Role of Thrombospondin-1 in Control of von Willebrand Factor Multimer Size in Mice*

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Plasma von Willebrand factor (VWF) is a multimeric glycoprotein from endothelial cells and platelets that mediates adhesion of platelets to sites of vascular injury. In the shear force of flowing blood, however, only the very large VWF multimers are effective in capturing platelets. The multimeric size of VWF can be controlled by proteolysis at the Tyr453–Met454 peptide bond by ADAMTS13 or cleavage of the disulfide bonds that hold VWF multimers together by thrombospondin-1 (TSP-1). The average multimer size of plasma VWF in TSP-1 null mice was significantly smaller than in wild type mice. In addition, the multimer size of VWF released from endothelium in vivo was reduced more rapidly in TSP-1 null mice than in wild type mice. TSP-1, like ADAMTS13, bound to the VWF A3 domain. TSP-1 in the wild type mice, therefore, may compete with ADAMTS13 for interaction with the A3 domain and slow the rate of VWF proteolysis. TSP-1 is stored in platelet α-granules and is released upon platelet activation. Significantly, platelet VWF multimer size was reduced upon lysis or activation of wild type murine platelets but not TSP-1 null platelets. This difference had functional consequences in that there was an increase in collagen- and VWF-mediated aggregation of the TSP-1 null platelets under both static and shear conditions. These findings indicate that TSP-1 influences plasma and platelet VWF multimeric size differently and may be more relevant for control of the VWF released from platelets.

von Willebrand factor (VWF) serves a critical role in hemostasis by facilitating the initial tethering of platelets to the subendothelial at high shear (reviewed in Ref. 1). VWF is synthesized by megakaryocytes and endothelial cells and circulates in blood as a series of multimers made up of a variable number of disulfide-linked 500-kDa homodimers (2). Only the larger multimers of VWF are effective in hemostasis, since a selective deficiency of these forms is associated with a bleeding diathesis, type IIA von Willebrand disease (3). The intracellular assembly of VWF multimers is a stepwise process. First, individual pro-VWF subunits are linked in a tail-to-tail orientation by C-terminal disulfide bonds to form pro-VWF dimers, which are then linked in a head-to-head orientation by N-terminal disulfide bonds to form VWF multimers. The largest VWF multimers have a molecular mass in excess of 20,000 kDa (reviewed in Ref. 4). The VWF secreted constitutively from endothelial cells is composed of dimers and small multimers in contrast to the ultralarge multimers that are released from the storage compartment (5). The Weibel-Palade bodies in endothelial cells and the α-granules of platelets release ultralarge VWF in response to vascular injury and platelet activation. The largest VWF multimers in plasma are smaller than those stored within endothelial cells and platelets (6). Two mechanisms operate in regulating VWF multimer size: shear-dependent hydrolysis of VWF multimers by the VWF-cleaving metalloproteinase, ADAMTS13 (7–9) (a disintegrin-like and metalloprotease with thrombospondin type 1 motif) and cleavage of the linking disulfides by the plasma and platelet glycoprotein, thrombospondin-1 (10).

A severe deficiency of ADAMTS13 is associated with congenital and acquired forms of thrombotic thrombocytopenic purpura (TTP) (11–13), a disorder characterized by thrombocytic hemolytic anemia, a consumptive thrombocytopenia, and variable degrees of renal and neurological impairment. The persistence of unprocessed ultralarge VWF multimers in the circulation is thought to precipitate platelet clumping in arterioles and capillaries, resulting in tissue ischemia (6). The full-length ADAMTS13 transcript is expressed predominantly in the liver (11, 14) and appears to cleave ultralarge VWF multimers as they are secreted from endothelial cells and undergo conformational change when exposed to high shear (15).

The thrombospondins are a family of extracellular glycoproteins that function in cell-cell and cell-matrix communication and modulate cellular phenotype (16). We reported that the supernatant of cultured endothelial cells possessed VWF reductase activity and identified TSP-1 as a VWF reductase (10, 17). More recently, we showed that the VWF-reducing activity of TSP-1 centers on a free thiol at Cys974 in the Ca2+-binding C-terminal sequence of the protein (18). The role of TSP-1 in regulating VWF multimer size in vivo and its relationship to ADAMTS13 activity was unknown.

