SUPPLEMENT MATERIAL

Detailed Methods

Materials

Prostaglandin E\(_1\) (PGE\(_1\)), prostaglandin I\(_2\) (PGI\(_2\)), bovine serum albumin (BSA), fatty acid-free human serum albumin (HSA), TRITC (tetramethylrhodamine isothiocyanate)-phalloidin and human FGN were purchased from Sigma-Aldrich (St. Louis, MO) and protein G-Sepharose from GE Healthcare (Little Chalfont, United Kingdom). 1-paraformaldehyde (PFA) was from Electron Microscopy Sciences (Hatfield, PA) and O-phenylenediamine from Thermo Scientific (Waltham, MA). Human VWF was isolated from factor VIII concentrates (EFS-Alsace, Strasbourg, France) according to the method of Toti et al.\(^1\) Botrocetin was purified from snake venom (Latoxan, Valence, France) as described previously.\(^2\) Experiments were performed with different batches of human TN-C from the U251 glioma cell line (Millipore, Billerica, MA). Western blotting, silver and Coomassie blue stainings and two different techniques of mass spectrometry (MALDI-MS and LC-MS/MS) were used to ensure that very pure TN-C was used. This TN-C was structurally related to that found in atherosclerotic vessels.\(^3\),\(^4\) In addition, similar results were obtained with a different source of TN-C (AbD Serotec, Raleigh, NC; data not shown). Apyrase was purified from potatoes as previously described.\(^5\) DM-BAPTA-AM (5,5’-dimethyl-1,2-bis(O-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid-tetra(acetoxymethyl) ester) and the indicators Oregon Green 488 BAPTA-AM-1 and Calcein red-orange-AM were from Molecular Probes (Eugene, OR). Acid citrate dextrose (ACD) solution was obtained from Bioluz (St-Jean-de-Luz, France), hirudin from Transgene (Illkirch-Graffenstaden, France) and heparin from Sanofi-Aventis (Paris, France). The integrin α\(_{IIb}\)β\(_3\) antagonist eptifibatide (Integrilin\(^6\)) was purchased from Schering-Plough (Kenilworth, NJ). The monoclonal antibody (mAb) DB7 against TN-C was from Millipore, horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) from Jackson Lab (Bar Harbor, ME) and the HRP-conjugated anti-VWF
antibody from Dako (Glostrup, Denmark). Mouse monoclonal blocking antibodies were as follows: anti-human integrin β₁, 4B4 (Beckman Coulter, Fullerton, CA) and P5D2 (Abcam, Cambridge, United Kingdom); anti-human GPIbα, AK2 (Abcam) and ALMA.12 (produced in our laboratory); anti-human integrin α₃β₃, LM609 (Millipore); anti-human integrin α₁β₃, chimeric mAb 7E3 Fab fragments abciximab (ReoPro®) (E. Lilly, Indianapolis, IN); anti-human VWF A1 domain, clone 701 kindly provided by Dr. C. Denis. Other antibodies used were: WM23, against the macroglycopeptide region of GPIbα (Pr. S. Jackson, Monash University, Melbourne, Australia) and MOPC-21, a mouse IgG₁κ isotype control (Biolegend, San Diego, CA).

**Mice**

Mice were backcrossed onto the C57BL/6 background for a minimum of 6 or 12 generations and were maintained in the animal facilities of the Etablissement Français du sang-Alsace. VWF-deficient (VWF⁻/⁻) mice were from Dr. C. Denis, β₁-null (β₁⁻/⁻) mice from Dr. R. Fässler (Max Planck Institute, Martinsried, Germany) and α₂-null (α₂⁻/⁻) mice from Dr. B. Eckes (University of Cologne, Cologne, Germany). A WT littermate was purchased from Charles River (Wilmington, MA).

**Blood Collection and Preparation of Washed Platelets and Red Blood Cells**

Blood was drawn from the abdominal aorta in 8-week-old mice (about 5 animals for each condition) anesthetized intraperitoneally with a mixture of xylazine (20 mg per kg body weight, Rompun®, Bayer, Leverkusen, Germany) and ketamine (100 mg per kg body weight, Imalgene 1,000®, Merial, Lyon, France). All experiments conformed to the French legislation for animal experimentation and followed the recommendations of the Guide for the Care and Use of Laboratory Animals.

