HHS Public Access

Author manuscript

J Thromb Haemost. Author manuscript; available in PMC 2023 September 01.

Published in final edited form as:

J Thromb Haemost. 2022 September ; 20(9): 2098–2108. doi:10.1111/jth.15804.

Contribution of ADAMTS13-independent VWF regulation in sickle cell disease

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Abstract

Background: Von Willebrand factor (VWF) is elevated in sickle cell disease (SCD) and contributes to vaso-occlusion through its thrombogenic properties. VWF is regulated by ADAMTS13, a plasma protease that cleaves VWF into less bioactive multimers. Independent investigations have shown VWF to be elevated in SCD, whereas measurements of ADAMTS13 have been variable.

Objectives: We assessed ADAMTS13 activity using multiple activity assays and measured levels of alternative VWF-cleaving proteases in SCD.

Methods/ Patients: Plasma samples were collected from adult patients with SCD ($n = 20$) at a single institution when presenting for routine red cell exchange transfusion therapy. ADAMTS13 activity was measured by FRETS-VWF73, Technozym ADAMTS-13 Activity ELISA kit and a full-length VWF digestion reaction. Alternative VWF-cleaving proteases were identified by ELISA. A cell culture model was used to study the impact of SCD stimuli on endothelial ADAMTS13 and alternative VWF-cleaving proteases.

Results: ADAMTS13 activity was found to be moderately deficient across the SCD cohort as assessed by activity assays using a VWF A2 domain peptide substrate. However, SCD plasma showed preserved ability to digest full-length VWF, suggesting assay-discrepant results. Neutrophil and endothelial-derived proteases were found to be elevated in SCD plasma. Matrix

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AUTHOR CONTRIBUTIONS

R.C. Hunt carried out laboratory experiments and drafted the manuscript; U. Katneni assisted with laboratory assays; A. Yalamanoglu assisted with laboratory assays and critical feedback of manuscript; F.E. Indig assisted with hypoxia-related laboratory work and review of manuscript; J.C. Ibla provided critical analysis and helped with conceptual framework of the study; and C. Kimchi-Sarfaty formed the framework of study, helped with preparation and review of manuscript.

CONFLICT OF INTEREST

The authors report no relevant conflicts of interest.

metalloproteinase 9 specifically showed preferential cleavage of full-length VWF. Upregulation of alternative VWF-cleaving proteases occurred in endothelial cells exposed to SCD stimuli such as heme and hypoxia.

Conclusions: This is the first demonstration of accessory plasma enzymes contributing to the regulation of VWF in a specific disease state and may have implications for assessing the VWF/ADAMTS13 axis in other settings.

Keywords

ADAMTS13 protein; matrix metalloproteinase 9; sickle cell anemia; thromboinflammation; von Willebrand factor

1 | INTRODUCTION

Sickle cell disease (SCD) is hemoglobinopathy characterized by hemolysis and recurrent episodes of vaso-occlusion. Vaso-occlusive crisis (VOC) is a common acute manifestation in SCD. The obstructive vasculopathy of SCD also produces accumulative, irreversible end-organ injury such as stroke, cerebral microinfarcts, renal disease, cardiopulmonary disease, and retinopathy among other pathology.[1] The polymerization of deoxygenated hemoglobin is the hallmark feature of SCD; however, several interconnected processes are now recognized to contribute to vaso-occlusion. The interactions between sickle red cells and the vascular endothelium have received much investigative attention given that endothelial activation is pervasive in SCD.[2] Stimuli characteristic to SCD such as leukocyte activation, oxidative stress, and ischemia/reperfusion lead to endothelial activation and propagation of VOC.[3-6] A variety of adhesive molecules are expressed following endothelial activation, including the multimeric plasma protein von Willebrand factor (VWF). VWF can remain attached to the endothelial cell surface[7] and in its nascent, high molecular weight form VWF is hyperadhesive. VWF promotes platelet aggregation under shear flow[8] and also augments the adhesion of sickle erythrocytes to the endothelium.[9] The ability for plasma exchange therapy to rescue multiorgan failure in SCD crisis refractory to standard therapy speaks to the importance of plasma proteins such as VWF in the propagation of VOC.[10] Published data have consistently shown elevated levels of VWF during so-called “steady state” and further increases in VWF quantity and reactivity occur during VOC.[11] The strong correlation between the quantity of plasma VWF and severity of hemolysis in SCD implicates VWF as key mechanistic molecule in VOC.[12]

In contrast, published data concerning the VWF-cleaving protease ADAMTS13 have been variable in SCD (summarized in Table 1). Although some studies have shown decreased ADAMTS13 activity,[11,13-16] others have found no difference among controls, steady state SCD and VOC.[12,17,18] One mechanism explaining ADAMTS13 deficiency in SCD has been inhibition of VWF cleavage by thrombospondin-1.[14,19] The impact of oxidative stress on both ADAMTS13 and VWF has also been explored. Exposure of ADAMTS13 to neutrophil-derived oxidants leads to oxidation of key methionine residues and loss of enzymatic activity.[20] Oxidative modification of VWF also renders it less susceptible to cleavage.[21] The reasons for inconsistent findings regarding ADAMTS13 activity in SCD are unclear. ADAMTS13 is the primary plasma protease responsible for

VWF cleavage.[22-24] Neutrophil-derived proteases have also shown capacity to cleave VWF near the ADAMTS13 cleavage site when tested *in vitro*[25]; however, their contribution to *in vivo* VWF regulation remains unproven. A limitation of past studies concerning ADAMTS13 in SCD is that a single activity assay has been used for ADAMTS13 activity determination. It is generally assumed that activity assays under static conditions that employ a peptide fragment spanning the ADAMTS13 cleavage site in the VWF A2 domain are reflective of full-length VWF multimer cleavage that occurs under physiologic shear stress conditions of the microvasculature. Although peptide-based ADAMTS13 activity assays offer high reproducibility/precision and ease of use, agreement with full-length VWF cleavage assays may vary.[26] We therefore analyzed a single cohort of SCD patients using multiple ADAMTS13 activity assays. We show the existence of assay-discrepant results of ADAMTS13 activity in SCD and explore the potential contributions of neutrophil and endothelial-derived proteases to the regulation of VWF in SCD.

