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Effects of a Single Sickling Event on the Mechanical Fragility of Sickle Trait Erythrocytes

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Abstract

Hemolysis contributes to the pathology associated with sickle cell disease. However, the mechanism of hemolysis or relative contribution of sickling due to hemoglobin polymerization vs. oxidative damage remains unknown. Earlier studies aimed at deciphering the relative importance of these two mechanisms have been complicated by the fact that sickle red cells (SS) have already been affected by multiple rounds of sickling and oxidative damage before they are collected. In our study, we examine the mechanical fragility of sickle cell trait cells, which do not sickle in vivo, but can be made to do so in vitro. Thus, our novel approach explores the effects of sickle hemoglobin polymerization on cells that have never been sickled before. We find that the mechanical fragility of these cells increases dramatically after a single sickling event, suggesting that a substantial amount of hemolysis in vivo probably occurs in polymer-containing cells.

Keywords

sickle cell disease; hemolysis; fragility; hemoglobin

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Declaration of Interest

Drs. Gladwin and Kim-Shapiro are listed as co-inventors of a provisional patent application entitled "Methods for treatment of hemolysis".

INTRODUCTION

Sickle cell anemia is an autosomal recessive hemoglobinopathy that is characterized by the hemoglobin S (HbS) variant of the β -globin gene. The primary cause of the disease is the replacement of glutamic acid by valine at the $\beta 6$ position (1). HbS polymerizes under hypoxic conditions which distorts the shape of the red blood cells and contributes to poor red cell deformability, increased fragility, microvascular occlusion and other deleterious consequences (2–11). Hemolysis in sickle cell anemia and other hemolytic anemias contribute to pathology via reduced nitric oxide bioavailability (12–21) and oxidative damage (22–28). About one-third of hemolysis in sickle cell disease is intravascular and two-thirds is extravascular (29).

The mechanism of intravascular hemolysis has been largely attributed to both the formation of polymerization, which leads to irreversible and reversible sickling of cells, and oxidative damage (30). Irreversibly sickled cells are formed after cycles of oxygenation and deoxygenation (31). These cells are rigid and easily lysed (32, 33). At low oxygen conditions, sickle cells become dehydrated and lose ions. Dehydration and ion loss lead to an increase in intracellular hemoglobin concentration, which enhances polymerization (34). Most likely due to repeated sickling, these cells gradually shed part of their plasma membrane and become poorly deformable, leading to the formation of irreversibly sickle cells (6, 35). Oxidative damage also contributes to the formation of irreversibly sickled cells (36). It is believed to result from the release of iron, heme and heme degradation products (37), compounded by dysregulation of anti-oxidant enzymes (16, 38, 39). Oxidation promotes membrane damage (40–48), which contributes to poor red cell deformability, even under oxygenated conditions and can thus lead to hemolysis of non-irreversibly sickled cells. (49). Therefore, both hemoglobin polymerization and oxidative damage contribute to hemolysis, often but not necessarily through the intermediacy of irreversibly sickled cells.

The fragility of sickle cells has been previously shown to improve upon oxygenation, but not to the level of erythrocytes from AA volunteers (4). Thus, cells that were not morphologically sickled (and hence not irreversibly sickled cells) still had increased fragility (4). The relative contributions of polymerization versus oxidative damage that directly cause hemolysis are complicated by the fact that sickle cells drawn from a patient have potentially undergone multiple cycles of sickling and unsickling in vivo. In order to gain insight into the cause of intravascular hemolysis, we measured mechanical fragility by subjecting the cells to shear during shaking in the presence of glass beads and red cell deformability using a flow channel assay. An alternative to ektacytometry, flow chamber deformability measures the capacity of the cell to change its shape under applied stress, whereas mechanical fragility tests the resistance of the erythrocytes to hemolysis (50). In this study, both the deformability and fragility of red blood cells (RBCs) from people with sickle cell trait (heterozygous in the sickle mutation, AS genotype, having about 60% HbA and 40% HbS (51)) were measured; these cells typically do not contain polymers under physiological conditions (normally above 25% oxygen saturation, even in hypoxic tissues) nor do they have increased fragility (52–56). Although there have been some reports of intravascular sickling in the kidney blood vessels resulting in renal infarction in sickle trait patients (57), these cases are rare; due to the delay time for polymerization, polymers do not form extensively in vivo during short transit times through hypoxic tissues. However, in vitro, polymerization of AS cells can be induced by very low oxygen tension for a prolonged period (56). Hence, we tested the effect of polymerization on the fragility of these “virgin” AS cells.