Human blood was collected from healthy volunteers who had not taken any antiplatelet medication in the preceding 2 weeks. Platelets were washed using ACD-anticoagulated whole blood as previously described. Briefly, the platelet-rich plasma (PRP) was obtained by centrifugating blood at
250 x g for 16 min. Following a 10 min rest period, the PRP was centrifuged at 2,200 x g for 16 min. The platelet poor plasma was then removed by aspiration and the pelleted platelets were gently resuspended in an equal volume of isotonic Tyrode’s Buffer (138 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO₃, 0.4 mmol/L NaH₂PO₄, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, 5 mmol/L Hepes, 3.5 mg/mL HSA, 5.5 mmol/L glucose, pH 7.3) supplemented with 10 U/mL heparin and 0.5 µmol/L PGI₂. Following another 10 min rest period, 0.5 µmol/L PGI₂ was added to the platelets before centrifugation at 1,900 x g for 8 min. This step was performed twice and platelets were finally resuspended to a final concentration of 3 x 10⁸ platelets/mL in Tyrode’s Buffer supplemented with 0.02 U/mL apyrase. Washed platelets were kept at 37°C for 30 min prior to experimentation in order for apyrase activity to decay and studies were achieved within 6 hours after blood collection. To prepare reconstituted blood, red blood cells (RBCs) were obtained after centrifugating whole blood at 250 x g for 16 min. The PRP and leukocyte layers were removed and the RBCs were washed twice in Tyrode’s buffer and treated with 0.02 U/mL apyrase, prior to reconstitution with autologous washed platelets (50% (v/v)) at a final concentration of 250 x 10⁸ platelets/mL.

**Static Adhesion Assay**

Glass coverslips were coated with 100 µg/mL TN-C, FGN or VWF for 2 hours at RT and blocked with PBS-10 mg/mL (1%) HSA for 60 min. We checked that the protein concentrations used for coating gave maximal platelet adhesion. Human platelets (4.5 x 10⁶/coverslip) in Tyrode's buffer were allowed to adhere to the coated surfaces at 37°C. After 20 or 40 min, non-adherent platelets were washed away and adherent cells were fixed with PBS-40 mg/mL (4%) PFA and imaged by differential interference contrast (DIC) microscopy. In the case of VWF surfaces, the modulator botrocetin (2 µg/mL) was added since this matrix supports weak adhesion in the absence of fluid shear. Where indicated, the platelets were pretreated for 20 min with PGE₁ (10 µmol/L) to keep them in a resting state and prevent granule secretion.
In Vitro Flow-Based Adhesion Assay

Rectangular glass microcapillaries (VitroCom, Mountain Lakes, NJ) were coated with 100 µg/mL TN-C, 300 µg/mL FGN or 100 µg/mL VWF overnight at 4°C and blocked with PBS-10 mg/mL (1%) BSA for 30 min at RT. Concerning the flow experiments in which a double matrix was used, we have preliminarily determined the lowest concentrations of VWF (20 µg/mL) and TN-C (100 µg/mL) providing maximal platelet adhesion. Hirudinated (100 U/mL) whole blood or reconstituted blood was perfused through the coated capillaries with a syringe pump (Harvard Apparatus, Holliston, MA) at 37°C and at various flow rates as previously described. Platelet adhesion was observed in real time under an inverted Leica DMI 4000 B microscope (Leica Microsystems, Wetzlar, Germany) using a 63x, 1.4 numerical aperture oil objective and a DIC technique. Images were acquired with a Photometrics charge-coupled device (CCD) camera (CoolSNAP HQ Monochrome, Photometrics, Tucson, AZ) and analyzed off-line using Metamorph software version 7.6 (Molecular Devices, Downingtown, PA). Platelet adhesive behavior was analyzed frame by frame (15 frames/s) and classified as (i) rolling when platelets constantly moved over the surface of the matrix, (ii) stationary adhesion when they did not move more than one half of a single cell diameter over a 10 s period, and (iii) detaching when detachment occurred within 2 s following the initial contact.

Morphologic Analyses

Hirudinated (100 U/mL) whole blood was perfused through glass microcapillaries coated with TN-C (100 µg/mL), FGN (300 µg/mL) or VWF (100 µg/mL) for 3 min at 300 s⁻¹, followed by washing with PBS at 300 s⁻¹ for 2 min. Adherent platelets were observed by DIC microscopy (Leica DMI 4000 B, 63x/1.4 oil objective). Change in cell shape was defined as the transformation of a resting discoid platelet into an activated spherical cell with filopodial projections more than 0.2 µm in length and a flattened “fried egg”-like morphology.
**Analysis of Cytosolic Ca$$^{2+}$$ Fluxes**