2 | METHODS

2.1 | SCD plasma samples

Plasma samples were collected from adult patients with SCD ($n = 20$) undergoing regular exchange transfusion therapy at the University of Colorado, Denver Anschutz Medical Campus, and stored at −80°C until analysis. The SCD patients in this cohort were assigned to regular exchange transfusion therapy primarily for the prevention of end-organ injury (e.g., recurrent stroke). Plasma samples were collected during routine clinical care and no associated demographic or clinical information was recorded per the original institutional review board study protocol. Citrate plasma samples were obtained at the time of automated erythrocyte apheresis therapy. The collection of blood/plasma was approved by the University of Colorado Denver Anschutz Medical Center institutional review board and by Food and Drug Administration's Research Involving Human Subjects Committee under a co-investigator in the Division of Plasma Protein Therapeutics (Dr. Paul W. Buehler). Age- and race-matched healthy volunteers served as controls ($n = 15$).

2.2 | ADAMTS13 peptide-based activity assays and antigen measurements

ADAMTS13 activity was determined by both an in-house activity assay using the FRETS-VWF73 substrate (Peptides International) and the Technozym ADAMTS13 Activity ELISA Kit (Technoclone). ADAMTS13 antigen in patient plasma was measured using Technozym ADAMTS13 Antigen ELISA (Technoclone).

2.3 | Full-length VWF digestion assay and multimer analysis

Multimeric digestion assays were performed as previously described,[27] briefly recombinant VWF (rVWF) 150 nM was incubated with 1.0 μl of plasma in reaction buffer (20 mM Hepes, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.5 mg/ml BSA, pH 7.5) under constant vortexing (2500 rpm) within a heated chamber at 37°C. In separate experiments, digestion of rVWF by control and sickle cell plasma was assessed under the influence of a small molecule matrix metalloproteinase 9 (MMP-9) inhibitor (CAS 193807-58-8, MMP-9 IC50 = 240 nM). Plasma samples were pre-incubated with the MMP-9 inhibitor (26 μM) at room temperature for 30 min followed by rVWF digestion under continuous vortexing for 15 min. Additional

rVWF digestion reactions were completed using recombinant MMP-9 (rMMP-9, R&D Systems) and recombinant ADAMTS13 (produced in-house by HEK293 cell line following lentiviral transduction of wild-type ADAMTS13 expression construct). To activate rMMP-9 the protein was incubated at 37°C overnight in 50 mM Tris HCl pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl with APMA added to a final concentration of 1 mM. The VWF digestion reaction was stopped by cessation of vortexing and analyzed for residual collagen binding activity using a Technozym vWF:CBA ELISA kit (Diapharma). For VWF multimer gel analysis, 20 μl of the reaction mixture was diluted with 980 μl of sample buffer (80 mM Tris-acetate, 0.2% EDTA, 0.2% SDS, 20% glycerol, 0.2% bromophenol blue). Ten microliters of the diluted digestion reaction was subsequently loaded onto a 0.6% SDS-agarose gel. Protein was transferred to Immobilon-P PVDF membrane (Millipore) and immunodetection was achieved using anti-VWF rabbit polyclonal antibody (Abcam) followed by incubation with HRP-conjugated secondary antibody (LSBio). Visualization VWF multimeric structure was achieved on a molecular imager (Carestream) by chemiluminescence.

2.4 | Plasma protease measurements

Commercially available ELISAs were used to measure the quantity of polymorphonuclear elastase, proteinase 3 (PR3), and MMP-9 per manufacturer instructions (all purchased from Abcam).

2.5 | Gel zymography

Plasma samples (2 μl) were loaded into a Novex 10% zymogram plus gelatin protein gel (Invitrogen). Following electrophoresis, gels were incubated in renaturing buffer for 30 min at room temperature followed by incubation in developing buffer at 37°C for 48 h. Gels were stained with colloidal blue to visualize areas of protease activity.

2.6 | Endothelial cell culture

Low passage (P2) human umbilical vein endothelial cells (HUVECs) were purchased from iXCells Biotechnologies and expanded under standard cell culture conditions in endothelial basal medium supplemented with endothelial growth supplements per supplier instructions. Cells were expanded on Primaria cell culture dishes (Corning).

2.7 | *In vitro* hypoxia and heme exposure

Upon reaching 80% to 90% confluency, HUVEC cells were subsequently cultured for 24 h under 21% O_2 or under hypoxic conditions (1% O_2) in a hypoxia chamber glove box (Plas-Labs) flushed with nitrogen to achieve 1% O_2/ 5% CO_2 at 37°C. Cellular supernatants were collected and concentrated 100× using Amicon Ultra-15 centrifugal filter units with 10 kDa nominal molecular weight limit before further analysis. Supernatants were buffer exchanged to FRETS buffer and applied directly to FRETS-VWF73 activity assay without freezing of samples. VWF within concentrated cell culture supernatant was measured using a VWF ELISA kit (Abcam). Separately, HUVEC cells were studied under exposure to various concentrations of heme added to the cell culture supernatant. A 4-mM heme-albumin stock solution was prepared by dissolving 65 mg Hemin (Sigma) in 100 mM NaOH at 37°C

and incubating for 1 h at 37°C with 20% human serum albumin. The pH was adjusted to 7.45 with 14 mM orthophosphoric acid/317mM NaCl followed by sterile filtration.

2.8 | SDS-PAGE/ Western blotting

SDS-PAGE of concentrated cell culture supernatants was performed under reducing conditions on NuPAGE Novex 4% to 12% Bis-Tris gels (Invitrogen) followed by Western blotting analysis using a mouse primary monoclonal antibody to detect ADAMTS13 (Wh2-11-1).[28] For heme exposure experiments, intracellular endothelial lysates were analyzed by SDS-PAGE/Western blotting using anti-MMP9 (Santa Cruz), anti-β-actin (Abcam), and anti-HO-1 (Enzo life sciences) antibodies.