MATERIALS AND METHODS

Reagents

Sodium hydrosulfite (dithionite) and all other chemicals unless otherwise noted were purchased from Sigma Chemical Co. Both the AS and SS blood were obtained from volunteers using ACD tubes and the blood was used for experiments within 2–3 days of being drawn. AA blood was freshly drawn into EDTA blood tubes (pilot tubes additionally drawn at the time of blood donation), purchased from the Interstate Blood Bank (Memphis, TN) and used for experiments within 2–3 days of being collected. The blood was collected by standard phlebotomy procedures for each experiment from volunteer subjects previously documented by hemoglobin high performance liquid chromatography to have homozygous sickle cell disease, heterozygous sickle trait, or normal hemoglobin A. Samples were transferred by overnight express shipping. The blood donations from volunteers received official institutional approval according to the IRB.

Mechanical fragility

Fragility was measured by subjecting blood to mechanical shear and subsequently measuring hemolysis. 100 μ l of blood was added to 9.9 ml of normal saline and centrifuged at 2,000 rpm for 5 minutes. The supernatant was removed and saved as the “blank”. A 2 ml volume of blood and 4 glass beads (4 mm diameter) were placed in each tube. The tubes were stoppered and rotated vertically at 33 rpm for 1, 2 and 3 hours at room temperature. 100 μ l of the rotated blood was added to 9.9 ml of normal saline and placed at room temperature for 5 minutes and subsequently centrifuged at 2,000 rpm for 5 minutes. The saline supernatant was decanted and the amount of hemolysis was determined spectrophotometrically. Absorption was measured from 700 nm to 450 nm. The hemoglobin concentration was determined using the absorption differences between A_{590} - A_{577} and A_{541} - A_{518} , and by calculating the concentration using known extinction coefficients (58). The percentage of hemolysis was obtained by taking the ratio of total concentration of hemoglobin in the supernatant for each condition to the total hemoglobin concentration. These data are an average of several trials taken from three different blood samples from three separate donors ($n=3$). The total hemoglobin concentrations for each condition are given in Table 1. These concentrations are in heme, where 1 g/dL = 625 μ M in heme. Thus, 16 g/dL = 10 mM.

Spectrophotometry

Absorption spectroscopy for Hb in the absence of red blood cells (supernatant) was measured using a Cary 50 bio-spectrometer in the visible wavelength range (Varian, Inc.). Absorption spectroscopy on turbid samples containing red blood cells was performed using a Perkin-Elmer Life Sciences Lambda 9 spectrometer, equipped with an integrating sphere to detect scattered light. For the deoxygenated samples, septum-capped cells were used for the measurements.

Deformability

Blood hemoglobin concentrations were obtained spectrophotometrically using the Perkin Elmer spectrophotometer. The desired Hb concentration for deformability measurements was 0.066 mM, so the blood was diluted into deformability buffer with a total volume of 20 ml. Deformability buffer consisted of 50 g dextran, 34 ml distilled water, 10 ml OptiPrep Density Gradient Medium (60% w/v solution of iodixanol in H_2O) and 200 ml of 0.01 M Phosphate Buffered Saline (NaCl-0.138 M, KCl-0.0027 M, 2 mg/ml glucose). The red cells in the viscous buffer were passed through a narrow chamber at controlled flow rates to induce shear as described previously (59, 60). These flow rates included a range from 0.00

ml/min to 20.00 ml/min, totaling 14 flow rates. Sampling different flow rates allowed us to make sure that our results were consistent over a large range of shear. The images were acquired using a digital camera and an argon ion laser (National Laser Company; Salt Lake City, UT). The laser beam was directed through the chamber, forming a diffraction image that is used to determine the extent of cell deformation similar to the procedure in ektacytometry. The images were averaged and analyzed using Matlab (The MathWorks, Inc; Natick, MA), to obtain the deformability constant (DC). The DC is defined in terms of the length (L) and the width (W) of the diffraction image at each flow rate. DC is a numerical representation of red cell deformability, defined by: $DC = L/W$; where the DC increases as the red cell deformability increases (61, 62). The flow rates were correlated to the amount of shear stress and plotted with respect to the deformability constant [63]. The data are an average of several trials taken from three different blood samples from three separate donors.

