Supplemental Material

Materials and Methods

Constructs and protein purification

Recombinant human ADAMTS13 (rA13) was expressed in human embryonic kidney (HEK) 293 cells. Recombinant C-terminal fragment of ADAMTS13 consisting of TSP1 2-8 plus CUB (T2C) or TSP1 5-8 plus CUB (T5C) was stably expressed in Chinese hamster ovary (CHO) cells as described previously [1, 2]. The recombinant proteins were purified to homogeneity using chromatography as described previously [1, 2]. A SDS-polyacrylamide gel (PAGE) with coomassie blue staining determined the purity and integrity of purified rA13, T2C, and T5C.

Surface free thiol labeling and detection by mass spectrometry

Purified rA13, T2C, and T5C were incubated at 4 ^{o}C for 12 hours with N-ethylmaleimide (NEM) (Pierce, Rockford, IL) at final concentration of 5 mM in a buffer containing 0.1M phosphate, 0.15M sodium chloride, pH 7.4 according to the method described previously [3]. The residual NEM was removed by a mini-spin desalting column (Precision separation). The protein concentration was then determined by absorbance at 280 nm (1OD=0.74 mg/ml) and verified by Coomassie blue staining. The labeled r13 and C-terminal fragments were boiled at 100 ºC for 5 min with SDS-sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) and fractionated on a 10% SDS-PAGE and stained with coomassie blue. The specific protein bands were cut out and sent for trypsin digestion and peptide identification using a Nano LC-tandem mass spectrometer (Thermo Scientific, Waltham, MA) at the Proteomic Core, the University of Pennsylvania.

Surface free thiols blocking and gel filtration

Purified rA13 and its C-terminal fragments (100 μg) were incubated with 5 mM of NEM in 0.1M phosphate, pH 7.4, 0.15 M NaCl at 4 °C for 18 hours [3]. The residual NEM was removed by a mini-spin desalting column (Precision separation). The protein concentration was determined by absorbance at 280 nm according to the extinction coefficients determined previously[2].

Proteolytic activity

Proteolytic activity of purified rA13 with or without NEM treatment was determined by cleavage of a fluorescein-labeled VWF73 peptide (FRETS-VWF73) [4, 5] and a multimeric VWF under denaturing conditions [1, 4] as described previously.

Microplate binding assay

A microplate was coated with 100 μl (10 μg/ml) soluble type 1 collagen (BioData Corp, Horsham, PA) or human plasma-derive VWF. After being blocked with 200 μl of 2.5% BSA, rA13, T2C, and T5C at various concentrations in 20 mM Tris-HCl, pH 7.5, 150 mM

NaCl (TBS) containing 0.5% BSA were added and incubated for 2 hours at room temperature. The bound rA13 or the C-terminal fragment was determined by incubation with 100 μl biotin-conjugated rabbit anti-ADAMTS13 IgG (1:1,000) in 0.5% BSA in TBS, followed by 100 μl of streptavidin-peroxidase (1:3,000) (Vector Labs, Burlingame, CA). The color reaction was carried by addition of 100 μl of 3,3', 5,5'-Tetramethylbenzidine (TMB) substrate. The absorbance at 450 nm was determined with a SpectraMax 190 microplate reader after the reaction was stopped with 50 μl of 0.5 M H_2SO_4. The data were then fit into a non-linear isotherm to determine the dissociation constants using SigmaPlot software.

Collagen-induced platelet aggregation

Washed platelets isolated from platelet-rich plasma of KO mice (0.5 ml, $4x10^8$/ml) in a Tyrode's buffer were incubated at 37 °C with type 1 collagen (5 μg/ml) in the absence or in the presence of rA13 (10 nM), T2C (100 nM), and T5C (100 nM). The change in light transmission was monitored with a PAP-4 light scattering platelet aggregometer (BioData Corporation, Horsham, PA) for 3 min. The percentage of change in the light transmission was expressed as a function of time.

