Research Paper

A new role for oxidative stress in aging: The accelerated aging phenotype in Sod1−/− mice is correlated to increased cellular senescence

Yiqiang Zhang, Archana Unnikrishnan, Sathyaseelan S. Deepa, Yuhong Liu, Yan Li, Yuji Ikeno, Danuta Sosnowska, Holly Van Remmen, Arlan Richardson

1. Introduction

The Free Radical or Oxidative Stress Theory of Aging postulates that reactive oxygen species (ROS) formed exogenously or endogenously from normal metabolic processes play a role in the aging process. The imbalance of pro-oxidants and antioxidants leads to an age-related accumulation of oxidative damage in macromolecules, resulting in a progressive loss in function and aging [1]. Over the past three decades, the Oxidative Stress Theory of Aging has become one of the most popular theories to explain the biological/molecular mechanism underlying aging because several lines of evidence support the theory. First, the levels of oxidative damage to lipid, DNA, and protein have been reported to increase with age in a wide variety of tissues and animal models [2]. Second, studies with animal models showing increased longevity are consistent with the Oxidative Stress Theory of Aging. Longer-lived animals show reduced oxidative damage and/or increased resistance to oxidative stress, e.g., dietary restriction in rodents and genetic manipulations that increase lifespan in invertebrates (C. elegans and Drosophila) and in mice [3]. Thus, the observations that experimental manipulations that increase lifespan in invertebrates and rodents were correlated to increased resistance to oxidative stress or reduced oxidative damage provided strong support for the Oxidative Stress Theory of Aging.

In contrast to other mouse models that are deficient in antioxidant enzymes, mice null for Cu/Zn-superoxide dismutase (Sod1−/− mice) show a major decrease in lifespan and several accelerated aging phenotypes. The goal of this study was to determine if cell senescence might be a contributing factor in the accelerated aging phenotype observed in the Sod1−/− mice. We focused on kidney because it is a tissue that has been shown to a significant increase in senescent cells with age. The Sod1−/− mice are characterized by high levels of DNA oxidation in the kidney, which is attenuated by DR. The kidney of the Sod1−/− mice also have higher levels of double strand DNA breaks than wild type (WT) mice. Expression (mRNA and protein) of p16 and p21, two of the markers of cellular senescence, which increased with age, are increased significantly in the kidney of Sod1−/− mice as is β-gal staining cells. In addition, the senescence associated secretory phenotype was also increased significantly in the kidney of Sod1−/− mice compared to WT mice as measured by the expression of transcripts for IL-6 and IL-1β. Dietary restriction of the Sod1−/− mice attenuated the increase in DNA damage, cellular senescence, and expression of IL-6 and IL-1β. Interestingly, the Sod1−/− mice showed higher levels of circulating cytokines than WT mice, suggesting that the accelerated aging phenotype shown by the Sod1−/− mice could result from increased inflammation arising from an accelerated accumulation of senescent cells. Based on our data with Sod1−/− mice, we propose that various bouts of increased oxidative stress over the lifespan of an animal leads to the accumulation of senescent cells. The accumulation of senescent cells in turn leads to increased inflammation, which plays a major role in the loss of function and increased pathology that are hallmark features of aging.

ARTICLE INFO

Keywords:
Cellular senescence
Superoxide dismutase
Aging
Inflammation
DNA damage
Dietary restriction
Oxidative stress

ABSTRACT

In contrast to other mouse models that are deficient in antioxidant enzymes, mice null for Cu/Zn-superoxide dismutase (Sod1−/− mice) show a major decrease in lifespan and several accelerated aging phenotypes. The goal of this study was to determine if cell senescence might be a contributing factor in the accelerated aging phenotype observed in the Sod1−/− mice. We focused on kidney because it is a tissue that has been shown to a significant increase in senescent cells with age. The Sod1−/− mice are characterized by high levels of DNA oxidation in the kidney, which is attenuated by DR. The kidney of the Sod1−/− mice also have higher levels of double strand DNA breaks than wild type (WT) mice. Expression (mRNA and protein) of p16 and p21, two of the markers of cellular senescence, which increased with age, are increased significantly in the kidney of Sod1−/− mice as is β-gal staining cells. In addition, the senescence associated secretory phenotype was also increased significantly in the kidney of Sod1−/− mice compared to WT mice as measured by the expression of transcripts for IL-6 and IL-1β. Dietary restriction of the Sod1−/− mice attenuated the increase in DNA damage, cellular senescence, and expression of IL-6 and IL-1β. Interestingly, the Sod1−/− mice showed higher levels of circulating cytokines than WT mice, suggesting that the accelerated aging phenotype shown by the Sod1−/− mice could result from increased inflammation arising from an accelerated accumulation of senescent cells. Based on our data with Sod1−/− mice, we propose that various bouts of increased oxidative stress over the lifespan of an animal leads to the accumulation of senescent cells. The accumulation of senescent cells in turn leads to increased inflammation, which plays a major role in the loss of function and increased pathology that are hallmark features of aging.
Stress Theory of Aging. However, all of the experimental manipulations that increase lifespan also alter processes other than oxidative stress/damage; therefore, the increase in longevity in these animal models could arise through another mechanism.

