Extracellular Hemoglobin Regulation of von Willebrand Factor Activity in Plasma of Patients with Sickle Cell Disease

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Abstract

Elevated levels of ultralarge (UL) von Willebrand factor (VWF) multimers in plasma play an important role in cell adhesion and vascular occlusion in sickle cell disease (SCD). Recently, we have shown that the binding of extracellular hemoglobin (ECHb) to the A2 domain of VWF significantly blocks VWF cleavage by the metalloprotease ADAMTS13 *in vitro*. We speculated that on release from the inflamed endothelium, the VWF multimers maintain the UL structure in plasma if ECHb prevents their cleavage. We observed that a subpopulation of VWF multimers that are bound to ECHb (HbVWF), which accounted for about 14 % of the total VWF in the plasma of SCD patients. The plasma HbVWF level is parallely correlated with the ECHb and VWF-antigen levels. The HbVWF multimers are resistant to the metalloprotease ADAMTS13 *in vitro* and are more adhesive to platelets and collagen compared with their Hb-free counterpart. Therefore, we speculate that the HbVWF, which are probably UL multimers, play an important role in tethering and stably adhering blood cells to the vascular endothelium and culminate in vascular occlusion/thrombosis/strokes in SCD patients. Thus, this article provides a new insight into the molecular pathophysiology of SCD.

Sickle cell disease (SCD) is a common hemolytic anemia caused by a single gene mutation in the β-subunit of hemoglobin (Hb),[1] affects millions of people worldwide, and is associated with significant morbidity and mortality. An estimated 83,000 US citizens have been diagnosed with the disease and more than two million carry the genes for related disorders, including sickle cell anemia and sickle-beta thalassemia.[2] Although the clinical manifestations of the disease are quite heterogeneous, periodic vasoocclusive crises, and chronic intravascular hemolysis are common in SCD patients. Vascular occlusion causes more than 33 % of all deaths in SCD, resulting in acute chest pain, arterial/venous thrombosis, and ischemic strokes.[3] Strokes and transient ischemic attacks occurred mostly in SCD patients between five to ten years of age. An estimated stroke alone resulted in 20 % mortality, with 70 % of the victims having a motor deficit and significant neurocognitive complications; 70 % of them had a recurrent stroke within the next three years.[4]

Investigations have also suggested that clinical events such as thromboembolisms and pulmonary hypertension are also associated with the short-life expectancy of SCD patients.[3,5] Microvascular thrombus formation is another clinical event that also occurred during crisis in SCD patients.[6,7]

