Human bulbar conjunctival hemodynamics in hemoglobin SS and SC disease

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The known biophysical variations of hemoglobin (Hb) S and Hb C may result in hemodynamic differences between subjects with SS and SC disease. The purpose of this study was to measure and compare conjunctival hemodynamics between subjects with Hb SS and SC hemoglobinopathies. Image sequences of the conjunctival microcirculation were acquired in 9 healthy control subjects (Hb AA), 24 subjects with SC disease, and 18 subjects with SS disease, using a prototype imaging system. Diameter (*D*) and blood velocity (*V*) measurements were obtained in multiple venules of each subject. Data were categorized according to venule caliber by averaging *V* and *D* for venules with diameters less than (vessel size 1) or greater than (vessel size 2) 15 μ m. *V* in vessel size 2 was significantly greater than *V* in vessel size 1 in the AA and SS groups ($P \geq 0.009$), but not in the SC group ($P = 0.1$). *V* was significantly lower in the SC group as compared to the SS group ($P = 0.03$). In AA and SS groups, *V* correlated with *D* ($P \leq 0.005$), but the correlation was not statistically significant in the SC group ($P = 0.08$). *V* was inversely correlated with hematocrit in the SS group for large vessels ($P = 0.03$); however, no significant correlation was found in the SC group ($P \geq 0.2$). Quantitative assessment of conjunctival microvascular hemodynamics in SS and SC disease may advance understanding of sickle cell disease pathophysiology and thereby improve therapeutic interventions. Am. J. Hematol. 88:661–664, 2013. © 2013 Wiley Periodicals, Inc.

Introduction

Sickle cell disease (SCD) is a hereditary hematologic disorder characterized by abnormalities of red blood cells (RBC) as a result of an inherited single amino acid substitution in the beta globin chain of hemoglobin (Hb) [1] assigned to chromosome region 11p1205–11p1208 [2]. The substitution of valine for glutamic acid results in a form of hemoglobin (Hb S) that upon deoxygenation polymerizes into rod like structures that favor the formation of “sickle” shaped erythrocytes [1,3]. Individuals with homozygous variant S alleles, or Hb SS (SS) disease, develop anemia with severe complications typified by chronic hemolysis syndrome [4], as well as pulmonary hypertension and stroke. A heterozygote variant of SCD is characterized by the possession of a Hb S variant allele with another beta globin variant, such as Hb C, in which lysine is substituted for glutamic acid resulting in compound heterozygous Hb SC (SC) disease [5,6].

Both SS and SC disease result in anemia and may significantly limit life span [7]. Complications associated with Hb SS occur with Hb SC albeit at a much lower rate of disease severity [7]. SC disease is generally a milder anemia with less frequent pain crisis compared to SS disease [8]. Conversely, subjects with SC disease are at increased risk for thromboembolic events, renal papillary necrosis, and retinopathy [9–11] compared with SS disease. Subjects with SC disease typically display greater blood viscosity compared to SS hemoglobinopathy in part due to the constellation of increased hematocrit (HCT) and erythrocyte Hb concentration [12,13].

The phenotypic differences between SS and SC diseases may be due to biophysical properties associated with each respective Hb abnormality. Pathologies associated with SS disease are thought to arise secondary to the polymerization of Hb S within erythrocytes because of deoxygenation, and can be increased by hypoxia, hyperosmolarity, and/or acidotic conditions [13,14]. Complications in SC disease are impart due to the contribution of Hb C, which unlike Hb S, is predisposed to intraerythrocytic crystallization when oxygenated, which results in increased cell density due to K^+ efflux and cellular dehydration [12,13,15]. Variability in erythrocyte morphology is a further

distinguishing characteristic between Hb SS and Hb SC [13,16]. Therefore, based on these biophysical differences of erythrocytes, microvascular flow properties between SS and SC diseases may differ. In this study, we used a noninvasive in-vivo optical imaging system to compare conjunctival microvascular hemodynamics among healthy control subjects (Hb AA) and subjects with Hb SS or SC disease.