2.9 | Statistical analysis

Data are presented as means ± SD unless otherwise noted. A two-tailed *t*-test was used to compare between control and SCD group. Pearson's correlation coefficient was used to assess the relationship between activity assays. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software).

3 | RESULTS

3.1 | ADAMTS13 activity in SCD is assay-dependent

Plasma samples were obtained from steady state adult SCD patients undergoing regular exchange transfusion ($n = 20$) at a single institution (University of Colorado, Denver) and healthy control patients ($n = 15$). Plasmatic ADAMTS13 activity was determined by two peptide-based assays that use a synthetic VWF A2 domain peptide encompassing the ADAMTS13 cleavage site. FRETS-VWF73 is a synthetic 73 amino acid peptide that, when cleaved, generates fluorescence from loss of the quenching molecule flanking the cleavage site.[29] Because bilirubin absorbs light near the same wavelength as the fluorophore in FRETS-VWF73, a notable limitation of this assay is the artifactual decrease in ADAMTS13 activity measurement in the setting of hyperbilirubinemia,[30] which can occur in SCD following brisk hemolysis. The Technozym ADAMTS-13 Activity Assay is commercially available ELISA kit that relies on antibody recognition of a neo-epitope exposed after VWF73 cleavage. The FRETS-VWF73 assay showed a mean ADAMTS13 activity of 54% of pooled normal plasma for SCD vs 103% for control patients ($p < .001$). The Technozym ADAMTS-13 activity assay showed a mean SCD activity 64% relative to control patients (0.70 IU/ml for SCD versus 1.11 IU/ml for control patients, $p < .001$) (Figure 1A). No SCD patients showed severe ADAMTS13 deficiency (<10%). There was a positive correlation between these two assays ($r = 0.76$); however, the strength of correlation was lower than anticipated given the high precision and reproducibility of these assays. ADAMTS13 antigen in plasma was also diminished in the SCD cohort (mean antigen 0.83 IU/ml for SCD vs 1.45 IU/ml for control patients, $p < .001$) (Figure 1B).

Given that the peptide-based activity assays showed lower levels of ADAMTS13 activity, we next assessed ADAMTS13 activity using a multimeric digest assay that uses full-length recombinant VWF under continuous shear stress. In contrast to the peptide-based ADAMTS13 activity assays under static conditions, VWF multimer digestion is generally

thought to be more reflective of physiologic conditions of the microvasculature. Following cessation of multimeric rVWF digestion, the residual collagen binding activity of digested rVWF can be used to evaluate the degree of digestion by the test plasma sample (more robust cleavage of the rVWF substrate results in less residual collagen binding activity). VWF multimer analysis of the rVWF digest products by low-resolution SDS-agarose gel electrophoresis also provides a qualitative/visual representation of VWF digestion. As digestion of the rVWF substrate progresses across time, there is a loss of multimeric VWF signal by low resolution SDS-agarose gel electrophoresis/Western blot.

In our SCD cohort, the ability for SCD plasma to digest multimeric VWF under shear conditions was preserved (mean residual CBA 0.18 IU/ml for SCD vs 0.15 IU/ml for controls, $p = .41$), although there was clearly more variability across the SCD cohort (Figure 2A). Some individuals in the SCD cohort showed digestion of full-length VWF exceeding that of control patients. We also performed VWF multimer analysis of select rVWF digest reactions by low-resolution SDS-agarose gel electrophoresis. Heightened VWF digestion by three SCD plasma samples could been seen (Figure 2B) after 30 min of digestion. When extending the reaction to 60 min, the VWF multimer substrate was nearly completely digested into lower molecular weight VWF (not visible by low-resolution SDS-agarose electrophoresis). In the absence of shear-induced unfolding of VWF, no digestion was seen when incubating the rVWF substrate with test plasma for 30 min at 37°C (Figure 2B, right panel). In sum, the results of full-length VWF digestion were not in agreement with peptide-based ADAMTS13 activity assays that showed more consistent depressed levels of ADAMTS13 activity in SCD. Altogether, this raised the possibility that additional plasma proteases in SCD may help regulate VWF under shear conditions.

3.2 | Neutrophil enzymes in SCD plasma with VWF-cleaving capacity

Given the assay-discrepant findings of ADAMTS13 activity, we hypothesized that additional proteases may be present in SCD plasma with capacity for VWF cleavage. Prior studies have shown that neutrophil enzymes are able to cleave VWF *in vitro*.[25,31] Knowing that neutrophil activation is pervasive in SCD,[32,33] we measured levels of three neutrophil proteases within SCD plasma: polymorphonuclear (PMN) elastase, proteinase 3 (PR3), and matrix metalloproteinase-9 (MMP-9). Levels of MMP-9 and PR3 measured by ELISA were elevated in SCD plasma relative to controls. However, only MMP-9 was increased with statistical significance (127 ng/ml for SCD vs 50 ng/ml for controls, $p = .0008$) (Figure 3A). Gel zymography of select plasma samples confirmed that MMP-9 in SCD plasma is functionally active (Figure 3B). To directly demonstrate the contribution of MMP-9 to VWF cleavage in SCD, we also performed rVWF digestion reactions in the presence of a small molecule MMP-9 inhibitor (CAS 193807-58-8, MMP-9 IC50 = 240 nM). We intentionally chose two sickle cell plasma samples with high concentrations of MMP-9 to test the impact of MMP-9 inhibition. Although control plasma was able to digest rVWF effectively in the presence of the MMP-9 inhibitor, SCD plasma showed less robust rVWF digestion under MMP-9 inhibition (Figure 3C). Importantly, this MMP-9 inhibitor showed no negative impact on ADAMTS13 activity at the concentration used (26 μM). Over a range of concentrations tested (2.6–112 μM), there was no impact on recombinant ADAMTS13 activity (data not shown).

