Organ-Specific Increase in Mutation Accumulation and Apoptosis Rate in CuZn-Superoxide Dismutase–Deficient Mice

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Abstract

Reactive oxygen species have been implicated as a cause of cancer and aging in mammals. Mice deficient for the antioxidant enzyme CuZn-superoxide dismutase (*Sod1*) have a decreased life span and an elevated incidence of liver cancer. To test the hypothesis that the cancer-prone phenotype in such mice is due to accelerated spontaneous mutation accumulation, we crossed these mutants with mice harboring a neutral *lacZ* mutation reporter gene. At 2 months of age, the *lacZ* mutation frequency in the liver of the hybrid animals was already twice as high as in littermate controls of the same age. This difference in mutation frequency increased to >3-fold at 6 months of age, after which it did not increase any further. Characterization of the mutation spectra in liver of the *Sod1*-null mice indicated mainly GC-to-TA transversions and GC-to-AT transitions, signature mutations of oxidative stress. The accelerated mutation accumulation in liver was accompanied by an increased frequency of apoptotic cells, as indicated by an increase in both terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling– and caspase 3–stained cells at 6 and 12 months of age. In kidney, an elevated mutation frequency above controls of ~2.5-fold was found not earlier than at 6 months. No increased mutation accumulation was observed in brain or spleen. These results support the hypothesis, that oxidative stress is an important causal factor of cancer in mammals. (Cancer Res 2005; 65(24): 11271-5)

Introduction

Accumulation of somatic mutations, driven by reactive oxygen species, has been implicated in the increased incidence of cancer with age (1). To limit the adverse effects of free radicals, cells possess a variety of defense mechanisms including antioxidant enzymes. One of the major antioxidant defense pathways consists of the superoxide dismutases (SODs), which reduce superoxide anions to hydrogen peroxide. CuZnSOD (SOD1), located in the cytosol, is responsible for the majority (90%) of total SOD activity (2). It has previously been shown that mice deficient in *Sod1* exhibit a reduced maximum life span of ~25 months as compared with ~36 months in the control groups ($Sod1^{+/-}$ and $Sod1^{+/+}$; ref. 3). This reduction of life span in the *Sod1*-null mice was correlated with a high incidence of liver tumors at ~20 months of age. In all mutant animals from 16 months onwards, various forms of hepatocyte injury were observed (3). Of note, increased tumor incidence has thus far only been found in liver, not in other organs, despite the ubiquitous nature of the *Sod1* deficiency (3). Here we show that the increased incidence of liver cancer in the *Sod1*-deficient mice correlates with significantly increased mutagenesis at a *lacZ* reporter locus, with virtually all such mutations comprising transition and transversion mutations at GC, hallmarks of oxidative stress.

Materials and Methods

Transgenic animals. $Sod1^{+/-}$ mice (4) on a C57BL/6J background were crossed with C57BL/6J pUR288-(*lacZ*)-transgenic mice, line 30 (integration site on chromosome 11; ref. 5) and bred among each other to generate $Sod1^{-/-}$ animals hemizygous for pUR288 (*lacZ*). $Sod1^{+/-}$ and $Sod1^{+/+}$ littermate animals served as controls. The animals were maintained in the animal facilities of the University of Texas Health Science Center at San Antonio. The mice were maintained on a 14-hour light/10-hour dark cycle at a standard temperature of 23°C. Standard lab chow (Harlan Teklad, Madison WI) and water were supplied *ad libitum*. Animals were sacrificed by CO_2 inhalation followed by cervical dislocation at 2, 6, or 12 months of age.

Plasmid rescue and mutation analysis. DNA was extracted by routine phenol/chloroform extractions. Complete protocols for plasmid rescue, mutant frequency determinations, and mutant analysis with this model have been described elsewhere (5–7). To characterize the mutations, the complete *lacZ* gene of ~50 mutants per time point was sequenced. Sequence reactions of purified mutant plasmids were outsourced to Davis Sequencing (Davis, CA). The returned chromatograms were analyzed with Sequencher (Gene Codes, Ann Arbor, MI). The primers used for the sequence reactions were the same as previously described (6).