To clarify these issues, we have characterized the plasma
and platelet VWF multimer pattern in TSP-1+/− and TSP-1+/+ C57BL/6 mice. Incubation of VWF with TSP-1 in vitro results in smaller VWF multimers. Surprisingly, TSP-1 contributes to the persistence of larger VWF multimers in the circulation possibly by negatively regulating ADAMTS13 activity. On the other hand, platelet VWF multimer size was reduced upon lysis or activation of wild type platelets but not TSP-1 null platelets. The data from functional assays as the TSP-1 null platelets exhibited an increase in collagen- and VWF-mediated aggregation under static and shear conditions. We discuss the implications of these findings with regard to the initiation and development of an arterial thrombus.

EXPERIMENTAL PROCEDURES

Proteins and Reagents—Leupeptin, β-Pho-Pro-Arg-chloromethyl ketone, and 4-2-aminomethyl)-benzenesulfonuyl fluoride (AEBSF) were from Calbiochem, and bovine thrombin, N-ethylmaleimide (NEM), EDTA, 1,10-phenanthroline, phenylmethanesulfonuyl fluoride, triton X-100, and Tween 20 were from Sigma. Aprotinin was from Bayer AG (Leverkusen, Germany), and 1-desamino-8-arginine vasopressin (desmopressin) was from Ferring AB (Limhamn, Sweden). TSP-1 was purified from human platelet concentrates (19); the recombinant TSP-1 fragments, CP123-1 (residues 294–529) and E3CaG-1 (residues 630–1117) were expressed in insect cells by Dr. Dean Mosher (20). The residue numbers are for the mature protein. The recombinant VWF A3 domain was expressed in E. coli and purified from bacterial inclusion bodies and was a gift from Dr. Miguel Cruz (21). The anti-TSP-1 monoclonal antibody, mAb 133, was a gift from Dr. Joanne Murphy-Ullrich, and HB8432 was produced from a murine hybridoma cell line obtained from American Type Culture Collection (Manassas, VA). All other reagents were of analytical grade.

Animals—Wild type (TSP-1+/+) and TSP-1 null (TSP-1−/−) C57BL/6 mice were used in this study. TSP-1−/− mice (22) were generated in Dr. Jack Lawler’s laboratory (Boston, MA) by homologous recombination in 129/Sv-derivatized ES cells implanted in C57BL/6 blastocysts. Chimeras were bred to C57BL/6 mice, the offspring were genotyped, and heterozygotes were back-crossed (N9) to a C57BL6 background (99.6%). A breeding program was established at the biological resource center at the University of New South Wales (Sydney, Australia), and animals aged between 6 weeks and 6 months and of both sexes were used. The experimental procedures were approved by the Animal Care and Ethics Committee of the University of New South Wales.

Preparation of Plasma—The mice were anaesthetized with isoflurane (Abbott), and blood was collected by cardiac puncture using a 18-gauge needle and a 20-ml syringe (Abbott), and blood was collected by cardiac puncture using a 1-ml syringe (BD) for an in vivo experiment. Murine plasma, platelet lysate, and platelet releasate were diluted 10-fold in a 200 ml Tris, pH 7.6, buffer containing 10% glycerol, 1% SDS, 2 mM EDTA, and 0.01% bromophenol blue and warmed to 56 °C for 20 min. 20 μl of the mix was loaded onto a 1% agarose gel (Seakem™ G-TGHP)-agarose; Cambrex, Santa Rosa, CA) in Tris-glycine-SDS electrophoresis buffer, pH 8.3, and electrophoresed at 4 °C at 125 V for 5 h. The gels were fixed, and the nonspecific binding sites were blocked by incubating wells with 200 ml 5% milk. The gels were then washed in distilled water, dried, and fixed on GelBond film (BMA, Rockland, ME), and exposed to Eastman Kodak Co. BMOX MS film. The autoradiographs were developed at 48 h. Densitometry was performed using a Fluor-S Multimag and Quantity One software from Bio-Rad.

