Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging

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Van Remmen, Holly, Yuji Ikeno, Michelle Hamilton, Mohammad Pahlavani, Norman Wolf, Suzanne R. Thorpe, Nathan L. Alderson, John W. Baynes, Charles J. Epstein, Ting-Ting Huang, James Nelson, Randy Strong, and Arlan Richardson. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiol Genomics 16: 29–37, 2003; 10.1152/physiolgenomics.00122.2003.—Mice heterozygous for the Sod2 gene (Sod2+/− mice) have been used to study the phenotype of life-long reduced Mn-superoxide dismutase (Mn-SOD) activity. The Sod2+/− mice have reduced MnSOD activity (~50%) in all tissues throughout life. The Sod2+/− mice have increased oxidative damage as demonstrated by significantly elevated levels of 8-oxo-2-deoxyguanosine (8oxodG) in nuclear DNA in all tissues of Sod2+/− mice studied. The levels of 8oxodG in nuclear DNA increased with age in all tissues of Sod2+/− and wild-type (WT) mice, and at 26 mo of age, the levels of 8oxodG in nuclear DNA were significantly higher (from 15% in heart to over 60% in liver) in the Sod2+/− mice compared with WT mice. The level of 8oxodG was also higher in mitochondrial DNA isolated from liver and brain in Sod2+/− mice compared with WT mice. The increased oxidative damage to DNA in the Sod2+/− mice is associated with a 100% increase in tumor incidence (the number of mice with tumors) in old Sod2+/− mice compared with the old WT mice. However, the life spans (mean and maximum survival) of the Sod2+/− and WT mice were identical. In addition, biomarkers of aging, such as catarract formation, immune response, and formation of glycoxidation products carboxymethyl lysine and pentosidine in skin collagen changed with age to the same extent in both WT and Sod2+/− mice. Thus life-long reduction of MnSOD activity leads to increased levels of oxidative damage to DNA and increased cancer incidence but does not appear to affect aging.

oxidative damage; mitochondria

A COMPLEX ANTIOXIDANT DEFENSE system, including antioxidants, antioxidant enzymes, and a variety of pathways to repair oxidative damage, has evolved to protect cells from oxidative stress. The major antioxidant enzymes found in eukaryotes are the superoxide dismutases (SODs), the glutathione peroxidases (GPxs), and catalase. The SODs play a critical role in protecting cells from oxidative stress by catalyzing the dismutation of superoxide anions to hydrogen peroxide. There are three mammalian SODs: cytosolic CuZnSOD, which is the predominant SOD in most cells/tissues (70–80% of cellular SOD activity); extracellular SOD (EC-SOD), which is a minor form of the enzyme expressed in significant amounts in only a limited number of tissues (lung, kidney, and fat tissue); and MnSOD, which is located in the mitochondrial matrix of all cells and contributes 10–20% of the total SOD activity in the cell. The cellular location of MnSOD means that it is the major antioxidant defense system involved in protecting mitochondria from superoxide anions that are produced as a byproduct of the respiratory chain.

Two independent laboratories have generated Sod2 knockout mouse models by deletion of different segments of the Sod2 gene. The Sod2+/− phenotype is lethal in both knockout models, and mice lacking MnSOD die within 1–18 days from dilated cardiomyopathy or neurodegeneration, depending on the genetic background (18, 19). The Sod2−/− mutants produced by Epstein and colleagues (19) have a distinct phenotype characterized by small size and pale appearance compared with either wild-type (WT) or Sod2+/− mice. The Sod2−/− mice are also hypotonic, hypothermic, and suffer from accumulation of lipid in liver and skeletal muscle and metabolic acidosis. At death, the Sod2−/− mice have enlarged hearts with a dilated left ventricular cavity, consistent with dilated cardiomyopathy. In contrast, the Sod2−/− mice produced by Lebovitz et al. (18) could not be distinguished from their littermate controls at birth. However, between day 2 and day 7, they showed a slowing of growth that continued until death, which usually occurred within 18 days after birth. They exhibited severe anemia and degenerative injury to large CNS neurons, particularly in the basal ganglia and brain stem, motor disturbances, and evidence of mitochondrial damage. Interestingly, only 10% of these Sod2−/− mice showed signs of cardiomyopathy.

