Absence of Mitochondrial Superoxide Dismutase Results in a Murine Hemolytic Anemia Responsive to Therapy with a Catalytic Antioxidant

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Abstract

Manganese superoxide dismutase 2 (SOD2) is a critical component of the mitochondrial pathway for detoxification of O_2^-, and targeted disruption of this locus leads to embryonic or neonatal lethality in mice. To follow the effects of SOD2 deficiency in cells over a longer time course, we created hematopoietic chimeras in which all blood cells are derived from fetal liver stem cells of *Sod2* knockout, heterozygous, or wild-type littermates. Stem cells of each genotype efficiently rescued hematopoiesis and allowed long-term survival of lethally irradiated host animals. Peripheral blood analysis of leukocyte populations revealed no differences in reconstitution kinetics of T cells, B cells, or myeloid cells when comparing $Sod2^{+/+}$, $Sod2^{-/-}$, and $Sod2^{+/-}$ fetal liver recipients. However, animals receiving $Sod2^{-/-}$ cells were persistently anemic, with findings suggestive of a hemolytic process. Loss of SOD2 in erythroid progenitor cells results in enhanced protein oxidative damage, altered membrane deformation, and reduced survival of red cells. Treatment of anemic animals with Euk-8, a catalytic antioxidant with both SOD and catalase activities, significantly corrected this oxidative stress–induced condition. Such therapy may prove useful in treatment of human disorders such as sideroblastic anemia, which SOD2 deficiency most closely resembles.

Key words: transplantation (fetal liver) • oxidative stress • antioxidant • stem cells • SOD2

Introduction

Manganese superoxide dismutase 2 (SOD2)[1] is a nuclear-encoded mitochondrial protein that converts superoxide radicals to H_2O_2, which is then acted upon by glutathione peroxidase or catalase to yield nontoxic products (1). Similar enzymes have been isolated from the cytoplasm (SOD1 or CuZnSOD) and extracellular fluids (SOD3; reference 2). Together, these enzyme systems are responsible for protecting cells from reactive oxygen species (ROS) generated from endogenous and exogenous sources. Endogenous ROS produced by mitochondria as a by-product of respiration are proposed to be a major causal factor in organismal aging and cellular senescence. Support for this hypothesis comes from studies of model organisms in which increased longevity has been correlated with the enhanced ability to withstand oxidative stress (3–5), or with decreased endogenous production of ROS (6, 7). Conversely, impairment of an organism's ability to withstand oxidative stress, or increased generation of endogenous ROS, leads to a shortened life span characterized by accelerated aging phenotypes (8, 9). The relevance of oxidative stress as a determinant of mammalian longevity is supported by a recent study demonstrating that an oxidative stress–activated signal transduction pathway is linked to apoptosis and regulates life span in mice (10). The possibility of altering longevity through pharmacological intervention to ameliorate ROS-induced damage has also been explored in a recent study in which *Caenorhabiditis elegans*

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[1]*Abbreviations used in this paper:* CBC, complete blood count; MCV, mean corpuscular volume; ROS, reactive oxygen species; SA, sideroblastic anemia; SOD, superoxide dismutase.

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were treated with a novel class of antioxidants placed directly into the growth medium, resulting in extension of mean life span by >40% (11).

Sod2 knockout mice were originally produced independently on two separate strain backgrounds (12, 13), both of which demonstrate a lethal phenotype in which the time of death is dependent on the genetic background. $Sod2^{-/-}$ animals have pathologic evidence of mitochondrial injury, with corresponding evidence of damage to cardiac muscle and neural tissues as well as metabolic derangement including acidosis and lipid accumulation. A partial rescue of this phenotype has been reported by using the synthetic SOD mimetic manganese 5, 10, 15, 20-tetrakis(4-benzoic acid) porphorin (MnTBAP), although treated animals succumb to neural degeneration within several weeks of birth, presumably because of failure of this agent to cross the blood–brain barrier (14). In addition to early lethality, $Sod2^{-/-}$ animals have increased oxidative DNA damage and respiratory chain defects in mitochondria (15). Heterozygous animals appear normal, but also show oxidative damage to mitochondrial DNA, decreased levels of reduced glutathione, and altered mitochondrial function (16, 17). Thus, *Sod2*-deficient mice demonstrate evidence of increased damage from mitochondrial ROS, but because of the severe and pleiotropic nature of the defect in $Sod2^{-/-}$ animals, it has not been possible to study the effects of SOD2 deficiency in cells over long periods of time in vivo.

To circumvent this problem, we constructed a transplant system in which $Sod2^{-/-}$ cells replace host hematopoietic cells and can be maintained for several months in vivo. This system places *Sod2*-deficient cells in a metabolically normal host animal, allowing for the assessment of cell autonomous phenotypes caused by increased intracellular damage from mitochondrial ROS. This makes possible an assessment of the specific role of mitochondrial antioxidant protective systems on immune and hematopoietic cell reconstitution and function. We report that murine fetal liver stem cells deficient in *Sod2* are capable of efficiently rescuing lethally irradiated host animals. However, whereas lymphoid and myeloid engraftment kinetics and durability are identical across all *Sod2* fetal liver genotypes (+/+, −/−, and +/−) there is a selective defect in erythroid reconstitution of $Sod2^{-/-}$ fetal liver recipients. This defect is similar to that seen clinically in hereditary and acquired sideroblastic anemias (SAs), which are believed to involve abnormal oxidation of red cell proteins and which may be caused by defects in mitochondria (18, 19).

Materials and Methods

Generation of Donor Cells for Fetal Liver Transplantation. Heterozygous B6-$Sod2^{tm1Cje}$ mice (12) were bred in timed matings in order to obtain $Sod2^{-/-}$, $Sod2^{+/-}$, and $Sod2^{+/+}$ littermates at E13.5-16.5 of development to serve as donors for fetal liver cell transplantation. Animals (two females, one male) were placed together for two or three consecutive nights, after which time males were removed. Pregnant females were killed on the 17th morning after the introduction of the male. Embryos were harvested, and fetal livers were dissected. All fetal livers were mechanically dissociated into a single cell suspension by pipetting up and down using an Eppendorf p-1000 pipette in 1 cc of culture medium (RPMI, 7% FCS, glutamine, penicillin-streptomycin, and 10^{-5} M betamercapto-ethanol). 5% of each sample was used for DNA extraction followed by PCR analysis for genotyping. Knockout ($Sod2^{-/-}$) fetuses were often more pale than +/− or +/+ littermates at the time of harvest, but were otherwise indistinguishable. At E13.5-16.5, 15% of genotyped animals were $Sod2^{-/-}$, consistent with a previous report of decreased frequency of $Sod2^{-/-}$ pups at birth on the C57BL/6 background (20). Variations in the time of fertilization do lead to differences in the size and developmental stage of harvested fetuses. However, we have not noted any variation between experiments in the ability of a fixed dose of 10^6 fetal liver cells to rescue lethally irradiated host animals.

