Erythrocyte Aggregation and Blood Viscosity is Similar in Homozygous Sickle Cell Disease Patients with and without Leg Ulcers

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Abstract

Background There is no consensus regarding the role of red blood cell (RBC) aggregation in the pathogenesis of leg ulcers (LUs) in sickle cell disease (SCD).

Objectives We sought to evaluate whether the cross-sectional determination of RBC aggregation and hematological indices were associated with the presence of LU in homozygous SCD.

Methods Twenty-seven patients with LU and 23 with no history of ulceration were recruited into the study. A laser-assisted rotational red cell analyzer (LoRRca) was used in the determination of the aggregation index (AI), aggregation half-time ($t_{1/2}$), and the RBC aggregate strength (AMP). Hematological indices were determined using a CELL-DYN Ruby analyzer. Whole blood viscosity (WBV) and plasma viscosity (PV) were measured using a Vilastic bioprofiler. The data were presented as means $\pm$ standard deviation or median, interquartile range. Two-sample *t*-test was used to test for associations between the AIs, WBV, and PV in patients with and without LU. Statistical significance was taken as $p < 0.05$. All analyses were conducted using Stata/SE v. 12.1 (StataCorp, College Station, TX).

Results The AI was comparable in the group with and without ulcers (68.6, 16.7 versus 67.7, 16.9; $p = 0.74$); $t_{1/2}$ (1.7, 1.3 versus 1.8, 1.3; $p = 0.71$); AMP (18.8, 14.5 versus 19.1, 13.3; $p = 0.84$), WBV (3.8, 1.2 versus 3.8, 0.7; $p = 0.77$); and the PV (1.3, 0.08 versus 1.4, 0.1; $p = 0.31$) and were also not statistically different between the groups of participants.

Conclusion RBC aggregation and aggregate strength are not associated with leg ulceration in SCD.

Keywords

- red blood cell (RBC)
- aggregation
- aggregation index (AI)
- leg ulcer (LU)
- sickle cell disease (SCD)
- hemorheology

Sickle cell disease (SCD) is characterized by a diverse pathophysiology including abnormal erythrocyte morphology upon deoxygenation,¹ a pro-inflammatory phenotype,² and abnormal rates of hemolysis.³ These mechanisms in turn are associated with many SCD comorbidities, including SCD leg ulcers (LUs), which affect the dorsum of the foot and the shin.^{3–5} In Jamaica, the prevalence and cumulative incidence of LU are estimated at 29.5 and 16.7%, respectively.³ A reduction in

hemoglobin concentration,⁶ high leucocyte counts,³ and poor socioeconomic status have been suggested as further correlates of LU in people with SCD.

Erythrocyte aggregation is believed to serve homeostatic functions under normal conditions.⁷ It may, however, become upregulated with diseases and infectious states due to a rise in the plasma concentration of acute-phase proteins such as fibrinogen.^{8–10} One theory of red blood cell (RBC) aggregation

posits a fibrinogen-mediated bridging of sialic acid residues on the surfaces of neighboring erythrocytes.¹¹ The resulting structures, termed rouleaux, promotes vascular stasis especially in areas of low-shear blood flow.^{12–14} Indeed, low-shear micro-circulatory flow is characteristic of SCD rheological behavior and is suggested to bear some association with a preponderance of oxidant-mediated etiology.^{15–20} The inherent susceptibility of RBCs to oxidant stress coupled with the deoxygenation-related increases in the membrane fragility of sickle RBCs could underlie some aspects of the abnormal flow profile seen in this condition.^{15,17,19} In spite of these findings, the kinetics of RBC aggregation in SCD and its role on blood flow dynamics *in vivo* is still poorly understood. Moreover, the available literature regarding the relationships between whole blood viscosity (WBV) and RBC aggregation in SCD patients with LU is confounding and warrants further clarification.^{6,21,22}

We therefore investigated whether there was an association between the presence of LU and low-shear WBV, plasma viscosity (PV), and RBC aggregation indices in Jamaican SCD patients with and without LUs using a laser-assisted rotational red cell analyzer and viscometry (LoRRca, Mechatronic, the Netherlands).

