Red blood cell aggregation, aggregate strength and oxygen transport potential of blood are abnormal in both homozygous sickle cell anemia and sickle-hemoglobin C disease

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ABSTRACT

Background

Recent evidence suggests that red blood cell aggregation and the ratio of hematocrit to blood viscosity (HVR), an index of the oxygen transport potential of blood, might considerably modulate blood flow dynamics in the microcirculation. It thus seems likely that these factors could play a role in sickle cell disease.

Design and Methods

We compared red blood cell aggregation characteristics, blood viscosity and HVR at different shear rates between sickle cell anemia and sickle cell hemoglobin C disease (SCC) patients, sickle cell trait carriers (AS) and control individuals (AA).

Results

Blood viscosity determined at high shear rate was lower in sickle cell anemia (n=21) than in AA (n=52), AS (n=33) or SCC (n=21), and was markedly increased in both SCC and AS. Despite differences in blood viscosity, both sickle cell anemia and SCC had similar low HVR values compared to both AA and AS. Sickle cell anemia (n=21) and SCC (n=19) subjects had a lower red blood cell aggregation index and longer time for red blood cell aggregates formation than AA (n=16) and AS (n=15), and a 2 to 3 fold greater shear rate required to disperse red blood cell aggregates.

Conclusions

The low HVR levels found in sickle cell anemia and SCC indicates a comparable low oxygen transport potential of blood in both genotypes. Red blood cell aggregation properties are likely to be involved in the pathophysiology of sickle cell disease: the increased shear forces needed to disperse red blood cell aggregates may disturb blood flow, especially at the microcirculatory level, since red blood cell are only able to pass through narrow capillaries as single cells rather than as aggregates.

Key words: sickle cell disease, red blood cell aggregation, viscosity, red blood cell deformability.

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Introduction

Red blood cell (RBC) deformability is known to be reduced in patients with sickle cell anemia (SCA; SS genotype) and sickle cell hemoglobin C disease (SCC; SC genotype).[1-3] When deoxygenated in certain areas of the circulatory system, these rigid, distorted RBC can prompt vascular occlusions leading to painful crises. Although the genetic mutation is well described and is universal in all patients with SCA, the clinical severity and complications of the disease show significant inter-individual variability. The underlying pathomechanisms for this observation are not yet understood, but studies suggest the importance of hemorheological factors other than reduced RBC deformability.[1,4] For example, increased RBC aggregation, elevated blood viscosity and the ratio of hematocrit (Hct) to blood viscosity (HVR, an index to the oxygen transport potential of blood) have been shown to significantly affect *in vivo* microcirculatory flow dynamics.[5-7] In a recent study, Alexy *et al*. described impaired HVR in patients with SCA and suggest that the reduced HVR might play a role in the pathophysiology of the disease.[8] Unfortunately, similar studies are lacking for patients with SCC and sickle cell trait (AS).

The present investigation was designed to evaluate and compare hematologic and selected hemorheological parameters among healthy controls (AA) and individuals with SCA, SCC and AS genotypes. Particular emphasis was directed toward differences between RBC aggregation parameters and HVR.

Design and Methods

Subjects and blood sampling

Twenty-one patients with SCA (SCA group; hemoglobin S concentration: 82.9±5.6%), 21 patients with sickle cell hemoglobin C disease (SCC group; hemoglobin S and C concentrations: 47.9±2.6% and 45.2±3.1%, respectively), 33 sickle trait carriers (AS group, hemoglobin S concentration: 38.4±2.2%) and 52 subjects with the normal AA genotype (control group) were enrolled in the present study. Controls were matched for age, gender and ethnicity with the other groups. All patients were in their steady state condition and had not been transfused or in crisis for at least 90 days prior to enrollment. Patients with α thalassemia and on hydroxyurea therapy were excluded from the present study since they are known to have abnormal RBC rheology.[9-12] All subjects provided informed consent and the ethics committee of the Academic Hospitals of Pointe-à-Pitre approved the study.

To test for the hemoglobin variant, venous blood was drawn into tubes containing EDTA and screened by isoelectric focusing. Results were confirmed by citrate agar electrophoresis. Hemoglobin variants were isolated and quantified by high performance liquid chromatography (HPLC). Solubility test was also performed to confirm the presence of HbS. To test for α thalassemia, we used the technique described by Chong *et al*.[13] with a single-tube multiplex-PCR assay that is capable of detecting any combination of the six common single and double gene deletions in α thalassemia.