Genotyping at Sod2 Locus Using PCR. DNA was extracted from fetal liver cell suspensions using a DNA isolation kit according to the manufacturer's instructions (Gentra Systems). PCR was performed using the following primer pairs: wild-type specific sequence 5′-AGG GCT CAG GTT TGT CCA GAA AAT-3′ and common primer 5′-CGA GGG GCA TCT AGT GGA GAA GT-3′; SOD mutation 5′-TTT GTC CTA CGC ATC GGT AAT GAA-3′ and common primer as above. PCR conditions were: 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, with a final extension at 72°C of 4 min.

Host Animal Conditioning and Fetal Liver Transplantation. C57BL/6 mice congenic at the Ly5 locus (B6.SJL-$Ptprc^aPep^{3b}$/BoyJ[Ly5.1]) were obtained from the National Cancer Institute and were used as recipients in order to allow for differentiation of host (Ly5.1) versus donor (Ly5.2) hematopoietic cells. Ly5.1 host animals (age 11–15 wk) were subjected to 10.50 cGy (1,050 rad) of gamma irradiation divided into two equal doses 4 h apart on the day of transplant. 10^6 fetal liver cells, prepared as noted above, were injected intravenously (either into tail vein or infraorbital sinus) into irradiated Ly5.1 congenic recipients. Animals were maintained on chlorinated water for 21 d after irradiation. All experiments were approved by the Animal Use Committee.

Analysis of Fetal Liver Cell Engraftment. After recovery from transplantation, mice were bled at 3–4 wk and every 4–8 wk thereafter to assess the donor contribution to peripheral T cells, B cells, granulocytes, and macrophages. Transplanted Ly5.1 mice were bled from the ocular venus sinus, and ~250 μl of blood was collected into 0.5 cc of PBS/EDTA (3 mg/ml EDTA). Samples were then mixed with 0.5 cc of dextran T-500 in PBS (2% dextran) and allowed to sediment for 30 min at 37°C. Leukocytes were recovered in the supernatant and were mixed with one volume of a 1:4 dilution of saline/water to lyse remaining red cells. After 5 min, one volume of 1.9% saline was added, and the samples were spun down to pellet leukocytes. Cells were resuspended in FACS® wash solution (PBS, 3% heat inactivated bovine serum, 0.02% sodium azide) with Fc block (BD PharMingen). After 15 min, the cells were mixed with an equal volume of biotinylated anti–mouse CD45.2 (Ly 5.2) antibody (BD PharMingen). After 30 min, the cells were washed, spun down, and aliquoted into mixtures containing streptavidin R-PE (Southern Biotechnology Associates, Inc.) and either FITC-labeled CD90.2 for T cells, FITC-labeled CD45RA for B cells, or a combination of FITC-labeled CD11b and FITC-labeled Ly-6G for myeloid cells (all from BD PharMingen). After 30 min, cells were washed and resuspended in FACS® wash solution plus propidium iodide for FACS® analysis on a FACStar™ machine (Becton Dickinson).

Western Blot Analysis of MnSOD, CuZnSOD, and Porin Proteins. Polyclonal rabbit antisera specific for CuZnSOD and MnSOD (StressGen Biotechnologies) were used to blot whole cell lysate and red cell lysate obtained from fetal liver transplant recipients. Anti-porin antibody was obtained from Calbiochem. Bone marrow from transplanted animals was used as a source of nucleated cells in order to obtain an estimate of the ratio between porin and CuZnSOD and porin and MnSOD. Second stage horseradish peroxidase–conjugated anti–rabbit antibody and anti–mouse antibody were obtained from Amersham Pharmacia Biotech.

Protein Oxidation in Red Cells of Transplanted Animals. Red cell lysate proteins (20 μg protein/reaction) from $Sod2^{+/+}$, $Sod2^{+/-}$, and $Sod2^{-/-}$ transplant recipients were reacted with 2,4-dinitrophenylhydrazine to derivatize carbonyl groups formed through protein oxidation using oxyblot reagents (Intergen). Control samples were treated identically, except 2,4-dinitropheylhydrazine was omitted. Proteins were then subjected to SDS-PAGE followed by Western blot detection of derivatized residues according to the oxyblot protocol. Samples were normalized for protein concentration and verified by secondary blotting for SOD1.

Therapeutic Trial of EUK-8 in Transplanted Mice. EUK-8 was provided by Eukarion. EUK-8 powder was resuspended in sterile 5% dextrose at a concentration of 3 mg/ml and passed through a 0.2-micron sterile filter. For the experiment detailed in Fig. 6, mice were transplanted 13 wk before initiation of Euk-8 therapy. Peripheral blood samples were obtained at 4 and 8 wk after transplant to verify reconstitution with donor (Ly5.2)-derived cells. Mice were weighed and bled at 13 wk to determine EUK-8 dosage and to obtain a baseline complete blood count (CBC). Animals received either 30 mg/kg EUK-8 3 d/wk, or vehicle alone via intraperitoneal injection, on the same schedule for a total of 8 wk. Mice were bled again at 4 and 8 wk for hematocrit, reticulocyte count, and blood smear.

Measurement of RBC Half-life. Animals were transplanted 4 mo before RBC labeling to ensure that no residual pretransplant host red cells remained. Animals were bled 5 wk before RBC labeling and demonstrated 100% donor-derived B cells, ~85% donor-derived T cells, and ~90% donor-derived myeloid cells. *N*-hydroxysuccinimide (NHS)-biotin (E-Z link; Pierce Chemical Co.) was suspended in sterile saline at a concentration of 4 mg/ml and injected intravenously into fetal liver transplant recipients. Animals received a total dose of 30–40 mg/kg of body weight in a volume of 200–250 μl. Animals were first bled ~36 h after injection, and then at 2–3-d intervals for the first 2 wk, followed by 3–4-d intervals for the third and fourth weeks, then weekly twice more. Care was taken to obtain the smallest amount of blood possible, typically 5–10 μl with each blood sampling. Labeled cells were analyzed by FACS® after staining with streptavidin R-PE. The exponential curve showing both age-dependent and -independent processes was fitted to the equation: $A(t) = A_0[1 - (t/T)]e^{-kt}$ (reference 21), where $A(t)$ is the fraction of labeled RBCs present at time (t), A_0 is the initial fraction of labeled RBCs at $t = 0$, T is the time of senescent death of RBC (extinction time), and k is the fraction of cells which are removed independent of RBC age (hemolysis). In this experiment, $k = 0$ for $Sod2^{+/+}$ and $Sod2^{+/-}$ cells, and $k = 0.04$ for $Sod2^{-/-}$ cells.

