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Forum Review

SOD2-Deficiency Sideroblastic Anemia and Red Blood Cell Oxidative Stress

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ABSTRACT

Iron overload is a feature of an array of human disorders such as sideroblastic anemias, a heterogeneous group of erythropoietic disorders without identified cause in most cases. However, sideroblastic anemias appear to result from a disturbance at the interface between mitochondrial function and iron metabolism. A defining feature is excessive iron deposition within mitochondria of developing red cells, the consequences of which are an increase in cellular free radicals production, increased damage to proteins, and reduced cell survival. Because of its mitochondrial location, superoxide dismutase (SOD2) is the principal defense against the toxicity of superoxide anions generated by the oxidative phosphorylation. We have used hematopoietic stem cell transplantation to study blood cells lacking SOD2. We became interested in the role SOD2 plays in the metabolism of superoxide anions during erythroid development, as anemia is the major phenotype in transplanted animals. Our exploration of this model suggests that oxidative stress—and in particular, mitochondrial-derived oxidants—plays an important role in the pathogenesis of the human disorder, sideroblastic anemia. Here we review the relation between mitochondrial dysfunction and sideroblastic anemia, describe several methods for assessing oxidative damage to mature or developing red cells, present data on, and discuss the potential of antioxidant therapy for this disorder. *Antioxid. Redox Signal.* 8, 1217–1225.

INTRODUCTION

IRON OVERLOAD IS A FEATURE of an array of human disorders including sideroblastic anemia (SA), hemochromatosis, Friedreich ataxia, and Parkinson disease. Excess iron is toxic because it can catalyze the generation of reactive oxygen species (ROS) that are detrimental to cellular macromolecules in mammals (11). In particular, mitochondrial iron accumulation results in cellular damage, tissue injury, and organ failure (43). A variety of tissue injuries, mitochondrial disorders, and diseases are associated with elevated production of ROS. Oxidative damage has been reported to contribute to hemolysis in a variety of anemias, such as sickle cell anemia, β-thalassemia, erythropoietic protoporphyria, G6PD deficiency, and malaria (26, 32, 47). To buffer excessive oxidation, mammalian red blood cells (RBCs) are equipped with multiple antioxidant systems (25). Among them, three SOD enzymatic systems act synergistically to protect cells against superoxide from endogenous and exogenous sources. Mammalian manganese superoxide dismutase (SOD2) is a nuclear-encoded intramitochondrial antioxidant enzyme (53). Two related family members are the cytosolic copper-zinc SOD1 and the extracellular SOD3 (34, 37). Because of its mitochondrial location, SOD2 is the principal defense against the toxicity of superoxide anion radicals ($O2^{\bullet -}$) generated as a by-product of oxidative phosphorylation. It converts $O2^{\bullet -}$ to hydrogen peroxide (H_2O_2), which is then converted to water by catalase or glutathione (GSH) peroxidase (53). We have used hematopoietic stem cell transplantation to study blood cells deficient in SOD2. We became interested in the role that SOD2 plays in the metabolism of $O_2^{\bullet -}$ during erythroid development, as anemia is the major phenotype in transplanted animals (15). This result was somewhat surprising, given that mature erythrocytes do not possess mi-

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tochondria. Our exploration of this model suggests that oxidative stress—and in particular, mitochondrial-derived oxidants—play an important role in the pathogenesis of the human disorder SA.

We review the relation between mitochondrial dysfunction and SA, describe several methods for assessing oxidative damage to mature or developing RBCs, and present data on and discuss potential of antioxidant therapy for this disorder.

SOD DEFICIENCIES AND ANTIOXIDANT TREATMENT

Although the genetic inactivation in mice of *Sod1* and *Sod3* have generated mild phenotypes and normal life span (7, 46), inactivation of *Sod2* results in embryonic or neonatal lethality, depending on the strain background (27, 30). The SOD2-deficient ($Sod2^{-/-}$) phenotype is associated with pathologic evidence of mitochondrial injury, metabolic disorders, oxidative stress-induced damage to macromolecules, as well as severe damage to cardiac muscle and neural tissue (27, 30, 38, 41). Because of the unambiguous role of enhanced oxidative stress in the lethality resulting from SOD2 deficiency, therapeutic trials using synthetic SOD or combined SOD/catalase-mimetic compounds have been performed. A partial rescue was reported with the synthetic SOD-mimetic manganese 5, 10, 15, 20-tetrakis (4-benzoic acid) porphyrin (MnTBAP); treated mice succumbed to neural degeneration within several weeks of birth, showing a slight increase in life span (41). Synthetic salen–manganese complexes demonstrating both SOD and catalase activities have also been tried (Euk compounds; Eukarion, Inc., Bedford, MA). Such antioxidants provide partial protection in a variety of conditions that involve overproduction of oxygen free radicals. They extended the life span of prematurely aging *Caenorhabditis elegans*, demonstrating the importance of ROS as a major factor in limiting life span in this system (40). They prevented acidosis and anoxia damage in hippocampal homogenates *in vitro* (42); *in vivo*, they protected rat kidneys from ischemia–reperfusion-induced damage (17), and rat brains against ischemic injury (4). Encouragingly, when tested in $Sod2^{-/-}$ mice, these compounds appear to have higher *in vivo* bioactivity than agents such as MnTBAP, although their therapeutic effect is only partial (19, 39).

