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Effects of altered levels of extracellular superoxide dismutase and irradiation on hippocampal neurogenesis in female mice

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Abstract

Purpose—Altered levels of extracellular superoxide dismutase (EC-SOD) and cranial irradiation have been shown to affect hippocampal neurogenesis. However, previous studies were only conducted in male mice, and it was not clear if there was a difference between males and females. Therefore, female mice were studied and the results compared to those generated in male mice from an earlier study.

Methods and materials—Female wild type (WT), EC-SOD null (KO), and EC-SOD bi-genic mice with neuronal-specific expression of EC-SOD (OE) were subjected to a single dose of 5 Gy gamma rays to the head at 8 weeks of age. Progenitor cell proliferation, differentiation, and long-term survival of newborn neurons were determined.

Results—Similar to results from male mice, EC-SOD deficiency and irradiation both resulted in significant reductions in mature newborn neurons in female mice. EC-SOD deficiency reduced long-term survival of newborn neurons while irradiation reduced progenitor cell proliferation. Overexpression of EC-SOD corrected the negative impacts from EC-SOD deficiency and irradiation and normalized the production of newborn neurons in OE mice. Expression of neurotrophic factors, BDNF and NTF3, were significantly reduced by irradiation in WT mice, but the levels were not changed in KO and OE mice even though both cohorts started out with a lower baseline level.

Conclusion—In terms of hippocampal neurogenesis, EC-SOD deficiency and irradiation have the same overall effects in males and females at the age the studies were conducted.

Introduction

Cranial irradiation is widely used as a treatment modality for patients with primary and secondary brain tumors. Although effective, cranial irradiation can lead to serious

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complications, including impairments in the cognitive functions of learning and memory [1]. Reduced hippocampal neurogenesis has been considered a major contributor to radiation-induced cognitive impairments [2], although other factors, such as reduced dendritic spine densities [3,4], neuronal apoptosis [5], and hypoxic and ischemic injury [6] have all been proposed as possible causes for cognitive decline following radiation therapy.

Clinical studies suggest that radiation response may differ as a function of gender. In pediatric patients, cranial irradiation usually leads to higher frequency and greater cognitive damage in female patients [7,8]. In adults, the data are less conclusive. In animal studies, female mice, examined at 14 days [9] or 2 months [10] of age, also appear to show age-dependent sensitivity to irradiation. Based on differences in radiation sensitivity, requirements for clinical treatment and post-irradiation management may need to be varied at different ages between male and female patients to achieve optimal outcomes. Therefore, it is important to identify the underlying mechanisms of gender-based differences in radiation response.

Ionizing irradiation leads to acute and chronically elevated levels of oxidative stress. Male transgenic mice expressing higher levels of the antioxidant enzyme, extracellular superoxide dismutase (EC-SOD), showed that high levels of EC-SOD can promote dendritic development and long-term survival of newborn neurons and preserve neurocognitive functions after cranial irradiation [11]. To understand how altered EC-SOD levels and irradiation affected hippocampal neurogenesis in females, we used young adult mice with altered EC-SOD levels and investigated different stages of hippocampal neurogenesis in sham and irradiated EC-SOD null mice with either ubiquitous EC-SOD deficiency (KO) [12], or with high levels of EC-SOD reconstituted to excitatory neurons (OE) [11]. Comparison of results with that obtained from male mice examined at the same age [11] suggested a similar outcome in hippocampal neurogenesis in response to EC-SOD deficiency and irradiation.

Methods and Materials

Animals

Generation and genotyping of EC-SOD knockout (KO) and TRE-*Sod3*-GFP/CaMKII-tTA double transgenic mice on the EC-SOD null background (OE) had been described [11]. C57BL/6J mice generated in parallel were used as wild type (WT) controls. All mice were housed in a barrier facility with a 12-hour dark-light cycle, given food and water *ad libitum*. All animal procedures were approved by the Subcommittee on Animal Studies at the VA Palo Alto Health Care System and in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals.