Membrane Deformability. Cellular deformability was monitored by ektacytometry using a Technicon ektacytometer (Bayer Diagnostics). The osmotic deformability curve was recorded as described previously (22).

Results

Reconstitution and Engraftment Kinetics. Fetal liver cells for transplantation were generated by crossing $Sod2^{+/-}$ animals. Initial transplants compared the reconstituting ability of fetal liver cells of each genotype (wild-type, heterozygous, or knockout at the *Sod2* locus). Survival was 100% in all groups. Regardless of the genotype of the donor cells, >95% of peripheral blood B cells and >83% of peripheral blood myeloid cells were derived from the donor fetal liver cells at 3 wk after transplant. T cell engraftment was more gradual, with donor T cells first appearing in peripheral blood 3–4 wk after transplant. Donor-derived T cells continued to increase and reached 80–90% of all peripheral blood T cells by 3 mo after transplant. Throughout this experiment, B cell and myeloid engraftment has remained >90% and T cell engraftment >80% up to 1 yr after transplant, with no evidence of a difference in kinetics or duration of engraftment related to donor genotype. Thus, $Sod2^{-/-}$ hematopoietic stem cells are capable of giving rise to normal numbers of T cells, B cells, and myeloid cells, and can maintain this cellular output for up to 1 yr with no evidence of a decline in graft function.

Reconstitution of Spleen, Bone Marrow, and Thymus. Hematolymphoid tissues from transplanted animals were harvested to investigate in more detail engraftment among recipients of $Sod2^{-/-}$, $Sod2^{+/-}$, and $Sod2^{+/+}$ fetal liver. Recipients of $Sod2^{-/-}$ cells had larger spleens than animals receiving wild-type or heterozygous cells (4.2×10^8 vs. 2.3×10^8 cells/spleen; $P = 0.02$). There was no enlargement of the thymus, lymph nodes, or Peyer's patches compared with control transplanted animals. Fig. 1 A shows flow cytometric profiles of representative spleens from transplanted animals in which cells are simultaneously stained for the donor-specific marker CD45.2 (Ly5.2) and for one of the following lineage markers: Ly6G and CD11b for myeloid cells, CD45RA for B cells, and CD90.2 for T cells. Spleens from animals receiving $Sod2^{-/-}$ cells had a large population of cells which did not stain for markers of B cells, T cells, granulocytes, or macrophages, but which did stain for the erythroid marker Ter119 (Fig. 1 B). These data demonstrated effective reconstitution in all lineages (as expected from the peripheral blood analysis) and revealed a greatly expanded population of erythroid precursors in the spleens of animals transplanted with $Sod2^{-/-}$ fetal liver cells. This population represented 25–40% of the nucleated cells present in spleens from $Sod2^{-/-}$ fetal liver recipients, compared with 1–5% of nucleated spleen cells from $Sod2^{+/+}$ fetal liver recipients. Splenic red cell precursors ($Ter119^+$) were stained with CD45.2 (Ly5.2 or donor antigen) or CD45.1 (Ly5.1 or host antigen) to demonstrate that erythroid cells were also donor derived (Fig. 1 C). Analysis of the bone marrow revealed additional evidence for increased erythropoiesis in animals which received $Sod2^{-/-}$ cells, with the Ter119 population representing 40–50% of nucleated marrow cells versus 20–30% of marrow cells in recipients of $Sod2^{+/+}$ or $Sod2^{+/-}$ fetal liver cells (Fig. 1 B). Total marrow cell number in recipients of $Sod2^{-/-}$ fetal

