonal antibody RAM.1 was produced in our laboratory\(^3\) and the rat IgG isotype control was obtained from Becton Dickinson (Le Pont de Claix, France). The anti-GPIIbα antibody, Xia.B2, was from Emfret Analytics (Eibelstadt, Germany). Acid-soluble type I fibrillar collagen (ASC) solution was prepared from bovine Achilles tendon as previously described.\(^4\) Fatty acid-free human serum albumin (HSA), TRITC-phalloidin, fibrinogen, bovine serum albumin (BSA), U46619, adenosine 5'-diphosphate (ADP) and bovine thrombin were from Sigma-Aldrich (Lyon, France). The GPIIb-IIIa antagonist eptifibatide (Integrilin\(^\text{®}\)) was obtained from Millennium Pharma (San Francisco, CA). 3,3'-dihexyloxacarbocyanine iodide (DIOC\(_6\)), Oregon Green 488 BAPTA-AM-1 and Calcein red-orange-AM were from Molecular Probes (Eugene, OR). Xylasine (Rompun\(^\text{®}\)) and ketamine (Imalgen\(^\text{®}\)) were provided by Bayer (Puteaux, France) and Merial (Lyon, France), respectively. Calibrated automated thrombin (CAT) reagents were from STAGO (Asnières, France) and the fluorogenic substrate Z-GGR-AMC was from Bachem (Bubendorf, Switzerland). Fluoresceinisothiocyanate (FITC)—conjugated annexin V was from Roche Diagnostics (Meylan, France) and recombinant human tissue factor (Innovin) was from Dade Behring. Cross-linked collagen-related peptide (CRP) was obtained from Dr. R.W. Farndale (University of Cambridge, Cambridge, UK). Ca\(^{2+}\) ionophore A23187 was from Calbiochem (La Jolla, California). CHO cells transfected with human GPIb-IX complex were kindly provided by Dr J. Lopez (Houston, TX).
**Preparation of F(\text{ab}')_2 fragments**

F(\text{ab}')_2 fragments were prepared as described with minor modifications.\textsuperscript{5} Monoclonal antibody (MoAb) RAM.1 was dialyzed overnight against a 0.1 mol/L sodium acetate buffer (pH 4). The antibody (2.4 mg/mL) was digested by incubation with pepsin (Sigma-Aldrich (Lyon, France); 1 part pepsin to 50 parts MoAb) for 2.5 hours at 37°C. Digestion was stopped by adding 1 volume of a 2 mol/L Tris buffer (pH 9). Digested IgG were dialysed against 20 mmol/L Tris buffer (pH8) and F(\text{ab}')_2 fragments were further purified on a monoQ column.

**Platelet count**

Whole blood was collected into EDTA (6 mmol/L) after severing the tail of an anesthetized mouse. The platelet count was determined in a Scil Vet ABC automatic cell counter (Scil Animal Care Company, Holtzheim, France) set to murine parameters.

**Static adhesion assay**

Glass coverslips were coated for 2 h with human VWF (10 µg/mL) and the surface was blocked with PBS-1% HSA for 1 h. Adhesion of CHO cells transfected with the GPIb-IX complex to a VWF surface was studied as previously described.\textsuperscript{6} Briefly, 10\textsuperscript{5} cells were allowed to adhere to VWF in the presence of botrocetin (1 µg/mL) and EDTA (5 mmol/L) at 37°C for 30 min. After a washing step, the adherent cells were fixed with 4% PFA, stained with TRITC-phalloidin (2 µg/mL) and viewed by epifluorescence microscopy. The number of adherent cells and the percentage of cells extending at least one filopod were quantified. Adhesion of platelets to VWF was investigated as described elsewhere.\textsuperscript{7} Briefly, mouse platelets in Tyrode's buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO\textsubscript{3}, 0.4 mmol/L NaH\textsubscript{2}PO\textsubscript{4}, 1 mmol/L MgCl\textsubscript{2}, 2 mmol/L CaCl\textsubscript{2}, 5 mmol/L Hepes, 3.5 mg/mL HSA, 5.5
mmol/L glucose, pH 7.3), preincubated for 15 min with RAM.1 or an isotype control, were allowed to adhere to VWF (9 x 10^6 cells/cover slip) in the presence of botrocetin (1 μg/mL) and Integrilin (40 μg/mL) at 37°C. After 15 min, non-adherent platelets were removed by washing and adherent platelets were fixed with 4% PFA, stained with TRITC-phalloidin (2 μg/mL) and viewed by epifluorescence microscopy. The number of adherent platelets and of those extending filopodia were determined. Alternatively, the adherent platelets were fixed with 2.5% glutaraldehyde and processed for scanning electron microscopy as previously described.\(^7\)

**Measurement of cytosolic Ca\(^{2+}\) concentrations**

Intracellular Ca\(^{2+}\) concentrations were monitored using a quantitative dual-dye ratiometric assay as previously described.\(^8\) Briefly, mouse platelets resuspended at 5 x 10^8/mL after a first washing step were simultaneously loaded with the membrane-permeating non ratiometric Ca\(^{2+}\) indicator dye Oregon Green 488 BAPTA-AM-1 (5 μmol/L) and the morphological dye Calcein red-orange-AM (4 μmol/L) for 30 min at 37°C. The dye-loaded platelets were washed a second time and finally resuspended in Tyrode’s buffer containing apyrase (0.02 U/mL). The increases in platelet and Ca\(^{2+}\)-dependent fluorescence intensity upon adhesion of the cells were measured in the ranges 572-700 nm and 495-535 nm, respectively, by confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany) (1.85 frames/s for 10 min). The ratio of the signal intensities in the two dye channels was converted into intracellular Ca\(^{2+}\) concentrations (Leica TCS SP5 LASAF software).