Apoptosis detection. Two core tissue biopsies (2 mm in diameter) were taken from each individual paraffin-embedded tissue sample (donor blocks) and arranged in a new recipient paraffin block (tissue array block) using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). Each tissue array block contained up to 60 samples. Sections of 4 μm were cut from each tissue array block, deparaffinized, and dehydrated. Immunohistochemical detection of apoptosis was carried out using an *In situ* Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) following the procedures provided by the manufacturer (8). Immunohistochemical staining against caspase 3 (1:100; Cell Signaling Technology, Beverly, MA) was done using a streptavidin peroxidase procedure.

Statistical analysis. Unpaired *t* test was used for all statistical analyses using the statistical program JMP (SAS Institutes, Inc., Cary, NC). $P < 0.05$ was considered significant.

Results

Using transgenic mice harboring a *lacZ* reporter gene, our laboratory has previously reported an age-related mutation accumulation in mouse tissues, including liver (5), heart and small intestine (9), spleen (10), and kidney (11). At the time, we predicted that such a spontaneous accumulation of mutations—likely to be due to oxidative stress, a generally recognized causal

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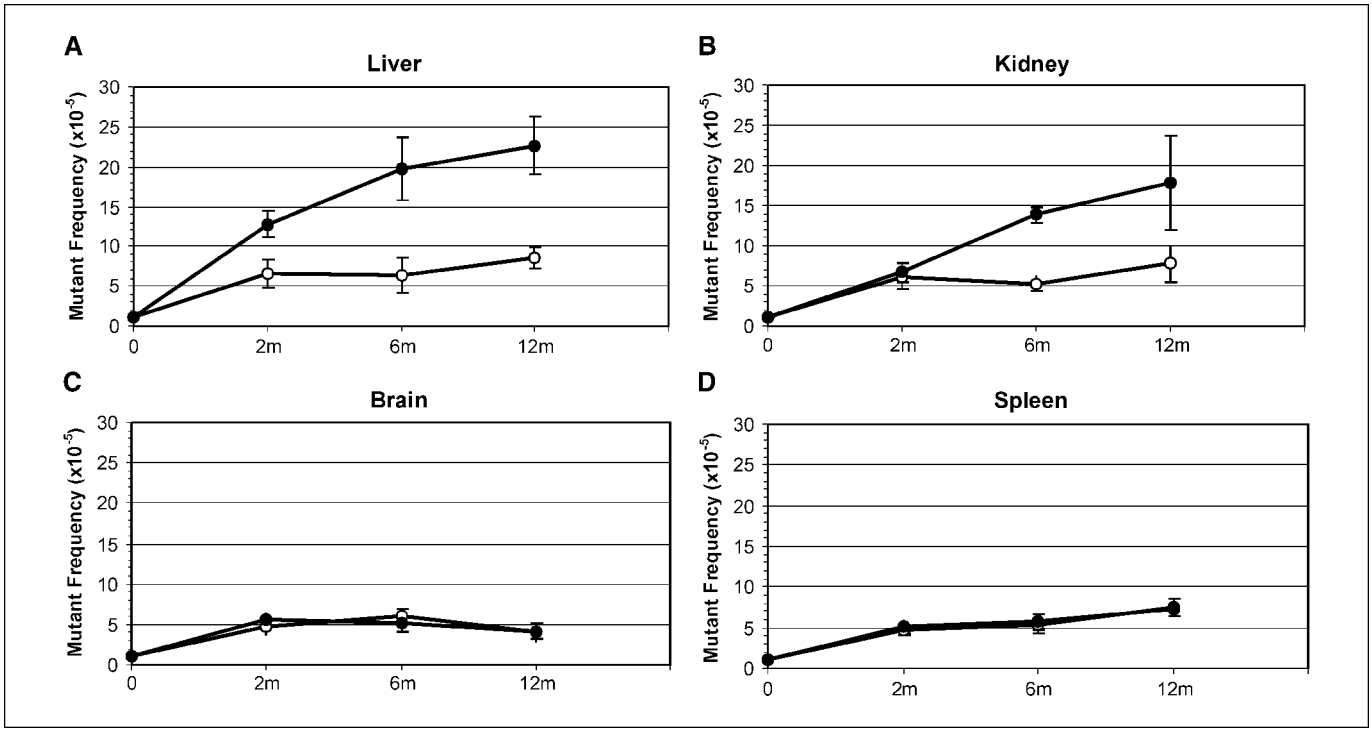