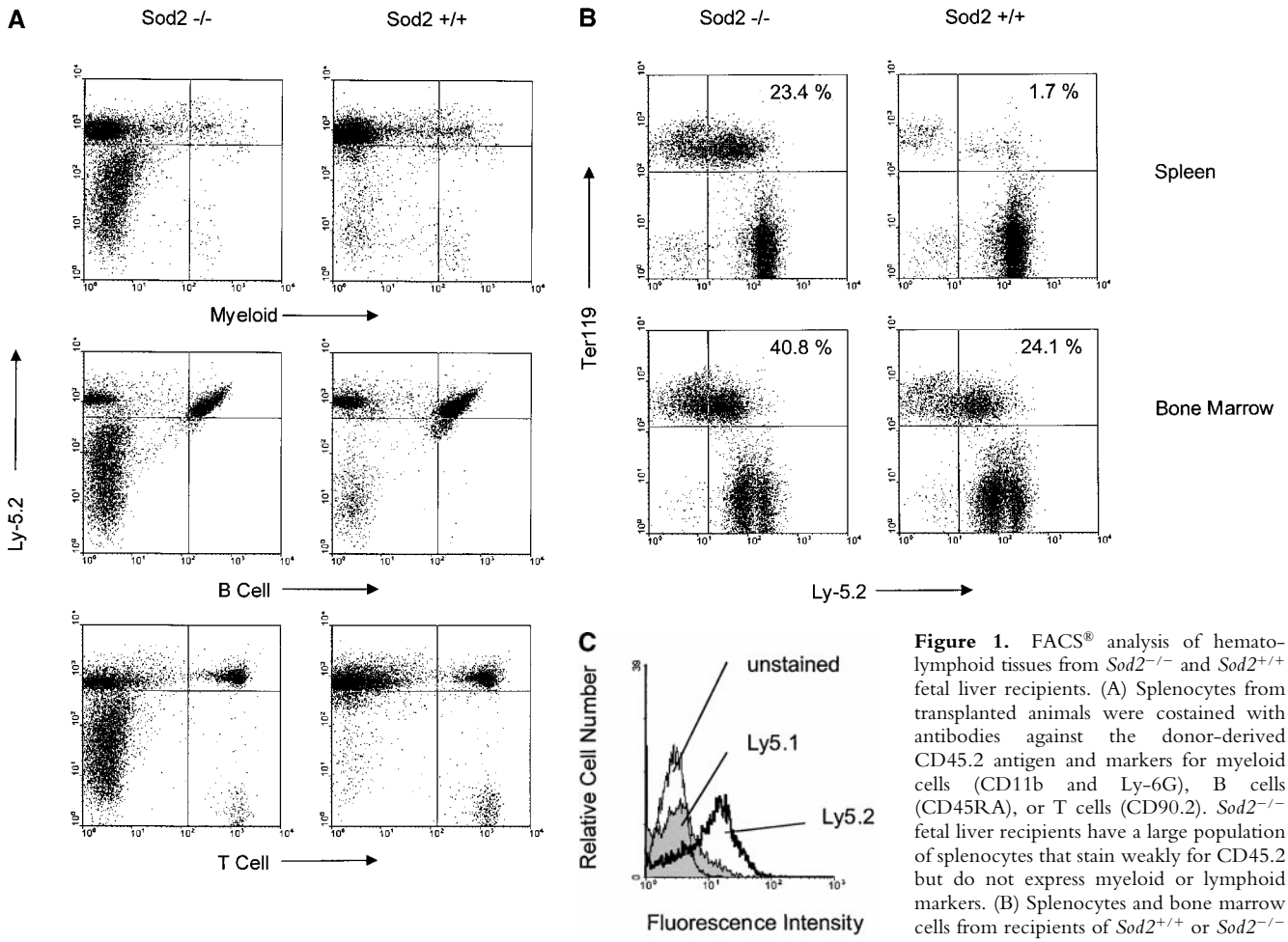

Figure 1. FACS® analysis of hematolymphoid tissues from *Sod2*$^{-/-}$ and *Sod2*$^{+/+}$ fetal liver recipients. (A) Splenocytes from transplanted animals were costained with antibodies against the donor-derived CD45.2 antigen and markers for myeloid cells (CD11b and Ly-6G), B cells (CD45RA), or T cells (CD90.2). *Sod2*$^{-/-}$ fetal liver recipients have a large population of splenocytes that stain weakly for CD45.2 but do not express myeloid or lymphoid markers. (B) Splenocytes and bone marrow cells from recipients of *Sod2*$^{+/+}$ or *Sod2*$^{-/-}$ fetal liver cells were costained with CD45.2 and the erythroid marker Ter119. The population shown in *Sod2*$^{-/-}$ samples in panel A represents abundant erythroid precursors (23.4% of sample), which are nearly absent from *Sod2*$^{+/+}$ samples (1.7%). Analysis of bone marrow with the same markers reveals that *Sod2*$^{-/-}$ recipients have increased erythroid progenitors in this compartment as well. (C) Erythroid lineage cells (Ter119^{+}) from the spleen of a *Sod2*$^{-/-}$ fetal liver recipient were costained with either FITC–anti-Ly5.1 (host) or FITC–anti-Ly5.2 (donor) to demonstrate that erythroid precursor cells are also donor derived.

liver was slightly increased compared with recipients of *Sod2*$^{+/+}$ fetal liver (range 1.0–1.25 times *Sod2*$^{+/+}$ cell number). Analysis of the thymus from reconstituted animals revealed no differences in cell number or CD4^{+} versus CD8^{+} profile, suggesting that lack of SOD2 does not significantly affect these measures of T cell reconstitution.

Sod2$^{-/-}$ *Fetal Liver Recipients Are Anemic.* Analysis of peripheral blood and hematolymphoid tissues revealed normal lymphopoiesis and myelopoiesis, but suggested abnormalities restricted to the erythroid lineage. This was substantiated by comparison of CBCs from transplant recipients (obtained 3 mo after transplant) that showed animals reconstituted with *Sod2*$^{-/-}$ fetal liver cells were anemic and had elevated circulating reticulocytes ($P < 0.001$). In addition to hematocrit, several parameters were abnormal in erythrocytes derived from *Sod2*$^{-/-}$ fetal liver (Table I). Mature red cells in *Sod2*$^{-/-}$ recipients were smaller and possessed less hemoglobin than those in control transplanted animals. Morphologic examination of peripheral blood smears (Fig. 2, Wright-Giemsa stain) suggested a hemolytic process affecting *Sod2*$^{-/-}$ red cells. There was a marked reticulocytosis in *Sod2*$^{-/-}$ peripheral blood, and variability in RBC size and shape. In addition, *Sod2*$^{-/-}$ red cells had a high frequency of basophilic intracellular inclusions that were absent from *Sod2*$^{+/+}$ cells, and which did not appear to be Howell-Jolly bodies. Specific staining for Heinz bodies revealed that *Sod2*$^{-/-}$–derived cells possess more of these inclusions, thought to represent accumulation of oxidized proteins within erythrocytes (data not shown). White blood cell count, differential (not shown), and platelet counts were not statistically different among recipients of *Sod2*$^{+/+}$, *Sod2*$^{-/-}$, or *Sod2*$^{+/-}$ fetal liver cells. We did not find consistent morphologic changes in leukocytes or platelets between *Sod2*$^{-/-}$ and *Sod2*$^{+/+}$ smears. Together, the examination of peripheral blood from *Sod2*$^{-/-}$ fetal liver recipients demonstrated an anemia with evidence of an ongoing hemolytic process.

Red Cell Half-life Is Decreased for Cells That Lack SOD2. The anemia observed in *Sod2*$^{-/-}$ fetal liver recipients could be due to increased red cell destruction (hemolysis) or defective red cell maturation (ineffective erythropoiesis) or a combination of both processes. To measure more directly

Table I. *CBC Comparison between Transplanted Animals*

	$Sod2^{+/+}$	$Sod2^{-/-}$	$Sod2^{+/-}$
Percent hematocrit	48.1 ± 0.6	31.8 ± 1.6*	48.1 ± 1.2
Percent reticulocyte	2.8 ± 0.2	16 ± 2.8*	2.6 ± 0.4
Mean corpuscular volume (fl)	50.1 ± 0.3	46.7 ± 1.0*	48.1 ± 1.3**
Red cell count ($\times 10^6$)	9.6 ± 0.2	6.8 ± 0.3*	9.8 ± 0.3
White cell count ($\times 10^3$)	12.3 ± 1.7	12 ± 2.7	9.0 ± 2.6
Platelet count ($\times 10^3$)	1,075 ± 139	1,262 ± 137	1,062 ± 103
Mean corpuscular hemoglobin (pg)	14.9 ± 0.1	13.8 ± 0.4*	14.5 ± 0.3
Red cell distribution width	13.2 ± 0.2	21.8 ± 0.9*	13.2 ± 0.3

