

Extreme Sensitivity of Adult Neurogenesis to Low Doses of X-Irradiation¹

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ABSTRACT

Therapeutic irradiation of the brain is associated with a number of adverse effects, including cognitive impairment. Although the pathogenesis of radiation-induced cognitive injury is unknown, it may involve loss of neural precursor cells from the subgranular zone (SGZ) of the hippocampal dentate gyrus and alterations in new cell production (neurogenesis). Young adult male C57BL mice received whole brain irradiation, and 6–48 h later, hippocampal tissue was assessed using immunohistochemistry for detection of apoptosis and numbers of proliferating cells and immature neurons. Apoptosis peaked 12 h after irradiation, and its extent was dose dependent. Forty-eight h after irradiation, proliferating SGZ cells were reduced by 93–96%; immature neurons were decreased from 40 to 60% in a dose-dependent fashion. To determine whether acute cell sensitivity translated into long-term changes, we quantified neurogenesis 2 months after irradiation with 0, 2, 5, or 10 Gy. Multiple injections of BrdUrd were given to label proliferating cells, and 3 weeks later, confocal microscopy was used to determine the percentage of BrdUrd-labeled cells that showed mature cell phenotypes. The production of new neurons was significantly reduced by X-rays; that change was dose dependent. In contrast, there were no apparent effects on the production of new astrocytes or oligodendrocytes. Measures of activated microglia indicated that changes in neurogenesis were associated with a significant inflammatory response. Given the known effects of radiation on cognitive function and the relationship between hippocampal neurogenesis and associated memory formation, our data suggest that precursor cell radiation response and altered neurogenesis may play a contributory if not causative role in radiation-induced cognitive impairment.

INTRODUCTION

Therapeutic irradiation of the brain can result in significant injury to normal brain structures. Although severe structural and functional injury generally occur after relatively high radiation doses (1–4), lower doses can lead to cognitive dysfunction without inducing significant morphological changes (5–10). Such cognitive changes can occur in both pediatric and adult patients and are often manifest as deficits in hippocampal-dependent functions of learning, memory, and spatial information processing (5, 7, 10–12).

Within the hippocampus, memory functions are associated with the principal cells of the hippocampal formation, *i.e.*, the pyramidal and granule cells of the dentate gyrus (13). New granule cells are produced from mitotically active neural precursor/stem cells in the SGZ;³ this production of new cells occurs in all adult mammals, including humans (14–19). Newly born cells migrate into the GCL (20), develop granule cell morphology and neuronal markers (15), and connect with their target area, CA3 (21, 22). Recent studies show that

these new cells become functionally integrated into the dentate gyrus and have passive membrane properties, action potentials, and functional synaptic inputs similar to those found in mature dentate granule cells (23). Most importantly, the new neurons play a significant role in synaptic plasticity (24), which can be considered as a cellular substrate for learning. Furthermore, reductions in the number of newly generated neurons using the toxin methylazoxymethanal acetate impair learning (25), and recently, investigators using a hippocampal slice model showed that radiation-induced reductions in dentate neurogenesis were associated with an inhibition of long-term potentiation, a type of synaptic plasticity (26). Thus, any agent that damages neuronal precursor cells or their progeny, such as ionizing irradiation, could have a significant impact on neurogenesis and ultimately on specific cognitive functions associated with the hippocampus.

We (27–29) and others (30–32) have addressed the radiation response of cells in the dentate gyrus. In the rat, proliferating SGZ precursor cells undergo apoptosis after irradiation (27, 28), and reductions in precursor cell proliferation are still observed months after exposure (28). Furthermore, a single 10 Gy dose of X-rays to the rat brain almost completely abolishes the production of new neurons, whereas surviving precursor cells adopt a glial phenotype (29). Given the relationship between hippocampal neurogenesis and memory (19), and the significant effects of irradiation on SGZ precursor cells, it may be that radiation-induced impairment of SGZ neurogenesis plays a contributory if not causative role in the pathogenesis of cognitive dysfunction after irradiation. In the current study, we were interested in determining if there was a dose response relationship in terms of radiation-induced alterations in neurogenesis and, secondly, if acute changes in the SGZ were predictive of later developing changes in neurogenesis. Understanding how irradiation affects neurogenesis may provide useful insight into potential approaches/strategies to reduce cognitive impairment after cranial irradiation.