From each participant 7 mL of venous blood was collected; 5 mL of which were drawn into a vacuum tube containing EDTA (1.5 mg/mL) and were used for the hematologic and hemorheological tests, and 2 mL were collected into 3.2% citrate to measure plasma fibrinogen concentration. All samples were carefully oxygenated according to the method described by Hardeman *et al*. prior to measurements.[14]

Hematologic parameters

The following hematologic parameters were determined using an automated hematology analyzer (Max M-retic, Coulter, USA): hemoglobin concentration (Hb), Hct, mean cell volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and percent reticulocytes (% Ret). Plasma fibrinogen was determined using the Clauss method.

Hemorheological parameters

Blood viscosity (ηb) was measured at native hematocrit at room temperature (≈25°C) using a cone-plate viscometer (Brookfield DVII+ with CPE40 spindle) at shear rates of 45 s^{-1}, 90 s^{-1} and 225 s^{-1}. HVR was calculated using the blood viscosity data obtained at each of the three different shear rates and plotted as a function of Hct. A polynomial curve fitting procedure was employed to determine the Optimal Hct value (i.e., the Hct with the highest calculated HVR value) for each patient group at each shear rate.

Due to an initial lack of suitable instrumentation, RBC aggregation and deformability were determined for a sub-set of the patients: 21 SCA, 19 SCC, 15 AS and 20 AA individuals. RBC deformability was measured at 37°C using a Laser assisted Optical Rotational Cell Analyzer (LORCA, RR Mechatronics, Hoorn, The Netherlands). Based on laser diffraction patterns obtained at user-defined shear stress values (3 and 30 Pa in the present study), the LORCA system determines RBC elongation under shear and reports an elongation index (EI) that increases with increased RBC deformability.[15]

In addition to measuring RBC deformability, the LORCA system was also utilized to determine RBC aggregation. For this test, the Hct of each sample was adjusted to 40% by the appropriate combination of RBC and autologous plasma. In one mode of operation, the sample is initially sheared to disperse pre-existing aggregates, following which the shear is abruptly reduced to zero. Three indices of RBC aggregation are then measured: (i) total change in aggregation signal (AMP); (ii) time required for half maximal change in aggregation signal (aggregation half time; $t_{1/2}$); (iii) the extent of RBC aggregation (aggregation index; AI). In the other mode, the shear rate is varied in a defined sequence of steps to determine the minimal shear rate (γ_{min}) required to disperse RBC aggregates.[14]

Statistical analysis

Results are presented as mean ± SD. Hematologic and hemorheological parameters were compared between controls and the study groups using one-way analysis of variance (ANOVA) and the post-hoc Tukey test. All statistical tests were performed using Statistica (v. 5.5, Statsoft, USA) and the significance level was defined as $p<0.05$.

Results

Hematologic parameters

In agreement with reports in literature, Hb and Hct values followed the rank order of SCA<SCC<AS=AA and the proportion of reticulocytes showed a reverse trend (SCA>SCC>AA=AS) (Tables 1 and 2). MCV was very similar for the AA, AS and SCA groups but significantly lower for SCC subjects. MCHC was significantly elevated in all patient groups when compared to the controls with the following rank order: AA<AS<SCA<SCC. It is interesting to note that the highest MCHC value was for the SCC group (Table 1). There were no significant differences in plasma fibrinogen concentration between the study groups (Table 3).

Hemorheological parameters

Average blood viscosity of the SCA group was significantly less than the other groups at all three shear rates (Table 2). Despite the considerable difference in their average Hct, the viscosity values for the AS and SCC subjects were similar at all shear rates and, except for the lowest shear, they were significantly above the values measured for the AA population.

As shown in Figure 1, no difference in HVR was found between the AA and AS groups at any shear rate. In addition, their calculated HVR values at all shear rates were significantly higher than those for the SCA and SCC groups. Optimal Hct values (i.e., Hct at maximum HVR), obtained via curve fitting HVR versus Hct data, had the following rank order: AA=AS>SCC>SCA (Table 4). While optimal Hct was not influenced by shear rate in AA, AS and SCC subjects, it increased slightly with increasing shear rates in the SCA group.