Aliquots of murine plasma, platelet lysate, and platelet releasate were diluted 10-fold in 20% mm imidazole, pH 7.3, buffer containing 0.125 M NaCl, 5 mM citric acid (ELISA buffer) and 5% bovine serum albumin (BSA), and the VWF collagen binding affinity and VWF antigen level were determined as described by Favaloro et al. (23). Briefly, aliquots of murine and human plasma, platelet lysate, and releasate were diluted 10-fold in a 200 ml Tris, pH 8.6, buffer containing 10% glycerol, 1% SDS, 2 mM EDTA, and 0.01% bromophenol blue and warmed to 56 °C for 20 min. 20 μl of the loaded onto a 1% agarose gel (Seakem™ G-TGHP)-agarose; Cambrex, Santa Rosa, CA) in Tris-glycine-SDS electrophoresis buffer, pH 8.3, and electrophoresed at 4 °C at 125 V for 5 h. The gels were fixed, and the nonspecific binding sites were blocked with skim milk, followed by an overnight incubation with 109-labeled anti-human VWF polyclonal antibodies (Dako, Carpinteria, CA). The gels were then washed in distilled water, dried, and fixed on GelBond film (BMA, Rockland, ME), and exposed to Eastman Kodak Co. BMOX MS film. The autoradiographs were developed at 48 h. Densi-
TSP-1 in electrophoresis. **, pE was infused over 30 min to three TSP-1 mice. In separate experiments, desmopressin was infused over 30 min via the tail vein using a 10-ml syringe, "Flowline" Springfusor syringe driver, and Springfusor flow control tubing (Pacific Medical Supplies, Victoria, Australia) and a 30-gauge 0.5-inch needle. For the time course experiment, the concentration of desmopressin was adjusted to deliver 3 mg/kg at a fixed volume of 250 ml over 30 min to six TSP-1 or TSP-1 mice. Blood was sampled 1 h before desmopressin infusion and at 1 and 6 h postinfusion. The first two collections were by saphenous vein bleeds, and the third was by cardiac puncture. In separate experiments, desmopressin was infused over 30 min to three TSP-1 or TSP-1 mice, and blood was collected by cardiac puncture at 1 h in one study and 6 h in another.

Statistics—Comparative data are presented as means ± S.D. Statistical significance was calculated with Student’s t test for all analyses.

RESULTS

The average multimer size of VWF in plasma and platelet samples was estimated using two different measures. Samples were resolved on 1% agarose gel electrophoresis and the VWF was detected using 125I-labeled anti-human VWF polyclonal antibodies. The densities of the resulting multimer patterns were quantified and expressed as band intensity as a function of size. To compensate for small variations in protein loading and to better compare average VWF multimer size in a given experiment, the optical densities of the individual lanes were normalized so that the total density for each lane was the same. The CBA was also measured and expressed relative to the total VWF concentration (VWF/Ag) in the sample. The CBA/VWFAg ratio correlates with the average molecular weight of the intermediate and high VWF multimer forms for a given concentration of VWF. The overall error for the CBA/VWFAg ratio was calculated by adding the relative errors (one S.D.) for the individual CBA and VWFAg measures.

Shear-induced Platelet Aggregometry—1 ml of whole blood was collected by cardiac puncture from six 12-week-old female TSP-1 and TSP-1 mice and added to 140 ml of citrate-phosphate-dextrose anticoagulant. Shear-induced platelet aggregation was performed using a PFA-100 test system (Dade Behring) and a cartridge coated with collagen (fibrillar type 1 equine tendon) and ADP.