Increasing evidence suggests that elevated von Willebrand factor (VWF) antigen,[8,9] including ultralarge (UL) multimers,[9-11] is associated with the development of complications such as vasoocclusive pain crises, thrombosis, and ischemic strokes in SCD.[6,8,12] Our recent observation also shows that the ULVWF multimers are prevalent in 60–70 % of SCD patients who are in stable conditions of their health. As in SCD, activation of the vascular endothelium is primarily triggered by the inflammatory cytokines tumor necrosis factor alpha (TNFα), interleukin (IL)-1β/6/8/10,[13] and mediators such as superoxide[14] and cell-free Hb.[15] The inflamed endothelium constitutively secretes VWF to maintain a high antigen level in the plasma of patients. Several *in vitro*[16,17] and *in vivo*[12] studies have suggested a crucial role of VWF in sickled-red blood cell(RBC)/platelet adhesion to the vascular endothelium in SCD. Likewise, studies have shown that adhesion of sickled-RBCs to the vascular endothelium creates a nidus for thrombus formation in the presence of VWF.[6] An *ex vivo* experiment has shown that VWF secreted from desmopressin-stimulated endothelium in wild-type mice agglutinated infused sickled-RBCs in microvessels; the agglutination was significantly inhibited by an anti-VWF antibody.[8] VWF binds potently to the $\alpha_4\beta_1$ integrin[18] and sulfatide[19,20] on sickled-RBCs to promote cell adhesion to the vascular endothelium. The UL forms of VWF multimers, which are significantly higher in SCD plasma of patients,[10,11] are known to
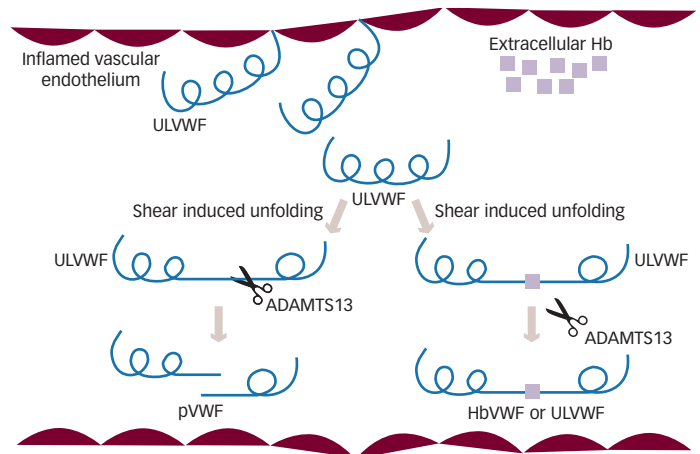
aggregate platelets spontaneously by forming high-strength bonds with its platelet-receptor glycoprotein Ib (GPIb).[21,22] The ULVWF multimers are also known to mediate leukocyte rolling and adhesion on inflamed endothelium through binding to P-selectin GP ligand-1 (PSGL-1) and $\alpha_M\beta_2$ receptors on leukocytes.[23] Thus, VWF multimers contain all of the determinants necessary for RBCs, platelets, and leukocytes to tether and stably adhere to the endothelium.

Considering the implicated role of VWF in SCD pathophysiology, we have recently investigated the function of the plasma metalloprotease ADAMTS13, which determines VWF activity. This is particularly significant given that the ULVWF multimers secreted from endothelial cells might accumulate in plasma and on endothelium if not properly cleaved by the ADAMTS13, and are not only extremely large, but also hyperadhesive. We have described that a moderate deficiency in ADAMTS13 activity (about 40 %) existed in SCD patients who were in stable, healthly conditions.[24] In addition, we have also described that the extracellular Hb (ECHb) significantly inhibited VWF cleavage by ADAMTS13 by blocking the enzyme-cleavage site on VWFA2 *in vitro*.[24] The study has also shown that the presence of 100 µg/ml of ECHb in buffer completely inhibited VWF cleavage by ADAMTS13 under physiological flow-shear conditions.[24] Therefore, we speculated that a similar mechanism might exist *in vivo* under pathophysiological conditions in SCD. The excessive ECHb in the plasma of patients[24–26] might inhibit the cleavage of VWF multimers by blocking the enzyme cleavage site on the VWFA2 domain and promoting the accumulation of ULVWF in plasma and on the endothelium (see *Figure 1*). We have isolated and characterized from the plasma of SCD patients a subpopulation of VWF multimers that are bound to ECHb.

Extracellular Hemoglobin-bound Von Willebrand Factor Multimers are Prevalent in the Plasma of Sickle Cell Disease Patients