Over the past two decades, our group has directly tested the role of oxidative damage/stress in aging by genetically manipulating the antioxidant status of a wide variety of antioxidant genes to increase or reduce the level of oxidative stress/damage and determine what affect these manipulations had on lifespan. Our research with 18 different genetic manipulations in the antioxidant defense system shows that only the mouse model null for Cu/Zn-superoxide dismutase (Sod1) had an effect on lifespan (in this case a decrease in lifespan) as predicted by the Oxidative Stress Theory of Aging [4]. Because Elchuri et al. reported that more than 70% of Sod1−/− mice developed liver hyperplasia and hepatocellular carcinoma later in life, it was initially believed that the 30% decrease in the lifespan of Sod1−/− mice was not due to accelerated aging but was the result of a dramatic increase in hepatocellular carcinoma, which is rare in C57BL/6 mice [5]. In a more recent study, we found a similar 30% decrease in lifespan of the Sod1−/− mice; however, in our study, only about 30% of Sod1−/− mice developed hepatocellular carcinoma later in life [6]. In addition, we showed that dietary restriction (DR), which is a manipulation that retards aging in rodents, increased the lifespan of the Sod1−/− mice to that of normal, wild type (WT) mice. These data combined with studies showing that Sod1−/− mice exhibited various accelerated aging phenotypes [e.g., muscle atrophy and loss of fat mass, hearing loss [7], cataracts [8], skin thinning and delayed wound healing [9] lead us to conclude that the Sod1−/− mice exhibit accelerated aging. This then raised the question of why we observed a significant decrease in lifespan and accelerated aging in only the Sod1−/− mice and not in other mouse models with compromised antioxidant defense systems that showed changes in oxidative stress/damage.

Sod1−/− mice show a much higher level DNA oxidation (i.e., 8-oxodG levels) in tissues than any of the mouse models we have studied, which all have deficiencies in one or more of the antioxidant genes [4]. In addition, DNA mutations have been reported to increase significantly in several tissues in Sod1−/− mice [10]. Because the DNA damage response has been shown to play a central role in the generation of senescent cells [11] and because Van Deursen’s laboratory has shown that clearance of senescent cells delays aging-associated disorders and increases lifespan in a progeroid mouse model [12] as well as normal, WT mice [13], we hypothesized that the increased oxidative damage to DNA in tissues of Sod1−/− mice could activate the DNA damage response and drive cells into becoming senescent. To test

![Fig. 1. Cellular senescence is increased in kidney of Sod1−/− mice. (A) Transcript levels of p16INK4a and p21 in kidney measured by qRT-PCR and normalized to GAPDH. (B) The level of p16INK4a and p21 protein in kidney as measured by Western blot (top panel). Quantification of p16INK4a and p21 normalized to β-tubulin is shown in the bottom panel. (C) Images of SA β-Gal positive staining cells in kidney is shown in the left panel (arrow points to β-Gal positive cells). Percentage of SA β-Gal positive cells is quantified and graphically represented in the right panel. Four groups of mice were studied: young (4–6 month-old) WT (YWT, turquoise bar); old (24 month-old) mice (OWT, blue bar); young (4–6 month-old) Sod1−/− mice (YKO, red bar); young (6-month-old) Sod1−/− mice on DR (YKODR, yellow bar). The data are the mean ± SEM of 4 mice per group and were statistically analyzed by one-way ANOVA followed by student T-test. The asterisk (*) indicates a significance (P < 0.05) difference between either young WT mice or young Sod1−/− mice on DR and old WT mice or young Sod1−/− mice on DR. There were no significant differences between the old WT and young Sod1−/− mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

![Diagram with graphs and images showing gene expression and protein levels for p16 and p21 in different mouse models: YWT, OWT, YKO, YKODR.](image-url)
our hypothesis, we measured various markers of cellular senescence in kidney tissue, a tissue that shows a significant increase in senescent cells with age [14]. We compared kidney from young-adult and old WT mice and young-adult Sod1−/− mice fed ad libitum or a DR diet. Our data clearly demonstrate that the level of senescent cells is dramatically increased in the kidney of young-adult Sod1−/− mice compared to young-adult WT mice and are at a level comparable to old WT mice. In addition, we observed that the increase in cellular senescence observed in the Sod1−/− mice was attenuated by DR. Interestingly, the increase in cellular senescence in the Sod1−/− mice was correlated to increased circulating cytokines. Thus, our data suggest that increased cellular senescence could play a role in the accelerated aging phenotype we have observed in the Sod1−/− mice.