Desmopressin—Desmopressin was diluted in sterile saline and infused over 30 min via the tail vein using a 10-ml syringe, “Flowline” Springfusor syringe driver, and Springfusor flow control tubing (Pacific Medical Supplies, Victoria, Australia) and a 30-gauge 0.5-inch needle. For the time course experiment, the concentration of desmopressin was adjusted to deliver 3 mg/kg at a fixed volume of 250 ml over 30 min to six TSP-1 or TSP-1 mice. Blood was sampled 1 h before desmopressin infusion and at 1 and 6 h postinfusion. The first two collections were by saphenous vein bleeds, and the third was by cardiac puncture. In separate experiments, desmopressin was infused over 30 min to three TSP-1 or TSP-1 mice, and blood was collected by cardiac puncture at 1 h in one study and 6 h in another.

Statistics—Comparative data are presented as means ± S.D. Statistical significance was calculated with Student’s t test for all analyses.

**Plasma VWF Multimer Size Was Smaller in TSP-1 Null than in Wild Type Mice**—To evaluate the contribution of TSP-1 to the control of plasma VWF multimer size, we measured VWF multimer size in the plasmas of TSP-1 and TSP-1 mice. Based on the in vitro evidence that TSP-1 reduces VWF multimer size (10), we anticipated that in its absence the multimers might be larger in the TSP-1 mice. To the contrary, in both individual and pooled murine plasma, VWF multimer size was significantly smaller in TSP-1 mouse plasma.
The ultra-large VWF multimers in the plasmas of individual mice were larger in TSP-1\(^{-/-}\) mice than in TSP-1\(^{+/+}\) mice (Fig. 1A). This difference was confirmed by densitometry (Fig. 1B). The CBA to VWFAg ratios of the TSP-1\(^{-/-}\) cohort plasmas was also significantly higher than for the TSP-1\(^{+/+}\) cohort (Fig. 1C).

To allow for individual variations in multimer size between mice within a cohort, VWF multimer size was also measured in pooled plasma. Blood was collected by cardiac puncture from 12 (six male and six female) TSP-1\(^{-/-}\) or TSP-1\(^{+/+}\) mice and an equal volume of blood from each mouse was mixed to prepare pooled plasma. This was repeated using three other groups of mice. The VWF multimer size in the pooled plasmas of the TSP-1\(^{-/-}\) mice was significantly smaller than in the TSP-1\(^{+/+}\) mice in each group (Fig. 1, D and E).

The multimer size of endothelium-derived VWF was reduced more rapidly in TSP-1 null than in wild type mice—Desmopressin is a synthetic analog of arginine vasopressin. The infusion of desmopressin to mice (28) and humans (29) results in a rapid increase in plasma VWF, which persists for 6 h or more. Desmopressin is thought to act via vasopressin receptors on endothelial cells to stimulate the release of VWF from endogenous stores. The increase in plasma VWF is accompanied by an increase in the concentration of ultra-large VWF multimers in the circulation (30).

The intravenous infusion of desmopressin to TSP-1\(^{-/-}\) and TSP-1\(^{+/+}\) mice resulted in an increase in plasma VWF levels, which persisted at 6 h. The increase in plasma VWF concentration in TSP-1\(^{-/-}\) mice (Fig. 2A), however, was accompanied by a greater relative increase in the ultra-large VWF multimers than was observed in TSP-1\(^{+/+}\) mice (Fig. 2B). The average multimer size of plasma VWF was larger in TSP-1\(^{-/-}\) than TSP-1\(^{+/+}\) mice at both 1 h (Fig. 2C) and 6 h (Fig. 2D) after desmopressin treatment. This result indicated that ultra-large VWF multimers released from stimulated endothelial cells in vivo are more efficiently processed in the absence of plasma TSP-1.

