2-2010

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EFFECTS OF A SINGLE SICKLING EVENT ON THE MECHANICAL FRAGILITY OF SICKLE CELL 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 (Hb) 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 Hb 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 (Hb)

INTRODUCTION

Sickle cell anemia is an autosomal recessive hemoglobinopathy that is characterized by the Hb S variant of the β-globin gene. The primary cause of the disease is the replacement of glutamic acid by valine at the β6 position (1). Hb S polymerizes under hypoxic conditions which distorts the shape of the red blood cells (RBCs) 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 (NO) 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 (Hb) 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 Hb 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 *vs.* 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 RBCs from people with sickle cell trait [heterozygous in the sickle mutation, AS genotype, having about 60% Hb A and 40% Hb S (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

Sodium hydrosulfite (dithionite) and all other chemicals unless otherwise noted were purchased from Sigma Chemical Co., (**St. Louis, MO, USA**). 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, USA) 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 Hb high performance liquid chromatography (HPLC) to have homozygous sickle cell disease, heterozygous sickle trait, or normal Hb A. Samples were transferred by overnight express shipping. The blood donations from volunteers received official institutional approval according to the **Institutional Review Board (IRB, Wake Forest University)**.

Fragility was measured by subjecting blood to mechanical shear and subsequently measuring hemolysis. One hundred μL of blood was added to 9.9 mL of normal saline and centrifuged at 2,000 rpm for 5 min. The supernatant was removed and saved as the "blank". A 2 mL volume of blood and four 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. One hundred μL of the rotated blood was added to 9.9 mL of normal saline and placed at room temperature for 5 min. and subsequently centrifuged at 2,000 rpm for 5 min. The saline supernatant was decanted and the amount of hemolysis was determined spectrophotometrically. Absorption was measured from 700 nm to 450 nm. The Hb 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 Hb in the supernatant for each condition to the total Hb concentration. These data are an average of several trials taken from three different blood samples from three separate donors ($n = 3$). The total Hb concentrations for each condition are given in Table 1. These concentrations are in heme, where 1 g/dL = 625 μM in heme. Thus, 16 g/dL = 10 mM.

Absorption spectroscopy for Hb in the absence of RBCs (supernatant) was measured using a Cary 50 bio-spectrometer in the visible wavelength range (Varian Inc., **Palo Alto, CA, USA**). Absorption spectroscopy on turbid samples containing RBCs was performed using a Perkin-

Elmer Life Sciences Lambda 9 spectrometer **(Perkin-Elmer Corp., Norwalk, CT, USA)**, equipped with an integrating sphere to detect scattered light. For the deoxygenated samples, septum-capped cells were used for the measurements.

Blood Hb concentrations were obtained spectrophotometrically using the Perkin Elmer spectrophotometer **(Perkin-Elmer Corp., Norwalk, CT, USA)**. 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, USA). 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, USA), 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. The DC is a numerical representation of red cell deformability, defined by: $DC = L/W$; where the DC increases as the red cell deformablity increases (61, 62). The flow rates were correlated to the amount of shear stress and plotted with respect to the deformability constant (62). The data are an average of several trials taken from three different blood samples from three separate donors.

For the oxygenated conditions, the blood was equilibrated with room air. To maintain deoxygenated conditions in mechanical fragilty 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 fragilty of deoxygenated cells.

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, USA) 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 Hb oxygen saturation was confirmed.

Data are presented as means ± SD (standard deviation). 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 Hb 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 Hb 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 1 hour of exposure to shear, there was $5.2 \pm 1.6\%$ hemolysis for oxy *vs.* $11.1 \pm 5.9\%$ 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 Hb measured in the supernatant, while taking into account a 100-fold dilution of shear-exposed blood and subsequent sedimentation. After 3 hours of shear, the percent hemolysis was $18.5 \pm 6.1\%$ and $23.3 \pm 19.5\%$ ($n = 3$) 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 Hb S polymerization makes the SS cells more fragile and prone to hemolysis. The 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 Hb 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 Hb S 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 Hb A/Hb S mixture compared to Hb S 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 <u>Hb</u> 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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SHORT TITLE: Lysis of Hb S-Containing Red Blood Cells

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FIGURE LEGENDS

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 cell disease) 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 $5.2 \pm 1.6\%$ for 1 hour, $11.7 \pm 2.7\%$ for 2 hours, and $18.5 \pm 6.1\%$ for 3 hours. When the cells were deoxygenated, % hemolysis = 11.1 ± 5.9 % for 1 hour, 18.7 ± 15.7 % for 2 hours, and 23.3 ± 19.5 % for 3 hours. Upon reoxygenation via dialysis, % hemolysis = $13.2 \pm 9.0\%$ for 1 hour, $26.1 \pm 19.6\%$ for 2 hours, and $38.7 \pm 26.9\%$ for 3 hours. 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 hour, 8.0 ± 1.6% for 2 hours, and 12.4 ± 2.0 % for 3 hours. 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 hour, 9.9 ± 0.4% for 2 hours, and 16.8 ± 2.0% for 3 hours. In the absence of oxygen, hemolysis increased nearly three-fold in comparison to an oxy environment. For 1 hour, the % hemolysis = 36.2 ± 14.2%. The lysis increased over time to 52.9 ± 23.8% and 67.4 ± 41.8% for 2 and 3 hours, respectively. Once the cells were reoxygenated, the hemolysis was significantly reduced to 11.5 ± 9.5% for 1 hour, 19.0 ± 12.5% for 2 hours, and 29.4 ± 11.5% for 3 hours. For dialyzed oxy, % hemolysis = 5.1 ± 2.4% for 1 hour, 10.5 ± 4.1% for 2 hours, and 14.5 % ± 5.1% for 3 hours (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 hour, 18.1 ± 12.2% for 2 hours, and 25.2 ± 15.1% for 3 hours. For deoxy, % hemolysis = 28.1 ± 10.2% for 1 hour, 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 hour, 16.4 ± 7.2% for 2 hours, and 16.8 ± 3.1% for 3 hours. These values are comparable to the dialyzed oxy conditions (% hemolysis = 4.5 ± 1.9% for 1 hour, 7.4 ± 3.3% for 2 hours, and 12.4 ± 3.8% for 3 hours). *p <0.05 for a t-test and one-way ANOVA, deoxy compared to oxy or reoxy compared to dialyzed oxy.

TABLE 1 The Average Total Hemoglobin Concentration of the Blood is Tabulated for Each Condition. Variations Seen Are Due to Differences in the Initial Hematocrit and Changes Due to Analysis (these concentrations are in heme where 1 g/dL = μM in heme)

Condition	Oxy (mM)	Deoxy (mM)	Reoxy (mM)	Dialyze 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