In contrast to the null mice, mice heterozygous for the Sod2 gene (Sod2+/−) appear normal but have ~50% of the MnSOD activity of WT mice in all tissues (42). Because mitochondria are a primary site of reactive oxygen species (ROS) production, reduced antioxidant protection in this cellular compartment would be predicted to contribute significantly to oxidative damage/stress in cells and tissues. In previous studies, we have...
shown that the Sod2+/− mice exhibit alterations in mitochondrial function and increased mitochondrial oxidative damage as early as 2–4 mo of age (43, 46). For example, the activities of aconitase and NADH-oxidoreductase, mitochondrial enzymes that are sensitive to inactivation by oxidative stress, are significantly decreased in mitochondria from liver and heart. In addition, mitochondrial respiration is altered in the young Sod2+/− mice as shown by a significant decrease in the respiratory control ratio for substrates metabolized by complexes I, II, and III for liver mitochondria and complex I and II for heart mitochondria isolated from Sod2+/− mice compared with WT mice (43, 46). Thus mitochondria from young Sod2+/− mice show alterations that are consistent with increased oxidative stress in cells/tissues of Sod2+/− mice, even though the mice appear normal. In this series of experiments, we studied the Sod2+/− mice over their entire life span to determine whether they show any phenotype that would be predicted to arise from life-long increased oxidative stress, e.g., an increase in the incidence of disease or accelerated aging.

METHODS

Sod2+/− knockout mice. Sod2+/− mice, designated Sod2min/Cryp, were originally produced in the CD1 strain of mice (19). The mice used for this study have been backcrossed to C57BL/6 for 13 generations. All procedures followed the guidelines approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and South Texas Veterans Health Care System, Audie L. Murphy Division. The colony of Sod2+/− mice used for this study was generated by breeding heterozygous male Sod2+/− mice to female WT C57BL/6 mice purchased from Jackson Laboratories (Bar Harbor, ME). The mice were screened at 4–5 wk of age for the Sod2 mutation by PCR analysis of DNA obtained from tail clips of these animals as previously described (19). The mice were maintained under barrier conditions in a temperature-controlled environment and fed a commercial mouse chow (Teklad Diet LM485) ad libitum. There was no significant difference in body weights or food consumption of the Sod2+/− and WT mice over their life spans (data not shown). Tissues were collected from female mice and frozen immediately in liquid nitrogen. The tissues were stored at −80°C until used to measure enzyme activities or oxidized DNA levels. For the life span studies, female WT and Sod2+/− mice were housed four per cage following weaning and fed a commercial mouse chow (Teklad Diet LM485) ad libitum and age at natural death recorded.

Antioxidant enzyme activities. The activities of CuZnSOD and MnSOD were measured in tissue extracts from liver, heart, brain, and kidney of female WT and Sod2+/− mice using native gels as previously described (42). Extracts containing 40–80 μg protein were separated on a 10% polyacrylamide gel, and the gel was soaked in a solution containing nitroblue tetrazolium, riboflavin, and TEMED. The riboflavin is activated to oxidize an electron donor (TEMED). The gel image was recorded with a digital-camera imager system (ImageMaster VDS, Amersham Pharmacia Biotech, NJ) and analyzed using ImageQuant Software (Sunnyvale, CA) to quantitate the intensity of the regions representing the CuZnSOD and MnSOD activity. GPX and catalase activities were measured using activity gels as described by Sun et al. (40), using cumene hydroperoxide (0.008%) and hydrogen peroxide (0.003%), respectively. The activities of the enzymes were determined using concentrations of the tissue extracts in which the assays were linear and expressed as relative units per milligram of protein.

Oxidative damage to DNA. Nuclear DNA (nDNA) was isolated using the DNA Extractor WB Kit (Wako Chemicals, Richmond, VA). The liver, brain, and spleen were homogenized in a Dounce homogenizer in ice-cold lysis solution, and the heart was homogenized in a ground-glass homogenizer in ice-cold lysis solution. Nuclei were collected by centrifuging the homogenate at 10,000 g for 20 s, and the nuclear pellets were resuspended in the enzyme reaction solution and proteinase K (10 μg/ml) provided with the kit. RNase cocktail (Ambion, Austin, TX) was then added to a final concentration of 20 μg/ml. Mitochondrial DNA (mtDNA) was isolated using mtDNA Extractor CT Kit (Wako Chemicals). Briefly, mitochondria were isolated from livers pooled from five mice or brains from nine mice. The homogenates were centrifuged at 1,200 g for 10 min. The supernatants were collected and centrifuged at 10,000 g for 10 min. The pellets, which contained mitochondria, were treated with the solutions in the mtDNA extractor kit.