TSP-1 Bound to the A3 Domain of VWF—The interaction of TSP-1 and TSP-1 fragments with the A3 domain of VWF was measured using a competitive binding technique. In this technique, A3 immobilized on polystyrene was used as a probe to monitor the solution phase binding of TSP-1 or E3CaG-1 (which contains the Ca\(^{2+}\)-binding repeats and C-terminal sequence and harbors the VWF reductase activity) to A3. Interaction of ligands with protein acceptors immobilized on plastic often introduces artifacts due to varying degrees of denaturation of the plastic-bound protein. This was probably the cause of the lack of saturable binding of TSP-1 (Fig. 3B) and E3CaG-1 (Fig. 3D) to immobilized VWF A3. Soluble A3 competed for the binding of TSP-1 (Fig. 3C) and E3CaG-1 (Fig. 3E) to immobilized A3, however, which implied that the interaction was specific. Apparent dissociation constants for binding of TSP-1 or E3CaG-1 to soluble A3 of \(-3\) and \(-6\) \(\mu M\), respectively, were estimated from the competition experiments. As expected, soluble E3CaG-1, but not the CP123-1 fragment (which contains the procollagen-like module and three properdin-like or type 1 modules), competed with TSP-1 for binding to immobilized A3 (Fig. 3, E and F). These results indicate that TSP-1, like ADAMTS13 (31), interacts with the A3 domain of VWF.

The Average Multimer Size of the VWF in Human Platelets Is Smaller than in Murine Platelets—Variation in the pattern and processing of VWF multimer size in human and murine platelets was evaluated by lysing platelets in the absence or presence of the TSP-1 inhibitors, EDTA and NEM. There was a partial reduction in multimer size of both human and murine platelet VWF upon lysis in the absence of the TSP-1 inhibitors (Fig. 4). Notably, the VWF multimers in human platelets are significantly smaller than in C57BL/6 murine platelets.
but not in TSP-1\(^{-/-}\) platelets when lysed without the TSP-1 inhibitors (Fig. 5). The reduction in multimer size was more obvious when measured by collagen avidity, which preferentially detects high \(M_r\) multimers (27). Lysis of TSP-1\(^{-/-}\) platelets in the presence of 10 \(\mu\)g/ml human platelet TSP-1 resulted in reduction of VWF multimer size (Fig. 5D). This result indicated that the addition of human TSP-1 corrects for the absence of endogenous murine TSP-1 with respect to control of VWF multimer size.

The Multimer Size of VWF in the Thrombin-activated Release of TSP-1 Null Platelets Was Larger than in Wild Type Platelet Release—The VWF multimers in thrombin-activated TSP-1\(^{-/-}\) platelet release were smaller than in TSP-1\(^{-/-}\) platelet release (Fig. 6, A–C). These observations are consistent with a role for TSP-1 in controlling the multimer size of the very large VWF multimers during platelet activation.

TSP-1 Null Platelets Exhibited an Increase in Collagen- and VWF-mediated Aggregation under Both Static and Shear Conditions—The aggregation of platelet-rich plasma pooled from TSP-1\(^{-/-}\) or TSP-1\(^{-/-}\) mice in response to increasing concentrations of collagen and ADP was measured by optical density in a platelet aggregometer (Fig. 7) on four separate occasions using four different groups of mice. The TSP-1\(^{-/-}\), but not TSP-1\(^{-/-}\), platelets aggregated in response to 5 \(\mu\)g/ml collagen and 5 \(\mu\)M ADP, although at these concentrations, the ADP-mediated aggregation was less pronounced than with collagen. Although both groups of platelets demonstrated sustained aggregation in response to 10 \(\mu\)M ADP (data not shown), it is noteworthy that TSP-1\(^{-/-}\), but not TSP-1\(^{-/-}\), platelets disaggregate following an initial wave of aggregation in response to lower concentrations of ADP. The aggregation in response to collagen was inhibited by anti-VWF antibodies, confirming a central role for VWF in the process but without distinguishing platelet from plasma VWF or excluding contributions from other plasma proteins such as fibrinogen. Considering that fibrinogen is the most important ligand mediating platelet aggregation in plasma (32), it was surprising that VWF played such a significant role in a fibrinogen-rich environment. It is possible that the binding of fibrinogen to mouse platelets is suppressed by TSP-1 and elimination of TSP-1 enhances plate-
let aggregability, although it has been well described that TSP-1 promotes platelet aggregation in the presence of fibrinogen (for example, see Ref. 33).

