Thrombospondin-1 inhibits ADAMTS13 activity in sickle cell disease

Sickle cell disease (SCD) is characterized by increased cellular adhesiveness, hemolytic anemia, hemostatic activation, vaso-occlusion and thrombosis. We found that patients with SCD in steady state have elevated plasma levels of the platelet protein thrombospondin-1 (TSP1) and that these levels are associated with vaso-occlusive complications and a history of acute chest syndrome (ACS).[1] Critically ill patients with ACS develop thrombocytopenia from platelet consumption[2] and have a high incidence of pulmonary embolism,[3] thereby suggesting that hemostatic activation plays a role in ACS.

TSP1, via its cognate receptor CD47, modulates vascular responses to hypoxia, regulates vaso-constriction, inhibits angiogenesis and nitric oxide, and promotes adhesion of sickle RBC to the endothelium, an inciting event of sickle vaso-occlusion.[4,5] *In vitro* studies have also shown that TSP1 binds to ultra-large von Willebrand factor (ul-VWF) multimers and prevents their degradation by the metalloproteinase ADAMTS13 by competitively inhibiting its activity.[6,7] Increased ul-VWF multimers have been documented in patients with SCD,[8,9] thereby suggesting the

Table 1. Associations of demographic, laboratory, and clinical characteristics by ADAMTS13 (0, >0) in SCD patients (all genotypes) at steady state.

		ADAMTS13 activity (%)			
		0		>0	
Characteristic	n.	median (IQR)[1]	n.	median (IQR)[1]	*P*[2]
Male gender, n. (%)	7	2 (28.6)	20	7 (35.0)	0.8
SS genotype, n. (%)	7	6 (85.7)	20	11 (55.0)	0.15
Age, years	7	26 (21-32)	19	37 (32-44)	**0.02**
Coagulation tests					
Thrombospondin-1 (ng/mL)	7	3573 (2869-6687)	20	374 (271-712)	**0.003**
ADAMTS13 inhibition, %	7	100.0 (100.0-100.0)	4	55.7 (36.9-82.8)	**0.02**
ADAMTS13 autoantibody (U/mL)	7	8.6 (6.7-10.9)	4	13.2 (8.7-21.9)	0.2
ADAMTS13 antigen (U/mL)	7	0.94 (0.69-1.34)	4	0.72 (0.65-0.90)	0.4
VWF:Ag (%)	6	218 (148-294)	13	175 (140-226)	0.4
VWF:CB (%)	5	150 (150-192)	11	150 (128-198)	0.7
VWF:CB:VWF:Ag ratio	5	0.95 (0.84-1.08)	11	0.91 (0.81-0.98)	0.8
ul-VWF multimers[3], n(%)	7	7 (100)	5	5 (100)	>0.999
Hematological parameters					
Red blood cells, 10^{12}/L	7	2.82 (2.32-3.60)	20	3.27 (2.09-4.01)	>0.999
Hemoglobin, g/L	7	110 (84-117)	20	99 (72-104)	0.09
Reticulocyte count, 10^{12}/L	7	0.18 (0.13-0.32)	20	0.17 (0.11-0.25)	0.5
Reticulocytes, proportion	7	0.044 (0.04-0.13)	20	0.054 (0.03-0.08)	0.7
White blood cells, x10^9/L	7	10.9 (7.5-12.2)	20	8.2 (6.9-10.3)	0.2
Neutrophils, x10^9/L	7	5.70 (4.10-9.00)	20	5.20 (3.90-6.55)	0.5
Platelets, x10^9/L	7	386 (236-423)	20	262 (197-360)	0.4
Hemoglobin F, %	5	8.4 (4.3-18.8)	14	3.14 (0.7-6.4)	0.12
Hemoglobin A, %	6	0.0 (0.0-5.1)	11	0.0 (0.0-4.6)	0.9
Hemoglobin S, %	5	77.9 (75.5-83.3)	19	51.4 (47.1-85.2)	0.08
Other clinical variables					
Systolic blood pressure, mmHg	7	110 (100-117)	19	108 (98-120)	0.9
Diastolic blood pressure, mmHg	7	70 (60-71)	19	70 (62-78)	0.5
Lactate dehydrogenase, U/L	7	254 (205-271)	20	292 (234-416)	0.08
Total bilirubin, µmol/L	6	35.9 (29-41)	20	35.9 (24-55)	0.8
Creatinine, µmol/L	7	53.0 (35-62)	17	71 (53-97)	**0.04**
Ferritin >1000, n.(%)	7	2 (28.6)	18	4 (22.2)	0.7
Hydroxyurea[4], n.(%)	7	3 (42.9)	20	7 (35.0)	0.7
Severe vaso-occlusive episodes, >0, n.(%)	6	6 (100.0)	16	9 (56.3)	**0.05**
Severe vaso-occlusive episodes >2, n.(%)	6	5 (83.3)	16	5 (31.3)	**0.03**
Acute chest syndrome, n.(%)	7	7 (100.0)	19	7 (36.8)	**0.004**
Chronic pain, n.(%)	7	3 (42.9)	20	8 (40.0)	0.9
Cerebrovascular accident[5], n.(%)	6	2 (33.3)	19	1 (5.3)	0.07
Leg ulcers, n. (%)	7	0 (0.0)	20	4 (20.0)	0.2
Priapism, n.(%)	2	2 (100.0)	7	2 (28.6)	0.07
Avascular necrosis, n. (%)	7	1 (14.3)	20	7 (35.0)	0.3
Retinopathy, n. (%)	7	1 (14.3)	19	4 (21.1)	0.7
Deep vein thrombosis, n. (%)	7	2 (28.6)	19	3 (15.8)	0.5
Pulmonary embolism, n. (%)	6	3 (50.0)	20	2 (10.0)	**0.03**