2. Results

2.1. Cellular senescence is induced in the kidney of Sod1−/− mice

Using several assays, we measured the level of cell senescence in the kidneys from four groups of mice: 4- to 6- and 24-month-old wild type (WT) mice and 6-month-old Sod1−/− mice fed ad libitum or a DR diet. We first measured the expression of two of the well-accepted markers of cellular senescence, p16INK4a and p21 [11]. As shown in Fig. 1A, the transcripts for both p16INK4a and p21 protein increased significantly with age in the WT mice. Berkenkamp et al. had previously reported a 3-fold increase in p16INK4a mRNA levels in the kidney of mice between 3–5 and 18 months of age [14]. More importantly, we observed a 2.5-fold increase in p16INK4a transcript levels in the Sod1−/− mice compared to age-matched WT mice, which is similar to the level of p16INK4a in the old WT mice. DR attenuated the increase in cell senescence in the Sod1−/− mice. The level of p16INK4a transcripts was significantly reduced in the Sod1−/− mice fed a DR diet for 4 months to a level similar to that observed in the young WT mice. As can be seen in Fig. 1A, similar changes were observed in the levels of p21, e.g., the Sod1−/− mice showed a 3-fold increase in p21 transcript levels, and DR reduced the expression of p21. We further determined if the changes in p16INK4a and p21 transcript levels were observed at the protein level. Fig. 1B shows the protein levels of p16INK4a and p21 in the kidneys from the four groups of mice. These data show similar changes in the expression of p16INK4a and p21 proteins, i.e., a dramatic increase in these two proteins in the kidneys from old mice and 6-month-old Sod1−/− mice, which is reduced by DR. Interestingly in our previous study, we observed no significant difference in p21 levels in liver from 6-month-old WT and Sod1−/− mice [6]. We believe that the most likely explanation for these differences in p21 expression in kidney and liver of the Sod1−/− mice is due to the physiological differences in how these tissues respond to increased oxidative damage/stress. In liver, increased oxidative stress has been shown to lead to activation of regeneration [15] and increased regenerative proliferation, which is linked to hepatocellular carcinoma [16]. Therefore, Elchuri et al. [5] proposed that oxidative damage/stress in liver results in cell injury/death leading to regeneration, chromosome instability, and eventually hepatocellular carcinoma. Based on our limited data, it would appear that oxidative stress/damage in kidney leads to cell senescence rather than regeneration and proliferation.

We next measured the senescence associated β-galactosidase (SA-β-gal) activity in kidney of Sod1−/− mice, which is the hallmark of cellular senescence [17]. As shown in Fig. 2C, very few cells positive for SA-β-gal were observed in the 6-month-old WT mice; however, the number of SA-β-gal positive cells was increased 3.5-fold in the kidney of Sod1−/− mice (0.7% vs. 0.2% of the cells were SA-β-gal positive for Sod1−/− and wild type mice respectively). Again, DR attenuated the increase of SA-β-gal positive cells in the Sod1−/− mice (0.35% of cells were SA-β-gal positive).

We previously reported a significant increase in DNA oxidation (measured as the level of 8-oxo-dG) in several tissues from Sod1−/− mice, e.g., skeletal muscle, liver, kidney and brain [7]. To determine if the changes in cell senescence in kidney correlated with changes in DNA damage, we measured the level of 8-oxo-dG in kidney of WT and Sod1−/− mice. As shown in Fig. 2A, we observed a significant increase (40%) in DNA oxidative damage in the Sod1−/− mice compared to the WT mice, and DR suppressed the increase of DNA oxidation in kidney of the Sod1−/− mice to the level observed in the WT mice. In a second cohort of mice, we measured the level of double strand DNA breaks (DSBs) in kidney tissue from 4- month-old WT and Sod1−/− mice as percentage of γ-H2AX nuclei, a widely used marker for DSBs [18]. As can be seen in Fig. 2B, kidney from the Sod1−/− mice showed a dramatic increase (52%) in DSBs compared to the WT mice. Thus, the increased damage to DNA in the Sod1−/− mice is correlated to an accumulation of DSBs in kidney.