SOD2-DEFICIENCY SIDEROBLASTIC ANEMIA

We have used hematopoietic cell transplant to study blood cells and circumvent the lethality of SOD2 deficiency. Murine fetal liver cells are used as a source of hematopoietic stem cells (HSCs) to reconstitute lethally irradiated congenic mice. $Sod2^{-/-}$ HSCs replace host hematopoietic cells and can be maintained *in vivo* with identical lymphoid and myeloid engraftment kinetics and durability when compared with wild-type (WT) HSCs (15). This system places $Sod2^{-/-}$ HSCs in a metabolically normal host, allowing the assessment of potential cell-autonomous phenotypes in hematopoietic tissues that lack the SOD2 protein over long periods. Whereas $Sod2^{-/-}$ HSCs rescue irradiated WT recipients, $Sod2^{-/-}$ chimeric mice show a persistent hemolytic anemia secondary to increased endogenous oxidative stress, with similarities to human hereditary and acquired SA (15). Loss of SOD2 in erythroid progenitors alters membrane deformation ability and shortens the life span of peripheral $Sod2^{-/-}$ RBCs (15). $Sod2^{-/-}$ nucleated marrow cells and peripheral RBCs show increased levels of oxidized protein residues after derivatization of protein carbonyls with 2,4-dinitrophenylhydrazine and Western blotting (14, 15). A flow-cytometry–based assay involving oxidation-sensitive dyes revealed that $Sod2^{-/-}$ RBCs produce enhanced ROS levels; we also showed that the higher the iron content of RBC, the higher the ROS production (14, 35). In addition, measurement of zinc protoporphyrin (ZPP) levels showed a defect in iron incorporation into heme (14). $Sod2^{-/-}$ reticulocytes (Rtc) show increased mitochondrial number and mass. Microscopy reveals abundant stainable iron deposits in Rtc and mature RBCs (14, 35). Importantly, electron microscopy reveals iron deposition within mitochondria—a diagnostic feature of human SA. The accumulation of ferrimagnetic minerals in human tissues has been reported (10). We reasoned that it may be possible to purify iron-laden cells by using magnetic columns. We recently developed a method allowing the rapid purification of $Sod2^{-/-}$ siderocytes (35) and human sideroblasts (unpublished results). Passage of $Sod2^{-/-}$ peripheral blood through magnetic affinity columns allowed retention of 2.8% of the total RBCs as iron-laden siderocytes. Flow cytometry and electron microscopy showed that $Sod2^{-/-}$ siderocytes are mainly Rtc with excess iron deposition within mitochondria, in the intermembrane space as well as in the mitochondrial matrix. Purified cells produce high levels of ROS and show enhanced protein oxidative damage, mitochondrial enrichment, and altered mitochondrial membrane potential ($\Delta\Psi_m$) (35).

A comparative proteome analysis based on 2-D gel separation and MALDI mass spectrometry identified 41 proteins, involved in folding/chaperone functions, redox regulation, adenosine triphosphate synthesis, and RBC metabolism, as differentially expressed when comparing membrane preparations from $Sod2^{+/+}$, $Sod2^{-/-}$, and antioxidant-treated $Sod2^{-/-}$ RBCs (14). Treatment of $Sod2^{-/-}$ chimeric mice with SOD/catalase-mimetic antioxidant Euk drugs significantly corrected the anemic phenotype as it improved hematocrit (Hct), decreased Rtc count, and increased RBC life span (14, 15). Antioxidant therapy also affected differential protein expression in some intriguing ways. For instance, several proteins involved in mitochondrial bioenergetics decreased in abundance with antioxidant therapy, while at the same time, several enzymes involved in metabolism increased (14)—this may indicate improved mitochondrial function in the presence of antioxidant. Also of interest, peroxiredoxin 2 (PRDX2), a member of the thioredoxin peroxidase family (23), was found to be decreased in $Sod2^{-/-}$ cells, but showed an increase with antioxidant treatment. Knockout of PRDX2 causes a mild hemolytic anemia (29) with evidence of increase oxidative damage to mature RBCs. This suggests that PRDX2 may be important target of oxidative damage in $Sod2^{-/-}$ cells. In support of this thesis, we have found that

PRDX2 is highly oxidized (as measured by protein carbonyl content) in Sod2$^{-/-}$ cells, and that SOD/catalase mimetics ameliorate change in its expression (14). Based on phenotypic similarity between Sod2$^{-/-}$ siderocytes and sideroblasts seen in human SA, we propose that oxidative stress is a critical event in the pathogenesis of SA and is sufficient to induce mitochondrial dysfunction and pathologic iron accumulation.

