Von Willebrand Disease

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.

Keywords

Extracellular hemoglobin (ECHb), Hb-bound VWF (HbVWF) multimers, sickle cell disease (SCD)

Disclosure: Zhou Zhou, MD, PhD, has no conflicts of interest to declare. Prasenjit Guchhait, PhD, has received research funding from the American Heart Association.

Acknowledgment: The authors acknowledge Molly Behymer’s technical assistance for this work.

Received: March 16, 2010 Accepted: July 28, 2011 Citation: US Oncology & Hematology, 2011;7(2):150–2. DOI: 10.17925/OHR.2011.07.2.150

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Sickle cell disease (SCD) is a common hemolytic anemia caused by a single gene mutation in the β-subunit of hemoglobin (Hb),7 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.7 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.5 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.8

Investigations have also suggested that clinical events such as thromboembolisms and pulmonary hypertension are also associated with the short-life expectancy of SCD patients.12 Microvascular thrombus formation is another clinical event that also occurred during crisis in SCD patients.6,11 Increasing evidence suggests that elevated von Willebrand factor (VWF) antigen,6,8 including ultralarge (UL) multimers,12,13 is associated with the development of complications such as vasoocclusive pain crises, thrombosis, and ischemic strokes in SCD.14,15 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)-6, IL-8, IL-10,10 and mediators such as superoxided and cell-free Hb.15 The inflamed endothelium constitutively secretes VWF to maintain a high antigen level in the plasma of patients. Several in vitro6,17 and in vivo18 studies have suggested a crucial role of VWF in sickle-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. An ex vivo experiment has shown that VWF secreted from desmopressin-stimulated endothelium in wild-type mice agglutinates infused sickled-RBCs in microvessels; the agglutination was significantly inhibited by an anti-VWF antibody. VWF binds potently to the α4β3 integrin and sulfatide15,20 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,6,11 are known to
aggregate platelets spontaneously by forming high-strength bonds with its platelet-receptor glycoprotein Ib (GPIb). 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 α4β1 receptors on leukocytes. Thus, VWF multimers contain all of the determinants necessary for RSCs, 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, healthy conditions. 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. 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. Therefore, we speculated that a similar mechanism might exist in vivo under pathophysiological conditions in SCD. The excessive ECHb in the plasma of patients 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. 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. 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. 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. Thus, the metalloprotease converts ULVWF to smaller circulating multimers with a wide range of size distributions. 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](image)

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

<table>
<thead>
<tr>
<th>Plasma Level of Hb-bound VWF</th>
<th>Normal</th>
<th>SCD</th>
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<tbody>
<tr>
<td>NI-NTA Assay</td>
<td>9.4±2.8 (%)</td>
<td>21.2±4 (%)</td>
</tr>
<tr>
<td>ELISA Assay</td>
<td>0.45±0.08 (OD)</td>
<td>1.05±0.13 (OD)</td>
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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 hemoglobin (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-Hb 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 Hb-bound VWF multimers in the plasma of SCD patients.

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

### A. VWF Cleavage

<table>
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<th>Hb-bound VWF (%)</th>
<th>Hb-free VWF (%)</th>
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<tr>
<td>18±6</td>
<td>87±6</td>
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### B. VWF Activity

| RCof assay | 117±6±9.3 | 79.3±13 |
| CBA assay  | 133±4±8.6 | 100±7.3 |

Panel A: The hemoglobin (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 ADAMTS13 (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 (HDWVF) multimers from plasma. We immunoprecipitated HDWVF multimers from plasma using either an anti-VWF or anti-Hb antibody. We also isolated HDWVF multimers from plasma using a
superose gel-filtration and nickel-nitrioltriacetic 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 aggregation (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.