Design and Methods

Subjects

The study was cross-sectional. Prospective volunteers were identified from the Sick Cell Unit's (SCU) patient management system database and recruited during the steady state at either the SCU, Tropical Metabolism Research Institute, University of the West Indies, Mona Campus while they were waiting to be seen by their physician or via telephone. Written informed consent was obtained from those who agreed to participate before enrolment into study. Patients who gave verbal consent by telephone were visited in their homes where they gave written consent and donated blood samples. The study included 23 participants with homozygous SCD and LU and 17 participants with SCD but no history of LU.

The minimum age for enrolment into the study was 18 years old. The LU group included patients with an active ulcer which affected the malleolar region of the lower leg, the dorsum of the foot, and/or the shin. Steady state was defined as not having had a blood transfusion within the previous 4 months and the absence of acute pain episodes for at least 1 month prior to enrolment into the study. Patients were excluded if they had documented cardiovascular, liver, or renal complications, were presently on hydroxyurea therapy, or were pregnant or breast-feeding.

Sample Collection

Five milliliters of venous blood was withdrawn from the antecubital vein into a Vacutainer test tube containing 0.057 mL of 15% solution of potassium ethylenediaminetetraacetic acid (K^+ EDTA) for use in hematological and rheological measurements. All samples were oxygenated before hemorheological measurements.²³ There was complete adherence to the guidelines for hemorheological and laboratory procedures.²⁴

Aggregation Index

All aggregation measurements were conducted at a standardized temperature of 37°C. A LoRRca (Mechatronics) was used in the determination of the aggregation index (AI).²² All samples were adequately oxygenated before investigation.²⁴ The LoRRca determined the AI by syllectometry; it assessed the relationship between laser backscatter versus time to determine an AI. The AI approximates the extent of RBC aggregation and was recorded as a percent (%)—an increase in the AI correlated with a rise in RBC aggregation. The magnitude of the syllectogram (AMP) was recorded in arbitrary units (au) and approximated the strength of RBC aggregates. The minimal shear rate required to disperse RBC aggregates, the aggregation halftime ($t_{1/2}$), or the disaggregation threshold was determined by a re-iterative process.

Whole Blood and Plasma Viscosity

A Vilastic bioprofiler (Vilastic Scientific Inc., Austin, TX) was used to determine PV and WBV.²⁵ The bioprofiler has the capacity to measure WBV in sample sizes between 0.5 and 1 mL. Its vessel-like geometry allowed for more precise simulation of *in vivo* conditions. Since RBC aggregation is generally a low-shear mechanism, WBV was investigated at a low shear rate of 2.51/second, while the PV was investigated at 37°C.

Statistical Analyses

Normally distributed descriptive data were presented as means and standard deviations and skewed data as medians and interquartile range. Two-sample *t*-test and the Kruskal-Wallis equality-of-populations tests were used to test for significant differences in the predictor variables between patients with active ulcers and those without a history of LUs.

Results

WBV at native hematocrit was similar in SCD patients with and without LUs at a shear rate of 2.51/second. PV at 37°C was also comparable in subjects with ulcers and those who have never had LUs. RBC aggregation, the AI, and the aggregation halftime, $t_{1/2}$, were not statistically different in patients with and without LU. Moreover, the disaggregation threshold and the RBC aggregate strength, AMP, were similar in both groups of patients (►Table 1).

Discussion

RBC aggregation determined as the AI was comparable in SCD subjects with and without LUs. Similarly, the shear force required to disperse RBC aggregates or to prevent the formation of new aggregates, $t_{1/2}$, and the RBC aggregate strength, AMP, were similar in both groups of participants. These findings were consistent with earlier reports involving 13 SCD patients with a history of ulceration and 62 patients with no records of ever having had a LU.²² Furthermore, the comparability of the low-shear WBV and the PV between subjects with and without LU were in accordance with the RBC aggregation data. Noteworthy, the relatively small sample data documented herein were the results of pilot

Table 1 The aggregation index, plasma viscosity at 37°C, and low-shear whole blood viscosity at the native hematocrit in SCD patients with and without leg ulcers