Quantities of 50–75 μg of nDNA and mtDNA were hydrolyzed as described by Kasai et al. (15). The 8-oxo-2-deoxyguanosine (8oxodG) and 2-deoxyguanosine (2dG) were resolved by high-pressure liquid chromatography (HPLC) and quantitated by electrochemical detection as described by Hamilton et al. (9) using a CoulArray electrochemical detection system (model 5500/5600; ESA, Chelmsford, MA) and a C-18 column (YMC, Wilmington, NC). To identify both 2dG and 8oxodG, standards were coinjected, and standards were run after every sixth sample for verification. The data were expressed as the ratio of nanomoles of 8oxodG to 103 nanomoles of 2dG.

Biological analysis. For comprehensive pathological analysis, brain, pituitary gland, lung, heart, liver, kidney, pancreas, spleen, thymus, testis, liver, spleen, blood, bone marrow, and lymph nodes were studied. The severity of lymphoma was graded as follows: grade 1 (primary site only); grade 2 (2–3 organs); grade 3 (multiple organs, more than 3 organs); and grade 4 (4 organs with additional pathological changes, e.g., effusion, hemorrhage). The level of cell proliferation in lymphoma from the Sod2−/− and WT mice was compared by measuring the percentage of proliferating cell nuclear antigen (PCNA)-positive cells using immunohistochemistry. Sections 5-μm thick of liver, spleen, and lymph nodes that showed neoplastic lymphocytes were placed on glass slides coated with poly-l-lysine (Sigma, Deisenhofen, Germany), then examined immunohistochemically using a PCNA monoclonal antibody purchased from DAKO (Carpinteria, CA). The samples were analyzed by the avidin-biotin complex (ABC) method (13) using VECTOR MOM immunodetection Kit (Vector Laboratories, Burlingame, CA). The image analysis system used to quantify the number of immunopositive cells consisted of a SPOT cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI), an Olympus AX 70 True Research System Microscope (Olympus America, Lake Success, NY), a Dell Dimension XPS M166x (Dell Computer, Round Rock, TX), a FlashPoint video graphics card (Integral Technologies, Indianapolis, IN), and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The image analysis was accomplished by a double-blind procedure without knowledge of the genotype of animals. The frequency of a lesion or grade of lesion was accomplished by a double-blind procedure without knowledge of the genotype of animals. The frequency of a lesion or grade of lesion was analyzed without knowledge of the animal's identity. Tissues were fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin as described previously (32). A tumor profile was constructed for each mouse. The frequency of a lesion or grade of lesion was analyzed without knowledge of the animal's identity. Tissues were fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin as described previously (32). A tumor profile was constructed for each mouse. The frequency of a lesion or grade of lesion was analyzed without knowledge of the animal's identity. Tissues were fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin as described previously (32). A tumor profile was constructed for each mouse. The frequency of a lesion or grade of lesion was analyzed without knowledge of the animal's identity. Tissues were fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin as described previously (32). A tumor profile was constructed for each mouse. The severity of lymphoma was graded as follows: grade 1 (primary site only); grade 2 (2–3 organs); grade 3 (multiple organs, more than 3 organs); and grade 4 (4 organs with additional pathological changes, e.g., effusion, hemorrhage). The level of cell proliferation in lymphoma from the Sod2−/− and WT mice was compared by measuring the percentage of proliferating cell nuclear antigen (PCNA)-positive cells using immunohistochemistry. Sections 5-μm thick of liver, spleen, and lymph nodes that showed neoplastic lymphocytes were placed on glass slides coated with poly-l-lysine (Sigma, Deisenhofen, Germany), then examined immunohistochemically using a PCNA monoclonal antibody purchased from DAKO (Carpinteria, CA). The samples were analyzed by the avidin-biotin complex (ABC) method (13) using VECTOR MOM immunodetection Kit (Vector Laboratories, Burlingame, CA). The image analysis system used to quantify the number of immunopositive cells consisted of a SPOT cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI), an Olympus AX 70 True Research System Microscope (Olympus America, Lake Success, NY), a Dell Dimension XPS M166x (Dell Computer, Round Rock, TX), a FlashPoint video graphics card (Integral Technologies, Indianapolis, IN), and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The image analysis was accomplished by a double-blind procedure without knowledge of the genotype of animals. The frequency of a lesion or grade of lesion was analyzed with a chi-square test (37). When the expected frequencies were too small for the chi-square test, the data were analyzed with Fisher’s exact test for 2 × 2 tables (37).
levels in skin collagen, cataract formation, and immune function. CML and pentosidine levels residues were measured in insoluble skin collagen by isotope dilution gas chromatography mass spectrometry and HPLC, respectively, as previously described (27, 33). To assess cataract formation, cataracts were read blindly using a hand-held slit lamp at a 30 degree angle after dilation with 1% tropicamide. Both eyes were scored on an opacity scale of 0, 1, 2, 3, or 4+, with 4 representing complete lens opacity as described previously (47, 48).