Given that MMP-9 showed the most consistent upregulation across our cohort, we next looked to see if MMP-9 showed a substrate preference between FRETS-VWF73 (a VWF A2 domain substrate) and full-length multimeric VWF. We first empirically determined concentrations of recombinant MMP-9 and recombinant ADAMTS13 that generate equivalent FRETS-VWF73 activity (Figure 4A). These enzyme amounts were subsequently applied to full-length VWF cleavage assays. When normalization for equal FRETS-VWF73 activity, rMMP-9 showed greater digestion of rVWF under shear conditions relative to rADAMTS13 (Figure 4B). Expectedly, there was no digestion of multimeric VWF by either rADAMTS13 or rMMP-9 without the presence of shear force (data not shown). This demonstrated that MMP-9 has relatively enhanced cleavage capacity against full-length VWF under shear stress as compared with the VWF A2 domain peptide substate.

3.3 | Impact of SCD stimuli on endothelial expression of VWF, ADAMTS13, and alternative VWF-cleaving proteases

The endothelium is subject to various insults in SCD and is also the site of VWF, ADAMTS13, and MMP-9 expression.[34,35] We therefore studied endothelial cells exposed to SCD stimuli, namely hypoxia and heme. Ischemia–reperfusion and tissue hypoxia are central to SCD pathophysiology[6] and mechanistically linked to endothelial activation resulting in release of adhesive molecules such as VWF and P-selectin from Weibel-Palade body exocytosis.[36] Heme generated from intravascular hemolysis also triggers endothelial activation and vaso-occlusion through TLR4 signaling.[3] We first assessed VWF and ADAMTS13 expression from HUVECs cultured under standard atmospheric cell culture conditions (21% O_2, 5% CO_2) alongside HUVECs cultured under hypoxic conditions (1% O_2, 5% CO_2). Exposure to hypoxia resulted in increased levels of VWF antigen in the cell culture media (hypoxia 161% ± 36% relative to normoxia, $p = .008$, Figure 5A). There was a modest decrease in extracellular ADAMTS13 expression between normoxic and hypoxic endothelial cells by 20 h as assessed by Western blotting of the concentrated cell culture media (Figure 5B). Despite decreased expression of ADAMTS13, the cell culture medium of hypoxic endothelial cells showed markedly enhanced VWF cleavage capacity as measured by FRETS-VWF73 (Figure 5C). These results revealed the capacity of endothelial cells to secrete VWF-cleaving proteases when exposed to stimuli central to SCD pathogenesis. To further test this concept, endothelial expression of MMP-9 was assessed in the presence of increasing concentrations of heme in the cell culture medium. Exposure to heme resulted in dose-dependent upregulation of endothelial MMP-9 (Figure 5D). The upregulation of MMP-9 appeared concurrent with increased expression of heme oxygenase-1, a cytoprotective enzyme that is upregulated in SCD following ischemia/reperfusion injury and oxidative stress.[37] Altogether, the endothelial cell culture model confirmed the mechanistic link between inflammatory/ oxidative mediators in SCD (e.g., heme/free hemoglobin) and the expression of alternative VWF-cleaving proteases, this concept is presented graphically in Figure 6.

4 | DISCUSSION

In this report, we set out to characterize ADAMTS13 function in SCD. While the level of ADAMTS13 activity as measured by peptide-based activity assays was deficient relative

to controls patients, the ability of SCD plasma to process multimeric VWF appeared on average to be preserved, albeit quite variably. Our data demonstrate that neutrophil and endothelial-derived proteases such as MMP-9 are upregulated in SCD and may help process VWF. The somewhat weak correlation ($r = -0.284$) between MMP-9 levels and residual collagen binding activity of digested full-length VWF suggests that additional plasma proteases in SCD are likely to collectively contribute to VWF digestion. Upregulation of enzymes with VWF-cleaving capacity in SCD is likely driven as a byproduct of processes central to SCD pathogenesis (summarized in Figure 6). We propose that endothelial and leukocyte activation in SCD collectively generate increased levels of VWF-cleaving enzymes within circulation. Our finding of elevated levels of MMP-9 within SCD plasma is in agreement with prior work showing that plasma levels of MMP-9 in SCD correlate with leukocyte counts, supporting leukocytes as the likely primary contributors to plasmatic MMP-9 in SCD.[38] Hemolytic by-products, including heme and free hemoglobin, also exert oxidative stress on the endothelium. Independent lines of investigation have shown that MMP-9 upregulation occurs in response to oxidative stress.[39-41] Oxidative stress also activates MMPs from their latent forms to active enzymes.[42]

Neutrophil proteases have shown capacity to cleave VWF *in vitro*.[25] Leukocyte-derived enzymes within the subendothelial glomerular space may help to maintain the balance of pro-thrombotic and anti-thrombotic forces within the kidney.[31] However, to our knowledge, data generated from this SCD cohort are the first demonstrations of ADAMTS13-independent cleavage contributing to VWF regulation in a specific patient population. The degree to which ADAMTS13-independent cleavage occurs during steady state versus VOC should be explored. Our SCD cohort consists of plasma samples derived from SCD patients presenting for regular/scheduled red cell exchange transfusion. The mechanisms leading to MMP-9 upregulation would be expected to be further stimulated during VOC (namely free heme/Hb-mediated oxidative stress and neutrophil activation). It would be reasonable therefore to anticipate that MMP-9 and other accessory VWF-cleaving enzymes would be further upregulated by VOC. Whether alternative VWF-cleaving enzymes play a role in disease states outside of SCD is also unclear.

From a technical standpoint, our findings also suggest that measurements of ADAMTS13 activity using peptide-based VWF substrates may not always reflect the full VWF-cleaving capacity of the test plasma under shear stress. It is possible that this phenomenon is isolated to SCD where accessory enzymes with VWF cleaving capacity are upregulated with unique VWF substrate preferences. In the healthy population, agreement among the various existing assays for ADAMTS13 activity should be expected. Multimer digest assays to determine ADAMTS13 activity are not used in the clinical setting because of onerous technical expertise, lower reproducibility relative to VWF peptide substrate assays, and challenges with quantification. Activity assays based on the VWF A2 domain peptide substrate remain robust diagnostic tools (e.g., FRETS-VWF73 has an inter-run coefficient of variation as low as 2.1%[29]). It is likely that elevated levels of bilirubin in SCD contributed to much of the inter-assay variability between our in-house fluorogenic FRETS-VWF73 assay and the commercially available chromogenic ADAMTS13 activity assay from Technoclone, which is not impacted by elevated bilirubin levels in the test sample.