HEMOLYTIC ANEMIAS, MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS

SAs are a heterogeneous group of acquired and inherited erythropoietic disorders without identified molecular cause in most cases; the etiology, epidemiology, pathophysiology, and treatment of these conditions differ vastly (1, 12, 36). However, two defining features are the presence of ringed sideroblasts in the bone marrow, abnormal erythroblasts with pathologic mitochondrial iron deposition, and impaired heme biosynthesis. Some genetic lesions have been identified as causes of hereditary or acquired SA (12). In cases with defined genetic lesions, dysfunction in one of several mitochondrial metabolic pathways has been observed: heme synthesis, iron homeostasis and transport, or electron transport. These lesions result in abnormal utilization of erythroid mitochondrial iron, causing pathologic iron deposition within mitochondria (43, 48). Identified lesions affect nuclear-encoded mitochondrial proteins or the mitochondrial genome. The heme biosynthetic pathway was identified as a primary cause of SA. However, other pathways, including mitochondrial oxidative phosphorylation, thiamine metabolism, and iron–sulfur cluster biosynthesis, were also identified as primary defects in SAs; they may secondarily affect heme metabolism (12, 48). Two X-linked sideroblastic anemias (XLSAs) exist, one caused by mutations of an erythroid-specific form of the heme biosynthetic enzyme aminolevulinic acid-synthase (ALAS2) (8), and one caused by mutation of a putative mitochondrial iron-transport protein, ATP-binding cassette, member 7 (ABC7) (2). Mutation of another mitochondrial membrane protein, flexed-tail, was reported to cause a transient neonatal form of SA in mice (13). Mitochondrial DNA (mtDNA) lesions are also involved in both acquired and inherited SA. Pearson marrow–pancreas syndrome results from a large deletion in mtDNA (44). Somatic point mutations in cytochrome *c* oxidase subunit 1 (COX1; *i.e.,* complex IV of the respiratory chain) have been found in patients with acquired idiopathic sideroblastic anemia (AISA) as a cause for respiratory chain dysfunction (16). Cases of acquired SA are also associated with specific drugs: the antibiotic chloramphenicol exerts its toxicity *via* mitochondrial dysfunction by inhibiting mitochondrial protein synthesis, suppressing the bone marrow, and inducing sideroblastic anemia (5); antituberculous therapy interferes with pyridoxine metabolism, a cofactor for ALAS2, resulting in mitochondrial dysfunction and SA (50, 55). These findings clearly suggest that mitochondrial dysfunction, in particular excessive ROS production and excess iron accumulation, plays a critical role in the etiology of SA. The specific location of iron deposits within the mitochondria of Sod2$^{-/-}$ siderocytes appear slightly different from those found in reported clinical biospecimens. To our knowledge, iron accumulation has not been reported to occur at the level of the intermembrane space in SA clinical samples or other disease models. On the contrary, iron deposits in human sideroblasts and Rtc are found predominantly within the mitochondrial matrix. Deposits are described as finely granular, electron-dense material resembling ferritin micelles, or amorphous accumulations of coarser granules (6, 51, 54). The observation that mice have siderocytes instead of sideroblasts is seen not only in SOD2 deficiency but also in other experimental forms of SA such as pyridoxine deficiency (24). We hypothesized that the aforementioned differences in the iron-deposition patterns and maturational stage (sideroblasts *vs.* siderocytes) at which abnormalities occur may be due to a species difference.

Oxidative stress is another critical factor in hemolysis in a variety of anemias. For instance, mice that lack SHP-1, a critical negative regulator of signal transduction in hematopoietic cells, show severe immune and inflammatory dysfunction, accompanied by hyperproliferation of myeloid cells, and a regenerative anemia. Their erythrocytes show increased oxidant damage susceptibility, as evidenced by a significant elevation of lipid peroxidation and diminished levels of GSH. Lyons *et al.* (33) hypothesized that as a consequence of severe inflammatory disease, SHP-1$^{-/-}$ erythrocytes are subject to exceptionally high oxidative stress, resulting in oxidation of phospholipids in the erythrocyte membrane with subsequent hemolysis (33). Lee *et al.* (28) reported that a long-term increase in oxidative stress due to decreased antioxidant capacity increases sequestration of oxidatively damaged erythrocytes and causes immune-mediated hemolytic anemia in mice lacking the basic leucine zipper transcription factor NF-E2^{p45}-related factor 2 (Nrf_2), suggesting a critical role of Nrf_2-antioxidant responsive element pathway in the cellular antioxidant defense system. Nrf_2 regulates the transcription of a wide array of antioxidant responsive element (ARE)-driven genes in various cell types including glutathione S-transferase and NAD(P)H:quinone oxidoreductase (22). Hamsters in the progressive anemic response at different stages of leishmanial infection show oxidative damage to erythrocytes including oxidative denaturation of hemoglobin and enhanced formation of malonyldialdehyde. Decreased activities of SOD and catalase in the infected animals are associated with decreases in the reduced GSH level along with the decreased activities of GSH-related enzymes during the postinfection period. A hypothesis is that enhanced degradation of cytoskeletal and integral membrane proteins induces membrane destabilization and early lysis of erythrocytes in this model (49).