Recently, we have shown that ECHb bound specifically to the A2 domain of VWF with high affinity (binding Kd~183nM, as measured by a surface Plasmon resonance assay) and inhibited VWF cleavage by ADAMTS13 *in vitro*.[24] We speculated that ECHb *in vivo* binds to the A2 domain of VWF multimers that are freshly released from the endothelium and prevent their cleavage by ADAMTS13. When the VWF multimers are released, they exist in UL forms.[27] The A2 domain of ULVWF multimers has unique features, such as the lack of protection by disulfide bonds within VWF and low resistance to unfolding that help the domain to be exposed easily for interaction with other proteins/ligands in circulation.[28] While VWF is UL, containing hundreds of monomers, when exposed to the intravascular hydrodynamic shear forces the tensile force on the long VWF multimer increases by the square of the multimer length, providing an efficient mechanism for regulation of VWF size distribution by force-induced A2 unfolding and cleavage by ADAMTS13.[28] Thus, the metalloprotease converts ULVWF to smaller circulating multimers with a wide range of size distributions.[27] Furthermore, the unfolding of the A2 domain becomes very limited in smaller plasma-forms of the VWF multimers. We speculated that a similar mechanism in which shear force-induced exposure of the A2 domain in freshly released VWF multimers probably facilitates VWF binding to ECHb. The ECHb binding to the ADAMTS13 cleavage-site on the A2 domain further prevents the ULVWF multimers from cleavage by the metalloprotease. Indeed, we have isolated a subpopulation of Hb-bound

Figure 1: Schematic Diagram Showing the Accumulation of Hemoglobin-bound von Willebrand Factor Multimers in Circulation

Hb = hemoglobin; HbVWF = hemoglobin-bound von Willebrand factor; pVWF = porcine von Willebrand factor; ULVWF = ultra-large von Willebrand factor.

Table 1: Quantification of Plasma Hemoglobin-bound von Willebrand Factor Multimers

Plasma Level of Hb-bound vWF		
	Normal	SCD
Ni-NTA Assay	9.4±2.8 (%)	21.2±4 (%)
ELISA Assay	0.45±0.08 (OD)	1.05±0.13 (OD)

Plasma was collected from sickle cell disease (SCD) or normal patients (n=5), and was passed through a Superose column (60 mm) to isolate von Willebrand factor (vWF) multimers. The pure vWF fraction was passed through a nickel-nitrilotriacetic acid (Ni-NTA) affinity column; flow-through was collected as hemaglobin (Hb)-free vWF and the eluted fraction as Hb-bound vWF multimers. The multimer was quantified using a commercial kit and expressed as a percent level of the assay control. In addition, a sandwich-ELISA assay was performed to directly measure Hb-bound vWF multimers from plasma. An anti-vWF antibody was coated on the enzyme-linked immunosorbent assay (ELISA) plate that traps the vWF multimers from plasma, which was detected by an anti-Hb antibody and expressed as absorbance. Both assays show high Hb-bound vWF multimers in the plasma of SCD patients. OD = optical density.

Table 2: Isolation and Characterization of Hemoglobin-bound von Willebrand Factor Multimers

A. vWF Cleavage		
Hb-bound vWF	Hb-free vWF (%)	
18±6	87±6	
B. vWF Activity		
	Hb-bound vWF (%)	Hb-free vWF (%)
RCof assay	117.6±9.3	79.3±13
CBA assay	133.4±8.6	100±7.3

Panel A: The hemaglobin (Hb)-bound and Hb-free von Willebrand factor (vWF) multimers were purified by the nickel-nitrilotriacetic acid (Ni-NTA) column as mentioned in Table 1, and each subtype (100 nM) was used for cleavage by rADAMTS13 (32 nM) under dialysis with 1.5 M urea. The 251 kDa monomer was cleaved into 164 and 121 kDa fragments, measured by western blot. The densitometry of the fragments was measured to calculate percent vWF cleavage. Panel B: vWF activity was measured for both subtypes using a ristocetin co-factor assay (RCof) kit and collagen-binding assay (CBA) and expressed as percent vWF activity. The data show that Hb-bound vWF multimers are significantly less cleavable and are hyperactive than their Hb-free counterpart.