2.2. Expression of inflammatory cytokines are induced in the kidney of Sod1−/− mice

Campisi’s laboratory made the important discovery that senescent cells secret biologically active proteins (e.g., growth factors, proteases, cytokines, and other factors) that have potent autocrine and paracrine activities [19,20]. Therefore, we measured the expression of several inflammatory cytokines in kidney that have been shown to be induced in senescent cells: IL-6, IL-1β and IL-8. As shown in Fig. 3A, IL-6 and IL-1β transcripts levels increased significantly (40-fold and 3-fold respectively) in kidney of old WT mice. Compared to age-matched WT mice, the Sod1−/− mice have significantly higher levels of IL-6 and IL-1β mRNA transcripts (20-fold and 2-fold respectively), which are comparable to the transcript levels observed in old WT mice. DR attenuated the increased expression of the transcripts for IL-6 and IL-1β mRNA in the Sod1−/− mice. Although we did not observe significant changes in the transcript levels of IL-8 mRNA because of the large animal to animal variation, a trend similar to IL-6 and IL-1β was observed in the old WT and Sod1−/− mice for IL-8. In a second group of mice, we also measured the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) p65 because cytokines secreted by senescent cells can activate NFκB in surrounding cells, leading to a wave of increased production of pro-inflammatory cytokines in a tissue [21]. The activation of NFκB p65 was measured by the phosphorylated levels of NFκB p65 (pS536) because phosphorylation of this serine residue in the C-terminal transactivation domain has been shown to be involved in nuclear import of p65 to stimulate the expression of specific target genes involved in inflammation. As shown in Fig. 3B, the ratio of NFκB p65 (pS536) to the total NFκB p65 is significantly increased in the Sod1−/− mice at both 4 and 9 months of age.

Because we observed changes in the expression of IL-6 in the kidney of the Sod1−/− mice, we were interested in determining if circulating levels of IL-6 were increased in the Sod1−/− mice. As shown in Fig. 3C, plasma levels of IL-6 increased 4-fold in the old WT mice compared to the young WT. A similar age-related increase in circulating levels of IL-6 has been reported in mice [22]. More importantly, the circulating levels of IL-6 were dramatically increased (over 6-fold) in the Sod1−/− mice, and this increase was completely attenuated by DR. Because of the dramatic changes in circulating levels of IL-6 in the Sod1−/− mice, we measured the levels of a panel of cytokines in the serum of a second group of 9-month-old WT and Sod1−/− mice and 25–29-month-old WT mice. In addition to IL-6, three of the ten cytokines we measured increased significantly in the Sod1−/− mice (Table 1). Although we observe a great deal of animal to animal variation in cytokine levels, it is striking that each of the Sod1−/− mice showed a global increase in circulating cytokine levels, similar to old WT mice. For example, the sum of the ten cytokine levels was 1.8-fold higher for the Sod1−/− mice compared to the WT mice.

2.3. Sod1−/− mice have increased renal pathology

To determine if the changes in cell senescence in the Sod1−/− mice had functional consequences, we measured the renal pathology in WT and Sod1−/− mice when they died. As shown in Table 2, the overall
Fig. 2. DNA damage is increased in the kidney of Sod1\(^{-/-}\) mice. (A) DNA oxidative damage (ratio of 8-oxo-dG to dG). (B) Kidney sections immunostained for γH2AX, a marker for DNA double strand breaks. The arrows point to γH2AX positive nuclei. (C) γH2AX nuclei were quantified, and data shown as mean percentage of nuclei positively stained for γH2AX. The following mice were studied: young (4–6 month-old) WT (turquoise bar); young (4–6 month-old) Sod1\(^{-/-}\) mice (red bar); young (6-month-old) Sod1\(^{-/-}\) mice on DR (yellow bar). The DNA oxidative damage data are the mean ± SEM of 4 mice per group and were statistically analyzed by one-way ANOVA followed by student T-test. The asterisk (*) indicates that the values for the Sod1\(^{-/-}\) mice are significantly different (P < 0.05) from the WT mice and DR Sod1\(^{-/-}\) mice. The DSB data are the mean ± SEM of 4 mice per group and were statistically analyzed by unpaired two-tailed T-test. The asterisk (*) indicates that the values for the Sod1\(^{-/-}\) mice are significantly different (P < 0.05) from the WT mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Expression of inflammatory cytokines are elevated in Sod1\(^{-/-}\) mice. (A) The levels IL6, IL-1β, and IL-8 mRNA in kidney was measured by qRT-PCR and normalized to GAPDH. (B) NFκB p65 (pS536) levels in kidney were measured using a Simple Step ELISA kit from Abcam and expressed as the ratio of NFκB p65 pS536/Total NFκB p65. (C) Quantification of IL-6 in plasma by ELISA. Four groups of mice were studied: young (4–6 month-old) (turquoise bar) and old (24 month-old) mice (blue bar) WT mice and young (4–6 month-old) Sod1\(^{-/-}\) mice fed ad libitum (red bar) or young (6-month-old) Sod1\(^{-/-}\) mice on DR (yellow bar). The cytokines data are the mean ± SEM of 4 mice per group and were statistically analyzed by one-way ANOVA followed by student T-test. The asterisks indicate statistical significance (*P < 0.05 and **P < 0.01) between either young WT mice or young DR Sod1\(^{-/-}\) mice and old wild type mice or young Sod1\(^{-/-}\) mice. There were no significant differences between the old WT and young Sod1\(^{-/-}\) mice or the young WT and young DR Sod1\(^{-/-}\) mice. The NFκB p65 (pS536) data are the mean ± SEM of 4 mice per group and were statistically analyzed by unpaired two-tailed T-test. The asterisk (*) indicates that the values for the Sod1\(^{-/-}\) mice are significantly different (P < 0.01) from the WT mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Table 1
Cytokine levels in the blood of WT Young, WT Old and Sod1−/− mice.