**Measurement of annexin V binding**

Measurement of annexin V binding was performed as previously described.\(^9\) Briefly, washed mouse platelets in Tyrode’s buffer were activated with CRP (10 μg/mL) or thrombin (1 U/mL) alone or together, or with A23187 (0.5 mmol/L) for 10 min at 37°C. A 2 μL aliquot of activated or resting platelets was then incubated with FITC-conjugated annexin V for 20 min and
analyzed by flow cytometry. A forward scatter/FL1 dot plot was performed to determine the ratio of activated to non-activated platelets. Activated annexin V-positive platelets were quantified as the percentage of total platelets.

**Calibrated automated thrombin generation analyses**

Calibrated automated thrombin (CAT) analyses were performed by the thrombogram method as previously described. Briefly, 20 μL of tissue factor (0.5 pmol/L Innovin 1/12 000 final dilution) or collagen (30 µg/mL) were added in triplicate to 80 μL of citrated (3.15%) mouse platelet-rich plasma (PRP) (1.3 × 10^8 platelets/mL final concentration) in a 96-well plate (Immuron 2 Dynex; Stago, Paris, France) maintained at 37°C. The accumulation of fluorescence from cleaved thrombin substrate: Z-GGR-AMC (41 μmol/L) in the presence of CaCl₂ (1.7 mmol/L) was measured continuously at excitation and emission wavelengths of 390 and 460 nm, respectively (Fluoroskan Ascent; ThermoLab Systems, Helsinki, Finland). The area under the curve, also called the endogenous thrombin potential (ETP), corresponds to the total amount of thrombin formed in the sample. Peak corresponds to the maximal concentration of thrombin generated.

**Measurement of the thrombus volume**

Rectangular glass microcapillaries (VitroCom, Mountain Lakes, NJ) were coated with type I collagen (2.5 mg/mL) overnight at 4°C and blocked with PBS-1% HSA for 30 min at room temperature. Hirudinated (100 U/mL) mouse whole blood labeled with DIOC₆ (1 μmol/L) was perfused through the collagen-coated channels at 37°C and various shear rates using a programmable syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA). To determine the thrombus volume, the fluorescence emitted in the range 490-595 nm after excitation with a 488 nm argon-ion laser was measured using a confocal Leica SP5 inverted microscope with a resonant scanner and a 40x oil objective. Series of optical sections in xyz were recorded from the base to the peak of the thrombi at designated time points. The images were then stacked and analyzed with ImageJ software (National Institute of Health,
Bethesda, MD) to determine the volume of the thrombi. 3D reconstructed images were obtained using Image surfer (Center for Computer-Integrated Systems for Microscopy and Manipulation, Chapel Hill, NC).

**Mice**

C57BL/6 WT mice were obtained from Charles River (L'Arbresle, France) and all mice were maintained in the animal facilities of the Etablissement Français du Sang-Alsace. We used 8-week-old male mice. All procedures for animal experiments were carried out in accordance with the Guide for Care and Use of Laboratory Animals as defined by European laws.

**Tail bleeding time**

The tail bleeding time was measured as previously reported. The time required for the arrest of bleeding and the volume of blood lost were determined over a 30 min period.

**Arterial thrombosis models**

Mice were anesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) and injected with a fluorescent dye (DIOC₆, 5 µL of a 100 µM solution/g of body weight) to label platelets and allow visualization of a thrombus. Mesenteric arterioles were injured with a laser and the resultant thrombus growth was monitored as previously described. Alternatively, thrombosis was induced mechanically by compression of the abdominal aorta with forceps for 60 s. Thrombus formation was then monitored in real time with a fluorescence microscope coupled to a charge-coupled device camera (Roper Scientific, Evry, France) and the images were analyzed with the Metamorph software (Molecular Devices, Roper Scientific). The mice were maintained and the experiments were performed in the animal facilities of the EFS-Alsace.
Statistics

All values are reported as the mean ± standard error of the mean (SEM) unless otherwise indicated. The data of Figures 1, 2, 3, 4, S1 and S2 were analysed with a two-tailed paired t-test. The data of Figures 5 and 6 were analysed with a Grubbs’ test followed with a two-tailed unpaired t-test. Differences were considered to be significant for p<0.05. All statistical tests were carried out using Prism software (GraphPad, La Jolla, CA).
REFERENCES