Cranial irradiation and neurogenesis study

Cranial irradiation with a single dose of 5 Gy of gamma rays was carried out at eight weeks of age as described [11]. The thymidine analog 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO) was used to label proliferating cells as depicted in Figure 1. At specified time points, brains were collected and processed for immunohistochemical staining as described [11] to detect BrdU+ cells, immature neurons (Dcx+), newborn neurons (BrdU+/NeuN+), and newborn astroglia (BrdU+/GFAP+) in the subgranular zone (SGZ) of the dentate gyrus (DG). The stereological counting principle of systematic, uniformly random sampling of sections was used for cell counting [4,11]. Categories E&F Dcx+ cells were identified based on the level of dendritic maturation [13].

Western blot and qRT-PCR analyses

Hippocampi were collected from individual mice 4 weeks after irradiation (Figure 1C). Protein levels of antioxidant enzymes were determined by western blot analyses, and expression levels of neurogenesis-related genes and vascular endothelial growth factor receptor 2 (VEGFR2) were determined by qRT-PCR as described [11]. Primers used in the study were listed in Table eI.

Quantification of CD31+ structures

To identify microvasculature, brain sections from the short-term study were stained with CD31 antibody (BD Biosciences, San Jose, CA), and neurons were counter stained with NeuN antibody (Chemicon, Temecula, CA). Stained sections were imaged with a LSM 700 Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging, Thornwood, NY). The area containing the entire DG (granule cell layer and the enclosed hilus) was calculated by Image J (National Institutes of Health, MD). Vascular structures at least 10 μm in length were manually counted within the DG using Image J. Vessels with a branch point were counted as 2. The average number of vessels per mm^2 from each mouse was then calculated.

Comparison of hippocampal neurogenesis between males and females

Total numbers of BrdU+, Dcx+, categories E&F Dcx+, BrdU+/NeuN+, and BrdU+/GFAP+ cells from female (this study) and male mice [11] were compared directly to identify gender effects. The female to male ratio of each cell population was then plotted to summarize the results.

Statistical analyses

Statistical analyses were carried out with GraphPad Prism® 4.03 for Windows (GraphPad Software, Inc. San Diego CA). Two-way ANOVA with Bonferroni post-test was performed to determine changes due to irradiation, variations in EC-SOD levels, gender, and genotype $\times$ treatment interaction (Table eII). Student's t test was also performed as noted in figure legends. All data were expressed as mean $\pm$ SEM; error bars in all figures signify SEM.

Results

Altered EC-SOD levels did not affect progenitor cell proliferation in the SGZ

The number of BrdU+ cells in the SGZ, which indicated the number of proliferating cells after short-term BrdU injection, was not significantly different in sham-irradiated WT, KO, and OE mice (Figures 2A–2B). Following irradiation, significant reductions in cell proliferation were observed in all three genotypes (Figure 2A), and no significant differences were observed among the three irradiated cohorts. Two-way ANOVA identified irradiation as the dominant factor in the reduction of BrdU+ cells, and there was no interaction between genotype and treatment (Table eII).

Irradiation and EC-SOD deficiency negatively affected early stage maturation of newborn neurons

No significant differences in the total numbers of Dcx+ cells were observed in sham-irradiated WT, KO, and OE mice. On the other hand, irradiation significantly reduced Dcx+ cells in WT and KO, but not in OE mice, with the largest reduction seen in irradiated KO mice (44% reduction) (Figure 2C). Categories E&F Dcx+ cells represent an important benchmark in the maturation process of newborn neurons. These cells are post-mitotic and have extensive dendritic arbors that reach into the molecular layer (Figure 2D) [11,13]. In sham-irradiated WT and OE mice, categories E&F Dcx+ cells accounted for 50% of total Dcx+ cells, but the number was significantly lower in sham-irradiated KO mice (Figures

2E). Irradiation reduced the number of categories E&F Dcx+ cells by 43%, 43%, and 21% in WT, KO, and OE mice, respectively. Because irradiation also reduced the number of total Dcx+ cells, the proportion of categories E&F Dcx+ cells to total Dcx+ cells in irradiated mice remained similar to that of their sham-irradiated counterparts (Table eIII).