The proliferative response of splenocytes was measured as described previously (24) in the presence of either T cell mitogens [concanavalin A (5 μg/ml) or anti-CD3 antibody (2 μg/ml)] or B cell mitogen lipopolysaccharide (LPS, 10 μg/ml), anti-CD3 antibody (2 μg/ml), or B cell mitogen LPS (10 μg/ml).

RESULTS

We have previously reported that the activity of MnSOD in tissues of young Sod2+/− mice is decreased 30–60% (42). To establish that MnSOD activity is reduced throughout the life span, i.e., that no compensatory upregulation or further decline occurs as the mice age, we measured the activity of MnSOD in WT and Sod2+/− mice at 6 mo and 26 mo of age. As shown in Fig. 1, the activity of MnSOD in Sod2+/− mice is reduced in the old animals to a level similar to that observed in young animals. In addition, we also measured the activities of the other major antioxidant enzymes (e.g., CuZnSOD, GPX, and catalase) to determine whether the reduced activity of MnSOD initiates a compensatory increase in other major antioxidant enzymes. No significant alteration in the activity of any of these enzymes was observed in any of the tissues from either young or old mice (Fig. 2).

To determine whether the tissues of the Sod2+/− mice have increased oxidative damage, we measured the level of 8oxodG in DNA isolated from various tissues of young and old Sod2+/− and WT mice. The data in Fig. 3 show 8oxodG levels in nDNA from several tissues and mtDNA from liver and brain of young and old Sod2+/− and WT mice. The levels of 8oxodG in nDNA and mtDNA increased significantly with age in both the Sod2+/− and WT mice. The levels of 8oxodG in nDNA increased more than 30% with age in liver and nearly 300% in brain in WT mice. In the Sod2+/− mice, 8oxodG levels increased more than 30% in liver and more than 40% in brain with age. In liver mtDNA, an ∼130–170% increase with age was observed in DNA isolated from both Sod2+/− and WT mice. More important, we observed significantly higher 8oxodG levels in the nDNA and mtDNA of both young and old Sod2+/− mice, compared with WT mice, i.e., the levels of 8oxodG were consistently higher in the Sod2+/− mice compared with the WT mice throughout the entire life span of the mice. For example, 8oxodG levels were ∼50–70% higher in the nDNA and mtDNA in the livers of young and old Sod2+/− mice compared with young and old WT mice. A similar trend was observed for 8oxodG levels in nDNA and mtDNA in brain and nDNA in heart and spleen (Fig. 3).

To test whether the decrease in MnSOD activity resulted in an increased sensitivity of the Sod2+/− mice to exogenous oxidative stress, we exposed the mice to paraquat, a compound that generates superoxide anions (1, 5). As shown in Fig. 4, the young WT mice survived this dose of paraquat, whereas more than 30% of the young Sod2+/− mice died within 4 days. The sensitivity of the old WT and Sod2+/− mice to paraquat was significantly greater than young mice. The old Sod2+/− mice tended to show a greater sensitivity to paraquat than the old WT mice (over 90% of the old Sod2+/− mice were dead by 5 days), but this difference was not statistically significant with the number of mice we used in this experiment.