CBC comparisons of $Sod2^{+/+}$, $Sod2^{+/-}$, and $Sod2^{-/-}$ fetal liver recipients. CBC was obtained 13 wk after fetal liver transplantation, and 5 wk after the most recent blood sampling for peripheral blood reconstitution assessment. For all parameters tested except MCV, there was no significant difference between $Sod2^{+/+}$ and $Sod2^{+/-}$ fetal liver recipients. $Sod2^{-/-}$ fetal liver recipients differ from $Sod2^{+/+}$ in hematocrit, reticulocyte count, MCV, mean corpuscular hemoglobin, and red cell distribution width.
*$P < 0.001$ compared with $Sod2^{+/+}$ sample.
**$P < 0.01$ compared with $Sod2^{+/+}$ sample.

whether the observed anemia was due to decreased production of mature RBCs or increased destruction of RBCs in the circulation, we measured RBC survival in transplanted animals, using direct in vivo biotin labeling (23). RBC survival in $Sod2^{-/-}$ and control fetal liver recipients was followed for 41 d in a random RBC labeling experiment. The survival curves plotted in Fig. 3 show that RBC removal was nearly linear for $Sod2^{+/+}$ and $Sod2^{+/-}$ fetal liver recipients with a disappearance rate of 2.6% RBCs per day and with an extinction time of ~40 d. In contrast, in the $Sod2^{-/-}$ fetal liver recipients, an exponential removal of RBCs was noted, indicating that one component of RBC destruction was independent of RBC age. The time required for loss of 50% of labeled $Sod2^{+/+}$ and $Sod2^{+/-}$ RBCs is 20 d, whereas the time required for loss of 50% of labeled $Sod2^{-/-}$ RBCs is 10 d.

Red cell membrane flexibility is required for RBC function, and alterations in membrane characteristics can affect cell survival (24). Therefore, we determined the osmotic deformability profile of RBCs from fetal liver recipients. The combined curves of three $Sod2^{+/+}$ fetal liver recipient and four $Sod2^{-/-}$ fetal liver recipient mice are shown (Fig. 4). A shift in the curve for $Sod2^{-/-}$ versus $Sod2^{+/+}$ RBCs is apparent, with the $Sod2^{-/-}$ cells showing reduced deformability under isotonic conditions. In addition to a smaller mean corpuscular volume (MCV), this shift may be due to a change in the mechanical properties of the membrane as indicated by the hypertonic arm of the curve, suggesting an overall increase in membrane stiffness. Altered membrane deformability and decreased red cell survival are both characteristics of the anemia that arises in animals transplanted with $Sod2^{-/-}$ fetal liver stem cells. This raises the interesting question of how deficiency in a mitochondrial enzyme can shorten the half-life of a cell that does not possess mitochondria.

SOD2 Protein Expression and Mitochondrial Distribution in Bone Marrow Cells and Erythrocytes of Transplanted Animals. To address the role of SOD2 protein deficiency as a cause of anemia, we determined the distribution of SOD2 and mitochondria in RBCs and marrow cells from transplanted animals (Fig. 5). We were unable to detect SOD2

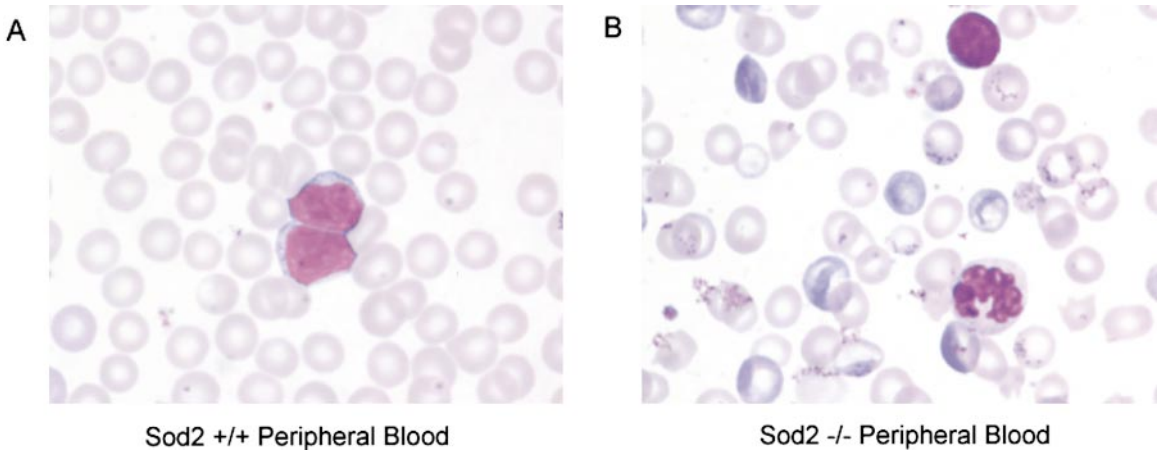

Figure 2. Wright-Giemsa stain of peripheral blood smears from transplanted animals. Morphologic comparison of peripheral blood demonstrates marked reticulocytosis and abnormal red cells in $Sod2^{-/-}$ fetal liver recipients. Many $Sod2^{-/-}$ RBCs have multiple prominent basophilic inclusions. There are many hypochromic cells, as well as variations in cell size and cell shape. Occasional Howell-Jolley bodies are seen in both the $Sod2^{+/+}$ and $Sod2^{-/-}$ red cells.

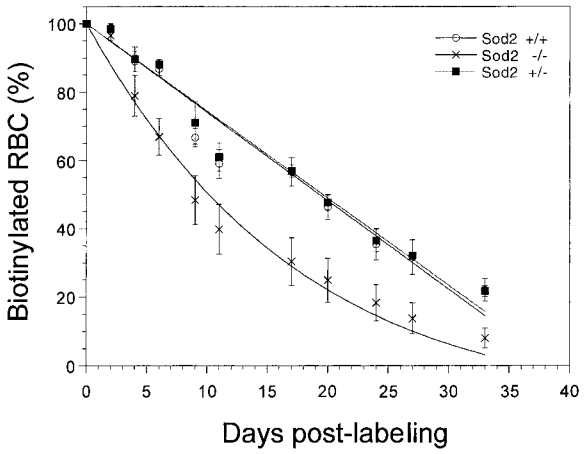

Figure 3. Red cell survival curve. In vivo biotin labeling was used to follow red cell survival kinetics over a 6-wk period. Small samples (5 μl) of peripheral blood were collected and stained using streptavidin-PE, followed by FACS® analysis, to determine the fraction of labeled RBCs remaining. *Sod2*$^{+/+}$ and *Sod2*$^{+/-}$ RBCs are lost in a linear fashion (2.6% per day) with an extinction time of ~40 d. *Sod2*$^{-/-}$ cells show a similar extinction time, but their removal curve has an exponential component.