VWF (HbVWF) multimers from plasma. We have immunoprecipitated HbVWF multimers from plasma using either an anti-VWF or anti-Hb antibody. We also isolated HbVWF multimers from plasma using a

superose gel-filtration and nickel-nitrilotriacetic acid (Ni-NTA) affinity purification method. The HbVWF multimers existed more in SCD plasma of patients, as accounted for by the 14% of total plasma VWF (HbVWF level 21% and total plasma VWF level 153 % compared with assay control). By contrast, normal individuals have only 6 % of plasma HbVWF (HbVWF level 9 % and total plasma VWF level 127 %, see *Table 1*). The plasma HbVWF level was also quantified in the same pool of patients/controls using a sandwich-enzyme linked immunosorbent assay (ELISA) assay. The data show a significantly increased level of HbVWF multimers in SCD patients than normal (see *Table 1*). Although the interaction between ECHb and VWF and their relationship *in vivo* is not clearly understood, our data show that increased HbVWF multimers co-existed with the high ECHb level (235.1±39 µg/ml) and VWF antigen (153 %) in the plasma of patients. By contrast, a low HbVWF multimer level was present concurrently with less ECHb (47.7±9.5 µg/ml) and VWF antigen (127 %) than normal plasma.

The Extracellular Hemoglobin-bound Von Willebrand Factor Multimers are Hyperadhesive

Furthermore, we examined whether the ECHb-bound VWF (HbVWF) multimers isolated from plasma are resistant to ADAMTS13, and whether the HbVWF multimers are hyperactive because of their UL size. Although it is yet to be determined that the circulating HbVWF multimers are actually the ULVWF, our data show that the HbVWF multimers are more hyperadhesive (about 35 %) to platelets than their Hb-free counterpart as measured by a conventional ristocetin cofactor (RCof) assay, which determines the VWF and platelet agglutination (see *Table 2*). In addition, a collagen-binding assay (CBA) also shows that the HbVWF multimers are about 33 % more hyperadhesive to immobilized collagen than the Hb-free subtype (see *Table 2*). Our observation also shows that HbVWF multimers are significantly less cleavable (about 80 %) by ADAMTS13 compared with Hb-free VWF multimers (see *Table 2*), suggesting that HbVWF multimers are hyperactive because they are resistant to the metalloprotease.

Summary

Thus we show that a subpopulation of VWF multimers exists in plasma that is bound to ECHb and is hyperactive. Elevation of the HbVWF multimers in plasma parallels the existence with levels of ECHb and VWF antigen in SCD patients. We suggest that the plasma HbVWF multimers may play a crucial role in the development of vasoocclusive and thrombotic complications in SCD. ■