The data in Table 1 show the incidence of tumors in the Sod2+/− and WT mice. More than 80% of the Sod2+/− mice had neoplastic lesions at 26–28 mo of age, compared with only 41% for their WT littermates. It is important to note that the neoplastic lesions observed in the Sod2+/− mice (potentially fatal tumors, e.g., lymphoma, pituitary adenoma, hemangioma, adenocarcinoma, and benign or occult tumors such as adenoma in thyroid and lung, and granulose-theca cell tumor in the ovary) have been reported to occur with age in C57BL/6 mice (28, 44), i.e., we observed no unusual neoplastic lesions in the Sod2+/− mice. The incidence of lymphoma was significantly higher in Sod2+/− mice (61%) compared with the WT mice (22%). The incidences of hemangioma and adenocarcinoma were also higher in the Sod2+/− mice; however, these differences were not significantly different because of the low incidence of these tumors. Both WT and Sod2+/− mice showed similar incidences of pituitary adenoma. In addition to the increased incidence of lymphoma, the number of animals that had multiple types of tumors was greater in the Sod2+/− mice compared with the WT mice, at 66.6% compared with 18.5% (Table 1).

Because the incidence of lymphoma was dramatically increased in the Sod2+/− mice, we measured the severity of lymphoma in the Sod2+/− and WT mice using the grading system described in the METHODS. The data in Fig. 5 show there was no evidence for a difference in the severity of the lym-
phoma between the Sod2+/− and WT mice, even though the incidence of lymphoma was greater in the Sod2+/− mice. We also compared the proliferative activity of the lymphoma from the Sod2+/− and WT mice by measuring the PCNA-positive cells. As shown in Fig. 5, the percent of PCNA-positive cells was similar for lymphoma from the Sod2+/− and WT mice.

To determine whether the age-related accumulation of oxidative damage to DNA is important in the aging process, we measured the survival of the WT and Sod2+/− mice under barrier conditions. There was no significant difference in body weights or food consumption of the Sod2+/− and WT mice over their life spans (data not shown). As shown in Fig. 6, the life spans of WT and Sod2+/− mice were indistinguishable, e.g., the mean survival was ~30 mo and the maximum survival was 40 mo. We also measured several age-sensitive chemical and physiological biomarkers of aging (e.g., CML and pentosidine levels in skin collagen, cataract formation, and immune function) to determine whether aging was altered in the Sod2+/− mice. These markers have been shown to change with age in a variety of animal models (25, 31, 47, 48) and to be reversed by dietary restriction (30, 47, 48). The data in Fig. 7 show that each of these parameters has the expected change with age in both the Sod2+/− and WT mice, i.e., an increase in CML and pentosidine levels in skin collagen, an increase in cataract formation, and a decline in splenocyte proliferation. However, none of these indices of aging were significantly different in the Sod2+/− and WT mice at any of the ages studied.

DISCUSSION
Sod2+/− mice, which have reduced MnSOD activity in all tissues throughout their life, exhibit no overt physical phenotype, e.g., their body weight, food consumption, and fecundity are similar to WT littermates. However, the Sod2+/− mice show increased sensitivity to the superoxide anion generator, paraquat, and tissues of the Sod2+/− mice showed higher levels of oxidative damage (8oxodG) to nDNA and mtDNA over their life span. This later observation, i.e., increased levels of 8oxodG, is of particular interest because 8oxodG is known to be a premutagenic lesion in mammalian cells, which could play a role in the initiation of cancer (17), and because an age-related increase in 8oxodG is a universal phenomenon in rodents (10) that has been proposed to be important in aging (2).

Our study demonstrates that the primary phenotype resulting from reduced MnSOD activity in the Sod2+/− mice is an increased incidence of cancer. It should be stressed that we observed no unusual age-related pathology for this particular...
strain of mice, i.e., the neoplastic lesions that normally occur with age in C57BL/6 mice were higher in the Sod2\(^{-/-}\) mice. More than 80% of the Sod2\(^{-/-}\) mice had neoplastic lesions at 26–28 mo of age, compared with only 41% for their WT littermates, and the incidence of lymphoma, the major neoplastic lesion, was significantly higher in Sod2\(^{-/-}\) mice (61%) compared with the WT mice (22%). We also compared growth/development of lymphoma in the Sod2\(^{-/-}\) and WT mice by measuring the severity and the cell proliferative activity of the lymphoma in these mice. The severity and percentage of PCNA-positive cells were similar for lymphoma from the Sod2\(^{-/-}\) and WT mice, suggesting that the progression/growth and cell proliferation of the lymphoma were not