RBC deformability and aggregation data are shown in Table 3. EI followed the rank order of SCA<SCC<AS=AA at both levels of shear stress. The amplitude of RBC aggregation and aggregation half time ($t_{1/2}$) were the highest in SCC individuals and were the lowest in the AA population. Interestingly, AI values measured for the SCA and SCC groups were significantly below the control values. The minimal shear rate (γ_{min}) required to disperse pre-existing aggregates was similar in patients with SCA and SCC genotypes and were significantly higher than for the AA and AS groups.

Table 1. Selected hematologic parameters.

	AA (n=52)	AS (n=33)	SCC (n=21)	SCA (n=21)
Hb (g/dL)	13.7±1.4	14.4±1.3	12.2±1.4[1,2]	8.2±1.2[1,2,3]
MCV (fL)	86.2±4.5	83.5±4.6	71.8±7.1[1,2]	84.3±6.7[3]
MCHC (g/dL)	33.5±1.2	34.6±0.8[1]	36.5±1.1[1,2]	35.6±1.1[1]
Ret (%)	0.9±0.3	0.9±0.3	3.1±1.2[1,2]	11.9±4.3[1,2,3]

Values represent mean ± SD. [1]different from AA group; [2]different from AS group; [3]different from SCC group.

Table 2. Hematocrit and blood viscosity data.

	AA (n=52)	AS (n=33)	SCC (n=21)	SCA (n=21)
Hct (%)	41.9±4.0	44.3±4.1	33.5±5.2[1,2]	24.5±4.8[1,2,3]
ηb (mPa/s; at 45 s^{-1})	6.81±1.08	7.48±1.0	7.8±1.94[1]	5.12±1.38[1,2,3]
ηb (mPa/s; at 90 s^{-1})	5.80±0.79	6.46±0.86[1]	6.69±1.55[2]	4.54±0.98[1,2,3]
ηb (mPa/s; at 225 s^{-1})	5.10±0.60	5.58±0.70[1]	5.99±1.30[1]	4.18±0.69[1,2,3]

Values represent mean ± SD. [1]different from AA group; [2]different from AS group; [3]different from SCC group.

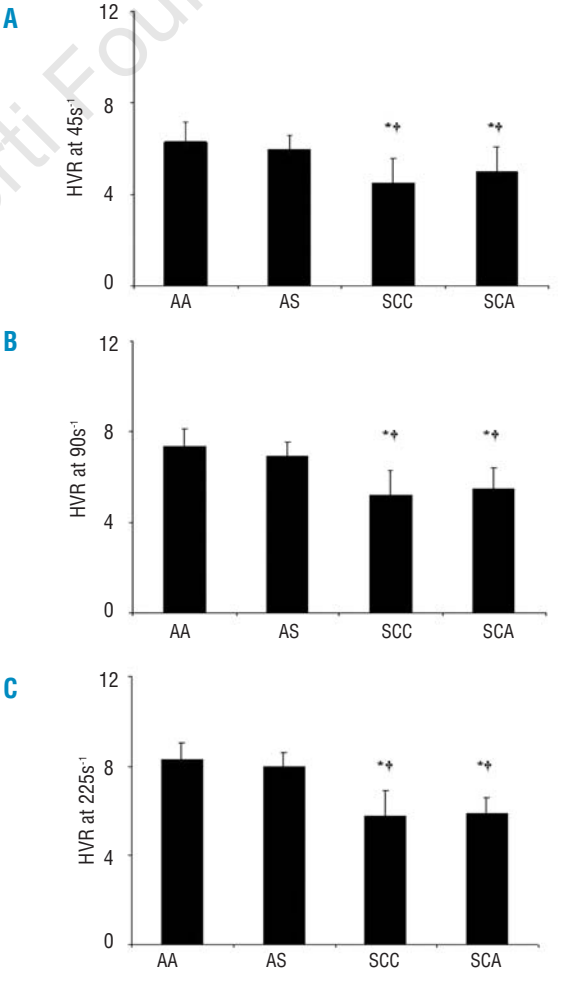

Figure 1. Calculated HVR results at 45, 90 and 225s^{-1}; *different from AA group; †different from AS group.

Discussion

It is well known that RBC deformability is significantly impaired in patients with SCA, even in steady state conditions,[2,3] and our results are consistent with these reports (Table 3). This abnormal rheological behavior is primarily due to the elevated internal viscosity of irreversibly sickled cells and the reduced membrane flexibility of both irreversibly and transiently sickled cells.[2] Our finding of reduced RBC deformability in the SCC group confirms previous data by Ballas *et al.* and may be explained by the presence of severely dehydrated, microcytic and hyperchromic RBC.[1,16] Note that, on average, RBC deformability for the SCC group was slightly better than for SCA patients at both shear stresses.