MATERIALS AND METHODS

Two-month-old male C57BL/J6 mice (~20 grams) were used in all studies. Mice were purchased from a commercial vendor (The Jackson Laboratory, Bar Harbor, ME) and housed and cared for in accordance with the United States Department of Health and Human Services Guide for the Care and Use of Laboratory Animals; all protocols were approved by the institutional Committee for Animal Research. Mice were kept in a temperature and light-controlled environment with a 12/12-h light/dark cycle and provided food and water *ad libitum*. All mice were anesthetized for irradiation and perfusion procedures; anesthesia consisted of an i.p. injection of ketamine (60 mg/kg) and a s.c. injection of medetomidine (0.25 mg/kg). Sham-irradiated mice were anesthetized as described.

Irradiation was done using a Phillips orthovoltage X-ray system as described previously (28, 33). Briefly, a special positioning jig was used so 4 animals could be irradiated simultaneously; the heads were centered in a 5 × 6 cm treatment field. The beam was directed down onto the head, and the body was shielded with lead. Dosimetry was done using a Keithley electrometer ionization chamber calibrated using lithium fluoride thermal luminescent dosimeters. The corrected dose rate was ~175 cGy/min at a source to skin distance of 21 cm.

Acute Radiation Response. To determine the time of peak apoptosis in the SGZ, groups of mice were irradiated with a single 10-Gy dose, and tissues were collected from 6 to 48 h later. Four sham-irradiated mice were killed at the time of irradiation. For determination of the radiation dose response for

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³ The abbreviations used are: SGZ, subgranular zone; GCL, granule cell layer; BrdUrd, 5-bromo-2'-deoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; Dcx, Doublecortin; NeuN, neuron-specific nuclear protein; ABC, avidin-biotinylated peroxidase complex; GFAP, glial fibrillary acidic protein; DAB, 3,3'-diaminobenzidine.

SGZ apoptosis, whole brain doses of 0, 1, 2, 5, and 10 Gy were given to groups of mice, and tissue was collected at the time of peak apoptosis; sham-irradiated mice were also killed at the time of peak apoptosis. To determine how radiation affected the cellular composition of the SGZ at a time when apoptosis was complete, groups of mice were irradiated with doses of 0, 2, 5, and 10 Gy, and tissues were collected 48 h later.

Mice were reanesthetized for tissue collection, and 50 ml of a 10% buffered formalin solution were infused into the ascending aorta using a mechanical pump (Masterflex Model 7014; Cole Parmer, Chicago, IL). After 5 min, mice were decapitated, and the brain was removed and immersed in a 10% buffered formalin solution for 3 days; tissue was stored in 70% ethanol until gross sectioning and paraffin embedding as described previously (28). A rotary microtome was used to cut 6- μ m-thick transverse sections that were placed on polylysine-coated glass microscope slides.