Methods

Subjects

The research study was approved by an institutional review board of the University of Illinois at Chicago. The study was described to the subjects, and informed consent was obtained according to the tenets of the Declaration of Helsinki. A total of 9 healthy control and 42 SCD subjects participated in the study. Of the 42 SCD subjects, 18 and 24 subjects had SS and SC disease, respectively. All SCD subjects were stable and not experiencing vaso-occlusive crisis. Conjunctival microcirculation imaging was performed with the use of our previously described optical imaging system (EyeFlow™) [17]. The subjects were asked to sit in front of the EyeFlow™ device and rest their head on a chin and forehead support. An external fixation target was presented during imaging to minimize eye motion.

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TABLE I. Age and Blood Pressure (BP) (mean $\pm$ SD) in AA, SS, and SC Groups

Variable	AA	SS	SC	P-Value
Age (years)	36 $\pm$ 9 (9)	39 $\pm$ 14 (18)	36 $\pm$ 13 (24)	0.79
Systolic BP (mmHg)	–	123 $\pm$ 14 (16)	124 $\pm$ 16 (22)	0.75
Diastolic BP (mmHg)	–	69 $\pm$ 10 (16)	72 $\pm$ 10 (21)	0.40

The number of subjects in each group is given in parenthesis. Statistical significance was accepted at $P < 0.05$.

TABLE II. Blood Chemistry Parameters (mean $\pm$ SD) in SS and SC Groups

Variable	SS (N = 18)	SC (N = 24)	P-Value
White Blood Cell Count ($10^6/\mu\text{L}$)	10.3 $\pm$ 3.2	8.4 $\pm$ 3.2	0.07
RBC Count ($10^6/\mu\text{L}$)	2.7 $\pm$ 0.5	3.2 $\pm$ 0.8	<0.01
Hb Concentration (g/dL)	8.6 $\pm$ 1.1	9.9 $\pm$ 1.7	0.01
HCT (%)	26.4 $\pm$ 3.1	29.6 $\pm$ 4.9	0.01
Mean Corpuscular Volume (10^{-15} L)	101 $\pm$ 13	93 $\pm$ 15	0.09
Mean Corpuscular Hb (10^{-12} g/cell)	33.1 $\pm$ 4.7	31.3 $\pm$ 4.9	0.24
Mean Corpuscular Hb Concentration (g/dL)	32.9 $\pm$ 1.2	33.7 $\pm$ 1.3	0.06
RBC Distribution Width (%)	20.1 $\pm$ 3.3	18.8 $\pm$ 3.3	0.23
Platelet Count ($10^3/\text{mm}^3$)	521 $\pm$ 179	357 $\pm$ 126	<0.01
Mean Platelet Volume (10^{-15} L)	7.4 $\pm$ 0.9	8.0 $\pm$ 0.8	0.03

Statistical significance was accepted at $P < 0.05$.

Magnified image sequences of the conjunctival microcirculation were captured with an electron multiplying charge coupled device camera (QuantEM, Photometrics, Tucson, AZ) at a rate of 60 Hz over a period of 2 sec. Several image sets were acquired in both eyes, typically requiring >15 min. The use of EyeFlowTM in a clinical setting was straightforward due to its noninvasive nature, the ease of operation, and the relatively short time period required for data acquisition. Clinical data including the subjects' ages, blood pressures, and systemic blood chemistry were also obtained. Blood pressure and chemistry data was not available in the AA group.

Image analysis

From each image sequence, a minimum of 10 consecutive frames were selected for registration based on image focus and the absence of large eye movements. Images were registered semiautomatically to correct for eye motion. Each image was comprised of 256×256 pixels with a pixel resolution of $1.5 \times 1.5 \mu\text{m}$. Conjunctival blood vessels were identified as venules by observing the direction of the blood flow and confirming that the vessels collected blood of the smaller connected microvessels. Conjunctival venules were selected for analysis since they are more numerous on the conjunctival surface, have minimal pulsatility, and are visualized with higher contrast as compared to arterioles. Venule diameter (D) and axial blood velocity (V) were determined from the registered image sequences as previously described [17].

Data and statistical analysis

Population statistics. The mean and standard deviation (SD) of age, blood pressure, and blood chemistry parameters were calculated in each group. A one way analysis of variance (ANOVA) was performed to compare differences in mean ages among AA, SS, and SC groups. An unpaired Student's t-test was used to compare blood pressure and blood chemistry parameters between SS and SC groups.

