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Further Identifying Free Radical Contributions to Sickle Cell Disease

by

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Abbreviations Used

CSSCD - Cooperative Study of Sickle Cell Disease;
EPR – electron paramagnetic resonance spectroscopy;
HbF – fetal hemoglobin;
HbS – sickling hemoglobin;
HU – hydroxyurea;
K⁺/Cl⁻ COT – potassium/chloride coupled transport or cotransport;
NADH: nicotinamide adenine dinucleotide;
NADPH – nicotinamide adenosine dinucleotide phosphate;
NHLBI: National Heart, Lung and Blood Institute;
Nfκ-B: nuclear factor κ-B;
NOS – nitric oxide synthase;
RBC – red blood cell;
RRI - ribonucleotide reductase inhibitors
SCD – sickle cell disorder;
TBARS – thiobarbituric acid reactive species

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Abstract

Sickle cell anemia is a genetic disorder which causes the expression of defective hemoglobin resulting irregularly shaped red blood cells, known as “sickle cells.” These sickle cells cause problems in the body, often blocking blood flow and causing painful attacks and sometimes stroke. Sickled cells have different biochemistry than normal red blood cells and are controlled by many free radical processes. This paper will review the disease state and discuss several therapies have been developed using free radical chemistry which show promise for long term treatment of these patients. A plan of research is proposed that will further prove the correlations between sickle cell disease and free radicals.

Introduction

Sickle cell disease (SCD) is an inherited blood disorder characterized by chronic anemia characterized by periodic episodes of pain. This disorder affects over 72,000 Americans and millions throughout the world, most of African descent. Approximately 1 in 12 African-Americans carry the trait for SCD and 1 of every 350 African-American infants born have the disorder and the incidence of the disorder in Africa is ten times higher [1,2,3]. Persons with sickle cell disorder inherit defective hemoglobin genes from both parents. Early research was funded by the National Heart, Lung and Blood Institute (NHLBI). The United States Congress passed the National Sickle Cell Disease Control Act in 1972 which called for the establishment of the National Sickle Cell Disease Program. Over the years, this program and others like the Cooperative Study of Sickle Cell Disease (CSSCD), established in 1979, has funded research that has elucidated much of what we know about the disease today [4].

Recently, it has been demonstrated that sickled red blood cells are more susceptible to oxidative damage than normal red blood cells and current treatments for SCD focus on applying free radical chemistry to sickled cells [5]. This information leads to the hypothesis that the symptoms of SCD are caused by extensive free radical damage and oxidative stress. This paper will discuss new methods for elucidating the role of free radicals in sickle cell disease.

Research Specific Aims

On the basis of previous research, it is hypothesized that oxidative stress in sickle red blood cells is the cause of the symptoms of sickle cell disease and the combination free radical biology and chemistry can lead to novel treatments of the disorder. In order to prove this hypothesis, experiments will be designed to address the following specific aims.

- 1. Demonstrate the role of oxidative stress in sickled cells.** Previous research shows that sickle red blood cells are more susceptible to oxidative lipid damage. Additional markers of oxidative damage should be identified to further provide evidence that oxidative stress is the major aggravator in sickle cell disorder. Sickle red blood cells will also be investigated in situations where ROS is scavenged to prove that oxidative stress is fundamental to problems associated with sickle cell disease.
- 2. Develop a system that counteracts the side effects of treatment.** Numerous treatments for the symptoms of SCD involve the generation of ROS. A novel systematic approach to treating the disease would involve treating SCD symptoms along with the side effects of the symptom therapy.

Background

Red blood cells (RBCs) carry oxygen from lungs to body in transport facilitated by hemoglobin (Hb). Hemoglobin constitutes 25–30% of the volume of a typical red cell and is composed of two α subunits and two denoted β subunits [6]. Individuals homozygous for the defective or sickling hemoglobin (HbS) will have sickle cell disorder. Sickling hemoglobin contains a mutational change from a glutamate to a valine in the 6th position of the β chains of hemoglobin [7,8]. Glutamate is a charged amino acid and valine is a neutral, hydrophobic molecule. This change in amino acids allows deoxygenated HbS to become “sticky” and polymerize to form fibers that cause rigidity and sickle shape [9]. The physical appearance of normal RBCs and sickled cells is very different as shown in **Figure 1**.