in RBC protein from *Sod2*$^{+/+}$ fetal liver recipients except after prolonged exposure (data not shown), demonstrating that there is very little SOD2 present in RBCs. SOD1, which is known to be abundant in RBCs, was highly and equally expressed in all samples. Examination of nucleated cells revealed that SOD2 was abundant in *Sod2*$^{+/+}$ reconstituted bone marrow. A residual amount of SOD2 was detected in the marrow of *Sod2*$^{-/-}$ fetal liver recipients, likely derived from radio-resistant host stromal elements or a small fraction of residual host hematopoietic cells. An intermediate level of SOD2 was detected in the protein fraction from the marrow of *Sod2*$^{+/-}$ reconstituted animals. Thus, the expression of SOD2 in bone marrow cells of transplanted animals was reflective of the number of wild-type copies of *Sod2* in the transplanted cells and suggests that nearly all marrow cells are donor derived.

The distribution of mitochondria in bone marrow and RBC samples was tracked through detection of the mitochondrial voltage-dependent anion channel (porin 31HL; reference 25). Porin was not seen in *Sod2*$^{+/+}$ or *Sod2*$^{+/-}$ RBCs. Conversely, porin was detected in *Sod2*$^{-/-}$ RBCs. Analysis of bone marrow cell proteins for porin expression shows slightly more porin in *Sod2*$^{-/-}$ compared with control fetal liver recipients. This finding suggests that SOD2-deficient cells have increased numbers of mitochondria. Alternatively, the increased porin levels in bone marrow may reflect the increased erythroid precursor frequency we observe in *Sod2*$^{-/-}$ fetal liver recipients. In peripheral blood, both the high reticulocyte count and increased numbers of mitochondria per cell may contribute to the porin band detected in *Sod2*$^{-/-}$–derived RBCs.

Sod2$^{-/-}$ *Red Cells Possess More Oxidized Protein.* Deficiency of SOD2 has been shown to lead to both structural and functional damage to mitochondria. We reasoned that lack of SOD2 either during erythroid development or in mature RBCs might be accompanied by increased protein oxidation. We used an indirect assay for protein oxidation that measures the amount of protein carbonyl groups formed through the oxidation of amino acid side chains (26). There was a slight increase in protein oxidation of *Sod2*$^{+/-}$ red cells compared with *Sod2*$^{+/+}$ red cells, which may be indicative of increased oxidative damage, although this damage is not sufficient to cause a decrease in survival of *Sod2*$^{+/-}$ RBCs (Fig. 3). In contrast, RBC proteins from *Sod2*$^{-/-}$ recipients demonstrated dramatically higher levels

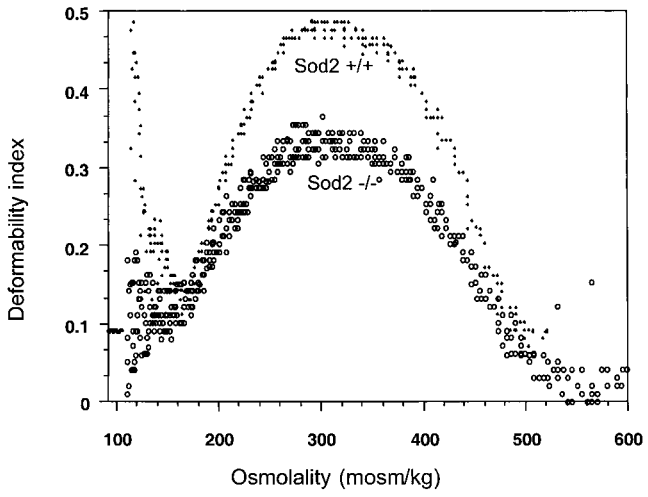

Figure 4. Red cell membrane osmotic deformability curve. Osmotic deformability was determined by ektacytometry. The combined curves of red cells from three *Sod2*$^{+/+}$ and four *Sod2*$^{-/-}$ fetal liver transplant recipients are shown. *Sod2*$^{-/-}$ cells have reduced deformability compared with controls.

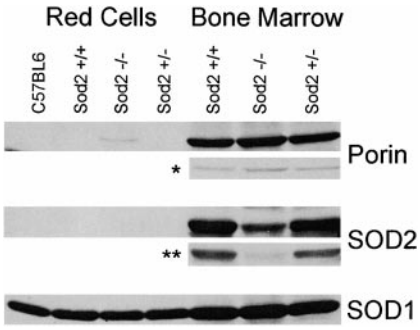

Figure 5. Western blot for expression of SOD1, SOD2, and porin in RBCs and bone marrow. 50 μg of total protein lysate from RBCs or bone marrow cells of transplanted animals was separated on a 12% SDS gel and blotted for protein expression. Red cell lysates contain abundant SOD1, but do not contain detectable amounts of SOD2. The mitochondrial protein porin is present in bone marrow cells, and can be detected in the RBC lysate of *Sod2*$^{-/-}$ transplant recipients. In a lighter exposure (*) of bone marrow–derived protein, porin is also more abundant in the sample from *Sod2*$^{-/-}$ transplant recipients. SOD2 can be detected in the bone marrow lysates, and with a lighter exposure (**) expression levels can be correlated with the genotype of transplanted cells: Sod2$^{+/+}$ lysates express the most, Sod2$^{+/-}$ lysates express less, and Sod2$^{-/-}$ lysates have <5% the level of SOD2 protein seen in Sod2$^{+/+}$ samples. Results shown for porin and SOD2 expression are representative of four separate determinations.

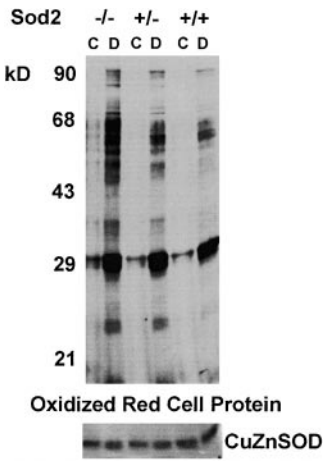

Figure 6. Comparison of oxidized protein among transplanted RBCs. 20 μg of protein from RBC lysates of *Sod2*$^{-/-}$, *Sod2*$^{+/+}$, and *Sod2*$^{+/-}$ fetal liver transplant recipients was reacted with 2,4-dinitrophenylhydrazine to derivatize (D) protein carbonyl groups, which were then detected using an anti-DNP antibody. 20 μg of each protein sample was incubated in reaction buffer alone to serve as a control (C). RBC proteins from *Sod2*$^{-/-}$ recipients have higher levels of oxidized protein residues. Secondary blotting using antisera against SOD1 was performed to verify equivalent protein loading (bottom panel).