| G-CSF | Eotaxin | GM-CSF | IL-1α | IL-1β | IL-6 | KC (IL-8) | MCP-1 | MIP-1 | TNFα | TOTAL |
|-------|---------|--------|-------|-------|------|----------|-------|-------|-------|-------|
| KO-1  | 2008    | 12071  | 60    | 227   | 0    | 70       | 752   | 450   | 98    | 27    | 15763 |
| KO-2  | 2938    | 14029  | 90    | 100   | 0    | 66       | 689   | 220   | 678   | 0     | 19579 |
| KO-3  | 3634    | 8168   | 199   | 100   | 0    | 36       | 890   | 828   | 594   | 29    | 15283 |
| KO-4  | 1686    | 10007  | 90    | 1733  | 238  | 0        | 774   | 572   | 594   | 67    | 15761 |
| KO-5  | 4009    | 7595   | 109   | 469   | 259  | 45       | 337   | 503   | 503   | 0     | 13364 |
| KO-6  | 2968    | 11733  | 0     | 1766  | 0    | 0        | 794   | 0     | 0     | 33    | 17294 |
| AVG   | 2874*   | 10601  | 43    | 1026  | 100  | 36       | 767   | 429   | 327   | 32    | 16174* |
| SEM   | 367     | 10008  | 0.08  | 2.1   | 0.2  | 0.05     | 0.03  | 0.14  | 0.47  | 0.06  | 0.0009 |
| P-value | 0.003  | 0.0008 | 0.08  | 0.21  | 0.21 | 0.05     | 0.03  | 0.14  | 0.47  | 0.06  | 0.0009 |

The levels of ten cytokines were measured in the serum collected from five 9-month-old WT (WT-Young), mice 26–29-month-old WT (WT-old) and six 9-month-old Sod1−/− (KO) mice.

The level of each cytokine is shown for each of the mice studied and is expressed as pg/ml (0 indicates that the level of the cytokine in the serum of that animal was undetectable). The data were analyzed using one-tailed student's T-test, and the p-values are given. Cytokines showing a significant increase in the Sod1−/− mice and old mice, compared to young-WT, are identified with an asterisk.

Table 2
Renal pathology in WT and Sod1−/− mice.

|                | WT (n=36) | KO (n=50) | KODR (n=47) |
|----------------|-----------|-----------|-------------|
| Incidence (%)  | 25 (69%)  | 44 (88%)  | 33 (70%)    |
| Incidence-free (percent): | 11 (31%)  | 6 (12%)   | 14 (30%)    |
| Total kidney disease incidence (average score per animal): | 24 (9.06) | 88 (1.76) | 38 (1.15) |
| Disease burden expressed as number of lesions per mouse | 0.96 | 1.76 | 1.15 |
| Glomerulonephritis Total incidence (percent): | 20 (56%)  | 37 (74%)  | 26 (55%)    |
| Total severity score (average score per animal): | 27 (1.35) | 64 (1.73) | 49 (1.88) |

The data presented are end of life pathology that were extrapolated and summarized from previous studies by our group [6,49].

*p < 0.05 denotes statistical significance between the KO (Sod1−/− mice) and the other two groups, WT (wild type) or KODR (Sod1−/− mice on DR).