Severe vaso-occlusive episode is defined as an acute pain episode leading to an emergency department visit or hospitalization in the 12 months prior to the study lab draw. [1]Unless otherwise indicated. [2]From Wilcoxon's rank sum test or Pearson's χ^2 test of independence. [3]Number (percentage) of patients with ul-VWF multimers. [4]Patients on hydroxyurea were on a stable dose over the preceding 3 months. [5]MRI-detectable cerebrovascular accidents (overt strokes and silent infarcts).

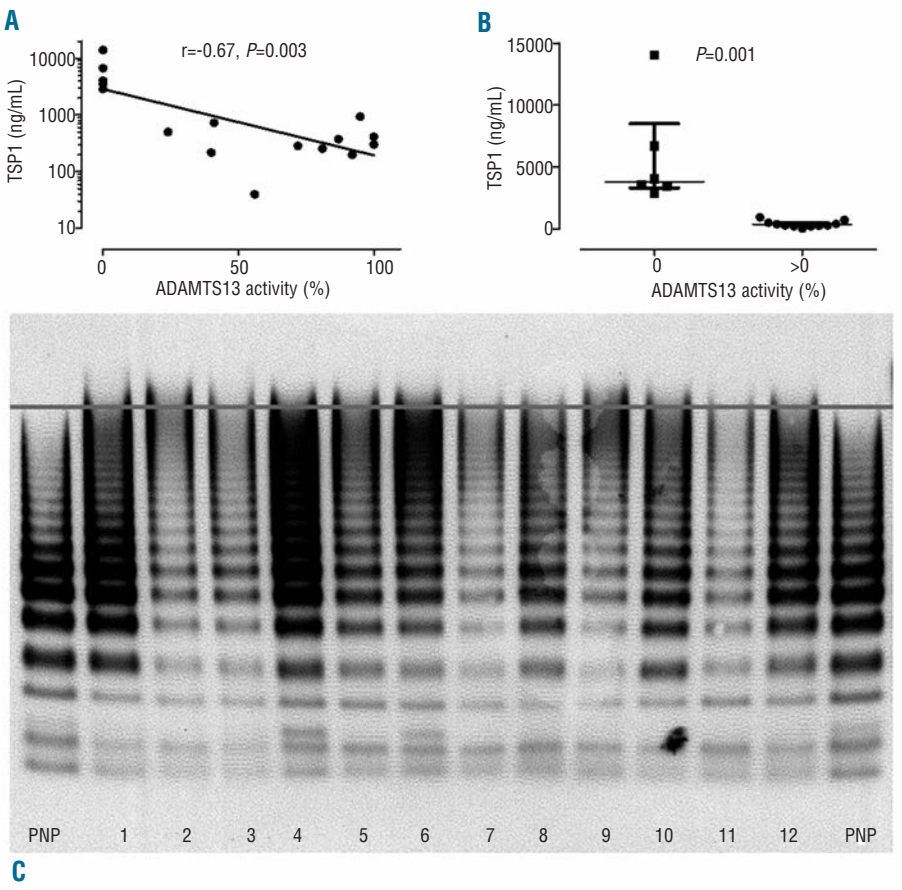

Figure 1. TSP1 inhibits ADAMTS13 activity in sickle cell disease. (A) Correlation of ADAMTS13 activity with TSP1 plasma levels in patients with HbSS (Spearman's correlation). (B) Scatter plot of TSP1 levels in HbSS patients with ADAMTS13 activity of 0 and >0 (error bars show median, IQ range, Wilcoxon's rank sum test). (C) The distribution of VWF multimers was evaluated in patients with undetectable ADAMTS13 activity (lanes 1-7) and detectable ADAMTS13 activity (lanes 8-12) and pooled normal plasma (PNP) by electrophoresis followed by Western blotting. The dashed line is drawn above the largest multimer in PNP. The multimers above the dashed line are considered ultralarge and were present in all patient's lanes.