SYNTHETIC SOD/CATALASE MIMETICS PARTIALLY RESCUE SOD2-DEFICIENCY ANEMIA

We reasoned that if oxidative stress is the etiologic agent of anemia due to loss of SOD2, treatment with antioxidant compounds should ameliorate the condition of Sod2$^{-/-}$

chimeric mice, (*i.e.,* rescue their phenotype). Euk-8 therapeutic trials of anemic Sod2$^{-/-}$ chimeric mice were initially performed. Transplanted mice received either Euk-8 at a dose of 30 mg/kg 3 days/week, or sham injections on the same schedule. Pretreatment Hct and Rtc counts were compared with samples obtained after 8 weeks of treatment with Euk-8. No effect of drug treatment was seen on the Hct of animals that received Sod2$^{+/+}$ HSCs. Recipients of Sod2$^{-/-}$ HSCs showed a significant increase in Hct in response to 8-week Euk-8 therapy, and a corresponding decrease in Rtc count. This demonstrates that enhanced protection from oxidative stress using a combined SOD/catalase mimetic can significantly ameliorate the stress-induced anemia of Sod2$^{-/-}$ fetal liver recipients (15). Optimization of the dose or compound design may yield more complete phenotypic rescue in mice, and such antioxidant therapy may prove useful in treatment of human disorders such as SA, should a direct role for oxidant stress in pathogenesis be established.

We then wanted to determine whether antioxidant therapy affects erythropoiesis, protects peripheral mature RBCs, or both (*i.e.,* to determine the effect of antioxidant therapy on RBC life span). A lipophilic analog of Euk-8, Euk-189, which was the most effective "Euk" drug in mice (39), was used in a study to determine at what stage of RBC development antioxidant therapy exerted a protective effect (14). The RBC survival study was performed by using an established *in vivo* biotinylation method (20). Frequency of injections and doses of Euk-189 were identical to those used in the previous trial (15), but Sod2$^{-/-}$ HSC transplanted mice were divided into four groups for RBC life-span measurement in the presence or absence of Euk-189: group 1, pretreatment for 4 weeks and treatment during RBC life-span measurement; group 2, pretreatment stopped at the time of RBC biotin-labeling; group 3, Euk-189 treatment starting at the time of RBC labeling; and group 4, sham injections. We showed that SOD/catalase mimetics act primarily on marrow erythroid progenitors (*i.e.,* increased RBC survival when given during RBC formation, but did not extend the survival of already circulating mature RBCs). Surprisingly, treatment starting coincidently with RBC labeling reproducibly resulted in a measurable decrease in survival of peripheral cells. In addition, antioxidant therapy had no significant effect on protein carbonyl formation, a protein-damage marker, or on steady-state ROS production by Rtc or mature RBCs *in vivo*. The effect of Euk drugs remain poorly understood. One interpretation is that whereas the net effect of catalytic antioxidant therapy is protective, it may be the sum of both positive and negative effects on developing and mature RBCs, respectively.