The role of oxidative stress in aging has been called into question by experiments from our group showing that in 18 different genetically modified mouse models, in which various components of the antioxidant defense system were altered to increase or decrease the level of oxidative stress/damage in tissues, only Sod1−/− mice showed a change in lifespan (a 30% decrease) and accelerated aging phenotypes as predicted by the Oxidative Stress Theory of Aging [23]. The focus of this study was to begin testing various mechanisms that would account for the accelerated aging in the Sod1−/− mice. Because DNA oxidation (i.e., 8-oxo-dG levels) is much higher in tissues from the Sod1−/− mice than in any of the mouse models we have studied, which all have deficiencies in one or more of the antioxidant genes [4], we hypothesized that the Sod1−/− mice might show increased cell senescence.

Consistent with this hypothesis, is our observation that DR, which increased the lifespan of Sod1−/− mice [6], reduces the level of DNA oxidation in the Sod1−/− mice. It is well established that DR dramatically increases the resistance of mice to oxidative stress, leading to reduced oxidative damage to various macromolecules [2], including the level of 8-oxo-dG in DNA of tissues from rats and mice [24]. By itself, 8-oxo-dG is not particularly mutagenic; however, when 8-oxo-dG residues are found in a cluster of lesions along with single strand breaks, double strand DNA breaks (DSBs) are generated when the cell attempts to repair these lesions [25–27]. Our data show that the level of DSBs is significantly increased in the kidney of Sod1−/− mice compared to WT mice. Although we did not study the effect of DR on the level of DSBs in Sod1−/− mice, Hallam et al., 2016, showed that the age related increase (~30%) in H2A.X foci in the central cornea of the mice was attenuated by DR [28]. DSBs have been shown to be involved in the generation of senescent cells through the formation of DNA-SCARS (DNA segments with chromatin alterations reinforcing senes-
ence) [29], which are persistent DNA damage foci that induce the DNA damage response signaling including the induction of two tumor suppressor pathways, e.g., p53/p21 and pRB/p16INK4a. Most senescent cells express p16INK4a, which is a cyclin-dependent kinase inhibitor that leads to pRB hypophosphorylation. The expression of p16INK4a has been shown to increase with age in mouse and human [30]. Our data clearly demonstrate that the level of senescent cells is dramatically increased in the kidney of young (6 months) Sod1−/− mice compared to young WT mice and is at a level comparable to old (24 months) mice, e.g., an increase in the expression of p16INK4a and p21 and an increase in β-gal positive cells was observed. In addition, the increase in cell senescence observed in the Sod1−/− mice was attenuated by DR, which we have shown increases the lifespan of the Sod1−/− mice [6]. Thus, we show that the decrease in the lifespan in the Sod1−/− mice or the increase in the lifespan of the Sod1−/− mice fed a DR diet, correlate to changes in cell senescence. Van Deursen’s laboratory has shown that clearance of p16INK4a positive senescent cells delays aging-associated disorders and increases lifespan in a progeroid mouse model as well as normal, WT mice [12,13]. Thus, cell senescence could play a role in the accelerated aging phenotype we have observed in the Sod1−/− mice.

Our data also point to a mechanism of how the increase in cell senescence might lead to accelerated aging in the Sod1−/− mice. Campisi’s laboratory has shown that senescent cells secret biologically active proteins (e.g., growth factors, proteases, cytokines, and other factors) that have potent autocrine and paracrine activities [19]; a chronic process termed the senescence associated secretory phenotype (SASP). The SASP includes several potent inflammatory cytokines including IL-1β, IL-6 and IL-8 which may serve as an important source of low-level chronic inflammation [31]. We showed that the expression of IL-6 and IL-1β are dramatically increased in the kidney of the Sod1−/− mice. We also measured the levels of NFκB p65 activation in kidney tissue because cytokines produced by senescent cells have been shown to activate NFκB in surrounding cells. Therefore, only a few senescent cells have the potential to lead to a wave of increased production of pro-inflammatory cytokines in a tissue [21]. The NFκB pathway is a well-established proinflammatory signaling pathway known to increase expression of cytokines and chemokines [32]. We showed that NFκB activation was higher in the kidney of the Sod1−/− mice. Because NFκB has been shown to act as a master regulator of the expression of SASP genes [33,34], the increase in NFκB activation in the Sod1−/− mice could further increase the production of pro-inflammatory cytokines by senescent cells.

Based on our study with kidney, we propose senescent cells also accumulate in other tissues of the Sod1−/− mice, and this accumulation of senescent cells in Sod1−/− mice plays a role in the accelerated aging and increased mortality observed in the Sod1−/− mice through the production of proinflammatory cytokines by senescent cells. Indeed, we showed that Sod1−/− mice had elevated circulating levels of many proinflammatory cytokines, demonstrating for the first time that inflammation is elevated in the Sod1−/− mice. Finch and Grimmins argue that senescent cells play an important role in increased inflammation that contributes to aging and age-related disease [35]. Chronic, low grade inflammation with age, often called ‘inflammaging’ is a prevalent feature of aging as well as many age-related diseases such as cardiovascular disease, type 2 diabetes, and dementia [36]. In addition, inflammaging is a substantial risk factor for both morbidity and mortality in the elderly people, e.g., epidemiological data show that inflammaging is associated with and predictive of aging phenotypes including frailty [36,37].