1. Ingram VM, Gene mutations in human hemoglobin: The chemical difference between normal and sickle cell hemoglobin, *Nature*, 1957;180:326.
2. Report from the Sickle Cell Disease Association of America, 2008.
3. Platt OS, Brambilla DJ, Rosse WF, et al., Mortality in sickle cell disease. Life expectancy and risk factors for early death, *N Engl J Med*, 1994;330(23):1639–44.
4. Steinberg MH, Management of Sickle Cell Disease, *N Engl J Med*, 1999;340(13):1021.
5. Balkaram B, Char G, Morris JS, et al., Stroke in a cohort of patients with homozygous sickle cell disease, *J Pediatr*, 1992;120:360.
6. Westerman MP, Cole ER, Wu K, The effect of spicules obtained from sickle red cells on clotting activity, *Br J Haematol*, 1984;56:557–62.
7. Stein PD, Beemath A, Meyers FA, et al., Deep venous thrombosis and pulmonary embolism in hospitalized patients with sickle cell disease, *Am J Med*, 2006;119:897–911.
8. Kaul DK, Nagel RL, Chen D, et al., Sickle erythrocytes-endothelial interactions in microcirculation: the role of von Willebrand factor and implications for vasoocclusion, *Blood*, 1993; 81:2429-38.
9. Schnog JJ, Hovinga JA, Krieg S, et al., ADAMTS-13 activity in sickle cell disease, *Am J Hematol*, 2006;81:492–8.
10. Wick TM, Moake JL, Udden M, et al., Unusually large von Willebrand factor multimers preferentially promote young sickle and nonsickle erythrocyte adhesion to endothelium cells, *Am J Hematol*, 1993;42:284–92.
11. Krishnan S, Siegel J, Pullen G, et al., Increased von Willebrand factor antigen and high molecular weight multimers in sickle cell disease associated with nocturnal hypoxemia, *Thromb Res*, 2008;122:455–8.
12. Kaul DK, Fabry ME, In vivo studies of sickle red blood cells, *Microcirculation*, 2004;11:153–65.
13. Makis AC, Hatzimichael EC, Bourantas KL, The role of cytokines in sickle cell disease, *Ann Hematol*, 2000;79:407–13.
14. Vischer UM, Jornot L, Wollheim CB, et al., Reactive oxygen intermediates induce regulated secretion of von Willebrand factor from cultured human vascular endothelial cells, *Blood*, 1995;85:3164–72.
15. Rother RP, Bell L, Hillmen P, et al., The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease, *JAMA*, 2005; 293:1653–62.
16. Wick TM, Moake JL, Udden MM, et al., Unusually large von Willebrand factor multimers increase adhesion of sickle erythrocytes to human endothelial cells under controlled flow, *J Clin Invest*, 1987;80:905–10.
17. Hebbel RP, Boogaerts MA, Eaton JW, et al., Erythrocyte adherence to endothelium in sickle-cell anemia. A possible determinant of disease severity, *N Engl J Med*, 1980;302:992–5.
18. Joneckis CC, Ackley RL, Orringer EP, et al., Integrin a4b1 and glycoprotein IV (CD36) are expressed on circulating reticulocytes in sickle cell anemia, *Blood*, 1993;82:3548–55.
19. Roberts DD, Rao CN, Liotta LA, et al., Comparison of the specificities of laminin, thrombospondin, and von Willebrand factor for binding to sulfated glycolipids, *J Biol Chem*, 1986; 261:6872–7.
20. Guchhait P, Lopez JA, Thiagarajan P, Characterization of autoantibodies against sulfatide from a V-gene phage-display library derived from patients with systemic lupus erythematosus, *J Immunol Methods*, 2004;295:129–37.
21. Wagner DD, Cell biology of von Willebrand factor, *Ann Rev Cell Biol*, 1990;6:217–46.
22. Furlan M, Lämmle B, Aetiology and pathogenesis of thrombotic thrombocytopenic purpura and haemolytic uraemic syndrome: the role of von Willebrand factor-cleaving protease, *Best Pract Res Clin Haematol*, 2001;14:437–54.
23. Pendu R, Terraube V, Christophe OD, et al., P-selectin glycoprotein ligand 1 and beta2-integrins cooperate in the adhesion of leukocytes to von Willebrand factor, *Blood*, 2006;108:3746–52.
24. Zhou Z, Han H, Cruz MA, et al., Hemoglobin regulation of von Willebrand factor proteolysis by ADAMTS13: a mechanism associated with sickle cell disease, *Thromb Hemost*, 2009;101:1070–7.
25. Naumann HN, Diggs LW, Barreras L, et al., Plasma hemoglobin and hemoglobin fractions in sickle cell crisis, *Am J Clin Pathol*, 1971;56:137–47.
26. Reiter CD, Wang X, Tanus-Santos JE, et al., Cell-free hemoglobin limits nitric oxide bioavailability in sickle cell disease, *Nat Med*, 2002;8:1383–9.
27. Batle J, Lopez-Fernandez MF, Lopez-Borrasca A, et al., Proteolytic degradation of von Willebrand factor after DDAVP administration in normal individuals, *Blood*, 1987;70:173–6.
28. Zhang X, Halvorsen K, Zhang CZ, et al., Mechanoenzymatic cleavage of the ultralarge vascular protein von Willebrand factor, *Science*, 2009;324:1330–4.