presence of impaired ul-VWF cleavage. We, therefore, hypothesized that high TSP1 levels in SCD lead to inhibition of the proteolytic inactivation of VWF by ADAMTS13 and may represent an unexpected pathway to the formation of ul-VWF multimers in this disease. We tested this hypothesis on samples collected from 27 adult patients with SCD (HbSS, HbSC and HbS/beta thalassemia) documented by high-pressure liquid chromatography in steady state, defined as usual state of health and at least two weeks following hospitalization or emergency department visit for a severe vaso-occlusive pain episode (VOE). All subjects were enrolled after informed consent under UPMC IRB protocol PRO08110422. Our cohort was a convenience sample of patients not on chronic transfusion and who had not been transfused in the three months prior to the study. Platelet poor plasma was collected and assessed for levels of TSP1, ADAMTS13 activity, ADAMTS13 antigen, ADAMTS13 neutralizing inhibition and ADAMTS13 autoantibody, VWF antigen (VWF:Ag), VWF collagen binding (VWF:CB) and ul-VWF multimers in the clinical laboratory of The Institute for Transfusion Medicine (ITXM^{SM}, Pittsburgh, PA, USA). ADAMTS13 activity and ADAMTS13 neutralizing inhibition (residual activity after incubation with normal pool plasma) tests were determined using a fluorescence resonance energy transfer system (Gen-Probe, Waukeska, WI, USA). The ADAMTS13 antigen (Technoclone, Vienna, Austria) and TSP1 were measured by ELISA (R&D Systems, Minneapolis, MN, USA), while VWF:Ag (Diagnostica Stago, France) was assessed on a BCS-XP automated coagulation instrument (Siemens, Marburg, Germany). VWF multimer pattern was evaluated by a discontinuous SDS-agarose gel electrophoresis and buffer system with Western blot using a polyclonal VWF antibody (Dako, Carpinteria, CA, USA; A0082). We analyzed correlations between ADAMTS13 activity and the variables of interest by Spearman's correlation coefficient. Differences between ADAMTS13 activity groups were measured by Wilcoxon's rank sum test or Pearson's χ^2 test of independence.

The median ADAMTS13 activity in our cohort was 66% (interquartile range (IQR): 0-87%) including: 7 patients with undetectable ADAMTS13 activity (0%); 8 patients with reduced but detectable ADAMTS13 activity (24-68%); and 12 patients with normal ADAMTS13 activity (>68%). Repeat confirmational testing was performed for samples with undetectable ADAMTS13 activity. In patients with HbSS, ADAMTS13 activity correlated inversely with TSP1 plasma levels (r = -0.67, P=0.003, Figure 1A) and ADAMTS13 inhibition (r = -0.98, P<0.0001). These correlations were present but attenuated when we tested all SCD patients regardless of their genotype (r = -0.36, P=0.06 and r = -0.84, P=0.001, respectively, data not shown). No other significant correlations were noted between ADAMTS13 activity and any of the laboratory or clinical variables we analyzed (data not shown). Patients with undetectable ADAMTS13 activity defined a group with distinctive phenotypic characteristics. TSP1 levels were approximately one order of magnitude higher than those with detectable ADAMTS13 levels (Table 1 and Figure 1B). Interestingly, these patients had normal levels of ADAMTS13 antigen, but 100% ADAMTS13 neutralizing inhibition. Although VWF:Ag levels were

slightly higher in this group, the difference was not significant. The lifetime prevalence of acute chest syndrome and the number of severe VOE (defined as an acute pain episode leading to an emergency department visit or hospitalization in the 12 months prior to the study lab draw) were significantly increased in this group (Table 1). ul-VWF multimers were increased regardless of the ADAMTS13 activity level (Figure 1C).

In summary, we have shown for the first time that there is a subset of patients with steady state SCD with a combination of undetectable ADAMTS13 activity, high circulating TSP1 and a history of vaso-occlusive complications (VOE, ACS). These patients do not have greater elevation of hemolytic markers, suggesting perhaps that hyperreactive VWF might accumulate as a consequence of TSP1 inhibition of ADAMTS13 cleavage, as an alternative to the proposed inhibitory effects of free hemoglobin on ADAMTS13.[10] Inhibition of ADAMTS13 activity by TSP1 has been shown in shear field experiments[6] and *in vitro* systems, where purified TSP1 bound to plasma derived VWF and restrained ADAMTS13 activity and cleavage.[7] Patients with high rates of baseline hemolysis may inhibit ul-VWF proteolysis by free hemoglobin binding to ADAMTS13 as previously shown,[10] whereas patients with high TSP1 may inhibit ul-VWF cleavage by TSP1 competitive inhibition of ADAMTS13. As TSP1 levels increase further in VOE,[1,11] and TSP1 and vWF are both proposed to participate in sickle cell adhesion, we can hypothesize that further ul-VWF inhibition in particularly severe crises/VOE also might promote ul-VWF-mediated thrombotic complications. Our steady state patients with undetectable ADAMTS13 did not have evidence of thrombotic thrombocytopenic purpura (TTP) or microangiopathic hemolytic anemia; there are, however, several reports of patients with vaso-occlusive complications who develop hallmark findings of TTP,[12-14] and similar to those with autoimmune TTP, appear to respond to plasma exchange.[15] In fact, an unexplained fall in platelet counts in ACS is associated with increased risk of neurological deterioration, a hallmark feature of the TTP pentad. While the mechanisms driving TTP in SCD are unknown, the current study presents a possible new mechanism, involving the inhibition of ADAMTS13 proteolysis of VWF by TSP1.

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