The significantly lower than control Hct, Hb and blood viscosity values found herein for SCA patients are similar to those reported previously by Serjeant *et al.*[17] and are the result of the ongoing hemolytic process in these subjects. Although SCC patients are also characterized by impaired RBC deformability (Table 3), their anemia is mild and thus reticulocytosis is less prominent; their near-normal Hct combined with reduced RBC deformability leads to markedly elevated blood viscosity. Although we did not investigate the percent of irreversibly sickled cells (ISC), their presence, in combination with individually variable Hct levels, can markedly affect blood viscosity and the occurrence of vaso-occlusive manifestations.[2,18]

Interestingly, our results showed that, except for the lowest shear rate, blood viscosity of AS subjects was similar to that of the SCC group. This is a surprising result because AS is usually considered a benign condition,[19] except when these individuals are subject to stressful conditions such as altitude hypoxia or extreme exercise.[20] However, recent reports by Tripette *et al.*[21,22] suggest that, besides abnormal blood rheology, AS subjects are characterized by the specific activation of L and P-selectins during strenuous exercise. Although microvascular complications are rarely observed in AS individuals, abnormal blood viscosity plus activation of certain selectins may contribute to such microcirculatory disturbances.

A novel finding of our study is that the RBC aggregation parameters $t_{1/2}$, AI and γ_{min} are similar for SCA and SCC patients and that these indices are significantly different from those for the AA and AS groups. In particular, SCA and SCC patients had significantly elevated γ_{min} levels and hence higher than normal shear forces are required to disperse preformed RBC aggregates. The lower extent of aggregation at stasis (AI) found in SCC and SCA patients in combination with the elevated γ_{min} may seem paradoxical. However, Baskurt *et al.*[23] have shown that exposure of RBC to superoxide anions decreases the extent of RBC aggregation but increases γ_{min} due to increased RBC aggregability. The increased γ_{min} observed herein does not seem to be related to plasma fibrinogen concentrations since the four groups had comparable values (Table 3). Fibrinogen is the plasma protein most involved in RBC aggregation and hence the finding of comparable levels suggests a role for cellular factors (e.g., glycocalyx properties).[5] Since RBC in SCD are exposed to increased oxidative stress,[24] it seems possible that such stress may have a role in the specific RBC aggregation pattern observed for SCA and SCC patients.

The pathogenic potential of RBC aggregation within the microcirculation is dependent on the extent of RBC aggregation and the cohesive forces within the aggregate (i.e., the resistance of aggregates to shear-induced disaggregation).[25] This increase of the required shear forces might have important physiological consequences, especially at the microcirculatory level, since RBC are only able to pass through small capillaries as single cells.[5] However, since γ_{min} for SCA and SCC patients were similar, differences of blood viscosity and RBC deformability between the two groups probably play a role in their unequal clinical severity.[26]

Although the forces required to disperse preformed RBC aggregates are elevated in SCA and SCC subjects, the extent of aggregation at stasis (i.e., AI) was lower than control in both groups; this finding contrasts with results obtained by Obiefuna *et al.*[27] on SCA patients but is consistent with results obtained by Chien *et al.*[2] The reasons for the discordant results for aggregation at stasis remain unknown, but may be due to different experimental methods: in the current study defined preshearing of blood samples to disperse pre-existing aggregates was employed whereas it was not part of the microscopic method used by Obiefuna *et al.*[27] Note that the present results indicate that time for formation of RBC aggregates is increased in SCA and SCC patients (Table 3), meaning that the formation of branched 3-dimension aggregates is slower in these subjects. The effects of RBC aggregation on *in vivo* flow dynamics and

Table 3. Red blood cell deformability, aggregation and plasma fibrinogen concentration.

	AA (n=16)	AS (n=15)	SCC (n=19)	SCA (n=21)
EI (at 3 Pa)	0.33±0.02	0.32±0.04	0.17±0.03[1,2]	0.09±0.10[1,2,3]
EI (at 30 Pa)	0.58±0.02	0.57±0.02	0.42±0.06[1,2]	0.33±0.16[1,2,3]
Amp	18.1±3.4	21.9±1.6[1]	26.1±2.5[1,2]	20.3±5.6[2]
$t_{1/2}$ (s)	2.6±1.1	3.0±1.6	4.2±1.7[1]	3.7±1.6
AI (%)	60.7±8.0	58.3±10.3	50.6±9.3[1]	53.2±9.7[1]
γmin (s^{-1})	134.4±47.0	168.3±45.6	382.6±182.9[1,2]	314.8±177.3[1,2]
Fibrinogen (g/L)	3.0±0.5	3.2±0.7	3.6±1.4	3.0±0.5

Values represent mean ± SD. [1]different from AA group; [2]different from AS group; [3]different from SCC group.