![Graph showing oxidative damage to nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) in tissues of Sod2\(^{-/-}\) and WT mice.](image)

Fig. 3. Oxidative damage to nuclear DNA (nDNA, top) and mitochondrial DNA (mtDNA, bottom) in tissues of Sod2\(^{-/-}\) and WT mice. The levels of 8-oxo-2-deoxyguanosine (8oxo-dG) in nDNA from liver, brain, heart, and spleen and mtDNA from brain and liver from 3- to 6-mo-old (open bars), 15- to 17-mo-old (gray bars), and 26-mo-old (solid black bars) female WT and Sod2\(^{-/-}\) mice are shown. The data are expressed as the ratio of nanomoles of 8oxoD G to 10\(^5\) nanomoles of 2deoxyguanosine (2dG). The values for the nDNA represent the means ± SE for 8 mice. For the liver mtDNA, the means ± SE were obtained from 3 samples with each sample pooled from the livers of 5 animals. For the brain mtDNA, the mean for 2 samples is given with each sample pooled from brain tissue collected from 9 animals. All values represent the means ± SE, and the data were analyzed using a one-way ANOVA with a Bonferroni test to show significance. Statistically significant differences (\(P < 0.05\)) within a given tissue are indicated by different lower case letters (a, b, c, d).

![Graph showing sensitivity of Sod2\(^{-/-}\) and WT mice to oxidative stress.](image)

Fig. 4. Sensitivity of Sod2\(^{-/-}\) and WT mice to oxidative stress. Young (6 mo) and old (26–28 mo) female mice (12 mice per group) were injected intraperitoneally with 50 mg/kg paraquat dissolved in saline. Following injection, mice were monitored twice daily for 2 wk for deaths. Data shown are for the first 10 days (no deaths occurred between 10 and 14 days). Data were analyzed using the Cox-Mantel log rank test. The survival of the young Sod2\(^{-/-}\) mice (solid line, at top) (\(P < 0.05\)). Survival in the two groups of old mice was different from the young mice (\(P < 0.05\)). Old WT mice are indicated by small-dotted line; old Sod2\(^{-/-}\) mice are indicated by large-dotted line.

### Table 1. Incidence of tumors in old Sod2\(^{-/-}\) and wild-type mice

<table>
<thead>
<tr>
<th>Tumor-Bearing Mice</th>
<th>WT</th>
<th>Sod2(^{-/-})</th>
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<tbody>
<tr>
<td>Tumor-bearing mice</td>
<td>11(41%)</td>
<td>15(83%)*</td>
</tr>
<tr>
<td>No. of mice with multiple tumors</td>
<td>5(18%)</td>
<td>12(67%)*</td>
</tr>
<tr>
<td>Incidence of potentially fatal tumors</td>
<td></td>
<td></td>
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<tr>
<td>Lymphoma</td>
<td>6(22%)</td>
<td>11(61%)*</td>
</tr>
<tr>
<td>Hemangioma</td>
<td>0</td>
<td>2(11%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>0</td>
<td>1(6%)</td>
</tr>
<tr>
<td>Pituitary adenoma</td>
<td>9(33%)</td>
<td>5(28%)</td>
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The number of tumor-bearing mice (i.e., mice with one or more tumors) and the number of mice with multiple tumors (i.e., mice with two or more different types of tumors) were determined in for Sod2\(^{-/-}\) mice (\(n = 18\)) and wild-type (WT, \(n = 27\)) mice as described in the METHODS. The data were analyzed by the chi-square test, and those values that are statistically different (\(*P < 0.01\) for Sod2\(^{-/-}\) mice when compared with the WT mice are shown.
altered in the Sod2−/− mice. Therefore, the enhanced lymphoma in the Sod2−/− mice appears to be primarily due to early stages (initiation, promotion, or both) in the development of lymphoma, which is consistent with the increased levels of endogenous oxidative damage to DNA in the Sod2−/− mice playing an important role in the increased incidence of tumors in these mice.