Figure 1. Spontaneous *lacZ* mutant frequencies with age in (*A*) liver, (*B*) kidney, (*C*) brain and (*D*) spleen of *Sod1*- deficient (●) and wild-type (○) mice. Time zero indicates the background mutant frequency of $\sim 1 \times 10^{-5}$ (16).

factor in aging and disease (1)—could contribute to the well-documented increased cancer incidence during aging. In an attempt to test if the observed increased incidence in liver tumors in *Sod1*-deficient mice was indeed due to an increased spontaneous mutation rate, we crossed the *lacZ* mice into the *Sod1*-null background. Mutant frequencies were determined in the liver, kidney, brain, and spleen of $Sod1^{-/-}/lacZ$, $Sod1^{+/-}/lacZ$, and $Sod1^{+/+}/lacZ$ mice at 2, 6, and 12 months of age. We observed no difference in mutant frequencies between the wild-type and heterozygous animals in any of the tissues studied (data not shown). Hence, these data were combined and are referred to in the text as wild-type.

A statistically significant increase in mutant frequency was observed in liver and kidney but not in brain or spleen (Fig. 1). The increase observed in liver was particularly dramatic. LacZ analysis of this organ at 2 months of age already showed a significant 2-fold ($P = 0.0007$) increase in mean mutant frequency (i.e., 6.5×10^{-5} in wild-type compared with 12.7×10^{-5} in knockout mice; Fig. 1*A*). This difference increased to >3-fold by the time the animals had reached 6 months of age ($P = 0.0051$). At 12 months of age, the difference had not further increased, suggesting that a plateau was reached (Fig. 1*A*).

Analysis of mutant frequencies in the kidney (Fig. 1*B*) showed no significant increase between wild-type and knockout animals at 2 months of age, but by the time the animals were 6 months old, mutant frequency in this organ was 2.5-fold higher than in control kidney (i.e., 5.3×10^{-5} in wild-type versus 13.8×10^{-5} in the *Sod1* mutant; $P = 0.0001$; Fig. 1*B*). As in liver, at 12 months of age, the difference in mutant frequency between knockout animals (17.8×10^{-5}) and controls (7.7×10^{-5}) had not further increased