Table 4. Calculated optimal hematocrit values for the four study groups at three shear rates.

	45 s^{-1}	90 s^{-1}	225 s^{-1}
AA (n=52)	41.7	41.2	41.8
AS (n=33)	43.6	42.5	42.8
SCC (n=21)	32.8	32.0	32.6
SCA (n=21)	26.4	27.5	29.1

flow resistance are complex[5,28] and further studies are warranted to better understand the association between RBC aggregation parameters and the severity of sickle cell disease. Although some hemorheological parameters differed between SCC and SCA patients, both have markedly reduced and essentially identical HVR levels at all shear rates (Figure 1); our calculated mean HVR values are consistent with the range reported by Serjeant *et al.*[29] Thus the oxygen transport effectiveness of blood in SCA and SCC subjects is lower than in controls, a finding also seen in other disease states.[6] Recent results obtained by Alexy *et al.*[8] also support our findings. They investigated HVR in mixtures of AA and SCA RBC and found that HVR decreased as the proportion of HbS increased; due to the non-Newtonian, shear-thinning flow behavior of blood, HVR also decreased as shear rate decreased. Our results indicating that HVR depends on shear rate confirm this prior report.[8] Note that HVR was not decreased in sickle cell trait carriers, and thus AA and AS groups have nearly identical HVR values (Figure 1). Although AS subjects have elevated blood viscosity as for SCC patients, the hemorheological oxygen transport potential seems to be well preserved in sickle cell trait.

The determination of an Optimal Hct that yields the greatest HVR is of clinical interest. For example, Schmalzer *et al.*,[30] and more recently Alexy *et al.*,[8] reported that the determination of an optimal Hct might be useful for transfusion therapy in SCA, and our viscosity results allowed determination of this optimal value. The present study also suggests an inverse relation between shear rate and the calculated Optimal Hct in SCA patients, whereas it was not shear rate dependent for the AA, AS and SCC groups (Table 4). Such an inverse relation has been previously demonstrated by Alexy *et al.*[8] for moderate to high shear as used in the present study, while for low rates of shear (e.g., 1 to 5 s^{-1}) no optimum was observed. The magnitude of Optimal Hct levels found in our SCA patients corresponds to that reported by Alexy *et al.*[8] and was lower than for SCC patients, AS individuals and the AA group. The results reported by Dupuy-Fons *et al.*[7] suggest that HVR may influence oxygen transfer to tissues, and thus the clinical status of patients may be affected by differences between native and Optimal Hct. Nevertheless, further studies are needed to investigate HVR and Optimal Hct in severe and non-severe SCA and SCC patients in order to address more clearly the clinical usefulness of these parameters in sickle cell disease.

In summary, the present study is the first to focus on the RBC aggregation characteristics and HVR in both SCA and SCC. Although the blood viscosity profile is very different between SCC and SCA, the two genotypes are marked by a low HVR as compared to control subjects and sickle cell trait carriers. The low HVR could play a role in tissue hypoxia and clinical status of patients. The greater minimal shear rate values required to disperse pre-formed RBC aggregates found in both SCA and SCC groups is of primary interest since it may modulate blood flow dynamics in the microcirculation. Nevertheless, further studies are clearly warranted to better understand the relationships between RBC aggregation and the clinical expression of sickle cell disease. Different markers of clinical severity, such as the presence of α thalassemia, the number of non-functional α genes or the hemoglobin haplotype, could aid in defining more accurately the role of blood rheology, and notably of RBC aggregation, in the disease.

Authorship and Disclosures

JT, MDHD and PC served as primary investigators: they elaborated the protocol, participated in measurements, data interpretation and prepared the manuscript. TA and HJM provided input related to blood rheology and helped in developing the final manuscript. DM and MEJ participated in protocol elaboration, patient recruitment and data analysis. EB, TC and RC participated in data collection and interpretation. OH contributed to the statistical analysis and discussion of the manuscript.

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