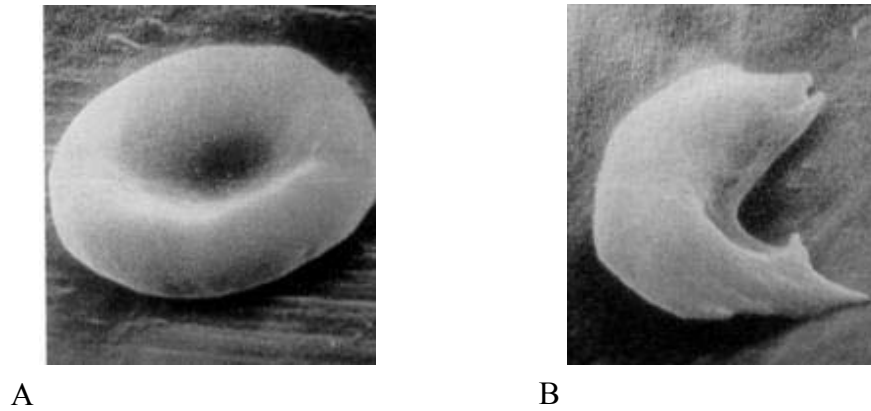


Figure 1. The normal red blood cell (A) is relatively round and flexible and moves easily through narrow blood vessels, while the sickle cell (B) is sickled-shaped and inflexible.¹

At times the stiff, sickled cells cannot squeeze through the narrow blood vessels and stack up to block blood flow. This blockage prevents oxygen from being transferred to tissue and organs which is known as ischemia. The loss of oxygen can result in tissue damage. Reperfusion after an ischemic event can be extremely painful and these events are common in SCD patients. The lifespan of sickle RBCs 10-20 days as compared to the 120-day lifespan of normal RBCs which results in chronic anemia in SCD patients [3]. This chronic hemolysis leads to the generation of many other molecules, some of them toxic.

Sickle cell disorder disproportionately affects humans of African and Middle-Eastern descent. Natural selection has favored the retention of HbS mutation in certain populations for individuals with heterozygous defective hemoglobin alleles have marked resistance to malaria. Only individuals homozygous HbS alleles are stricken with SCD. The HbS mutation did not develop in other populations because malaria is not prevalent in colder regions [10].

Symptoms of sickle cell disorder include Hand-foot syndrome, anemia symptoms such as fatigue and paleness, unpredictable episodes of pain, chronic inflammation, eye problems,

¹ Genetics © 2004, by Dr. S. Poethig, Dr. I. Waldron and J. Doherty, Department of Biology, University of Pennsylvania http://serendip.brynmawr.edu/sci_edu/waldron/genetics.html Accessed 5/1/05

jaundice, delayed growth in children, infections and sometimes stroke. Patients with SCD are also susceptible to diseases such as osteomyelitis and the spleen and kidneys of SCD patients are particularly susceptible to ischemic damage. Early diagnosis of SCD is key for proper treatment and without sufficient treatment SCD patients live to be less than 20 years of age [11]. Most states in the U.S. perform tests for HbS on all new-born infants, identifying individuals with SCD and carriers of sickle cell trait (those with heterozygous HbS allele).