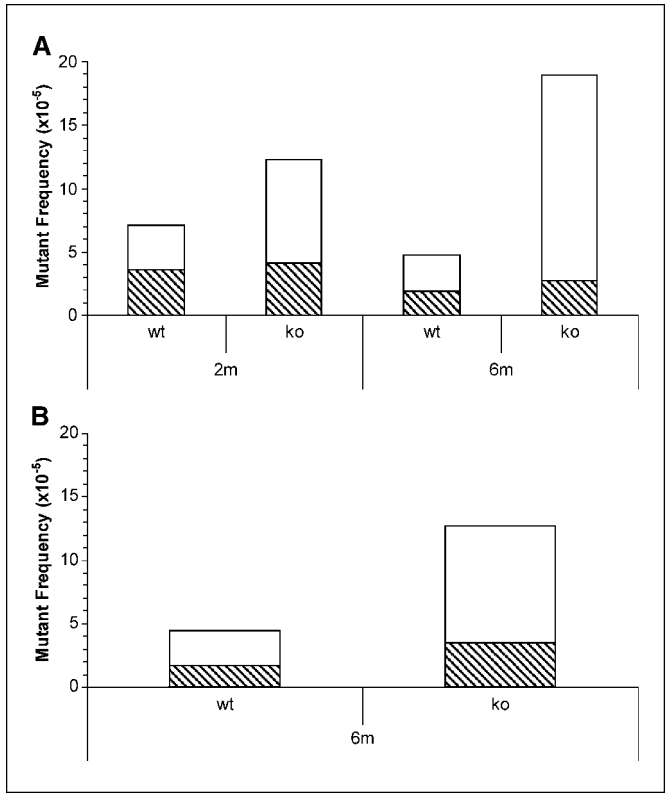

Figure 2. Mean mutant frequencies of no-change (open bars) and size-change (hatched bars) classes in (*A*) liver (2- and 6- months of age) and (*B*) kidney (6- months of age) of *Sod1* wild-type and knockout mice.

(P = 0.0119). In neither brain nor spleen was a significant elevation of the mutant frequency above controls observed (Fig. 1*C* and *D*). Although 12 months is still relatively early, in liver, kidney and spleen a trend towards an increased mutation frequency with age was observed, but not in the brain. In fact, this organ is one of the few organs in which no increase in spontaneous mutant frequency has been observed (5).

To gain further insight into the nature of the mutational events in the liver and kidney, we subsequently characterized *lacZ* mutants from 2- and 6-month-old livers, as well as those from the 6-month-old kidney, at the molecular level. Restriction analysis was used to classify the mutants into two subclasses: no-change mutations and size-change mutations. No-change mutations are those with similar gel migration patterns as wild-type control *lacZ* plasmids, representing point mutations (i.e., base substitutions and small insertions and deletions up to 50 bp). Size-change mutations are those which deviate from the wild-type restriction pattern, representing deletions >50 bp and other genome rearrangement events. In all cases, we found that the fraction of size-change mutations remained constant, with the increased mutant frequency in the *Sod1*-deficient animals entirely due to point mutations (Fig. 2*A* and *B*).

Sequence characterization of the point mutations was done to identify their likely cause. The predominant mutation types found to be increased in the liver of *Sod1* knockout mice were GC>AT transitions and GC>TA transversions (Fig. 3*A*), both of which are mutations associated with increased oxidative damage (12, 13). Indeed, these mutation spectra were almost identical to those observed when we previously placed embryonic fibroblasts from wild-type *lacZ* mice under conditions of high oxidative stress (20% O_2; ref. 6). Both GC>AT transition and GC>TA transversion mutations were also found to increase significantly in the kidney of 6-month-old animals (Fig. 3*B*) along with 1-bp deletion mutations.

To test if the observed increase in genomic instability was associated with an increase in spontaneous apoptosis, a common response to increased genomic stress, we did terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) and caspase-3 staining assays on the different organs. For this purpose, we used tissue arrays, which allowed direct comparison between tissues from different animals and different organs at 6 and 12 months of age. The results of both TUNEL and caspase-3 staining indicated increased levels of apoptosis only in the liver, the organ showing the most rapid increase in genomic instability (Fig. 4*A*). Quantitation of the caspase 3 data indicated an ~50-fold increase in the frequency of apoptotic cells at 6 months of age (Fig. 4*B*). Of note, this frequency was not further increased at 12 months and was even lower. It is possible that this result reflects the plateau in mutation accumulation that seems to have been reached at this time (Fig. 1*A*).