We recognize that increased oxidative stress in the Sod1−/− mice could lead to increased inflammation by mechanisms other than cell senescence. For example, necroptosis is a novel pathway of regulated necrosis that plays an important role in the development of inflammation and inflammatory diseases. Necroptosis can be induced by multiple factors such as death receptors, interferons, toll-like receptors or intracellular RNA, DNA sensors, and recent studies show that oxidative stress is an important initiator of necroptosis [38]. For example, absence of glutathione peroxidase 4 has been shown to induce necroptosis in mouse erythroid precursor cells [39], and knocking out a necrosis mediating protein, RIP3 (receptor-interacting protein 3) reduces inflammation and mortality in a mouse model of atherosclerosis [40,41]. Oxidative stress has also been linked to the development of inflammation through activation of NLRP3 inflammasome. The NLRP3 inflammasome is unique among innate immune sensors because it can be activated in response to a diverse array of endogenous metabolic “danger signals” to induce sterile inflammation in absence of overt infection. The generation of reactive oxygen species is one of the proposed mechanisms that triggers NLRP3 activation [42], and a recent study has shown that reduction of NLRP3 inflammasome activation enhances healthspan and reduces age-related functional decline in mice [43].

In summary, we believe that our data point to a new concept of how oxidative stress might lead to aging. Initially, it was proposed that oxidative damage (e.g., damage to lipid, protein, or DNA) was responsible for a deterioration of cellular function and eventually age-related pathologies and aging. However, cells have numerous pathways to repair damage that occurs to cellular macromolecules, i.e., macromolecular damage represents a steady state between the amount of damage occurring at one time and the ability of cells to repair the damage. Thus, a cell/tissue might experience a high level of oxidative stress resulting in high levels of damage, which could be repaired over time. Based on our data, we propose that in response to high levels of oxidative damage resulting in DSBs, a cell becomes senescent, which is irreversible. Velarde et al. [44] showed increased DSBs and cell senescence in the skin of very young mice null for Mn-superoxide dismutase (Sod2). Sod2−/− mice show massive levels of oxidative stress and die within a few days after birth [45]. We propose that a brief high level of oxidative damage could give rise to a senescent cell before it is repaired to steady state levels by the cell. Once generated, the senescent cell would remain in the tissue over the lifespan of the mouse. Thus, various bouts of oxidative stress over the lifespan of an animal has the potential lead to the accumulation of senescent cells even though the steady state levels of damage are increased only transiently. This concept is similar to the role that DNA damage plays in cancer. DNA damage, which can be continuously repaired, becomes important only when it leads to a permanent change in the genome because of a change in the DNA sequence resulting in a mutation. Therefore, we are proposing that oxidative damage plays a role in aging when the damage produced by increased ROS results in the generation of senescent cells, which can accumulate over the lifespan of an animal.

4. Experimental procedures

4.1. Animals

The Sod1−/− mice used in this study were generated by Dr. Charles Epstein and Ting-Ting Huang and were genotyped as previously described [5]. Sod1−/− mice were fed a standard NIH-31 chow (19.1% protein, 5.8% fat, 62.7% carbohydrate) obtained from Harlan Teklad, Madison, WI (Diet LM485) and were housed 4 mice/cage under barrier conditions in micro-isolator cages on a 12-h dark/light cycle. For tissue collection, animals were sacrificed by cervical dislocation, and the tissues were immediately followed by cervical dislocation, and the tissues were immediately excised and placed on ice or in liquid nitrogen depending on the procedures performed later. All tissues following collection were stored at −80 °C and analyzed within 30 days. Dietary restriction (DR) was initiated at 2 months of age by feeding the mice 60% of diet (by weight) consumed by the mice fed ad libitum as described previously [6]. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San
were determined using the Milliplex Map Kit: Mouse Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore Corporation, Billerica, MA) and a Luminex Bio-Plex 200 system.

4.6. DNA damage

4.6.1. 8-oxo-dG

Oxidative damage to DNA was measured as the level of 8-oxo-deoxyguanosine (8-oxo-dG) using the HPLC approach as described previously [47]. DNA was isolated from tissues by the Nai method using the DNA Extractor WB kit obtained from Wako Chemicals USA, Inc. (Richmond, VA). Results are expressed as the ratio of nanomoles of 8-oxo-dG to $10^5$ nmol of 2-deoxyguanosine.