Oxygenation

For the oxygenated conditions, the blood was equilibrated with room air. To maintain deoxygenated conditions in mechanical fragility experiments, the blood was treated with 50 mM dithionite. For deformability measurements of deoxygenated cells, 25 mM dithionite was added to the blood and 5 mM dithionite was added to the deformability buffer. In each case, the blood and the deformability buffer were equilibrated with nitrogen prior to the addition of dithionite. Reoxygenation of deoxygenated samples was performed by overnight dialysis in air-equilibrated buffer. As a control for these measurements, air-equilibrated blood that had never been deoxygenated was also dialyzed overnight. This condition is referred to as “dialyzed oxy”. Once any cells were used for an assay, they were not reused for another assay. For example, deoxygenated cells used for reoxygenation experiments were not the same ones that had been used for studies of mechanical fragility of deoxygenated cells.

Blood Dialysis

To reoxygenate or fully oxygenate the blood, dialysis was performed. 0.01 M of phosphate buffered saline (PBS) was prepared to a 2 L volume. The desired volume of blood was placed in Slide-A-Lyzer dialysis cassettes (Fisher Scientific; Rockford, IL) and the cassettes were placed in 2 L of PBS. The cassettes were stirred at a steady pace throughout the night at 4 °C. Upon complete dialysis, the whole blood concentration was measured spectrophotometrically and complete hemoglobin oxygen saturation was confirmed.

Statistical Analysis

Data are presented as means $\pm$ SD. Statistical analysis was performed using Student's *t*-test and one-way ANOVA. The general acceptance level of significance was $p < 0.05$ and significance was confirmed by both *t*-tests and ANOVA.

RESULTS

Red cell deformability was measured in AS, AA and SS cells by analyzing the diffraction images of cells sent through a flow-channel diffraction apparatus (Figure 1A). The flow rates were quantified and converted to the corresponding shear stress rates. As demonstrated in Figure 2B, the deformability of oxygenated AS cells is not significantly different from AA cells (standard deviations are given in the figure caption), supporting the notion that the rheological properties of AS cells are not impaired in vivo. On the other hand, the deformability of oxygenated SS cells is significantly lower than that for AA cells, indicating poor rheological properties stemming from cycles of hemoglobin polymerization and/or oxidative damage in vivo. The cell deformability coefficient for AA cells is not affected by

the presence or absence of oxygen (2.1 ± 0.5 for oxy and 1.9 ± 0.4 for deoxy). However, the ability of both the SS and AS cells to deform is greatly influenced by oxygen. Under completely deoxygenated conditions, the SS and AS cells exhibit reduced deformability (Figure 1). This confirms that both the AS and SS cells contain hemoglobin polymers under these conditions.

The mechanical fragility of RBCs from AA subjects was assessed at different oxygen tensions after being subjected to shear for 1, 2, and 3 hours (Figure 2). As expected, the partial pressure of oxygen had no significant effect on the mechanical fragility of AA cells (Figure 2). After one hour of exposure to shear, there was 5.2 ± 1.6 % hemolysis for oxy vs. 11.1 ± 5.9 % for deoxy, $n=3$. These data are an average of several trials taken from three different blood samples from three separate donors. It should be noted that these levels of hemolysis are based on the amount of hemoglobin measured in the supernatant, while taking into account a 100-fold dilution of shear-exposed blood and subsequent sedimentation. After three hours of shear, the percent hemolysis was 18.5 ± 6.1 % and 23.3 ± 19.5 % ($n=3$) for AA oxy cells and AA deoxy cells, respectively. In addition, no change in the mechanical fragility of RBCs from AA subjects was measured when deoxygenated cells were subsequently reoxygenated by dialysis against oxygenated buffer or when the cells that had not been deoxygenated were similarly dialyzed (labeled dialyzed oxy in Figure 2).

Deoxygenation greatly increased the fragility of SS cells compared to oxygenated cells as indicated by increased hemolysis at 1, 2, and 3 hours of exposure to shear (Figure 3). This result demonstrates that HbS polymerization makes the SS cells more fragile and prone to hemolysis. SS cells that were reoxygenated had similar fragility as cells that did not undergo deoxygenation (compare results for “Reoxy” vs “Dialyzed Oxy” in Figure 3). The increase in mechanical fragility observed upon deoxygenation of SS cells is most likely due to intracellular hemoglobin polymerization; however, the degree of this dramatic effect may be due to pre-existing damage accumulating from cycles of polymerization and depolymerization in vivo coupled with oxidative damage.