Biochemical Effects

There are several differences in the biochemistry of normal RBCs and sickle RBCs. Red blood cells have been used for decades to study membrane transport. One transport system of particular interest is the coupled transport of potassium and chloride ions (K^+/Cl^- COT). Several K^+/Cl^- COT membrane proteins help the cells shrink to regulate their volume [12]. In normal RBCs, the cell volume and K^+/Cl^- cotransport activation remains relatively constant with changes in pH, K^+ concentration, and urea concentration, but primarily dependent upon the partial pressure of oxygen (P_{O_2}) in the cell. In sickle cells, K^+/Cl^- COT activity is not as dependent on P_{O_2} and remains active even at low P_{O_2} . K^+/Cl^- COT activity is ten times higher in sickle cells than in normal RBCs causing a loss of KCl thereby inducing cell shrinkage. Shrunken RBCs with deoxygenated hemoglobin are more likely to sickle which increases the possibility of ischemia-reperfusion events. Ohnishi and colleagues have also focused their research on the role of membrane alteration in the adhesion properties of sickle cells. They have found that a cocktail of antioxidant nutritional supplements has a corrective effect on RBC membranes, preventing blockages and subsequently preventing ischemia-reperfusion injury [11].

Adragna and colleagues found that oxidants such as nitrite (NOO^-) can exacerbate the K^+/Cl^- COT hyperactivity in sheep RBCs with low potassium concentrations and potential do the

same to sickle cells. Nitrite oxidizes hemoglobin to methemoglobin (MetHb) by changing the heme iron oxidation state from ferrous (Fe(II)) to ferric (Fe(III)). The addition NOO^- to these cells also resulted in increased cellular oxidation, decreased glutathione (GSH) levels, and increased K^+/Cl^- COT activity which causes cell shrinkage [13]. These results are hypothesized to be similar in sickle RBCs.

Sickle cells have a specific cascade of gene expression in response to the constant hemolysis. There is a dramatic upregulation of pro-inflammatory genes, but an anti-inflammatory pathway that is induced upon sickle cell crises. The lifespan of sickle cells is at least six times shorter than that of normal RBCs, so the cell is constantly trying to make more than 30 g of potentially pro-oxidant Hb per day. Heme oxygenase-1 (HO-1), the inducible isoform of heme oxygenase, is an enzyme responsible for heme catabolism. Its expression is thought to be the major player in the anti-inflammatory pathway in sickle RBCs. Heme oxygenase-1 breaks down hemes to biliverdin, releasing carbon monoxide and iron. Then biliverdin is reduced to bilirubin by biliverdin reductase. Biliverdin reductase is an important antioxidant system in many cell types. The upregulation of HO-1 is not only in response to the high Hb catabolism, but it also protects sickle RBCs from inflammation and oxidants [14].

Hemoglobin polymerization only occurs when the protein is deoxygenated and several SCD therapies have been developed to prevent the loss of oxygen from hemoglobin molecules.

Free Radicals in Sickle Cell Disorder

There are numerous linkages between the excessive amounts of free radicals and the incidence of vascular and tissue injury caused by SCD. This section will first discuss the role of

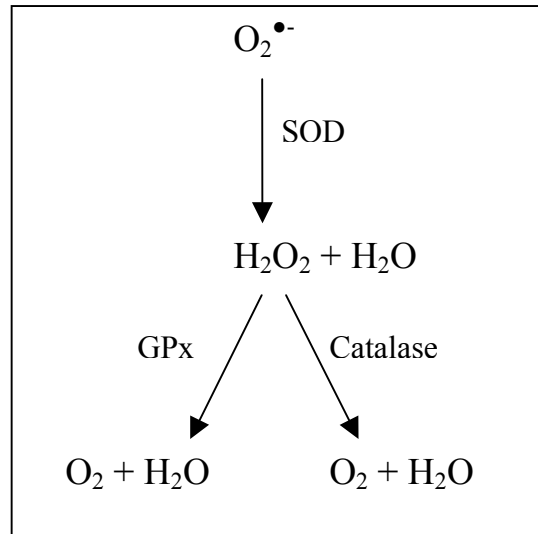
free radicals in RBCs, next examine the role of free radicals in the diseases and symptoms associated with sickle cell disorder and finally address the free radical chemistry involved in the treatment of SCD.