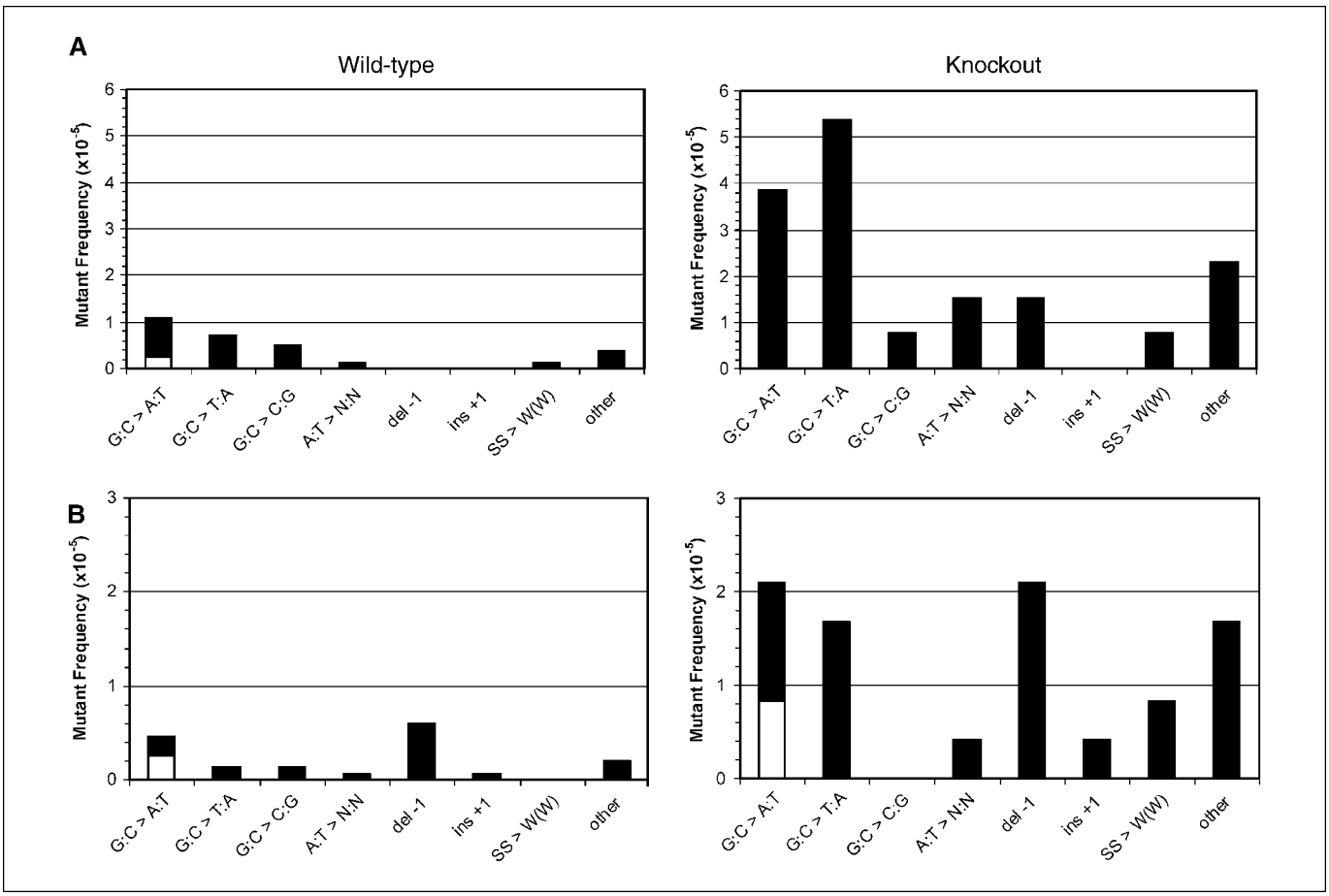

Figure 3. Point mutational spectra in (*A*) liver and (*B*) kidney of 6- month old *Sod1* deficient mice and their littermate controls. Open areas indicate mutations which occurred at CpG sites.