In order to study the potential role of both concurrent polymerization and oxidative damage in the phenomena observed for SS cells, we conducted studies using RBCs from volunteers with sickle cell trait (AS). The fragility of oxygenated AS cells was similar to that from the AA volunteers (compare Figure 4 to Figure 2, see captions for values). This result is consistent with the idea that the AS cells have not been previously damaged due to polymerization or oxidative damage, as the oxygen tension in vivo does not decline enough for HbS in AS cells to polymerize. However, by taking the AS cells to zero oxygen saturation, polymerization was induced and mechanical fragility significantly increased compared to oxygenated AS cells (Figure 4). When deoxygenated cells were reoxygenated, they regained fragility similar to the control cells (Figure 4, compare Reoxy AS to Dialyzed Oxy AS).

DISCUSSION

We have shown that irrespective of red cell type, an increased exposure to mechanical stress leads to enhanced lysis, as observed previously (63). As expected, normal cell lysis occurred similarly in the presence or absence of oxygen, confirming that AA cells remain unchanged when alterations in oxygen tension take place. Both AS and SS cells were more fragile under deoxygenated rather than oxygenated conditions. Importantly, a single polymerization event increased the mechanical fragility of AS cells, demonstrating them to be more fragile than oxygenated SS cells (that have undergone cycles of sickling and unsickling and oxidative damage in vivo). It is also important to consider that the degree of polymerization in AS cells at zero oxygen pressure is likely to be similar to or less than that encountered by

many SS cells in vivo due to the increased solubility of the HbA/HbS mixture compared to HbS alone. These results therefore imply that a substantial amount of intravascular hemolysis transpires in cells containing polymers.

It should be noted that although the percentage hemolysis between AS cells and SS cells is similar under deoxygenated conditions (Figures 3 and 4), the absolute amount of hemolysis is greater in AS cells due to their higher hematocrit (see Table 1). This may be due to fragile SS cells being “weeded out” in vivo due to intravascular hemolysis or phagocytosis. In general, the SS cells that persist through circulation may be more resistant to polymerization-induced decreases in fragility than AS cells.

Our in vitro study points out the utility of additional ones performed in vivo. We clearly show that RBCs that are sickled by deoxygenation for the first time are much more fragile than oxygenated sickle cells (SS) that have undergone cycles of sickling in vivo. This suggests that substantial hemolysis in vivo occurs in polymer containing cells. However, the extent of this hemolysis in vivo cannot be estimated from our in vitro experiments since the shear experienced by cells in vivo is likely to differ from that used in our studies. Moreover, the duration of the deoxygenation in vitro (up to 3 hours) does not correspond to the normal duration of deoxygenation in vivo, in which the deoxygenated red cells normally re-oxygenate when they reach the lungs.

Overall, these data support two important conclusions: (1) the observed poor rheology of SS cells under anaerobic conditions does not result from a single or prolonged sickling event, but rather is likely to include contributions from cumulative insults such as oxidative damage. This conclusion is based on the observation that rheological properties of deoxygenated AS cells return to normal following reoxygenation; (2) a substantial amount of intravascular hemolysis is likely to occur in vivo in cells that contain sickle cell hemoglobin polymers. This is suggested by the dramatic increase in mechanical fragility upon deoxygenation of both AS and SS cells. Thus, it is likely that many cells that hemolyze in vivo do so upon the first sickling event.

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A **B**

Figure 1. Deformability of AA, AS and SS cells

A) Images of red cell deformability for oxy and deoxy AA (control), AS (sickle cell trait) and SS (sickle) cells at a flow rate of 5 ml/min (106.1 Pa) B) The deformability coefficient (DC) is plotted for various shear stress. Oxygenation did not affect AA cells (oxygenated: 2.1 ± 0.5 ; deoxygenated 1.9 ± 0.4). Under oxygenated conditions, the AS cells average DC was 1.8 ± 0.4 ($n=3$), whereas the oxy SS cells average DC was 1.6 ± 0.3 ($n=3$). These data are an average of several trials taken from three different blood samples from three separate donors. Under deoxygenated conditions, the DC remains constant for both AS and SS cells at different shear (1.2 ± 0.04 and 1.3 ± 0.05 for AS and SS deoxy, respectively). This indicates that both cell types are poorly deformable due to polymerization.