Free Radicals in RBCs

Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$) and hydrogen (H_2O_2) are naturally generated in RBCs. The heme iron of Hb transfers an electron to oxygen during the binding process of oxygenated Hb. At times, Hb can auto-oxidize resulting in production methemoglobin (metHb) and superoxide. Sickle Hb is instable and is especially prone to auto-oxidation. This is the process responsible for the steady state levels of superoxide in RBCs which can dismutate to form hydrogen peroxide and molecular oxygen. The pathway of ROS removal is shown in **Figure 2**. The rate of hemoglobin auto-oxidation is 1.7 times greater in sickle RBCs than in normal RBCs resulting in twice as much ROS generation and lipid peroxidation products. The auto-oxidation of sickle RBCs denatures the protein often resulting in large amounts of free iron which can catalyze membrane lipid peroxidation [5]. Red blood cells reduce metHb back to active Hb with energy from nicotinamide adenine dinucleotide (NADH). Sickle RBCs have an impaired ability to reduce metHb to Hb because of insufficient levels of NADH [15].

While sickle cells have increased levels of superoxide dismutase, the glutathione peroxidase and catalase levels in sickle RBCs are lower than in normal RBCs. This leads to an abnormal accumulation of H_2O_2 in the cell and could one be cause of increased oxidative damage in sickle cells [5,16].

Figure 2. The removal of ROS is catalyzed by the antioxidant proteins superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. An imbalance in antioxidant proteins could lead to inefficient removal of ROS which could result in oxidative stress [16].



When normal RBCs undergo stress from transfusion or oxidation, glutathione and glutathione peroxidase levels decrease. However, when normal glutathione levels are artificially maintained, RBCs are protected from oxidative damage and chemokine scavenging [17]. In another study it was demonstrated that the interaction between sickle red blood cells and endothelial cells *in vitro* causes oxidative stress resulting in a fivefold increase in thiobarbituric acid reactive species (TBARS). This study also found that RBC adhesion to endothelial cells, (which is responsible for ischemic events), and Nfk-B activation, (which induces inflammatory response), increased as a result of the oxidative stress [18]. Oxidative stress can also be induced in RBCs by tert-butyl hydroperoxide and Hb oxidation is inhibited by the presence of antioxidant flavenoids [19].

Red blood cells with HbS are more prone to lipid peroxidation damage than cells with normal hemoglobin (Hb A), however ascorbate levels in sickled cells of SCD patients are not significantly different from those with normal RBCs. Nevertheless, SCD patient serum ascorbate levels were much lower than control patients and urine-excreted ascorbate was 36% higher in SCD patients than control patients [20]. Westerman and colleagues postulated that the normal levels of ascorbate in sickle RBCs is due to the recycling of ascorbate in the presence of

free radicals. What is strange is that α -tocopherol (Vitamin E) levels are decreased in sickle RBCs despite sufficient amount of ascorbate to recycle it. Redox balance in sickle RBCs is also very important. Levels of other non-enzymatic antioxidants are such as glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GRx), carotenoids and zinc are significantly lower in sickle cells than in normal RBCs [21].

Free radicals in SCD-related conditions and diseases

The ischemia-reperfusion episodes of SCD patients are also associated with free radicals. Mice with the sickle cell mutation generated excessive ROS in response to reperfusion after an ischemic event causing reperfusion injury. This injury was characterized by higher ethane excretion, indicative of lipid peroxidation, and greater conversion of salicylic acid to 2,3-dihydroxybenzoic acid, which is a marker of hydroxyl radical generation, when compared to mice with normal RBCs. Reoxygenation following hypoxia conditions caused nuclear factor κ -B (Nfk-B) activation in the kidney [23] and liver cells [23,22] sickle cell mice, but not in normal mice. The activation of the transcription factor Nfk-B causes the upregulation of pro-inflammatory molecules and recruitment of neutrophils responsible for the inflammation associated with reperfusion injury [23].

Ischemia-reperfusion events also cause the upregulation of nitric oxide synthases (NOS). In response to the blockage of blood flow, NOS proteins are upregulated in order to produce nitric oxide for vascular relaxation. The nitric oxide produced by NOS can react with the already elevated superoxide levels in ischemic events to form the highly oxidative molecule peroxynitrite (ONOO^-). The increase in ROS and reactive nitrogen species (RNS) can wreak havoc on cell membranes and proteins. This accounts for some ischemia-reperfusion injury [24].