ANTIOXIDANT PROPERTIES *VERSUS* TOXICITY OF MANGANESE

Mn is one of the essential trace elements required for all living organisms. Mn is a cofactor for various enzymes such as hydrolases, kinases, carboxylases, and transferases; in particular, it is a constituent of SOD2 and the catalytic center of Euk antioxidant drugs. It takes a part in certain physiologic functions such as brain development and metabolism (21). In animals, manganese deficiency is a cause of skeletal abnormalities and impaired growth as well as ataxia (45). Although, Mn is an essential element at physiologic levels, it produces toxic effects at higher dose (21). The toxicity of Mn has been associated with the general propensity of transition metals to produce cytotoxic levels of free radicals during redox cycling (18). Manganese, specifically in the divalent state, can act in both a pro- and antioxidant manner (18). In *Escherichia coli*, lacking cytoplasmic superoxide dismutases, Mn(II) enrichment of the culture medium stimulated bacterial growth. For these cells, resistance to the lethality of H_2O_2 and decrease in the mutation frequency were demonstrated. *E. coli* gain a SOD-like activity from growth in Mn(II)-supplemented medium. Also, Mn(II) can take place of the SODs in SOD-null *Lactobacillus plantarum*, yeast and *Bacillus subtilis* (3). Divalent manganese facilitates the disproportionation of H_2O_2 in a catalase-like manner. *In vivo*, this metal can also inhibit iron-induced lipid peroxidation and dopamine depletion in mammalian brain tissues, thus protecting neurons against oxidative stress induced by iron complexes (52). *In vitro* studies compared the ability of Mn in scavenging ROS to SOD2 activity in different brain regions in adult rats treated with $MnCl_2$ by incubating brain cells with different concentrations of Mn(II) and Mn(III). These experiments showed that in Mn-treated rat brain cells, nanomolar and micromolar concentrations effectively scavenged $O_2^{\bullet -}$ and hydroxyl radicals, respectively, acting in a fashion similar to SOD2. The results showed that SOD2 activity increased in Mn-treated animals (21).

SOD2-DEFICIENCY ANEMIA AND THERAPEUTIC TRIAL OF MANGANESE

Because Mn is the catalytic center in both SOD2 and Euk antioxidant drugs, we hypothesized that Mn alone could potentially alleviate the oxidative stress–induced anemic phenotype of the Sod2$^{-/-}$ chimeric mice.

Experimental design

The goal of this experiment was to treat Sod2$^{-/-}$ chimeric mice with amounts of Mn being stoichiometric to those founds in Euk drugs and to assess whether this treatment was enough to alleviate the anemic phenotype of mutant mice.

Manganese chloride ($MnCl_2 \bullet 4H_2O$; MCB Manufacturing Chemists, Cincinnati, OH) powder was resuspended in 5% dextrose (Harrel Medical, Canby, OR) at a 1.57 mg/ml concentration and 0.2-μm filter sterilized. Two cohorts of Sod2$^{-/-}$ *versus* Sod2$^{+/+}$ chimeric mice, generated as previously described (15), were weighted to determine $MnCl_2$ dosage, isoflurane-anesthetized, and bled at the retroorbital sinus to check anemia-related parameters (Hct and Rtc counts) and RBC ROS production at baseline. Before sampling, mice received 1 drop of an ophthalmic solution of 0.5% tetracaine hydrochloride (Miza Pharmaceuticals, Fairton, NJ). Mice received either 16 mg/kg $MnCl_2$ 3 times per week, or placebo (dextrose alone) *via* i.p. injection, for a month (16 injections total).

In the first part of the experiment, mice were bled to provide a baseline reading and were bled again after 1 month of treatment or placebo. Animals were then rested for 15 days before crossing over for a second month of treatment. Injections resumed on the same schedule, but mice previously $MnCl_2$ treated were switched to placebo treatment and *vice versa*; in a crossover design (cf. Fig. 1), as crossover studies often have greater statistical power than parallel-group designs that include a larger number of subjects) (31).

Hct, Rtc count, and ROS production were assayed at the beginning and the end of each part of the experiment. Hct levels were measured by using the spin method; Rtc counts and ROS production were assayed with flow cytometry by using a CellQuest Pro-equipped FACSCalibur (BD Biosciences, San Jose, CA), as described (35). For Rtc count, RBCs were stained with Retic-COUNT Reagent (thiazole orange; BD Biosciences) and analyzed according to manufacturer's instructions. For ROS-production assays, RBCs were washed in 0.5% BSA/2 m*M* EDTA/PBS FACS buffer and resuspended in prewarmed phenol red–free RPMI 1640 media (Invitrogen, Carlsbad, CA). We used the oxidation-sensitive dyes dihydroethidium (DHE), and chloromethyl, dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Molecular Probes, Eugene, OR). Cell samples were incubated in 10 μ*M* DHE/2 μ*M* CM-H2DCFDA/phenol red–free RPMI 1640 media at +37ºC, for 30 min in a humid 5% CO_2 incubator. Samples were washed with cold FACS buffer and analyzed by flow cytometry as reported (9, 35).

Analysis of variance (ANOVA) was performed by using GraphPad Prism version 4.0b for Macintosh (GraphPad Software, San Diego, CA); $p < 0.05$ was considered statistically significant.

$MnCl_2$ therapy partially rescues the oxidation-induced anemic phenotype of $Sod2^{-/-}$ chimeric mice

Under physiologic conditions, Hct was significantly decreased, and Rtc counts were significantly increased in $Sod2^{-/-}$ chimeric mice relative $Sod2^{+/+}$ recipients (Fig. 2; a***, $p < 0.001$), in accord with prior studies (15). In a similar manner, ROS production levels were enhanced by the lack of SOD2 (Fig. 3A and C; a***, $p < 0.001$).