The PFA-100™ analyzer measures whole blood flow through an agonist-coated capillary under high shear ($-6,000 \text{ s}^{-1}$). Collagen- and ADP-coated capillaries were used in this study. The machine has been validated as a sensitive measure of VWF multimer size and as a screening tool in the diagnosis of von Willebrand disease (34, 35). The closure time corresponds to the occlusion of capillary flow through the cartridge due to platelet aggregation. The closure time corresponds to the occlusion of flow through the capillary due to platelet aggregation

![Diagram](image)

**Fig. 4.** The average multimer size of the VWF in human platelets was smaller than in murine platelets. A, washed human (lanes 2 and 3) and wild type murine (lanes 5 and 6) platelets were lysed with a range of proteinase inhibitors with the addition of the TSP-1 inhibitors EDTA and NEM at 0 (lanes 2 and 5) or 5 (lanes 3 and 6) min, and VWF multimer size was measured by agarose gel electrophoresis. The 0-min time point is referred to as *platelet lysate + EDTA*, whereas the 5-min time point is referred to as *platelet lysate − EDTA*. Human (lane 1) and murine (lane 4) plasma is shown for comparison. B and C, densitometry traces of the VWF multimer patterns shown in A. D, VWF multimer size of the samples shown in A measured by the ratio of collagen binding to VWF antigen. The lysis of both human and murine platelets resulted in significant reduction in VWF multimer size. **, $p < 0.01$; ***, $p < 0.001$.

and is prolonged in the absence of ultralarge VWF multimers as in type 2A von Willebrand disease. VWF binding to glycoprotein Ib and/or $\alpha_{\text{IIb}}\beta_{\text{III}}$ appears to be the major determinant of closure time (36).

A closure time was not recorded for the six female TSP-1 $^{+/+}$ mice due to either the maximum test time of 300 s or the maximum syringe travel ($>250$ s in this sample) being reached. The failure of the wild type murine platelets to occlude the capillary was most likely a consequence of their small size. The machine is calibrated for human platelets, which are 2–3 times bigger than murine platelets (37). The closure time values for the TSP-1 $^{-/-}$ mice ranged from 160 to 200 s (mean ± S.D.;
Applying 250 s as the maximum closure time for the control group, $p$ is $<0.001$.

**DISCUSSION**

Plasma VWF is largely of endothelial cell origin (38). The smaller size and presence of proteolytic fragments in plasma support the premise that VWF stored in endothelial cells are modified after release (39, 40). TSP-1 reduces VWF multimer size; therefore, we anticipated that unless ADAMTS13 could adequately compensate for its absence, plasma VWF multimers in TSP-1 null mice would be larger than in wild type mice. To the contrary, we found that the plasma VWF multimer size was significantly smaller in TSP-1 null mice. In addition, endothelium-derived VWF was more efficiently processed in the

**FIG. 5. Platelet VWF multimer size was reduced upon lysis of wild type but not TSP-1 null platelets.** A, washed TSP-1$^{+/+}$ (lanes 2 and 3) and TSP-1$^{-/-}$ (lanes 5 and 6) mouse platelets were lysed with a range of protease inhibitors with the addition of the TSP-1 inhibitors EDTA and NEM at 0 (lanes 2 and 5) or 5 (lanes 3 and 6) min and VWF multimer size measured by agarose gel electrophoresis. The 0-min time point is referred to as platelet lysate + EDTA, whereas the 5-min time point is referred to as platelet lysate − EDTA. TSP-1$^{+/+}$ (lane 1) and TSP-1$^{-/-}$ (lane 4) mice plasma is shown for comparison. B and C, densitometry traces of the VWF multimer patterns shown in A. D, VWF multimer size of the samples shown in A measured by the ratio of collagen binding to VWF antigen. The lysis of TSP-1$^{+/+}$ platelets resulted in significant reduction in VWF multimer size, which was not evident in TSP-1$^{-/-}$ platelets or when TSP-1$^{+/+}$ platelets were lysed in the presence of 5 mM EDTA and 10 mM NEM. The lysis of TSP-1$^{-/-}$ platelets in the presence of 10 μg/ml human platelet TSP-1 resulted in reduction of VWF multimer size to the same extent as was observed for TSP-1$^{+/+}$ platelets. **, $p < 0.01$; ***, $p < 0.001$. 