Parameter	No history of ulcers (<i>N</i> = 17)	Active ulcers (<i>N</i> = 23)
AI (%)	67.67, 16.94	68.61, 16.73, <i>p</i> = 0.74
<i>t</i> _{1/2} (/s)	1.79, 1.32	1.74, 1.25, <i>p</i> = 0.71
AMP (au)	19.14, 13.3	18.78, 14.5, <i>p</i> = 0.84
Plasma viscosity (mPa.s)	1.39 ± 0.14	1.34 ± 0.08, <i>p</i> = 0.31
WBV at 2.51/s (mPa.s)	3.84 ± 0.76	3.84 ± 1.23, <i>p</i> = 0.77

Abbreviations: AI, aggregation index; AMP, amplitude; au, arbitrary unit; SCD, sickle cell disease; WBV, whole blood viscosity.

Note: Median, interquartile range; mean ± standard deviation. Statistical significance taken at *p* < 0.05.

investigations of an ongoing project. The diverse heterogeneity of SCD could have obscured the statistical strength of the various outcomes evaluated due to issues related to selection bias.

The present findings were inconsistent with the established vascular dysfunction associated with SCD, manifested as chronic inflammation,^{6,21} hypercoagulability,²⁶ and vasoocclusion.^{27–29} It is unknown whether environmental stimuli are influential in *in vivo* RBC aggregation processes. Painful crises, arguably the most common clinical expression of SCD, are favorably precipitated by cold climatic conditions.^{30,31} Nonetheless, there is evidence which demonstrates an increase in RBC aggregation velocities in response to a rise in temperature.^{32,33} In any case, the *in vitro* determination of the AI described herein would negate any meaningful temperature-related effects since the aggregometer temperature was standardized throughout the course of the study.

There is evidence to suggest that subjects with SCD and LU have a higher disease severity than those without LU as defined by certain pathogenic mechanisms, viz, increased red cell adhesiveness,^{18,19} increased hemolysis and reticulocyte counts,³ and higher concentrations of inflammatory biomarkers,⁶ all of which can lead to increased RBC aggregation by reducing the spatial distribution among adjacent RBCs. Acute-phase reactants such as fibrinogen and C-reactive protein are known to influence the protein-mediated non-Newtonian kinetics of blood by enhancing cell–cell interactions.^{34–36} It is reasonable to surmise that these mechanisms can lead to hypoperfusion in the microcirculatory beds of the skin covering the bony malleolus. However, owing to the chronicity of SCD, and the resilience of LU in SCD, the expression of acute plasmatic markers of disease severity may not be expected to vary widely among subpopulations of SCD.

Circulating concentrations of hemolytic markers were not evaluated herein, but could have provided more clues regarding the observed aggregation indices. It was previously suggested that lactate dehydrogenase, a hemolytic biomarker, had a significant positive association with the presence of LU in people with SCD.³ While chronic hemolysis and an inflammatory phenotype could foment a proulcer environment as evidenced by several hemorheological and physiological disturbances,^{6,16,22} it does not appear that RBC aggregation is similarly impacted in these individuals. This gives some credence to the assertion that LU in SCD may be

categorized into the hemolytic phenotype of SCD and not the vaso-occlusion phenotype which, instead, is marked by painful crises and the acute chest syndrome of SCD.¹⁶

The ulcer scar is susceptible to invasion by both aerobic and anaerobic bacterial flora^{37,38} and is thus an important source of infection. Notwithstanding the comparability in RBC aggregation between the SCD groups, we previously observed an upregulation in the concentration of the inflammatory cytokine IL-1β in homozygous SCD patients with LU compared with genotype-, age-, and sex-matched controls.⁶ The tendency toward abnormal RBC flow properties and the progression of LU in SCD may further be exacerbated in persons with existing vasculopathy such as incompetent calf veins or vascular abnormalities related to postural vasoconstrictor pathways.^{39,40}

We have shown that RBC aggregation assessed by the AI, AMP, and *t*_{1/2} was comparable in SCD subjects with and without LU. WBV and PV were also similar between the groups. These findings suggest that erythrocyte aggregation and blood fluidity are not associated with LU in SCD. Instead, blood flow disturbances related to the lowered flexibility of the RBC membrane may have a stronger impact on the pathogenesis of LU in persons with SCD.

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