One month of $MnCl_2$ treatment did not dramatically alter these parameters in $Sod2^{+/+}$ chimeric mice (Figs. 2 and 3A and B). Whereas $MnCl_2$ treatment only slightly improved Hct in $Sod2^{-/-}$ chimeric mice (not significant p value), it significantly decreased their Rtc counts and ROS production (Figs. 2B and 3C and D; b***, $p < 0.001$), suggesting an effect of manganese on RBC precursors rather than on mature RBCs. A similar effect was previously observed with Euk-189, while combining antioxidant treatment with measurement of RBC life span to determine whether antioxidant therapy exerts its effect during RBC development, in the periphery, or both.

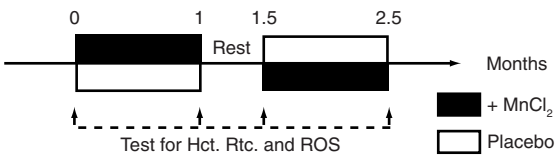

FIG. 1. Crossover study of $MnCl_2$ antioxidant properties in anemia. Mice were bled before and after each part of the experiment to assay Hct and Rtc levels and ROS production by red blood cells. *Rectangles,* Alternate treatment with $MnCl_2$ (250 μl of a 1.57-mg/ml $MnCl_2$•$4H_2O$/5% dextrose solution, i.p./3 times/wk) or dextrose alone (placebo).

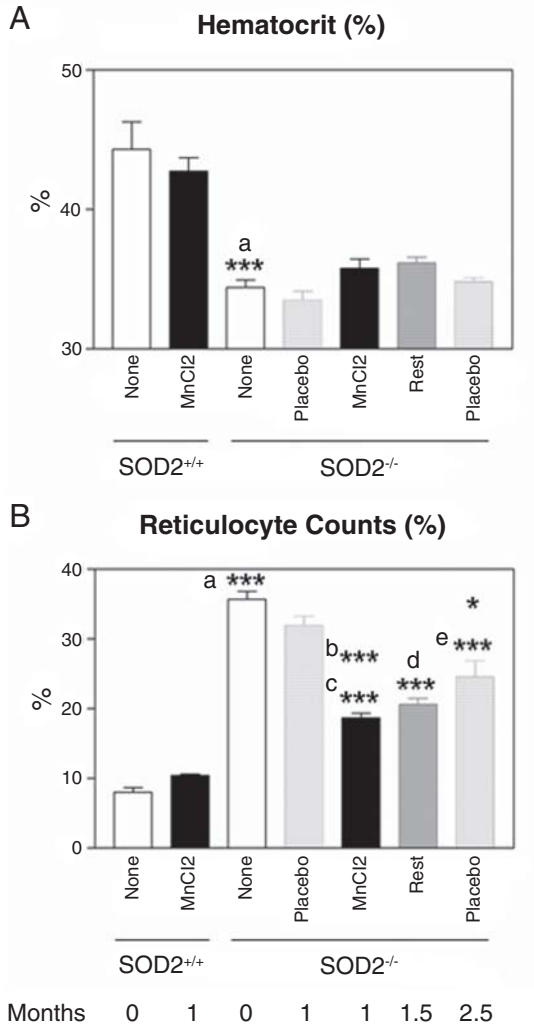

FIG. 2. $MnCl_2$ treatment of $Sod2^{-/-}$ chimeric mice partially rescues hematocrit and reticulocyte counts. Experimental controls including 1-month treatment of $Sod2^{-/-}$ and $Sod2^{+/+}$ (not shown) chimeric mice with vehicle alone (placebo) showed no significant effect on either Hct or Rtc counts; drug treatment of $Sod2^{+/+}$ chimeric mice did not alter these parameters. $MnCl_2$ treatment significantly decreased Rtc counts in $Sod2^{-/-}$ chimeric mice (b***). *Open bars*, baseline; *black bars*, 1-month $MnCl_2$ treatment; *light grey bars*, 1-month placebo treatment; *dark grey bars*, 15 days of rest; time is shown in months at the bottom. Data presented are mean (%) ± SEM; parametric two-way analysis of variance was used for statistical analysis with Tukey–Kramer multiple comparison posttest between groups ($Sod2^{+/+}$, $n = 3$; $Sod2^{-/-}$, $n = 4$–11). *$p < 0.05$; ***$p < 0.001$.