High levels of EC-SOD supported long-term survival of newborn cells in the SGZ following irradiation

Compared to sham-irradiated WT mice, a significantly lower number of surviving BrdU+ cells was observed in sham-irradiated KO mice (Figures 2F–2G); however, no significant difference was observed in sham-irradiated OE mice. Following irradiation, the number of BrdU+ cells was reduced by 53%, 48%, and 23% in WT, KO, and OE mice, respectively (Figure 2F). Taking into consideration reduced progenitor cell proliferation following irradiation, long-term survival of newborn cells was negatively impacted by irradiation in WT and KO, but not in OE mice (Table eIII).

High levels of EC-SOD preserved neuronal differentiation following irradiation

Similar to the number of surviving BrdU+ cells, sham-irradiated KO mice had a significantly lower number of newborn neurons than sham-irradiated WT mice (Figures 2H–2I). However, no significant difference was observed between sham-irradiated WT and OE mice. Following irradiation, significant reduction was observed in WT mice (61% reduction). A 56% and 19% reduction was also observed in irradiated KO and OE mice, respectively, although the reductions did not reach statistical significance. In contrast, the number of newborn astroglia in sham-irradiated KO and OE mice were significantly lower than that in sham-irradiated WT mice (Figures 2J–2K). Due to low abundance and large variance, the total numbers of newborn astroglia was not significantly different between sham and the irradiated counterparts (Figure 2J).

Altered EC-SOD levels did not affect expression of most antioxidant enzymes

EC-SOD protein levels in sham and irradiated OE mice were 2.9- and 4.1-fold higher, respectively, than that in the corresponding WT mice (Figures 3A–3B), and irradiation did not significantly change EC-SOD levels in WT or OE mice. Whether increased EC-SOD protein corresponds to a proportional increase in enzyme activity in the hippocampus is not clear due to difficulty in measuring EC-SOD from small quantity of tissues. CuZnSOD protein levels appeared to be lower in KO mice, with 29% and 24% reductions in sham and irradiated KO mice, respectively, compared to that of the corresponding WT controls (Figure 3C). All other antioxidant enzymes examined did not differ significantly among different cohorts (quantification data not shown).

Irradiation affected expression of neurogenesis-related genes

The expression levels of brain derived neurotrophic factor (Bdnf) and neurotrophic factor 3 (Ntf3) were significantly reduced by irradiation in WT mice (Figures 3D–3E). In contrast, Bdnf and Ntf3 expression in KO and OE mice did not change following irradiation, although the expression in sham-irradiated KO and OE mice were significantly lower than that in the corresponding WT controls. The homeobox transcription factor, Nkx2-2 (Figure 3F), showed an expression profile similar to that of neurogenesis (Figure 2H). Consistent with this finding, similar expression profile was also observed in the transcription factor NeuroD1 (Figure 3G), which is controlled by Nkx2-2 and is required for neuronal survival and differentiation in the hippocampus [14,15].

Irradiation and EC-SOD deficiency significantly reduced microvasculature in the DG

Significant reductions in the number of CD31+ vascular counts were observed in the DG and the hilus of KO and OE mice (Figures 4A–4B). Following irradiation, a significant reduction in the number of CD31+ microvessels was observed in WT mice, but no further reductions were observed in irradiated KO and OE mice (Figure 4B). Consistent with the reduction in the number of microvessels, expression levels of VEGFR2 were altered by EC-SOD deficiency and irradiation, with significantly lower expression levels in sham-irradiated KO and OE mice and a significant reduction in WT mice following irradiation (Figure 4C).

Gender was not a significant factor in radiation sensitivity

No significant difference was observed between males and females in the number of BrdU+ (both short-term and long-term), BrdU+/NeuN+, and BrdU+/GFAP+ cells (Figures 5A–5B, 5E–5F). Total number of Dcx+ cells, on the other hand, was significantly different in sham and irradiated WT and irradiated KO (Figure 5C). The number of categories E&F Dcx+ cells was also significantly different in sham-irradiated WT and irradiated KO (Figure 5D).