4.6.2. DNA double strand breaks (DSB)

DSBs were measured by immunocytochemistry as mean percentage of γ-H2AX positively stained nuclei [18]. Briefly, formalin-fixed paraffin embedded kidney sections were treated according to standard protocols. Anti-histone H2AX[13] from Abcam (Cambridge, MA) and a Luminex Bio-Plex 200 system.

Acknowledgements

This study was supported by NIH grants to HVR and AR (P01AG020591 and P01AG051442) and an NIH grant to AR and AU (R01 AG045693). AR and HVR are supported by the Senior Research Career Scientist awards from the Department of Veteran Affairs.

References

[1] R.S. Sohal, R. Weindruch, Oxidative stress, caloric restriction, and aging, Science 273 (1996) 59–63.
[2] A. Bokov, A. Chaudhuri, A. Richardson, The role of oxidative damage and stress in aging, Mech. Ageing Dev. 125 (2004) 811–826.
[3] H. Liang, E.J. Masoro, J.F. Nelson, R. Strong, C.A. McMahan, A. Richardson, Genetic mouse models of extended lifespan, Exp. Gerontol. 38 (2003) 1353–1364.
[4] V.I. Perez, A. Bokov, H. Van Remmen, J. Mele, Q. Ran, Y. Ikeno, A. Richardson, Is the oxidative stress theory of aging dead?, Biochim. Biophys. Acta 1790 (2009) 1005–1014.
[5] S. Elchuri, T.D. Oberley, W. Qi, R.S. Eisenstein, L. Jackson Roberts, H. Van Remmen, C.J. Epstein, T.T. Huang, CuZnSOD deficiency leads to persistent and widespread oxidative damage and hepatocarcinogenesis later in life, Oncogene 24 (2005) 367–380.
[6] Y. Zhang, Y. Ikeno, A. Bokov, J. Gelfond, C. Jaramillo, H.M. Zhang, Y. Liu, W. Qi, G. Hubbard, A. Richardson, H.V. Remmen, Dietary restriction attenuates the accelerated aging phenotype of Sod1−/− mice, Free Radic. Biol. Med. (2013).
[7] F.L. Muller, W. Song, Y. Liu, A. Chaudhuri, S. Pick–Dahli, R. Strong, T.T. Huang, C.J. Epstein, L.J. Roberts 2nd, M. Csete, J.A. Faulkner, H. Van Remmen, Absence of CuZn superoxide dismutase leads to elevated oxidative stress and acceleration of age-dependent skeletal muscle atrophy, Free Radic. Biol. Med. 40 (2006) 1014–2004.
[8] E.M. Olofsson, S.L. Marklund, A. Behndig, Glucose-induced cataract in CuZn-SOD null mice: an effect of nitric oxide?, Free Radic. Biol. Med. 42 (2007) 1098–1105.
[9] Y. Inuchi, D. Roy, F. Okada, N. Kibe, S. Tsunoda, S. Suzuki, M. Takahashi, H. Yokoyama, J. Yoshiizaka, S. Kondo, J. Fuji, Spontaneous skin damage and delayed wound healing in SOD1-deficient mice, Mol. Cell Biochem. 341 (2010) 181–194.
[10] R.A. Busuttil, A.M. Garcia, C. Calvera, A. Rodriguez, Y. Suh, W.H. Kim, T.T. Huang, J. Yijig, Organ-specific increase in mutation accumulation and apoptosis rate in CuZn-superoxide dismutase-deficient mice, Cancer Res. 65 (2005) 11271–11275.
[11] J. Campisi, F. d’Adda di Fagagna, Cellular senescence: when bad things happen to good cells, Nat. Rev. Mol. Cell Biol. 8 (2007) 725–740.
[12] D.J. Baker, T. Wijhake, T. Tchekonia, N.K. LeBrasseur, B.G. Childs, B. van de Sluis, J.L. Kirkland, J.M. van Deursen, Clearance of p16ink4a-positive senescent cells delays aging-associated disorders. Nature 479 (2011) 232–236.
[13] D.J. Baker, B.G. Childs, M. Durik, M.E. Wijers, C.J. Sieben, J. Zhang, R.A. Saltzman, K.B. Jeganathan, G.C. Verzosa, A. Pezeshki, K. Khazaie, J.D. Miller, J.M. van Deursen, Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan, Nature 530 (2016) 184–189.
[14] B. Berkenkamp, N. Susnik, A. Baisant, I. Kazmetova, C. Jacobi, I. Sorensen-Zender, V. Broecker, H. Haller, A. Melk, R. Schmitt, In vivo and in vitro analysis of age-associated changes and somatic cellular senescence in renal epithelial cells.