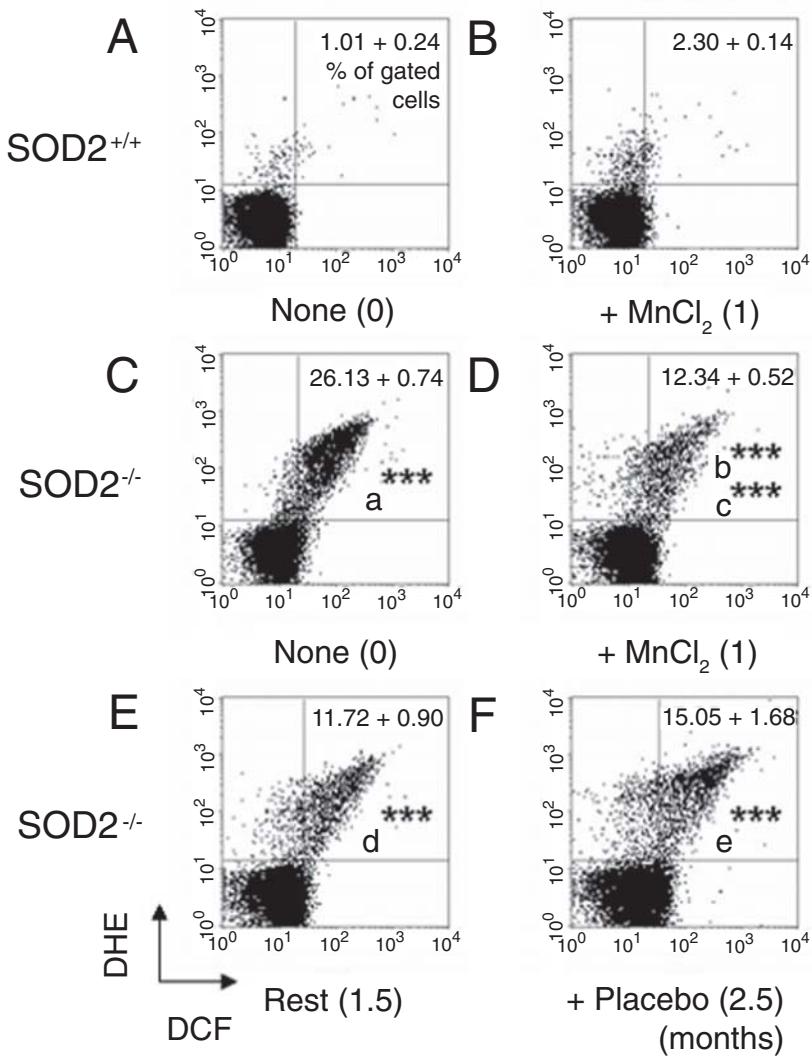

FIG. 3. $MnCl_2$ treatment of Sod2$^{-/-}$ chimeric mice partially rescues ROS levels in red blood cells. Controls including 1-month treatment of Sod2$^{-/-}$ and Sod2$^{+/+}$ chimeric mice with vehicle alone (placebo; not shown) showed no significant effect on ROS levels; drug treatment of Sod2$^{+/+}$ chimeric mice did not alter ROS production. $MnCl_2$ treatment significantly decreased ROS levels in Sod2$^{-/-}$ chimeric mice (b***); this effect is persistent after stopping $MnCl_2$ administration but shows partial reversal after several weeks. **A, C**, baseline; (**B, D**) 1-month $MnCl_2$ treatment; (**E**) +15 days of rest; (**F**) +1 month of placebo; time is shown in months in brackets. Density dot plots were drawn with CellQuest Pro (BD); data presented are mean ±SEM of DCF$^+$ DHE$^+$ double-positive RBCs in percentage of gated cells. Parametric two-way ANOVA was used for statistical analysis with Tukey–Kramer multiple comparison posttest between groups (Sod2$^{+/+}$, $n = 3$;

Our study showed that RBCs that develop in the presence of the catalytic Euk-189 drug exhibit extended peripheral survival (14). However, the $MnCl_2$-mediated rescue of the Sod2$^{-/-}$ Rtc counts was only partial, as $MnCl_2$-treated Sod2$^{-/-}$ chimeric mice still demonstrate higher Rtc counts relative to Sod2$^{+/+}$ mice (c***, $p < 0.001$).

Of interest, effects of manganese treatment on Hct, Rtc, and ROS production were sustained after 15 days of rest and 1 month of placebo. Differences in Rtc counts and ROS levels between time 1.5 and 2.5 months *versus* time 0 in Sod2$^{-/-}$ mice were still significant (Figs. 2B and 3E and F; d*** and e***). However, we observed a gradual return to normal Rtc counts (*, $p < 0.05$) and ROS levels (not significant p value) after placebo treatment (2.5 months), suggesting that the manganese effect on the anemic phenotype of Sod2$^{-/-}$ chimeras is slowly reversible.