Discussion

Female mice at 8 weeks of age were used in this study. This age in mouse was equivalent to early adulthood (17–18 years old) in humans [16]. We showed that: (a) EC-SOD deficiency did not change progenitor cell proliferation in the SGZ, but significantly reduced survival of newborn neurons in both sham and irradiated KO mice; (b) EC-SOD overexpression in granule cells preserved neurogenesis in OE mice after irradiation; (c) altered EC-SOD levels or irradiation suppressed expression of neurotrophic factors and transcription factors important for neurogenesis; and (d) compared to hippocampal neurogenesis in male mice examined at the same age, no significant difference in radiation sensitivity was observed between the two genders.

Most studies of irradiation and hippocampal neurogenesis used male mice or rats, and only a handful included females. Depending on age, there may or may not be a gender effect in progenitor cell proliferation or the production of newborn cells in the SGZ of the DG [17,18]. In an earlier study with male mice examined at the same age, we showed that altered EC-SOD levels affected how the different stages of neurogenesis responded to irradiation [11]. Given that the experimental system, the age, and the mouse strains used here were the same as in the previous study, these studies provided an opportunity to compare gender effects in hippocampal neurogenesis pertaining to altered EC-SOD levels and cranial irradiation.

When the numbers of BrdU+ cells and the number of newborn neurons and astroglia were compared between male and female mice, the overall effects of altered EC-SOD levels or irradiation on hippocampal neurogenesis were not significantly different between the genders. The data were consistent with previous publications on adult mice and supported the contention that there was no difference between genders at the age the studies were conducted. Differences in the number of immature neurons (Dcx+ cells) were noted, especially in WT mice (Figure 5C–5D). However, since variations in the number of these intermediate cells did not affect the final number of newborn neurons, there may not be biological significance associated with this observation.

BDNF expression in WT mice was reduced by 30% by irradiation in both genders. However, the expression patterns were not the same between male and female KO and OE mice. Expression patterns of a number of neurogenesis-related genes also appeared to be different between the two genders, suggesting that cell population changes during

neurogenesis may be regulated differently. Because the entire hippocampal formation, instead of specific cell populations, was used for the measurements, refined analyses will need to be performed to have a more definitive conclusion.

Irradiation can lead to marked reduction in microvasculature in the hippocampus [19]. In this study, we observed significant reduction in CD31+ vessel counts in WT mice 4 weeks after irradiation (Figure 4). This was a stark contrast to a more moderate reduction seen after a similar dose of whole brain irradiation in two-week-old male mice [20]. The discrepancy may be due to differences in the age, gender, and the interval between irradiation and tissue analysis. Nevertheless, the general trend was the same and the data supported the sensitive nature of microvasculature to irradiation. The CD31 study was not performed in age-matched male mice in the previous study [11], which precluded the opportunity for a direct comparison at this time. Given that CD31+ cells had an EC-SOD null phenotype in both KO and OE mice, the greatly diminished numbers of CD31+ vessel counts in sham-irradiated KO and OE mice suggested that EC-SOD was important for the formation and/or maintenance of the microvasculature. Although the exact mechanism for reduced microvascularization was not clear, reduced vascular endothelial growth factor (VEGF) signaling in the hippocampal formation may play a role. This was supported in part, by the significant reduction in VEGF receptor 2 (VEGFR2) expression in KO and OE mice.

In summary, when examined during the early adulthood, there was no significant difference in hippocampal neurogenesis between male and female mice in response to variations in EC-SOD levels or irradiation. The data also showed that high levels of EC-SOD were protective against irradiation in both genders, suggesting that EC-SOD-like compounds may be targeted for future therapeutic designs for enhancing or preserving neurogenesis to mitigate late effects resulted from cranial irradiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Cranial irradiation reduces hippocampal neurogenesis and the impact may be affected by the antioxidant enzyme EC-SOD. To determine gender differences in hippocampal neurogenesis pertaining to altered EC-SOD levels and irradiation, young adult female mice were examined. When the results were compared to that of male mice from a previous study, no significant difference was observed between the two genders in radiation sensitivity and in response to variations in EC-SOD levels.