Descriptive statistics. Due to the previously reported relationship between blood velocity and vessel diameter in conjunctival venules [18], data were categorized according to vessel caliber. Based on the mean ($15 \mu\text{m}$) and median ($14 \mu\text{m}$) of D measurements in our study sample of 536 venules, a diameter size of $15 \mu\text{m}$ was selected arbitrarily as an appropriate discriminator for classifying venular calibers as "small" or "large." Vessel size 1 (small) encompassed venules with diameters $>15 \mu\text{m}$, while vessel size 2 (large) comprised venules with diameters equal to or $>15 \mu\text{m}$. In both eyes of each subject, 2 or more measurements of D and V were averaged in the same vessel sizes, yielding either 1 or 2 data points per subject. For statistical analysis, the compiled data consisted of 18, 31, and 42 measurements in AA, SS, and SC groups, respectively. The mean and SD of V and D , categorized by vessel size, were determined in each group. Since 2 measurements (one from each

TABLE III. Conjunctival Venule Diameter and Blood Velocity (mean $\pm$ SD) in AA, SS, and SC Groups, Categorized by Vessel Size

Variable	Vessel Size	AA	SS	SC
Diameter (μm)	1	11.2 $\pm$ 0.9 (9)	11.7 $\pm$ 0.9 (15)	11.1 $\pm$ 1.3 (21)
	2	19.5 $\pm$ 3.0 (9)	20.5 $\pm$ 4.0 (16)	19.8 $\pm$ 2.5 (21)
P-Value		<0.001	<0.001	<0.001
Velocity (mm/s)	1	0.32 $\pm$ 0.08 (9)	0.48 $\pm$ 0.12 (15)	0.42 $\pm$ 0.16 (21)
	2	0.60 $\pm$ 0.18 (9)	0.66 $\pm$ 0.22 (16)	0.50 $\pm$ 0.20 (21)
P-Value		0.001	0.009	0.1

The number of subjects in each group is given in parenthesis. Statistical significance was accepted at $P < 0.05$.

Figure 1. Conjunctival blood velocity plotted as a function of venule diameter for (Top) AA, (Middle) SS, and (Bottom) SC groups.

vessel size group) were not available in all subjects, an unpaired t-test was used to compare V and D between vessel sizes 1 and 2 for each group separately. A two-way ANOVA was performed to determine the

Figure 2. Conjunctival blood velocity plotted as a function of HCT for: SS vessel size 1 (Top Left) and SS vessel size 2 (Top Right); SC vessel size 1 (Bottom Left) and SC vessel size 2 (Bottom Right).

effects of vessel caliber (vessel size 1 and vessel size 2) and group (AA, SS, and SC) on V . Post hoc Tukey test was performed for pairwise comparisons.

Linear regression analysis. Linear regression analysis was performed to relate V to D (combining data from both vessel sizes) in each group. Linear regression analysis was also performed to relate V and HCT (categorizing data by vessel size) in SS and SC groups. All statistical analyses were performed using Systat software (version 13.1). Statistical significance was accepted at $P < 0.05$.

Results

Subjects' mean age and blood pressure are listed in Table I. The ages of subjects in AA (36 ± 9 years; $N=9$), SS (39 ± 14 years; $N=18$), and SC (36 ± 13 years; $N=24$) groups were similar ($P=0.8$). Systolic blood pressure was similar between SS (123 ± 14 mmHg) and SC (124 ± 16 mmHg) groups ($P=0.8$). Likewise, diastolic blood pressure was not significantly different among SS (69 ± 10 mmHg) and SC (72 ± 10 mmHg) groups ($P=0.4$). Whole blood chemistry parameters of the SS and SC groups are listed in Table II. HCT in the SC group ($30 \pm 5\%$) was significantly higher than the SS group ($26 \pm 3\%$) ($P=0.01$). Statistically significant differences were also found in RBC count ($P<0.01$), Hb concentration ($P=0.01$), platelet count ($P<0.01$), and mean platelet volume ($P=0.03$) between SC and SS groups.

Mean values of D and V , categorized by vessel size, for AA, SS, and SC groups are listed in Table III. D was significantly larger in vessel size 2 as compared with vessel size 1 in all groups ($P<0.001$). D was not significantly different among AA, SS, and SC groups for both vessel size 1 ($P=0.2$) and vessel size 2 ($P=0.7$). V in vessel size 2 was significantly $<V$ in vessel size 1 in the AA and SS

groups ($P < 0.009$), but not in the SC group ($P=0.1$). There were significant effects of vessel size ($P<0.001$) and group ($P=0.02$) on V . In the SC group, V was significantly lower as compared to the SS group ($P=0.03$).