There are important limitations to address concerning this work. Because of the large volume of patient plasma samples received to carry out multiple independent investigations, precipitation during sample thawing was observed, rendering VWF measurements artifactually low. We therefore were not able to evaluate corresponding VWF measurements for each patient and evaluate the relationship between VWF, ADAMTS13 and accessory VWF-cleaving proteases. A second limitation of this study is that plasma samples were derived from SCD patients at a single institution undergoing regular red cell exchange transfusion therapy. Patients with SCD managed with exchange transfusion have generally manifested a more severe clinical phenotype such as stroke. It is possible that such patients may have greater leukocyte and endothelial activation as a direct consequence of increased disease activity. Therefore, the subset of SCD patients necessitating chronic exchange transfusion therapy may not be fully representative of the broader SCD patient population. The results presented here should therefore be interpreted within the context of this population and we are currently looking to replicate these findings within independent SCD cohorts. Nonetheless, given that leukocyte and endothelial activation appear to be fundamental components of SCD pathophysiology, we anticipate that these findings could be more applicable to the general SCD population.

Elevated plasma VWF, both at steady state and during VOC, has been documented across independent studies.[11,15,43] Studies from the 1980s showing that high molecular weight VWF multimers promote adhesion of sickle RBCs to the endothelium provided perhaps the first evidence that VWF is mechanistically involved in vaso-occlusion.[9] Given the strong correlation between quantity of active VWF in plasma and SCD disease activity,[12] elevated VWF in SCD is not merely a signal of endothelial activation but actively propagates vaso-occlusion though its adhesive properties. Non-ADAMTS13-mediated cleavage of VWF likely exists in SCD and may help mitigate the pathogenic impact of elevated VWF seen during steady state and the heightened levels of VWF reactivity seen in VOC. It should be emphasized that this accessory VWF cleavage is insufficient to balance the overwhelming level of VWF in SCD. Clinical trials aimed at addressing the imbalance of VWF/ADAMTS13 in SCD by recombinant ADAMTS13 infusion are currently under way: https://www.clinicaltrials.gov/ct2/show/NCT03997760.

ACKNOWLEDGMENTS

We acknowledge Dr. Paul W Buehler's laboratory while he was a principal investigator at FDA-CBER-Office of Blood Research and Review. Plasma test materials were obtained under a transfer agreement with RIHSC approval (17046-124419364) to Dr. Buehler from Dr. David C. Irwin located at the University of Colorado Anschutz Medical Center's Cardiovascular and Pulmonary Research Laboratory. We also would like to acknowledge Redab Alnifaidy for her contribution to this work.

Funding information

This work was supported by funds from the Hemostasis Branch/Division of Plasma Protein Therapeutics, Office of Tissues and Advanced Therapies, Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration (C.K.-S.).

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Essentials

- VWF is elevated in sickle cell disease (SCD) and contributes to vaso-occlusion.
- Prior assessments of ADAMTS13 activity in SCD have yielded variable results.
- ADAMTS13 activity shows assay-discrepant results in SCD.
- Alternative proteases such as MMP-9 help regulate the thrombogenicity of VWF in SCD.

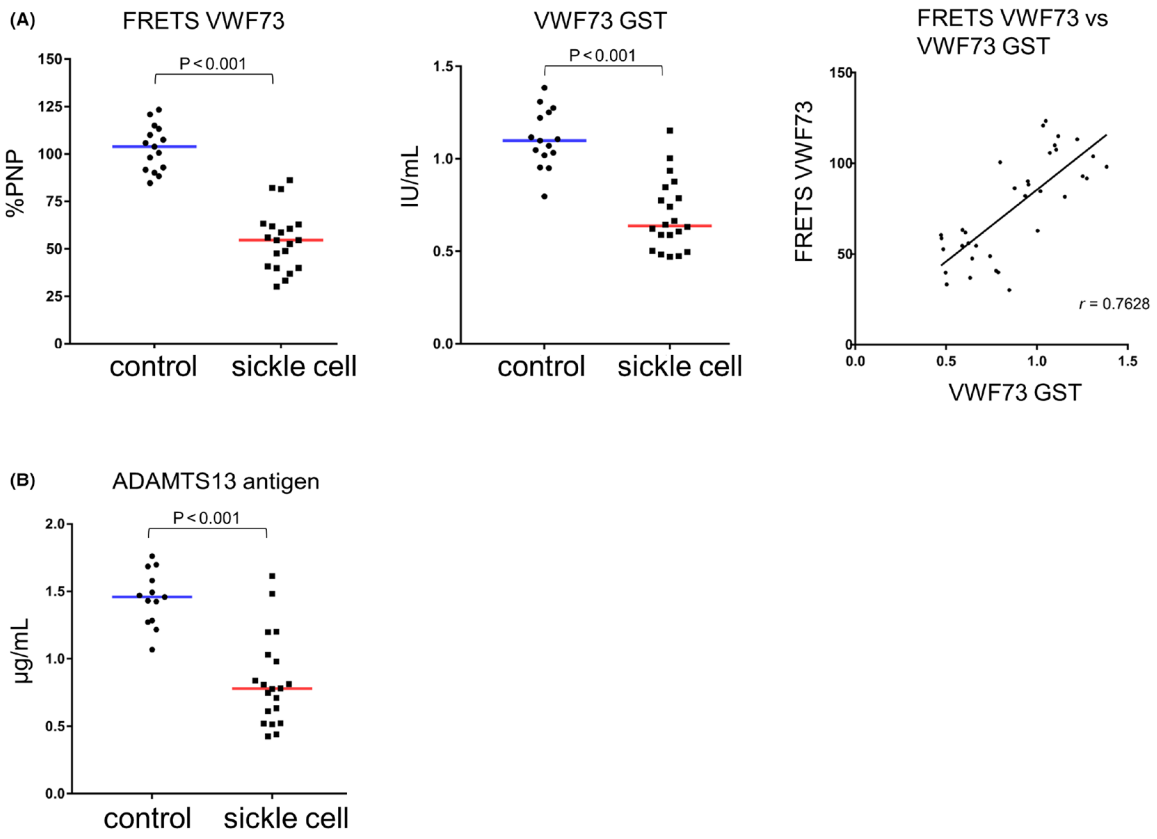

FIGURE 1.