Platelet intracellular Ca$$^{2+}$$ changes were monitored according to a modification of a previously published method. Briefly, human platelets resuspended at 500 x 10$$^6$/mL after the first washing step were simultaneously loaded with the membrane-permeating non ratiometric Ca$$^{2+}$$ indicator dye Oregon Green 488 BAPTA-AM-1 (4 µmol/L) and the morphological dye Calcein red-orange-AM (2.0 µmol/L) for 30 min at 37°C. The dye-loaded platelets were washed twice and finally resuspended in Tyrode’s buffer containing apyrase (0.02 U/mL). These platelets were incubated with either vehicle (1:1,000 DMSO) or DM-BAPTA-AM (50 µmol/L) for 10 min and reconstituted with 50% (v/v) autologous packed RBCs at 250 x 10$$^6$$ platelets/mL, prior to perfusion over TN-C (100 µg/mL), FGN (300 µg/mL) or VWF (100 µg/mL) at 300 s$$^{-1}$$. The increases in platelet and Ca$$^{2+}$$-dependent fluorescence intensity upon adhesion of the cells were measured in the ranges 572-700 nm and 495-535 nm, respectively, by confocal laser scanning microscopy (Leica TCS SP5, Leica Microsystems) (1.85 frames/s for 8 min). The mean fluorescence ratio R$$\text{max}$$ was determined from the signal of washed platelets preincubated with A23187 (5 mmol/L) + Ca$$^{2+}$$ (10 mmol/L) and allowed to adhere to glass coverslips (50 platelets per experiment), while R$$\text{min}$$ was calculated from that of platelets preincubated with DM-BAPTA-AM (70 µmol/L) + EGTA (ethyleneglycoltetraacetic acid) (2 mmol/L) and allowed to adhere to 10 mg/mL (1%) BSA-coated coverslips (50 platelets per experiment). The ratio of signal intensity between the two dye channels was converted to intracellular Ca$$^{2+}$$ concentrations as described previously (Leica TCS SP5 LASAF software). The estimated Ca$$^{2+}$$ concentration is indicated relative to the zero point set by DM-BAPTA-AM and EGTA Ca$$^{2+}$$ chelation. Basal Ca$$^{2+}$$ levels were determined by measuring the fluorescence ratio (mean ratio=0.6, n=45) in platelets adhering to non-reactive 10 mg/mL (1%) BSA-coated coverslips. A specific Ca$$^{2+}$$ signal was defined as a change in fluorescence ratio of more than two standard deviations ($2\sigma=0.38$, n=45) relative to the mean fluorescence ratio in resting platelets and corresponded to peaks ranging over 50 nmol/L.
**In Vitro TN-C Binding Assay**

Blocking solution composition: 1:1,000 Tween 20 and 1 mg/mL (0.1%) BSA in PBS.

**Immunoprecipitation**

Lysis buffer composition: 1:100 Triton X-100, 20 mmol/L Tris (tris(hydroxymethyl)aminomethane)-HCl, 5 mmol/L Na$_3$VO$_4$, 100 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L EGTA, 150 mmol/L NaCl, pH 7.4.

**References**


**Supplemental Figure Legends**

**Supplemental Figure I.** TN-C is highly expressed in mouse atherosclerotic plaques whereas it is almost absent in healthy vessel wall. Representative confocal images of transversal PFA-fixed cryosections of an atherosclerotic (*ApoE*−/− plaque) or healthy portion (*ApoE*−/− healthy) of a carotid artery from an ApoE-deficient mouse or of a carotid artery from a wild-type mouse (*WT*). TN-C (left panel) was detected with a specific mAb (TNC1.2, Dr. G. Orend), followed by a Cy5-conjugated secondary antibody (red). Sections were co-labeled with the nuclear marker DAPI (blue) to visualize the vessel wall. Scale bar, 75 µm. L indicates lumen.

**Supplemental Figure II.** Antibodies blocking collagen binding to α2β1 do not prevent platelet adhesion to TN-C. Hirudinated human whole blood was preincubated for 10 min with 10 µg/mL of irrelevant mouse IgG (Control) or 10 µg/mL of a mAb blocking collagen binding to α2β1 (*P1E6, BHA2.1*) and perfused through TN-C-coated (100 µg/mL) glass microcapillaries at 300 s⁻¹. Control experiments were performed to ensure that P1E6 and BHA2.1 inhibited collagen-induced platelet aggregation. Adherent platelets were counted in one random field for each condition after 7 min of perfusion and their number was expressed as a percentage of that in control set to 100%. Results are the mean ± S.E.M. of three separate experiments (*P1E6*, p=0.44, NS; *BHA2.1*, p=0.18, NS).
Supplemental Figure II

Platelet adhesion (% of control)

Control  P1E6  BHA2.1