Oxidative stress associated with the interaction between sickle RBCs and endothelial cells was previously discussed but the mechanism for this interaction was not. Wood and colleagues found that endothelial cell-derived NADPH oxidase is the enzyme that regulates the adhesion of RBCs to endothelial cells. The superoxide produced by NADPH oxidase and free iron catalyze a reaction that allows for the cell-to-cell adhesion resulting in ischemia. The overexpression of copper and zinc containing superoxide dismutase (CuZnSOD) in endothelial cells prevented the inflammatory response usually seen in ischemia-reperfusion [25].

Free radicals in SCD treatment

Hydroxyurea (HU) is one of the treatments used to prevent sickling in SCD patients. In cancer treatment, HU prevents DNA synthesis by inhibition ribonucleotide reductase, but in SCD, hydroxyurea prevents the polymerization of deoxygenate HbS by inducing fetal hemoglobin (HbF) expression in cells [26]. Hydroxyurea is oxidized by heme groups to produce nitric oxide *in vivo*. The release of NO[•] from hydroxyurea induces HbF through the soluble guanylyl cyclase pathway [26].

Several experiments have been performed to identify the oxidation products of HU. Molecule 1 in **Figure 3** is hydroxyurea [27]. Treatment of HU with H₂O₂ results in the nitroxide radical shown as molecule 2 in **Figure 3**. Molecule 3 is the product of the nitroxide radical and a donated electron. These three species were found in using electron paramagnetic spectroscopy (EPR).

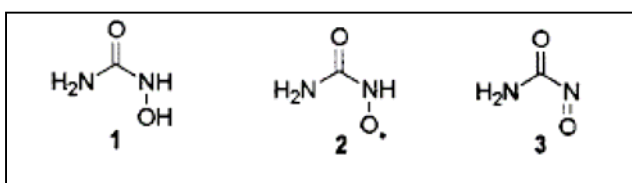


Figure 3. Hydroxyurea (1) is oxidized to a nitroxide radical (2) and a subsequent one electron reduction forms a C-nitroso

compound(3) [27].

Hydroxyurea been shown to inhibit tertiary-butyl hydroperoxide-induced and iron-induced lipid peroxidation in sickle cells [28], while it induces the generation of reactive species in large doses. Research has shown that treatment with ascorbic acid, D-mannitol and α -tocopherol can prevent HU-induced oxidative damage [29].

Table 1. Hydroxyurea protects RBC membranes from oxidative damage [28].

Addition to RBC Membrane	TBARS ($\mu\text{mol}/\text{mg}$ protein)
Membrane	1.21 +/- 0.07
tBOOH	2.60 +/- 0.15
tBOOH+Hydroxyurea	0.90 +/- 0.06
Hydroxyurea	0.66 +/- 0.05

A number of new drugs have been developed that produce the same effects as HU. One set of new drugs, ribonucleotide reductase inhibitors (RRIs) increase fetal hemoglobin, scavenge free radicals and prevent sickling of RBCs [30]. Antioxidant cocktail nutritional treatment for has also been suggested for sickle cell patients to inhibit dense cell formation [3,11]. The suggested cocktail includes aged garlic extract, ascorbic acid, α -lipoic acid, α -tocopherol, black and green tea extracts, coenzyme Q and β -carotene. Other physicians have had success treating SCD patients with inhaled S-nitrosated hemoglobin (iSNOHb) treatments.

Proposed Plan of Research

The following plan will address the specific aims aforementioned: **1. Demonstrate the role of oxidative stress in sickled cells, and 2. Develop a system that counteracts the side effects of treatment.**

Demonstrating the role of oxidative stress

Many experiments have shown that oxidative stress occurs in sickle cell disease crises and ischemia-reperfusion events, however, very few have identified oxidative stress as the cause of these traumatic events.

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