Microfluidic flow assay

Microfluidic flow channels in a BioFlux200 plate system (Fluxion Biosciences, South San Francisco, CA) [6] were coated with soluble collagen (Type I) (100 μg/ml) (Helena laboratories, Beaumont, TX) or fibrillar collagen (Type I) from equine tendons (Havertown, PA) (50 μg/ml) for 1 hour under 5 dyne/cm^2 shear for soluble collagen or 15 dyne/cm^2 shear for fibrillar collagen. The unbound surface was washed at 20-50 dyne/cm^2 with PBS for 3 min and blocked with 0.5% BSA in PBS for 30 min. Whole blood collected from healthy donors or wild-type and Adamts13-/- mice was anticoagulated with a thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK) (Sigma, St. Louis) (100 μM). PGE1 (1 μM) was used to inhibit platelet activation during preparation. The anticoagulated whole blood was incubated with a FITC-conjugated anti-human CD41 IgG for soluble one (1:10) or anti-mouse CD41 IgG for fibrillar one (1:1,000) to label platelets (BioLegend, San Diego, CA). A proteolytically active rA13, T2C, and T5C with or without NEM treatment at various concentrations were added into anticoagulated whole blood and incubated at 37 °C for 15 min. The whole blood mixtures were then flown over the microfluidic flow channels at various shear (~20-200 dyne/cm^2) for 1.5~3.0 min, depending on the flow rate. Kinetic increase in fluorescein-labeled platelets adhered to and aggregated on a collagen-coated surface was recorded every 2 seconds for the duration of flow under an inversed fluorescent microscope equipped with a high speed CCD camera (Nikon). The data analysis was performed off-line using the BioFlux Montage software. The area under the curve was determined using the GraphPad software. The relative percentage of surface coverage of the fluorescein-labeled platelets in the absence or in the presence of rA13 or a C-terminal fragment was determined. Each experiment was repeated for at least 3 times.

Intravital microscopy

Adamts13$^{-/-}$ and WT mice (12–15g) were anesthetized by intraperitoneal injection of sodium pentobarbital (50-80 mg/kg body weight). A jugular vein catheter was inserted using a standard surgical technique. Rhodamine 6G (1 μg/g body weight) (Invitrogen, Carlsbad, CA, USA) was injected intravenously via a retro orbital sinus perplex. An incision was made through the abdominal wall and mesenteric blood vessels were exposed and injured by a topical application of a filter paper soaked with 7.5% $FeCl_3$ for 1.5 min. After removal of the filter paper, venules immediately downstream of the application site were scanned to identify a region where platelet-decorated VWF strings were formed but no obvious thrombus formation was detected. VWF strings decorated with fluorescein-labeled platelets were visualized using a Nikon eclipse TE200 inverted microscope (Nikon Instruments, Melville, NY, USA) (20x) connected to a high speed CCD camera. Digital videos were recorded for 1 min prior to and after infusion of rA13 or variants using NIS Elements software (Melville, N.Y). The number and length of platelet-decorated VWF strings over time were determined per venule off-line using the Neurocida software. The average length of the platelet-decorated ULVWF strings was determined by the total length (μm) divided by the numbers of the ULVWF strings. The average length of the platelet-decorated ULVWF strings in first 10 seconds was arbitrarily defined as 100% (Initial length of the strings) and the relative lengths of these strings compared with the initial length of strings were determined for the entire duration of imaging analysis, one min each for pre- and post-injection of rA13 or C-terminal fragments as indicated in the figures. Also, the fluorescence intensity reflecting the accumulation of platelet/leukocyte aggregates in the entire field of each video was determined by NIS Elements Software. The fluorescence intensity in the first 10 seconds was defined as 100% (initial plt/leuk coverage) and the relative fluorescence intensity compared to the initial plt/leuk coverage was determined for each experiment. At least three independent experiments were performed to achieve statistical significance. The differences among various groups in the mean length of platelet-decorated VWF strings were determined by ANOVA one-way analysis of variants, defining $p<0.05$ and $p<0.01$ as statistically significant and highly significant, respectively.

Reference List

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