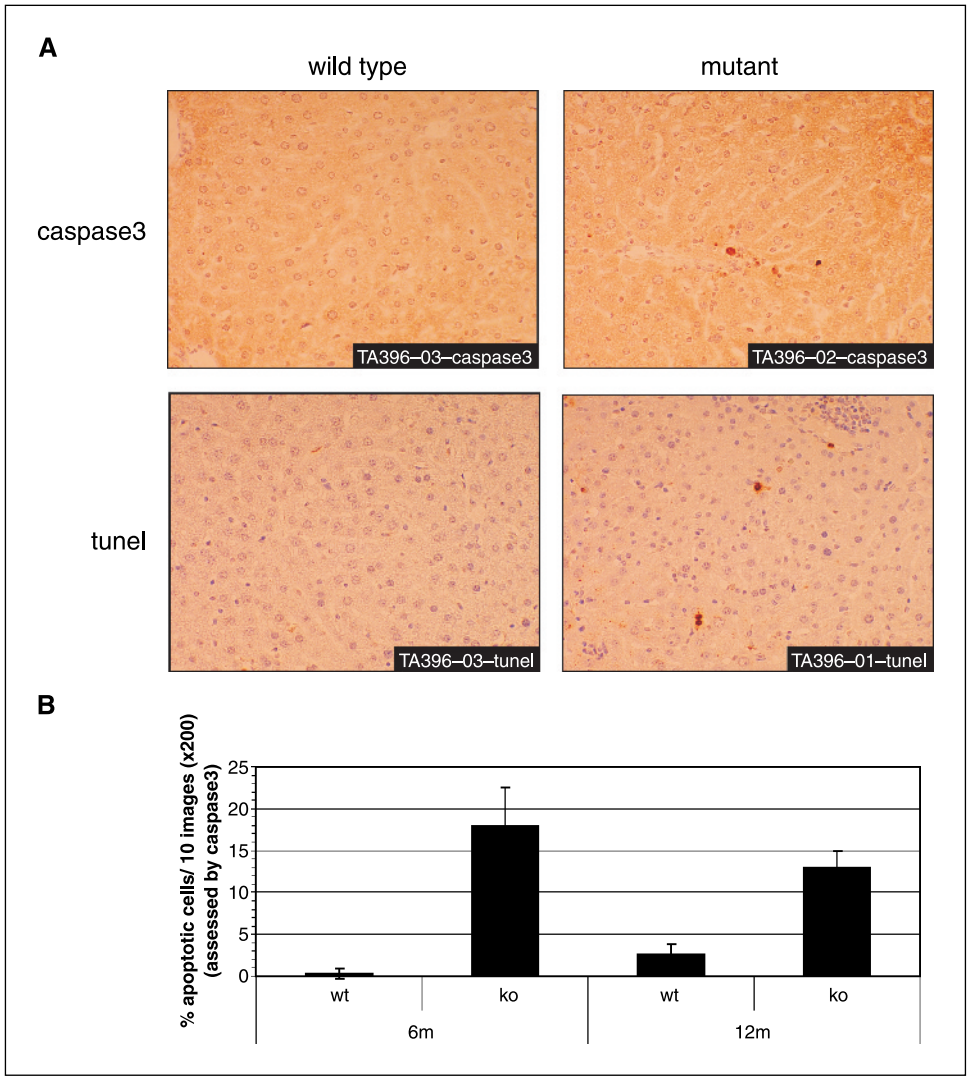

Figure 4. *A,* apoptosis was measured in both *Sod1* wild-type and knockout mice at age 12 months using both caspase 3 and TUNEL assays ($n = 3$). *B,* the percentage of caspase 3 positive cells per 10 images (×200) was determined for both 6- and 12- month old animals.