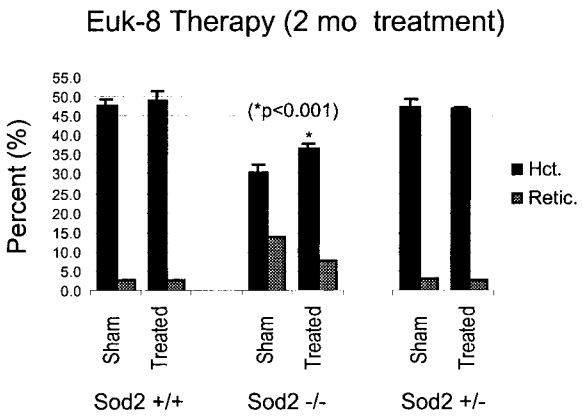

Figure 7. Partial correction of anemia using Euk-8, a synthetic SOD/catalase. Transplanted animals were divided into two groups that received either Euk-8 at a dose of 30 mg/kg 3 d/wk, or sham injections on the same schedule. Hematocrit (Hct.) and reticulocyte (Retic.) counts were determined after 8 wk of treatment. There was no effect of drug treatment on the hematocrit of animals that received *Sod2*$^{+/+}$ or *Sod2*$^{+/-}$ fetal liver cells. Recipients of *Sod2*$^{-/-}$ cells showed a significant increase in hematocrit in response to Euk-8 treatment after 8 wk (*$P < 0.001$) of therapy, and a corresponding decrease in reticulocyte count (P value not significant).

of oxidized protein compared with *Sod2*$^{+/-}$ or *Sod2*$^{+/+}$ fetal liver recipients (Fig. 6), supporting the hypothesis that accelerated RBC destruction in these animals is due to increased protein oxidation.

Partial Correction of Anemia Using a Synthetic SOD/Catalase Mimetic. We reasoned that if protein oxidation is central to the pathogenesis of anemia in recipients of *Sod2*$^{-/-}$ fetal liver cells, treatment with antioxidant compounds should ameliorate this condition. Indeed, previous work demonstrates that the antioxidant compound MnTBAP, which has SOD mimetic properties, can partially rescue the phenotype of *Sod2*$^{-/-}$ mice (14). EUK-8, a synthetic SOD mimetic that also possesses catalase activity, was tested in our transplant model because prior studies had demonstrated the efficacy of this compound in suppression of ROS-mediated damage in vivo (27, 28) and in vitro (29, 30). Transplanted animals received Euk-8 or vehicle alone by intraperitoneal injection three times/week for 2 mo. Pretreatment hematocrit and reticulocyte counts were compared with samples obtained after 4 and 8 wk of treatment with EUK-8. Treatment had no effect on the hematocrit or reticulocyte count in recipients of *Sod2*$^{+/+}$ or *Sod2*$^{+/-}$ fetal liver cells. After 4 wk of therapy, EUK-8–treated compared with sham-treated *Sod2*$^{-/-}$ fetal liver recipients had an increase in their hematocrit from 30.7 to 37.8% ($P = 0.01$). This increase was maintained after 8 wk of therapy (30.7 vs. 36.7%; $P < 0.001$), and was accompanied by a corresponding decrease in the reticulocyte count from 14% pretreatment to 8% (P value not significant) after 2 mo of therapy (Fig. 7). These results demonstrate that enhanced protection from oxidative stress using a combined SOD/catalase mimetic can significantly ameliorate the anemia observed in *Sod2*$^{-/-}$ fetal liver recipients.

Discussion

Sod2$^{-/-}$ *Fetal Liver Stem Cells Have Full Reconstituting Ability.* We studied the effect of chronic intracellular oxidative stress on immune/hematopoietic cells that lack SOD2, the enzyme responsible for detoxifying ROS generated during mitochondrial respiration. In a fetal liver transplant model, we found no obvious differences in radio protection or in long-term reconstituting ability of *Sod2*$^{-/-}$ stem cells compared with *Sod2*$^{+/+}$ or *Sod2*$^{+/-}$ cells. Further, 1 yr after transplant, there was no indication of bone marrow failure related to the lack of SOD2. To provide a more definitive answer as to whether lack of SOD2 can affect the proliferative potential of murine hematopoietic stem cells, with increasing age and/or under conditions of greater proliferative stress, the use of competitive repopulation assays and secondary transplantation are required.

Although we did not use hemoglobin electrophoretic variants or isozyme markers to quantitate donor-derived erythrocytes in our transplanted animals, several points from our data suggest that erythroid replacement was nearly complete. First, in normal hematopoietic cell reconstitution, myeloid and erythroid differentiation are closely connected, and thus it is likely that the near 100% donor-derived myeloid engraftment we observed is accompanied by a near 100% donor-derived erythroid engraftment (31). Next, when we stained bone marrow and spleen cells for the combination of CD45 and the erythroid-specific marker Ter119, we found Ter119^{+} nucleated cells that stain weakly (+/dim) for the donor CD45.2 marker (Fig. 1, B and C). However, the analogous (host-derived) Ter119^{+}, CD45.1$^{+/dim}$ population was absent, suggesting that erythroid precursors were of donor origin (Fig. 1 C). Finally, when we analyzed protein derived from bone marrow of transplanted animals for levels of SOD2, only a minimal amount of SOD2 could be detected in recipients of *Sod2*$^{-/-}$ fetal liver cells compared with recipients of *Sod2*$^{+/+}$ and *Sod2*$^{+/-}$ fetal liver (Fig. 5). Therefore, even in the absence of a donor-specific marker for mature erythrocytes, our data strongly suggest that virtually all red cells are donor derived.