ADAMTS13 activity and antigen assessments in SCD and healthy adults. (A) ADAMTS13 activity as determined by VWF A2 domain peptide-based assays: FRETS-VWF73 and VWF73 GST. From left to right: %PNP is the precent of ADAMTS13 activity relative to pooled normal plasma; IU/ml is calculated relative to manufacturer test internal control sample. The Pearson's correlation coefficient *r* between the two A2 domain activity assays is shown (right). (B) ADAMTS13 antigen by ELISA calculated relative to manufacturer internal control sample. P values are shown for unpaired two-sided *t*-tests.

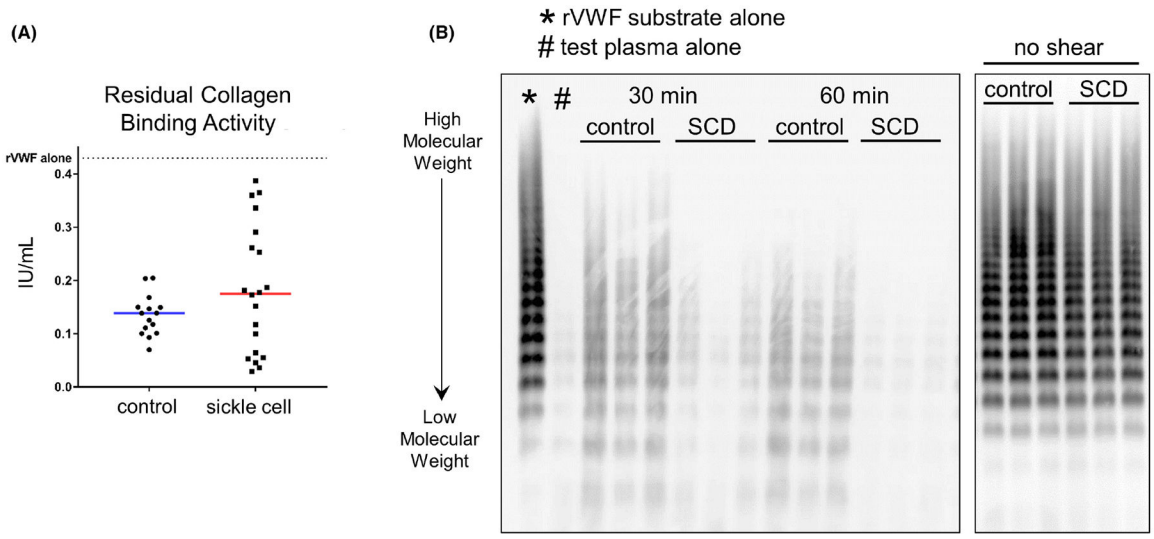

FIGURE 2.

Multimeric VWF digestion by SCD and control plasma. (A) Residual VWF collagen binding measurements following digestion of full-length recombinant VWF (rVWF) under continuous shear force by vortexing for 30 min. Lower residual collagen binding activity indicates greater digestion of rVWF substrate. As reference, rVWF alone without test plasma is included (dotted horizontal line). (B) Three SCD patients with elevated levels of rVWF digestion by residual collagen binding assay analyzed by VWF multimer analysis. Digestion reactions were stopped at 30 and 60 min and resolved by 0.6% SDS-agarose gel electrophoresis, transfer and immunoblotting for VWF. rVWF alone (without test plasma added) is indicated by an asterisk and test plasma alone (without rVWF) is indicated by a hashtag. Digestion reactions incubated for 30 min at 37°C without shear force are shown (right).

