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Increased von Willebrand Factor Antigen and High Molecular Weight Multimers in Sickle Cell Disease associated with Nocturnal Hypoxemia

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Introduction

Sickle cell disease (SCD) is a chronic hemolytic anemia characterized by episodic, localized, vascular occlusion termed “vaso-occlusive or painful crises”. Hypoxia causes oxyhemoglobin desaturation and sickle hemoglobin polymerization resulting in sickling of erythrocytes, and activation of platelets [1] and endothelium[2]. *In vitro* studies have shown that hypoxia is a strong agonist for endothelial release of von Willebrand factor (vWF)[3] including ultra large vWF (ULvWF) multimers[4]. vWF is a large multimeric glycoprotein critical for normal platelet function in primary hemostasis, its high molecular weight (HMW-vWF) multimers being the most hemostatically active subunits[5]. Sparse information is available regarding the relationship between vWF and clinical hypoxemia. Pinsky et al. demonstrated an increased proportion of HMW-vWF multimers during the acute hypoxia/ischemic period associated with aortic cross-clamping during coronary bypass surgery[6]. One recent report documents a correlation between increased vWF antigen (vWF:Ag) levels and severity of chronic hypoxemia in patients with COPD[7]. In recent years, SCD pathophysiology has been recognized to extend beyond the direct effects of polymerization of sickle hemoglobin, with vaso-occlusion resulting from complex interactions between sickle erythrocyte-endothelial adhesion, platelet activation, and the participation of adhesinogenic ligands, including vWF in the adhesive process[8]. Elevated vWF:Ag levels have been previously noted in steady state SCD and during vasoocclusive crises (VOC) [9] and several lines of experimental evidence suggest a pro-adhesive role for ULvWF multimers in sickle RBC-endothelial interactions [10,11]. We therefore sought to evaluate, in a pilot study, whether children and adolescents with documented nocturnal hypoxemia demonstrated differences in their vWF profiles that could potentially modulate the occurrence of vaso-occlusive events.

Methods

19 children with SCD (3–19 years) at St. Christopher's Hospital for Children were enrolled in an investigation of sleeping hypoxemia. Blood samples and pulse oximetry were obtained > 10 weeks post-transfusion, and > 2 weeks following acute illnesses/VOC. Exclusion criteria included chronic transfusions or hydroxyurea treatment. Blood samples were also obtained from ten age-matched controls. The study was approved by the Institutional Review Board and informed consent obtained. Waking blood oxygen saturations (SaO₂) were recorded by pulse oximetry, followed by overnight recordings at home (Scout 4500, In Vivo Research, Florida). The next morning, waking SaO₂s were repeated, histograms showing proportion of time spent at various SaO₂s constructed, and mean sleeping SaO₂s (averaged over time) calculated. The clinical assessment of hypoxemia in SCD is complicated by the right-shifted oxygen dissociation curve, a physiological adaptation to the chronic anemia of SCD. However, oxyhemoglobin desaturation (low SaO₂) in SCD patients at steady state could also reflect: (i) a state of chronic increased metabolic demand and oxygen utilization with limited reserve for further increases in oxygen demand and (ii) increased intra-erythrocytic HbS polymer concentration and risk for sickling. Accordingly Needleman et al. have shown spectrophotometric measurements of SaO₂ such as pulse oximetry, are accurate in SCD [12]. Therefore we have divided our patients in two groups based on sleeping SaO₂s. Using data from previous studies in children [13], those with SaO₂ <94% were assigned to the hypoxemic group (n=10); those with sleeping >94% comprised the normoxemic group (n=9). **vWF Profile:** (i) Platelet-poor citrated plasma was prepared and stored at -80 °C. (ii) **vWF Antigen (vWF:Ag)** was quantified by an automated immunoturbidometric assay (Instrumentation Laboratory, Massachusetts). (iii) **vWF Ristocetin Cofactor activity (vWF:RCO)** was measured on the Chrono-Log 480VS aggregometer (Chrono-Log, Pennsylvania) (iv) **Modified vWF Multimer analyses** was carried out as previously described [14,15]. Blots were then incubated with rabbit anti-human vWF polyclonal antibody (Dako, California) followed by anti-rabbit IgG-HRP conjugate (GE Healthcare, New Jersey), developed using ECL chemiluminescence (GE Healthcare, New Jersey). X-ray images were scanned and analyzed [16]. Optical density values representing the concentrations of vWF multimers of different molecular weights were plotted as densitographs using NIH ImageJ software (Table); individual peaks on the densitographic plot were identified as being separated by a clear trough between 2 adjacent peaks. All peaks beyond peak 10 (representing the 10th mer) were analyzed collectively as one peak "HMW-vWF multimers" ("X" in PNP, "A" in Hypoxemia, Table). Area under the curve was quantified by total pixel count (ImageJ); pixel count obtained for area under the curve for the collective >10-mer peak in pooled normal plasma (PNP) was used to normalize the proportion of HMW-vWF in each patient sample analyzed on the same gel. Differences in protein-loading between PNP and sample lanes were normalized by comparing the total area under the curve for each PNP-sample pair.

Statistical analysis

Multiple group comparison was done using one-way ANOVA. Association between any two variables was tested with the Pearson test (Sigmastat -Jandel Scientific, California).