Two important conclusions can be drawn from this study. First, we demonstrated that Sod2−/− mice, which have increased oxidative damage to DNA, show an increase in the incidence of spontaneous tumors, which occur normally with age. Although chemicals in the environment that generate oxidative damage to DNA are known to be potentially carcinogenic (16), we provide the first direct evidence that an increase in endogenously generated ROS, which arises from a defect in the mitochondrial antioxidant defense system, can increase oxidative damage to nDNA and the incidence of cancer. Thus our data point to the potential importance of endogenously generated ROS (in particular, those of mitochondrial origin) in spontaneous cancers that occur with age. These data are consistent with a recent study by Samper et al. (29) showing that increased production of ROS in mouse embryonic fibroblasts isolated from Sod2−/− mice is correlated to an increase in chromosomal alterations and genomic instability. We envision that the reduction MnSOD in the mitochondrial matrix could lead to oxidative damage in nDNA and cancer in two ways. First, increased superoxide anions in the mitochondrial matrix that arise from the reduced MnSOD activity leads to damaged mitochondria, which in turn release more ROS, increasing oxidative damage to the mitochondrial, cytosolic, and nuclear compartments (41). Second, the increased superoxide anion levels in the matrix generate other ROS species, e.g., hydrogen peroxide, that are able to transverse the mitochondrial membrane and generate oxidative damage in the nucleus.

Our data also suggest that mutations in the human MnSOD gene, which lead to reduced MnSOD activity, could increase the risk of cancer in the human population. Polymorphisms and mutations in the MnSOD gene have been reported (11, 34). For example, the Ile58Thr mutation in the human MnSOD gene has been shown to lead to an alteration in the structure of the Thr58 polypeptide, resulting in MnSOD occurring primarily as a dimer in solution with less activity and decreased thermostability compared with the MnSOD formed from the WT Ile58 polypeptide (4). In addition, polymorphisms in the Sod2 gene, resulting in a substitution of alanine for valine at the −9 codon position in the mitochondrial targeting sequence, have been reported to be associated with increased risk of breast, lung, and colon cancer in humans (22, 38, 45). These studies support our observations with Sod2−/− mice, suggesting that a reduction in MnSOD activity would lead to an increased incidence of cancer.

The second conclusion that can be drawn from our study is that an increase in oxidative damage to DNA over the life span of the Sod2−/− mice does not accelerate aging, as would be predicted from the oxidative stress theory of aging. This theory, which is based on the tenet that damage caused by ROS plays a critical role in determining life span, has been one of the most popular theories to explain the deterioration in biochemical and physiological processes that occur during the aging process. A large number of studies have produced correlative data in support of this theory, e.g., an increase in oxidative damage to lipid, protein, and DNA with age has been demonstrated in a variety of tissues and organisms (2), and dietary restriction, which has been shown to retard aging, has been shown to reduce the age-related increase in oxidative damage (8, 10, 50). Thus far, the most direct evidence for the oxidative stress theory of aging has come from studies with Drosophila, in which the life span of the flies has been increased by overexpressing CuZnSOD (26, 39); yet even these data do not prove that the extension in life span was due to altered oxidative damage. Therefore, conclusive data in

Fig. 6. Survival and life span characteristics of Sod2−/− and WT mice. The life spans of female WT and Sod2−/− mice were determined from the age of death as described in the METHODS.
support of this theory, linking alterations in oxidative damage with life span in mammals, are still lacking.

Surprisingly, our data show that the life span characteristics of the WT and Sod2+/− mice are nearly identical even though the Sod2+/− mice show an increase in oxidative damage and an increased incidence of cancer. A disconnect between the incidence of major pathological lesions, e.g., cancer, and life span is not unusual. For example, Garcia-Cao et al. (7) observed that transgenic mice with multiple copies of p53 show a major reduction in cancer but no change in life span. It should also be noted that the life spans of our Sod2+/− and WT mice are exceptionally long for C57BL/6 mice (3, 28, 44), which demonstrates the excellent conditions under which these mice were maintained. In other words, our life span data accurately reflect the aging of the Sod2+/− and WT mice and are not due to other complicating factors that arise from poor housing conditions, e.g., infectious disease or stress. In addition to the life span data, several measures of age-sensitive biomarkers, e.g., CML and pentosidine residues in skin collagen, cataract formation, and immune function, do not differ in the Sod2+/− and WT mice. Thus our data demonstrate that a reduction in MnSOD activity in the Sod2+/− mice that results in increased sensitivity to oxidative stress and increased oxidative damage (two phenotypes which are commonly associated with alterations in aging) does not lead to altered aging in the Sod2+/− mice. Thus our data with the Sod2+/− mice do not support the oxidative stress theory of aging.