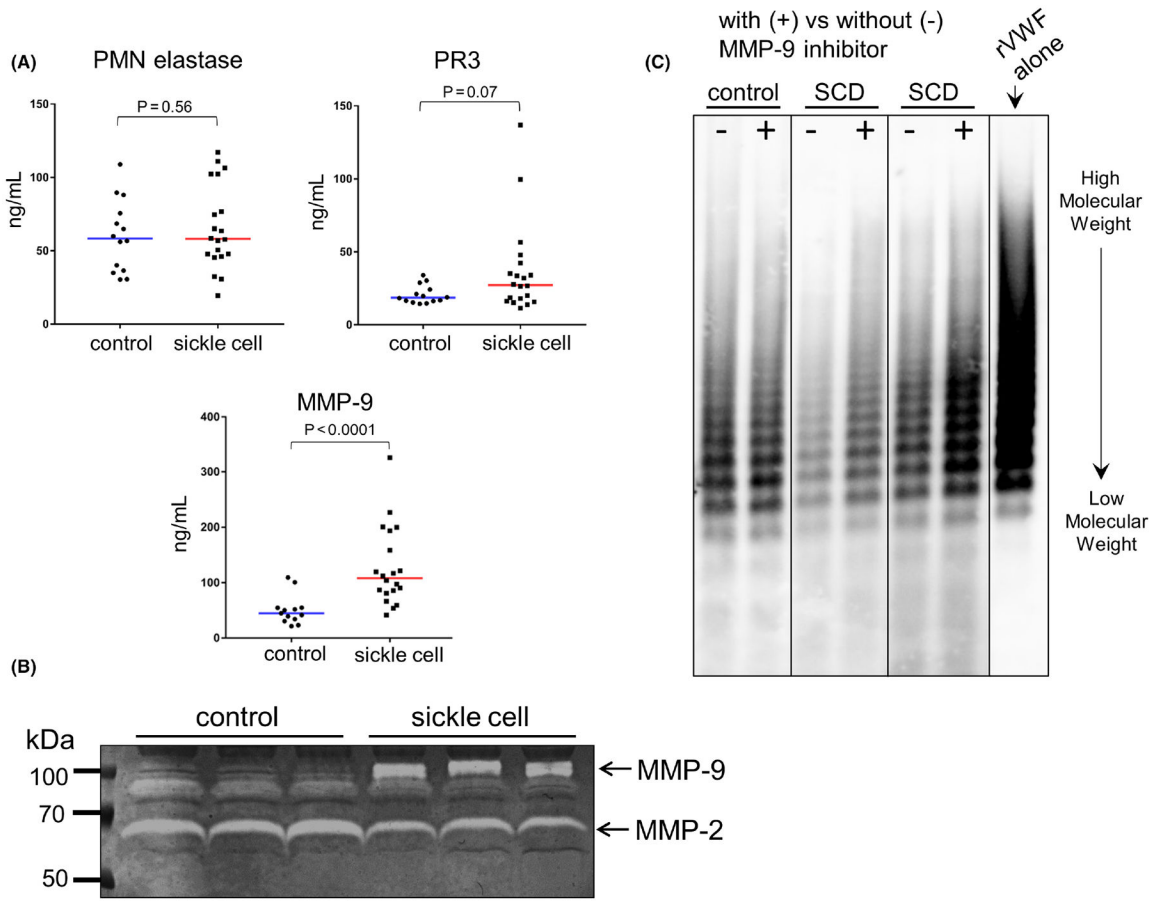

FIGURE 3.

Neutrophil proteases with capacity for VWF cleavage. (A) Quantification of neutrophil derived proteases within plasma determined by ELISA. (B) Gel zymography of randomly selected control and SCD plasma samples. Clearing of Coomassie blue stained gelatin indicates area of enzymatic activity. Bands corresponding to the molecular weight of MMP-9 and MMP-2 are indicated. (C) rVWF multimer digest reactions performed using control and SCD plasma samples preincubated with a small molecule MMP-9 inhibitor. Two SCD patients were chosen with high levels of MMP-9 to demonstrate the impact of MMP-9 inhibition on the digestion of rVWF. Following 15 min of digestion, all digestion reactions were analyzed by 0.6% SDS-agarose gel electrophoresis.

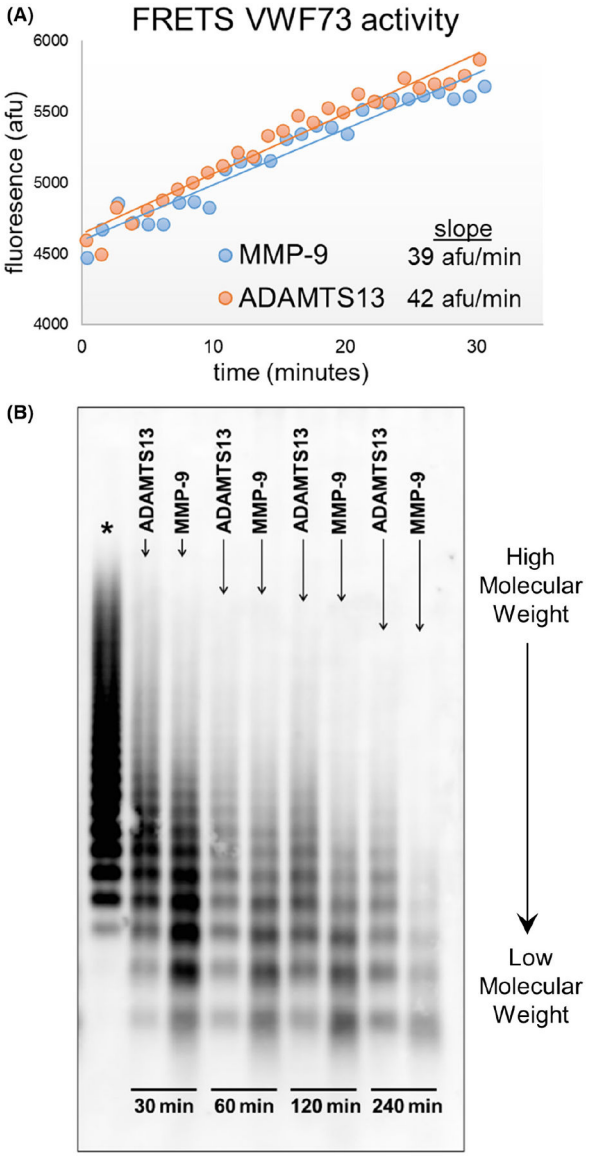

FIGURE 4.

MMP-9 substrate preference for multimeric VWF. (A) Recombinant ADAMTS13 and recombinant MMP-9 were first empirically normalized to achieve equivalent FRETS-VWF73 activity (top). (B) Subsequently the same quantities of enzyme were inputted into full-length rVWF digestion reactions. Independent digestions reactions were performed and stopped at various time points followed by VWF multimer analysis by 0.6% SDS-agarose gel electrophoresis (bottom).