Further to explore the effect of Mn on the production of specific ROS, we compared the individual DCF and DHE geometric mean fluorescence intensities (geoMFI) in treated mice. Although the effect of manganese on DCF oxidation was weak (not significant p values), 1 month of Mn treatment induced a significant decrease in DHE geoMFI (134.6 ± 18.51 *vs.* 72.75 ± 12.81 geoMFI, baseline *vs.* 1 month $MnCl_2$, respectively; mean ± SEM; $p < 0.05$) in Sod2$^{-/-}$ RBCs. One interpretation is that manganese exerts its antioxidant effect by scavenging $O2^{\bullet-}$ in Sod2$^{-/-}$ RBCs (*i.e.,* mimicking the absent SOD2 activity by converting superoxide to peroxide). This interpretation would be consistent with no significant change in DCF oxidation, as DCF is sensitive to peroxide (and mixed ROS). Alternatively, it may be that Mn exerts its effects indirectly, perhaps by competing with iron for transport into the mitochondria or at other sites within the cell. Inhibiting the accumulation of redox active iron in developing RBCs would have a protective effect.

As with prior studies involving Euk drugs, we followed Sod2$^{-/-}$ *versus* Sod2$^{+/+}$ RBC survival during the course of $MnCl_2$ therapy to determine whether treatment affected RBC survival. As opposed to results obtained with Euk-189 (14), we did not observe any Mn effect on RBC life span (not shown); however, this appears consistent with the weak positive effect of Mn on Hct in Sod2$^{-/-}$ chimeric mice and the slight decrease of Hct in Sod2$^{+/+}$ recipients (Fig. 2A; not sig-

nificant *p* values). As with the Euk antioxidant compounds, it appears that treatment with $MnCl_2$ has both positive and negative effect on erythroid cells. By measuring Rtc count and ROS production of SOD2-deficient cells, it is evident that $MnCl_2$ ameliorates these indices of pathology. However, this effect seems to be balanced by a weak negative effect on Hct in treated animals. This is reminiscent of the small but reproducible decrease in Hct that we have previously seen with SOD/catalase-mimetic therapy in this model (15). Although possible, it seems unlikely that these changes are due to experimental stress or repeated, small-volume blood sampling during RBC half-life experiments, as we do not see similar behavior in placebo treated animals within the same experiment.

Our results clearly demonstrate that manganese has a therapeutic effect on the $Sod2^{-/-}$ anemic phenotype under this experimental setup. However, unresolved questions still exist regarding the actual biologic function of manganese (*i.e.,* discriminating in whether manganese acts as an antioxidant, as a regulator of the iron metabolism, or both). Other obscurities include the optimal dose and the optimal route by which to give the drug. Although this remains to be investigated, we believe that giving $MnCl_2$ to mice in their drinking water may also significantly rescue their condition; also this route of administration will be of interest in the human disease.

SUMMARY

SA appears to result from a disturbance at the nexus of mitochondrial function and iron metabolism. It is fascinating that lesions affecting iron metabolism/utilization (ABC7 or ALAS) can have essentially the same phenotype as mutations affecting mitochondrial physiology (*Sod2* or *Cox1*). The common pathologic picture is tremendous accumulation of iron within the mitochondria. A consequence of this iron accumulation is a marked increase in cellular ROS production and increased damage to proteins, as measured by carbonyl formation. Clearly, unique features of developing erythrocytes make them susceptible to this type of iron accumulation, as other cell lineages harboring the same mutations do not show this type of manifestation. This may reflect the unsurpassed capacity of developing RBCs to uptake iron for heme biosynthesis, and suggest that regulatory elements of this uptake machinery may be directly affected by alterations in cellular redox status. Investigation of this possibility will be the focus of our future work.

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ABBREVIATIONS

ABC7, ATP-binding cassette, member 7; AISA, acquired idiopathic sideroblastic anemia; ALAS2, aminolevulinic acid-synthase; ANOVA, analysis of variance; ARE, antioxidant responsive element; CM-H2DCFDA, chloromethyl, dichlorodihydrofluorescein diacetate, acetyl ester; COX1, cytochrome *c* oxidase subunit 1; $\Delta\Psi_m$, mitochondrial membrane potential; DHE, dihydroethidium; geoMFI, geometric mean fluorescence intensity; GSH, glutathione; Hct, hematocrit; HSC, hematopoietic stem cell; H_2O_2, hydrogen peroxide; i.p., intraperitoneal; Mn, manganese; $MnCl_2$, manganese chloride; MnTBAP, manganese 5, 10, 15, 20-tetrakis (4-benzoic acid) porphyrin; mtDNA, mitochondrial DNA; Nrf_2, NF-E2^{p45}-related factor 2; RBC, red blood cell; ROS, reactive oxygen species; Rtc, reticulocyte; PRDX2, peroxiredoxin 2; SA, sideroblastic anemia; SOD2, manganese superoxide dismutase 2; $Sod2^{-/-}$, SOD2-deficient; $O2^{\bullet -}$, superoxide anion radicals; WT, wild type; XLSA, X-linked sideroblastic anemias; ZPP, zinc protoporphyrin.

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