Over the past five years, three other laboratories have used transgenic or knockout mice to test the oxidative stress theory of aging, and the results of these studies have been contradictory. For example, Epstein and colleagues (14) measured the life span of transgenic mice that overexpressed CuZnSOD. Although these mice show increased resistance to a variety of oxidative stresses (6, 12, 49), the increase in expression of CuZnSOD did not result in an extension of life span in the transgenic mice that overexpressed CuZnSOD did not result in an extension of life span in the transgenic mice compared with WT controls. Migliaccio et al. (21) reported that mice null for p66shc, which showed increased resistance to oxidative stress, had a 30% increase in life span. These data, in contrast to the studies with CuZnSOD transgenic mice, support the oxidative stress theory of aging; however, neither Epstein et al. (14) nor Migliaccio et al. (21) measured the effect of their genetic manipulation on the age-related accumulation of oxidative damage to macromolecules. More recently, Moskovitz et al. (23) produced mice mutated in the MsrA gene (MsrA−/−), which encodes methionine sulfoxide reductase, the enzyme responsible for reducing methionine sulfoxide residues in proteins. The MsrA−/− mice showed increased sensitivity to oxidative stress (hyperoxia) and increased protein oxidation (carbonyl groups) in kidney under normoxia and in liver, kidney, and lung in response to hyper-

Fig. 7. Changes in biomarkers of aging in WT and Sod2+/− mice. Three indices of aging were measured in female WT and Sod2+/− mice, and differences were determined using Student’s t-test. A: carboxymethyl lysine (CML) and pentosidine levels. Data are results for skin collagen taken from 7–9 mice in the three age groups shown. The increase in CML or pentosidine with age for the WT and Sod2+/− mice was significant at the P < 0.01 level, but not different between the two groups. B: cataract formation; each value is the mean ± SE for 7–14 mice. The increase in cataracts with age for both WT and Sod2+/− mice is significant at the P < 0.05 level. C: splenocyte proliferation; each point is the mean ± SD of data obtained from 6 spleens per group, and the decrease in proliferation with age for the three mitogens for both WT and Sod2+/− mice is significant at the P < 0.01 level. ConA, concanavalin A; LPS, lipopolysaccharide; Hyp, hydroxyproline.
oxia. Moskovitz et al. (23) reported that the Sod2−/− mice had a shorter life span under both normal and hypoxic conditions compared with WT mice. However, the studies by both Migliaccio et al. (21) and Moskovitz et al. (23) have two important limitations. First, the life spans of the mouse colonies used in the survival studies were relatively short (WT mice in both colonies lived only 800 to 850 days, compared with nearly 1,200 days for WT mice in our colony), suggesting that the animal colonies were not maintained under optimal conditions. Thus it is possible that the alterations in life span reported in these studies occurred because of the interaction of the genetic manipulations with the stressful conditions under which the mice were housed. Second, the only information on whether these genetic manipulations altered aging is the survival data; no data were presented on whether the genetic manipulations altered physiological processes that changed with age. Although survival is the ultimate endpoint for assessing aging, in this study, we also measured several age-sensitive chemical and physiological biomarkers of aging (e.g., CML and pentosidine levels in skin collagen, cataract formation, and immune function) to ask whether the characteristics of the aging process were altered in the Sod2−/− mice.

In summary, we have shown that tissues from the Sod2−/− mice show increased oxidative damage to DNA throughout the life span compared with tissues of their WT littermates. The increased levels of oxidative damage to DNA in the Sod2−/− mice were associated with increased incidence of cancer. We observed no difference in the life span of the Sod2−/− mice compared with their WT littermates and no alteration in three independent indices of aging. Thus we show in this study that the increased levels of oxidative damage to DNA throughout the life span of the Sod2−/− mice lead to increased incidence of cancer but do not appear to accelerate aging as would be predicted by the oxidative stress theory of aging.

REFERENCES