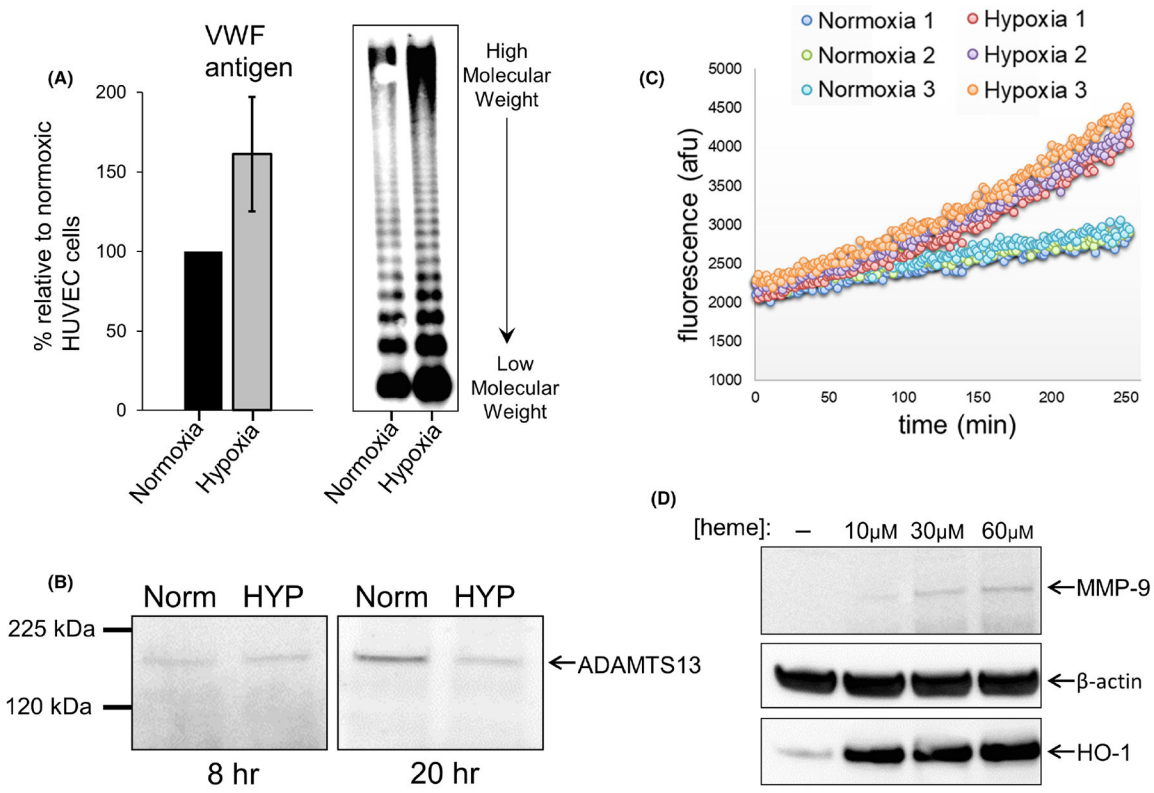

FIGURE 5.

Endothelial upregulation of VWF-cleaving proteases by hypoxia and heme-mediated oxidative stress. HUVEC cells were cultured under normoxia (21% O_2) or hypoxia (1% O_2) and were assessed for (A) VWF antigen by ELISA after 8 h. VWF multimer gel of normoxic and hypoxic HUVECcell culture media was performed (right). (B) ADAMTS13 expression in the HUVEC cell culture media was assessed by Western blot at 8 h and 20 h under normoxia or hypoxia. (C) HUVEC cell culture media collected at 20 h was assessed for VWF cleavage by FRETS-VWF73. The graph depicts raw 450 nm emission fluorescence readings over time from three technical replicates. (D) Endothelial cells cultured in the presence of 0, 10, 30, and 60 μM heme for 20 h were assessed for intracellular expression of MMP-9 (top) and heme oxygenase-1 (HO-1, bottom). β-actin included as loading control (middle).

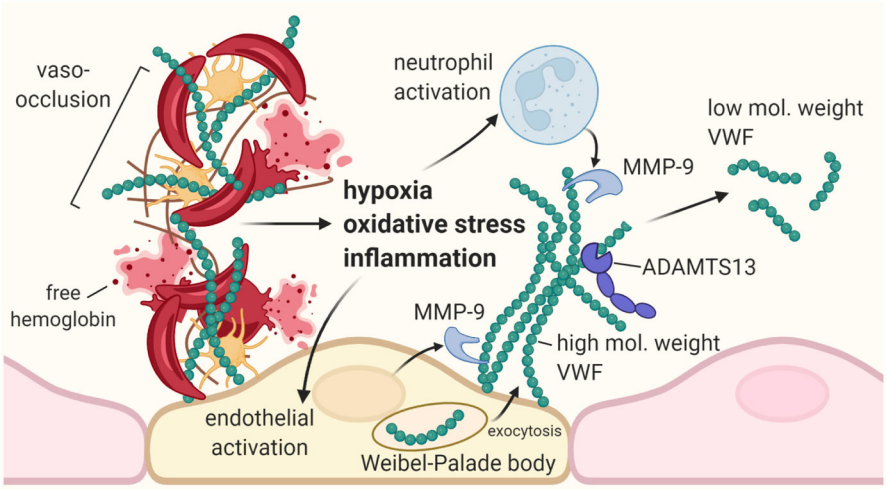

FIGURE 6.

The dynamics of VWF regulation in SCD. Vaso-occlusion produces local ischemia/reperfusion, inflammatory stimuli, and oxidative stress from free hemoglobin and heme. These stimuli collectively lead to endothelial and neutrophil activation and the release of alternative VWF-cleaving proteases. ADAMTS13 and alternative VWF-cleaving proteases such as MMP-9 help to regulate pathologically elevated levels of VWF in SCD.

TABLE 1

Summary of published literature on ADAMTS13 function in SCD

Study	ADAMTS13 findings	Method	Patients
Schnog et al., 2006	No difference among asymptomatic (77%), pain crisis (75%), and controls (62%)	Degradation of full-length urea-treated VWF under static conditions	*n* = 31 8 asymptomatic 23 pain crisis
Zhou et al., 2009	Reduced activity (mean 33%)	Recombinant VWF A2 domain	*n* = 10 steady state
Chen et at., 2011	No difference in activity between SCD and reference plasma	Recombinant VWF A2 domain substrate, HRP conjugate	*n* = 13 patients
Novelli et al., 2013	Reduced activity (median 66%) • 7 patients with undetectable activity • 8 patients with 24%–68% • 12 patients with >68%	FRETS-VWF73	*n* = 27 steady state
Al-Awadhi et al., 2017	No difference in ADAMTS13 antigen or activity levels in SCD adult patients compared with controls; increased levels of ADAMTS activity in SCD pediatric patients	Recombinant VWF A2 domain	*n* = 59 39 adults 20 children
Sins et al., 2017	Reduced activity during VOC vs. steady state	Degradation of full-length urea-treated VWF under static conditions	*n* = 24 all in VOC
Demagny et al., 2020	Reduced activity (median 70 IU/dL). No difference between asymptomatic and VOC/acute chest syndrome	FRETS-VWF73	*n* = 65 30 asymptomatic 35 VOC/ acute chest
Ladeira et al., 2021	Reduced activity (median 57.8%). No difference between hydroxyurea vs non-hydroxyurea users	FRET activity assay (ACTIFLUOR ADAMTS13 activity kit, American Diagnostica)	*n* = 40 40 steady state