The relationships between V and D in AA, SS, and SC groups are shown in Fig. 1. In the AA group, V was correlated with D ($R=0.6$, $P=0.005$, and $N=18$). Likewise, V was correlated with D in the SS group ($R=0.6$, $P<0.001$, and $N=31$); however, V was not significantly related to D in the SC group ($R=0.3$, $P=0.08$, and $N=42$). The relationships between V and HCT in SS and SC groups, categorized by vessel size, are shown in Fig. 2. In SS group, V was inversely correlated with HCT for vessel size 2 ($R=-0.6$, $P=0.03$, and $N=16$), but not for vessel size 1 ($R=0.03$, $P=0.9$, and $N=15$). In the SC group, the relationship between V and HCT was not statistically significant for vessel size 1 ($R=0.3$, $P=0.2$, and $N=21$) or vessel size 2 ($R=0.002$, $P=0.9$, and $N=21$).

Discussion

Hb is an essential metalloprotein within all mammalian erythrocytes that is responsible for oxygen transport. Mutations of the Hb protein are among the most common single gene defects in the world [19]. Among the structural hemoglobinopathies, SCD is the most lethal and common. Since biophysical variations of SS and SC erythrocytes may result in differences in microvascular flow properties, we compared hemodynamic properties within the bulbar conjunctival microvascular network among AA, SS, and SC groups. We identified a significantly lower blood velocity in the SC group compared with the SS group in the bulbar conjunctival microvasculature. Linear relationships between V and D were established in the SS and AA groups, but

F1

F2

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not in the SC group. In addition, V in large vessels was inversely related to HCT in the SS group, but not in the SC group.

Although abnormalities in the conjunctival microcirculation of subjects with SCD have been previously reported [20–24], to our knowledge, hemodynamics differences between subjects with SS and SC disease have not been demonstrated. The finding of lower V in the conjunctival microvasculature of the SC group as compared with the SS group may be due to the variety of SC erythrocyte shapes such as “pita bread,” “tricornis,” and “billiard ball” [16], in combination with the unique intraerythrocytic crystallization properties imparted by oxygenated Hb C [12,15]. Additionally, the higher systemic blood HCT in the SC group compared with the SS group would tend to increase microvascular flow resistance and decrease V . Although, it is noted that the systemic blood HCT may not be representative of HCT in the microcirculation.

A linear relationship between V and D was established in AA and SS groups, consistent with the previously reported trend of increased V with larger conjunctival venule D in healthy subjects [18]. Furthermore, in the SS group, V in the relatively large venules of the conjunctiva was inversely proportional to HCT, similar to the previously reported relationship between cerebral blood flow and HCT [25]. In contrast, a significant association between V and D (or HCT) was not observed in the SC group, specifically the expected increase in V with larger D and lower HCT was not present. Even though the relationships between V and HCT were examined in similar size venules, the findings may be limited due to the heterogeneity in V and HCT within microvascular networks [26].

Blood velocity in the microvasculature depends in part on HCT, which may be variably reduced within successive microvascular segments, contingent on the network topography [26]. Therefore, comparison of V among microvascular networks and subjects may be limited due to the lack of information about HCT within the venules, which is difficult to measure in-vivo. In this study, multiple V measurements in each subject were averaged to minimize the effect of heterogeneity within the microvascular networks examined. Furthermore, to assure comparisons between similar anatomical locations of the conjunctival microvascular networks among subjects, venules were categorized by vessel caliber. Comparison of V among groups and relationships between V and HCT were examined accounting for vessel caliber.

Future studies in a larger population are needed to determine and compare V in conjunctival arterioles, venules, and larger blood vessels between subjects with SS and SC disease. Evaluating hemodynamics in larger size vessels may also require other measurement techniques due to their larger blood velocity and volume. In summary, assessment of conjunctival microvascular hemodynamic properties may advance understanding of SCD pathophysiology and may improve pharmacotherapeutic and surgical interventions intended to optimize treatment outcomes. In addition, since conjunctival microvascular flow has been shown to correspond to middle cerebral artery flow [27], assessment of ocular microcirculation is a promising tool for identifying individuals at risk for stroke and evaluation of available and emerging treatment procedures for SCD.

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