Discussion

Spontaneous oxidative stress has been implicated in age-associated cellular degeneration and death. Especially cancer, a major aging-related disease, has been considered a result of increased genomic instability due to elevated levels of DNA damage induced by endogenous reactive oxygen species. In the C57BL/6J *lacZ* transgenic mice, we were never able to make a direct link between increased mutation frequencies and cancer. The most frequent spontaneous tumors in these animals are lymphomas and although mutations in the spleen, its major target organ, were found to increase with age (10), the increase in some other organs, such as liver and heart, was more prominent. Whereas liver tumors regularly occur in aged mice, although they are infrequent, tumors of the heart are completely absent. We ascribed this lack of a clear cause and effect relationship to other, organ-specific factors playing a major role in cancer etiology. In this respect, the observation by Elchuri et al. (3) of increased liver cancer in *Sod1*-deficient animals offered an ideal opportunity to further investigate the causal relationship between spontaneous mutagenesis and cancer.

Our present results make it clear that the increased incidence of liver cancer in the *Sod1*-deficient animals is likely to be caused by increased spontaneous mutagenesis as a consequence of the greatly increased level of oxidative stress due to the lack of a major antioxidant defense system. In a sense, this is not surprising because the liver has a high oxygen metabolism and is vulnerable to the effects of oxidative damage. This is, for example, indicated by the increased rate of accumulation of 8-oxoguanine in mice deficient in the base excision repair enzyme 8-oxoguanine DNA *N*-glycosylase in liver but in no other organ (14). In our case, it should be noted that most of the mutations in the *Sod1*-deficient liver were mutations at GCs, which are signature mutations for oxidative stress (1). Hence, our present results are in complete agreement with the hypothesis proposed by Elchuri et al. (3), suggesting that cell death in hepatocytes, induced by reactive oxygen species–mediated damage to DNA, would force hepatocytes to regenerate, thereby increasing the probability of DNA mutation accumulation ultimately leading to the hepatocarcinogenesis observed in these mice. This scenario is further supported by our finding of increased spontaneous apoptosis in the liver of *Sod1*-deficient mice, a sign of accelerated genomic instability.

The delayed increase in mutagenesis in kidney, as compared with liver, and its complete absence in spleen and brain are in keeping with both the much lower *Sod1* activity under normal

conditions and the lack of any obvious tissue injury or pathologic lesions in these organs.[7] Furthermore, spontaneous tumors in the kidney are extremely rare in aged animals whereas liver tumors are occasionally observed (15). Hence, these findings underscore the tissue specificity of cancer etiology, including cell type–specific molecular variables, such as antioxidant defense and genome maintenance.

Interestingly, whereas the difference in mutant frequency in liver between *Sod1*-null mice and the controls increased significantly between 2 and 6 months of age, the rate of mutation accumulation in the liver between 6 and 12 months of age was very similar in the knockout animals and controls. This may reflect a maximum mutation load that cells can tolerate. Further increases might be prevented by apoptosis, which was found to be significantly elevated at 6 months of age. Because we lack data from time points later than 12 months, we cannot formally exclude a reacceleration of the mutation rate in the knockout animals after that time.

In kidney, mutation accumulation was delayed as compared with liver, and at 12 months had still not reached the same level. Whereas the considerable animal-to-animal variation, which is not unusual in mutation frequency determinations, prevents us from drawing any definite conclusions, the kinetics of mutation accumulation suggests that also in this case a plateau will be reached. The slower mutation accumulation in the kidney may be responsible for the lack of an increase in the apoptosis rate. However, it is possible that at time points later than 12 months, such an increase would also become apparent.

In summary, our present findings strongly suggest that the increased mutation load observed in the liver of *Sod1* knockout mice is due to oxidative stress–associated DNA damage, which is in turn fixed into mutations that ultimately result in liver tumors that are the main cause of death in these animals. The complete lack of accelerated mutagenesis in spleen and brain, which corresponds to a lack of visible neoplasms, suggests that an elevated mutation load is necessary to increase spontaneous tumor development. Increased mutations, however, may not be sufficient to cause cancer as is suggested by the cancer resistance of kidney, which did show increased mutagenesis in the *Sod1*-deficient mice, albeit at a lower rate as compared with liver.

[7] T. Huang, unpublished results.

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