Results

One way ANOVA demonstrated significant differences in mean vWF:Ag (p=0.007; hypoxemia > normoxemia and controls) (Table). Of interest, we found significant correlations between nocturnal hypoxemia and vWF measurements. As depicted in Figure 1A and B, vWF:Ag levels correlated inversely with SaO₂ (r = -0.54, p = 0.01) and % HMW-vWF multimers also showed an inverse correlation with oxygen saturation (r=-0.62, p= 0.03). No difference in Blood group O representation was noted between the two SCD cohorts. While

mean vWF:RCo activity was higher in the hypoxemic group, this increase was not statistically significant. No correlation was noted between vWF:Ag levels and platelet counts.

Discussion

In this pilot study of extended vWF testing in children and adolescents with SCD in steady state, we demonstrate differences in vWF profiles in the presence of nocturnal hypoxemia. Normoxemic children and adolescents with SCD had vWF profiles that were similar to age matched controls. In contrast, sleep hypoxemia was associated with significant increases in vWF:Ag and a higher proportion of the more “active” HMW-vWF multimers. Our findings appear relevant in light of results from two recent studies: (i) In SCD children without prior history of stroke screened with overnight oximetry and CNS imaging, nocturnal hypoxemia is significantly associated with the risk of CNS events including stroke[17] (ii) children with low nocturnal oxygen saturation have a higher rate of painful crises requiring hospitalization[18].

Wick et al. first demonstrated that ULvWF multimers increased sickle RBCs adhesion to endothelial cells under flow conditions *in vitro*[9]. Using *ex vivo* rat mesoecum preparations, Kaul et al. showed that Desmopressin (an inducer of endothelial vWF release) increased sickle erythrocyte-endothelial adhesion[10]. Heterogenous adhesive complexes (including RBC-adhesive ligand-endothelial cell) contribute to sickle RBC adhesion. The functional significance of vWF as an adhesive ligand in such an interaction is highlighted by experimental inhibition of sickle-RBC –vWF-endothelial adhesion by monoclonal antibodies to $\alpha V\beta 3$, an endothelial integrin that appears to bind the vWF-erythrocyte complex [19]. Our novel clinical data, taken together with previously published experimental evidence, suggest a possible pathophysiological role for vWF: i.e. the increased presence of the more functionally active HMW-vWF multimers[5] with potential pro-coagulant and pro-adhesive effects. They thus provide a basis for further examination of hypoxemia-driven vWF-mediated pathophysiology in SCD.

Few clinical studies have systematically evaluated the role of vWF in the clinical complications of SCD. To our knowledge there is only one other published study [20] that performed extended vWF testing in a heterogeneous, adult group of SCD patients (n=14, mean age =29 years). This study also confirmed the observation (Hagger et al. [9]) of increased vWF:Ag levels at baseline with further elevations during VOC. The need for investigation of children and adolescents with sleep hypoxemia as a vulnerable sub-population at potentially greater risk for SCD vaso-occlusion is supported by our pilot findings of increased vWF:Ag levels and higher proportion of HMW-vWF multimers - suggestive of an increase in total functional vWF present - that are inversely correlated with SaO₂ (Fig 1). Mechanisms regulating the balance between latent and active vWF or causing undesired activation of vWF are just beginning to be critically assessed in the clinical context of vWF-mediated hypercoagulability [21]. We postulate that our findings in the hypoxemic SCD patient perhaps suggest a deficiency, relative or absolute, of ADAMTS13, the vWF-cleaving metalloproteinase ADAMTS13 that regulates vWF multimer size in physiology. However, mean vWF:RCo (reflecting platelet-vWF interaction) although increased in the hypoxemic cohort of patients, was not statistically significant and we did not further address the functional activity of the increased proportion of HMW-vWF multimers with respect to sickle RBC adhesion in this preliminary study.

In summary, our pilot findings demonstrate concerning evidence of hypoxemia-associated changes in vWF profiles with potential pro-coagulant and pro-adhesive effects upon SCD pathobiology. These observations are of interest both to the clinical hematologist and the translational researcher and support the use of expanded vWF testing as a tool to evaluate clinical pathology in the setting of hypoxemia-mediated vascular pathology.

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Fig 1A

Fig 1B

Figure 1.
Relationship between pulse oximetry and vWF in SCD

Table 1

vWF profile in children and adolescents with SCD : Differences related to nocturnal hypoxemia

	Oxygen Saturation (SaO ₂) [Pearson's Correlation (r)]	Controls Mean (SD)	SCD Normoxemia Mean (SD)	SCD Hypoxemia Mean (SD)
vWF:Ag	r= -0.54, p=0.01	127.5 (41.5)	120.8 (30.9)	191.9(69.6)* *p=0.007
vWF:RCo	r= -0.36, p=0.14	119.5 (44)	116.7 (36.8)	147.7 (71.3)
%HMW-vWF (densitographic analysis)	r= -0.62, p=0.03

Depicts mean (1SD) values for vWF:AG and vWF:RCo in control and SCD groups evaluated.

Normal range for vWF:Ag in our laboratory is 51% to 163% and for vWF:RCo is 49% to 166% (adults).

Representative densitographs: Total pixel count obtained for area under the curve HMW-vWF multimers peak (X; A) from patient samples were normalized by control pooled normal plasma lane (PNP) value; protein-loading differences between sample and PNP were normalized by comparing total area under the curves