Sod2$^{-/-}$ *Stem Cells Produce Defective Erythrocytes.* *Sod2*$^{-/-}$ fetal liver recipients were found to be persistently anemic, with erythroid hyperplasia in both the spleen and marrow as measured by the increased percentage of Ter119-positive cells. Anemia was noted as a characteristic of knockout mice of both the *Sod2*^{tm1BCM} strain (13) and the *Sod2*^{tm1Cje} strain used in this study. In analysis of the anemia, it was found that the bone marrow of *Sod2*$^{tm1BCM-/-}$ animals was hypocellular compared with control littermates. Yet, in our transplant model, we found marked erythroid hyperplasia in the bone marrow, with normal or increased total cell numbers. This suggests that the hypocellular marrow found in *Sod2*$^{tm1BCM-/-}$ animals is secondary to an environmental defect rather than a stem cell defect. However, when considering the erythroid lineage, defects are evident even when the metabolic environment of the host animal is normal. This clearly demonstrates an intrinsic defect in *Sod2*$^{-/-}$ erythroid progenitors. In addition, we show that whereas *Sod2*$^{-/-}$ progenitor cells are capable of expansion in both the spleen and marrow in response to anemia, they are only able to partially compensate for loss of red cells. Splenic erythropoiesis is increased in *Sod2*$^{-/-}$ fetal liver recipients, but the degree of erythroid hyperplasia observed is less than that seen in other murine anemia models with a similar hematocrit. This discordance between the degree of anemia and the response may result from intrinsic differences in the response of *Sod2*$^{-/-}$ stem cells or erythroid progenitors to homeostatic control of hematocrit through erythropoietin and other growth factors. Alternatively, the degree of erythroid hyperplasia possible in our model system may be limited due to irradiation of host tissues (specifically the spleen) before transplantation.

SOD2 deficiency anemia could be due to a defect incorporated into red cells during development that is unmasked upon exposure to oxidative stress in the circulation. The partial pressure of oxygen in the marrow (and spleen) is estimated to be quite low (24–40 mm Hg; reference 32), and therefore these environments may be relatively permissive for SOD2-deficient cells to develop with minimal oxidative damage. We speculate that most of the damage to RBCs occurs in the (arterial) circulation where the partial pressure of oxygen is the highest. In support of this conjecture, we see only a modest increase in total oxidized protein from bone marrow cells of *Sod2*$^{-/-}$ reconstituted animals, whereas there is a dramatic increase in oxidized protein in circulating RBCs of the same animals. We have also noted that *Sod2*$^{-/-}$ marrow and peripheral blood cells have elevated levels of the mitochondrial protein porin, indicating that these cells have a greater number or mass of mitochondria than found in *Sod2*$^{+/+}$ or *Sod2*$^{+/-}$ cells. The accumulation of abnormal mitochondria in *Sod2*$^{-/-}$ red cell precursors and their persistence in circulating reticulocytes may be both necessary and sufficient to explain the observed increase in oxidative damage to *Sod2*$^{-/-}$ red cells. In such a model, the mitochondria serve as the locus for ROS production, and those cells maintaining the most mitochondria for the longest period of time in the circulation sustain the most damage. Cells that successfully extrude or degrade their mitochondria rapidly would be relatively spared. This model is in accord with our observed red cell survival data, as some *Sod2*$^{-/-}$ cells are removed prematurely whereas other cells have a normal extinction time (Fig. 3). The magnitude of the premature removal effect is most pronounced at the early time points, suggesting a limited window during which young RBCs may accumulate "fatal" oxidative damage.

We have observed two consequences secondary to accumulation of oxidized protein in SOD2-deficient cells: decreased RBC survival and altered RBC membrane deformation. Decreased RBC survival and reduced membrane deformability are common characteristics of a wide range of hemolytic processes. However, protein oxidation, as shown by the increased protein carbonyl content of SOD2-deficient cells, may be a useful marker when applied to the classification of other anemias, and may help to define those conditions most likely to respond to antioxidant therapy.

Characterization of anemia secondary to SOD2 deficiency has the potential to further our understanding of, and therapeutic approach to, a subset of human erythrocyte disorders. Interestingly, there are morphologic similarities between SOD2-deficient RBCs and red cells from patients with SA, particularly the prominence of basophilic inclusions within immature RBCs, and the increased frequency of Heinz bodies. However, there are notable differences between SOD2 deficiency in mice and human SA. In particular, the morphologic abnormalities in human SA involve red cell precursors in the marrow, and patients with SA do not have a prominent reticulocytosis. In our model of SOD2 deficiency, we observe "siderocytes" in the peripheral blood, with very few sideroblasts evident in either the marrow or the erythropoietically active spleen. Despite these differences, the pathogenesis of anemia in both the SOD2-deficient model and in human SA involves mitochondrial dysfunction.

Two types of congenital SA have been shown to involve mitochondrial pathology. In Pearson marrow pancreas syndrome, large deletions are found in mitochondrial DNA (19, 33). In X-linked sideroblastic anemia with cerebellar ataxia, the human ABC7 transporter gene, which is involved in the maturation of iron-sulfur cluster-containing proteins, is mutated (18). There is also evidence that acquired SA may be caused by de novo mutations in mitochondrial DNA, specifically in subunit I of cytochrome C oxidase (34). Ultrastructural analysis of RBC precursors in SA reveals deposition of iron within mitochondria (35). Iron accumulation is proposed to occur in dysfunctional mitochondria when Fe^{3+} cannot be reduced to Fe^{2+}, which is required for heme synthesis (36). A possible unifying feature between these disorders and SOD2 deficiency is an abnormality in mitochondrial iron homeostasis. Abnormal iron homeostasis in *Sod2*-deficient cells is suggested by the measurement of severely depressed enzymatic activity of several iron-sulfur cluster-containing proteins in mitochondria from tissues of *Sod2*$^{-/-}$ mice (15). SA would then be a common morphologic pattern seen as a consequence

of the inability of dysfunctional mitochondria (representing many different primary defects) to successfully incorporate iron into heme.

Because of the unambiguous role of increased oxidative stress as the etiologic agent of anemia due to loss of SOD2, we performed a therapeutic trial using a synthetic SOD/catalase mimetic compound. Euk-8 and related compounds have advantages over traditional free radical scavengers such as ascorbate, as they possess catalytic SOD and catalase activities, and thus are able to degrade both superoxide anions and peroxides without being consumed in the reactions (37, 38). In our system, we have seen a partial, though highly significant correction of the anemia when using a single dose, route, and frequency of administration of Euk-8. Because none of these parameters has been optimized, it is likely that a more complete correction of the anemia will be possible. If catalytic antioxidant compounds such as Euk-8 prove to be useful in the treatment of human conditions like SA, they have the potential to reduce transfusion requirements and the toxicity associated with iron overloading, and would represent a welcome additional therapeutic.

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