Figure 2. Measure of hemolysis in AA cells

AA (control) cells were rotated at 33 rpm in the presence of glass beads. The percentage hemolysis was measured and plotted with respect to the time the cells were exposed to shear. No significant difference in fragility in the presence or absence of oxygen is displayed, where the data is an average of numerous measurements ($n=3$). These data are an average of several trials taken from three different blood samples from three separate donors. The lysis after one hour of shear for these oxy AA cells was 5.2 ± 1.6 % for 1 hr, 11.7 ± 2.7 % for 2 h, and 18.5 ± 6.1 % for 3 h. When the cells were deoxygenated, % hemolysis = 11.1 ± 5.9 % for 1 h, 18.7 ± 15.7 % for 2 h, and 23.3 ± 19.5 % for 3 h. Upon reoxygenation via dialysis, % hemolysis = 13.2 ± 9.0 % for 1 h, 26.1 ± 19.6 % for 2 h, and 38.7 ± 26.9 % for 3 h. As a control, another set of cells was treated identically as the reoxygenated ones except without any deoxygenation. These are referred to as “dialyzed oxy”. For dialyzed oxy, % hemolysis = 3.5 ± 0.6 % for 1 h, 8.0 ± 1.6 % for 2 h, and 12.4 ± 2.0 % for 3 h. No significant differences were observed when comparing oxygenated to deoxygenated conditions or dialyzed oxygenated conditions to reoxygenated conditions ($p > 0.05$, using t-test and one-way ANOVA).

Figure 3. Mechanical fragility of SS cells

SS (sickle) cells were rotated against glass beads under both oxy and deoxy conditions. Percent Hemolysis was measured and plotted as a function of the time of the exposure to mechanical stress. Under oxygenated conditions, % hemolysis = 5.6 ± 1.3 % for 1 h, 9.9 ± 0.4 % for 2 h, and 16.8 ± 2.0 % for 3 h. In the absence of oxygen, hemolysis increased nearly three-fold in comparison to an oxy environment. For one hour, the % hemolysis = 36.2 ± 14.2 %. The lysis increased over time to 52.9 ± 23.8 % and 67.4 ± 41.8 % for 2 h and 3 h, respectively. Once the cells were reoxygenated, the hemolysis was significantly reduced to 11.5 ± 9.5 % for 1 h, 19.0 ± 12.5 % for 2 h, and 29.4 ± 11.5 % for 3 h. For dialyzed oxy, % hemolysis = 5.1 ± 2.4 % for 1 h, 10.5 ± 4.1 % for 2 h, and 14.5 ± 5.1 % for 3 h (n=3). These data are an average of several trials taken from three different blood samples from three separate donors. *P < 0.05 for a t-test and one-way ANOVA, deoxy compared to oxy or reoxy compared to dialyzed oxy.

Figure 4. Mechanical Fragility of AS cells

AS (sickle trait) cells were subjected to mechanical stress similar to the SS (sickle) cells and hemolysis was measured. For oxy, % hemolysis = 10.9 ± 8.1 % for 1 h, 18.1 ± 12.2 % for 2 h, and 25.2 ± 15.1 % for 3 h. For deoxy, % hemolysis = 28.1 ± 10.2 % for 1 h, 47.2 ± 18.6 % for 2 h, and 84.4 ± 28.4 % for 3 h. (n=3). These data are an average of several trials taken from three different blood samples from three separate donors. When reoxygenated, the cells recovered to 4.9 ± 2.2 % for 1 h, 16.4 ± 7.2 % for 2 h, and 16.8 ± 3.1 % for 3 h. These values are comparable to the dialyzed oxy conditions (% hemolysis = 4.5 ± 1.9 % for 1 h, 7.4 ± 3.3 % for 2 h, and 12.4 ± 3.8 % for 3 h). *P < 0.05 for a t-test and one-way ANOVA, deoxy compared to oxy or reoxy compared to dialyzed oxy.

Table 1

Total Hemoglobin concentrations

The average total hemoglobin concentration of the blood is tabulated for each condition. Variation seen are due to differences in the initial hematocrit and changes due to dialysis.

Condition	Oxy (mM)	Deoxy (mM)	Reoxy (mM)	Dialyzed Oxy (mM)
AA	11.63 ± 4.12	9.56 ± 3.94	7.47 ± 3.37	13.01 ± 1.44
AS	10.74 ± 1.11	13.24 ± 2.14	6.47 ± 2.20	9.27 ± 1.59
SS	3.27 ± 1.83	2.86 ± 1.39	1.02 ± 0.91	1.64 ± 1.33

* Note: These concentrations are in heme, where 1g/dL = 625 µM in heme.