174.8 ± 16.8 s). Applying 250 s as the maximum closure time for the control group, $p$ is $<0.001$. 

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plasma of TSP-1 null mice than in wild type mice. These findings indicate that TSP-1 possibly inhibits the activity of ADAMTS13 in vivo. A preliminary report of the in vitro inhibition of ADAMTS13 by TSP-1 supports this premise (41).

Mechanisms by which TSP-1 inhibits ADAMTS13 are speculative and include interference with ADAMTS13 anchorage to

Fig. 6. The multimer size of VWF in the thrombin-activated releasate of TSP-1 null platelets was larger than in wild type platelet releasate. A, suspensions of washed TSP-1+/+ (lanes 1 and 3) or TSP-1−/− (lanes 2 and 4) mice platelets were activated with thrombin for 2 min, and the TSP-1 inhibitors EDTA and NEM were added after 5 min. The releasate (lanes 1 and 2) was separated from the aggregated platelets (lanes 3 and 4), which were lysed with proteinase inhibitors and EDTA and NEM, and VWF multimer size was measured by agarose gel electrophoresis. B, densitometry traces of the VWF multimer patterns shown in A. C, VWF multimer size of the samples shown in A measured by the ratio of collagen binding to VWF antigen. The average multimer size of the VWF in the TSP-1+/+ platelet releasate was significantly smaller than in the TSP-1−/− platelet releasate. **, p < 0.01.

Fig. 7. TSP-1 null platelets aggregated in response to a lower concentration of collagen. The aggregation of platelet-rich plasma pooled from six female TSP-1+/+ or TSP-1−/− mice in response to increasing concentrations of collagen and ADP is shown in A. The TSP-1−/−, but not TSP-1+/+ platelets, aggregated in response to 5 μg/ml collagen and 5 μM ADP. The aggregation in response to collagen was inhibited by anti-VWF antibodies (B).
the endothelial cell surface, binding to and inhibition of ADAMTS13 activity, and/or the inhibition of its interaction with VWF. Binding of ADAMTS13 to the A3 domain of VWF exposed to the shear force of flowing blood has been proposed to precede cleavage of the Tyr-Arg-Met peptide bond in the adjacent A2 domain. Our finding that TSP-1 also binds the A3 domain of VWF suggests a mechanism by which TSP-1 may compete with ADAMTS13 for interaction with VWF. The C-terminal TSP-1 repeats are not required for activity in static assays but may be necessary for anchorage of ADAMTS13 to endothelial cells and VWF proteolysis in vivo. The regulation of enzyme activity by TSP-1 is not without precedent. TSP-1 has been shown to bind and inhibit the activities of the neutrophil enzymes, neutrophil elastase, cathepsin G, and the fibrinolytic enzymes, plasmin and urokinase. We reported earlier that intraperitoneal administration of human TSP-1 to Balb/c mice (to achieve plasma concentration of \(-1 \mu g/ml\)) results in a lower average plasma VWF multimer size. It would appear, therefore, that although supraphysiological concentrations of TSP-1 result in reduction of plasma VWF multimer size, physiological plasma concentrations \((-0.02 \mu g/ml\) in humans) are inhibitory. On the other hand, there is a dramatic increase \((-1000\)-fold) in TSP-1 concentration at sites of vascular injury as a result of platelet release. It is conceivable that different concentrations of TSP-1 either complement or inhibit ADAMTS13 activity. It is also important to note that although the C-terminal region that contains the VWF reductase activity is highly conserved between human and murine TSP-1, variations in other regions could result in species differences.