Figure 1.

Experimental timeline. A, short-term study to determine progenitor cell proliferation in the SGZ. Four weeks after cranial irradiation, two doses of BrdU were injected 8 hours apart, and mice were sacrificed for tissue collection 16 hours after the second BrdU injection. B, long-term study to determine long-term survival of newborn cells in the SGZ. Daily BrdU injection was carried out four weeks after cranial irradiation, and mice were sacrificed for tissue collection three weeks after the last BrdU injection. C, Hippocampi were collected from sham and irradiated mice four weeks after cranial irradiation for western blot and qRT-PCR analyses.

Figure 2.

Effects of altered EC-SOD levels and cranial irradiation on hippocampal neurogenesis in female mice. A, total number of proliferating cells in the SGZ measured as BrdU+ cells from the short-term study. B, representative image of BrdU+ cells (brown color) from the short-term study. C, total number of immature neurons identified as Dcx+ cells in the SGZ. D, representative image of Dcx+ cells in the SGZ. Three categories E&F Dcx+ cells (arrow heads) are shown in the middle of the image. E, total number of categories E&F Dcx+ cells. F, total number of newborn cells in the SGZ measured as BrdU+ cells from the long-term study. G, representative image of BrdU+ cells (brown color) from the long-term study. H, total number of newborn neurons in the SGZ. I, representative image of newborn neurons identified as BrdU+/NeuN+ cells. J, total number of newborn astroglia in the SGZ. K, representative image of newborn astroglia identified as BrdU+/GFAP+ cells. Two-way ANOVA with Bonferroni post-test was carried out. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ comparing sham and irradiated mice within the same genotype. $N = 5-12$ per genotype, per treatment. SGZ, subgranular zone; GCL, granular cell layer. B and G, 10 $\times$; D, I, and K, 20 $\times$ objectives. I and K: BrdU, red; NeuN, green; GFAP, blue.

Figure 3.

Protein levels of antioxidant enzymes and message levels of neurogenesis-related genes in the hippocampal formation. A, western blot images showing identification of antioxidant enzymes. Each lane represents results from an individual animal. B–C, quantification of EC-SOD and CuZnSOD. All protein levels were normalized to that of β-actin. D–G, qRT-PCR analyses of Bdnf, Ntf3, Nkx2-2, and NeuroD1. *, $p < 0.05$; **, $p < 0.01$ two-way ANOVA with Bonferroni post-test comparing sham and irradiated mice within the same genotype. *a*, $p = 0.0006$, Student's *t* test comparing sham and irradiated WT mice. N=4–5 (A–C) or 5–11 (D–G) per genotype, per treatment.

Figure 4.

Analysis of vascular network in the DG of hippocampus. A, representative images from sham and irradiated WT, KO, and OE mice showing vessels stained in red (CD31+) and granule cells stained in green (NeuN+). B, average vessel counts; C, VEGFR2 message levels. Two-way ANOVA with Bonferroni post-test was carried out. **, $p < 0.01$; ***, $p < 0.001$ comparing sham and irradiated mice within the same genotype. $N = 4-5$ per genotype, per treatment for vessel counts; $n = 4-6$ per genotype, per treatment for VEGFR2 message levels.

Figure 5.

Comparison of neurogenesis between males and females. The female to male ratio of each cell population was calculated from population means. A ratio of 1 is expected with similar cell numbers between the two genders. Deviation from 1 would indicate higher (>1) or lower (<1) cell numbers, respectively in females. Two-way ANOVA with Bonferroni post-test was performed on actual cell numbers. *, $p < 0.05$; ***, $p < 0.001$ comparing males and females.