Platelet α-granules are described as consisting of nucleoid and electron-lucent zones. Based on electron density, the electron-lucent matrix can be further subdivided into intermediate and light zones. Platelet VWF is localized within tubular structures in the light zone located at one pole of the α-granule. TSP-1 along with fibrinogen and fibronectin, on the other hand, appears to be dispersed within the intermediate zone. This compartmentalization of VWF has been reported in both human and murine platelets. The concentration of VWF and TSP-1 within separate but closely related compartments raises the possibility that these glycoproteins mix during platelet degranulation. Considering that platelet lysates do not contain ADAMTS13 activity, we investigated a role for TSP-1 in regulating platelet VWF multimer size. To account for possible contamination of our platelet preparations with plasma ADAMTS13, we included the metalloproteinase inhibitor phenanthroline (8) in our mixture of protease inhibitors. Phenanthroline does not inhibit the VWF reductase activity of TSP-1 (data not shown). When human and wild type murine platelets were lysed or activated with thrombin, there was a reduction in VWF multimer size, which was not observed with TSP-1 null platelets. These results indicated that the multimer size of platelet α-granule VWF is altered by TSP-1-dependent process during platelet lysis and activation.

Notably, the average multimer size of human platelet VWF is significantly smaller than the murine protein, although both are processed by endogenous TSP-1. This result suggests that the human and murine proteins are processed to different degrees, which should be kept in mind when using mice to answer questions about control of human VWF multimer size. It is possible that the degree of processing of VWF also varies between mouse strains.

Endothelial cell derived (plasma) VWF is required for the initial tethering of platelets to the subendothelium at high shear by its association with collagen and the platelet glycoprotein Ib-V-IX complex. Platelet VWF, on the other hand, plays an important role in platelet aggregation by mediating the tethering and translocation of platelets on platelets. The individual contribution of the different VWF compartments is controversial. Chimeric pigs with normal platelet VWF but low plasma VWF have markedly prolonged bleeding times, whereas pigs with the reverse phenotype have mildly prolonged bleeding times. This is consistent with observations in humans with normal plasma and low platelet VWF who have either normal or mildly prolonged bleeding times. Platelet VWF can compensate for deficiency in plasma VWF to some extent. It has been estimated that 10–20% plasma VWF activity is required for platelet VWF to compensate for the plasma deficiency and sustain normal thrombus growth.

The deficiency in processing of VWF multimer size following activation of TSP-1 null platelets was reflected as more pronounced aggregation in response to collagen and/or ADP under both static and shear conditions. The TSP-1 null platelets aggregated in response to lower concentrations of collagen or ADP than wild type platelets, and this was inhibited by anti-VWF antibodies. The PFA-100 is highly specific for aberrations in platelet function under high shear and abnormalities in VWF-GPib and VWF-α3β3 interaction. We recorded a shorter closure time for TSP-1 null whole blood using the ADP/collagen cartridge. It is possible that the PFA-100 analyzer detects changes in platelet VWF in preference to plasma VWF, which would help explain the faster rate of aggregation in association with the larger VWF multimers released from TSP-1 null platelets despite the presence of smaller VWF multimers in TSP-1 null plasma. The normal tail bleeding times reported in TSP-1 null mice despite the lower average plasma VWF multimer size could represent a degree of compensation by platelet VWF. Alternatively, the difference in plasma and platelet VWF multimer size may be too subtle to be detected by this measure.

The presence of ultralarge VWF at sites of vascular injury is important for the initial tethering of platelets to the subendothelial matrix. Inhibiting ADAMTS13 activity at these sites would be desirable in facilitating these initial events. TSP-1 is very adhesive and binds to collagen (56), laminin (57), fibronectin, fibrin, and proteoglycans (58) and is well suited to interacting with other proteins that work in the extracellular matrix. As platelet activation and aggregation follow, there is a progressive release of platelet VWF. The exponential increase in TSP-1 that accompanies platelet activation could help regulate the rate of thrombus growth by controlling platelet VWF multimer size in the developing platelet thrombus. The relatively subtle differences in control of VWF multimer size observed herein are consistent with the lack of an obvious hemostatic disorder in the TSP-1 null mice. However, it remains to be determined whether these changes and the complex interactions of TSP-1 with other proteins attain greater significance in determining the rate of thrombus growth in damaged or diseased arteries.

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