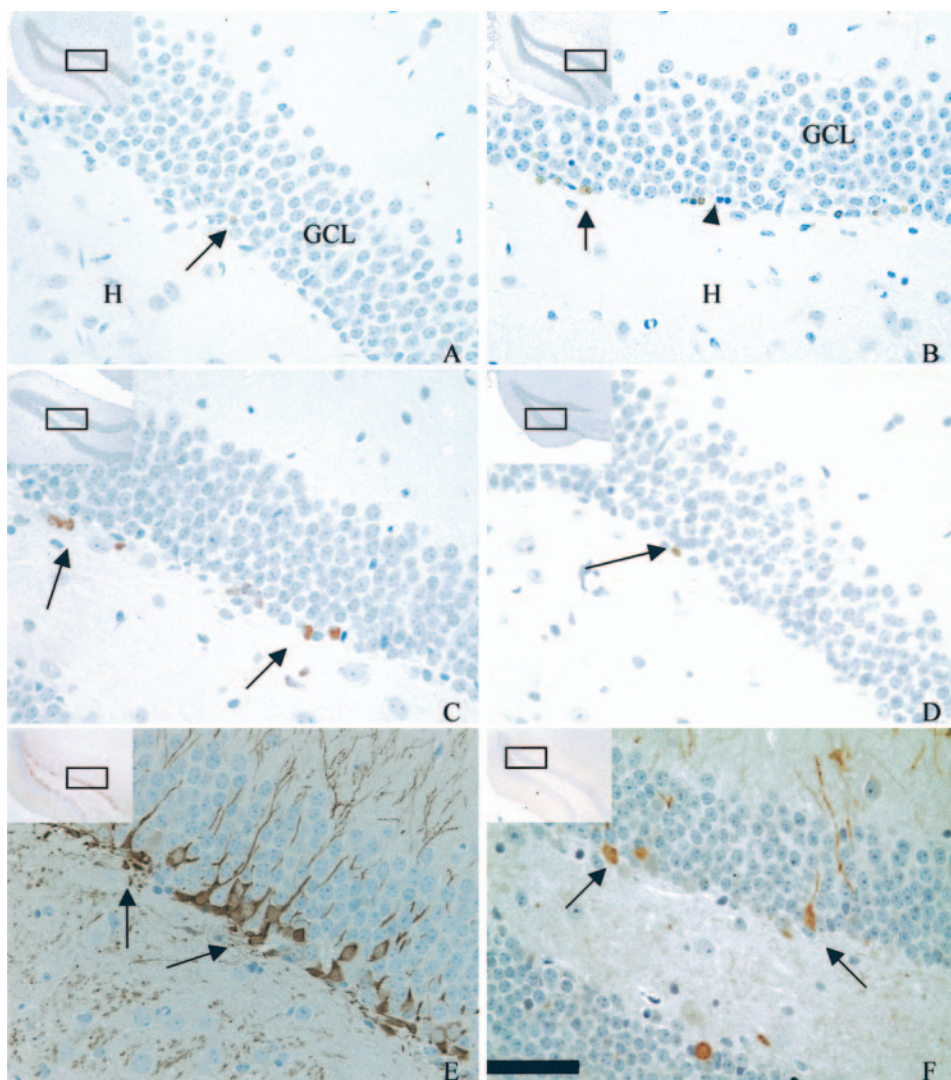
In the SGZ, apoptosis is characterized by cells showing morphological changes and/or TUNEL staining (28); only rarely does a given cell show both characteristics. Therefore, to get an estimate of the total number of apoptotic cells at a given time, both criteria were used in the present study. TUNEL-positive cells appeared as highly stained brown nuclei against the hematoxylin counterstain (Fig. 1, A and B). For the TUNEL procedure, all reagents were part of a kit (Apotag; Serological Corp., Norcross, GA), and the procedures were carried out as described previously (28, 34). Morphological changes included fragmentation, or the compaction of chromatin into two or more dense, lobulated masses, and pyknosis, which was characterized by small, round, darkly staining nuclei. To minimize the impact of including any normal cell profiles (*i.e.*, glia) in our counts of apoptosis, if any cytoplasm was

observed in conjunction with a small, dense nucleus, that cell was not considered as apoptotic.

To determine radiation-induced changes in the cellular composition of the SGZ, proliferating cells were labeled with an antibody against Ki-67, a nuclear antigen that is expressed during all stages of the cell cycle except G_0 (35, 36). Immature neurons were detected using an antibody against Dcx, the predicted gene product of the *XLIS* gene (37) that is associated with neuronal or neuroblast migration (37–39). For all immunostaining, binding of biotinylated secondary antibodies was detected using an ABC system (Vector, Burlingame, CA). To quench endogenous peroxidase activity, deparaffinized specimens were soaked for 30 min in 0.3% H_2O_2 (Sigma, St. Louis, MO) in 70% ethanol. After the primary and secondary antibodies were applied, the specimens were incubated with the ABC reagent for 30 min and developed with 0.025% DAB (Sigma) dissolved in double distilled water containing 0.005% H_2O_2 . Sections were then counterstained with Gill's hematoxylin, dehydrated, and mounted.

Ki67. After deparaffinization and quenching of endogenous peroxidase, tissue sections were soaked in 10 mM sodium citrate buffer (pH 6.0) and boiled for 10 min using a microwave oven. Sections were left in the citrate buffer for another 20 min, washed in PBS, and then incubated with 2% normal rabbit serum for 30 min. Sections were incubated overnight at 4°C with primary antibody (DakoCytomation, Carpinteria, CA) diluted 1:100 in PBS with 2% normal rabbit serum. After washing, sections were incubated for 30 min at room temperature with biotin-conjugated rabbit anti-rat IgG (Vector) diluted 1:200 in PBS with 2% normal rabbit serum. Finally, the specimens were incubated with ABC reagent, developed with DAB, and counterstained.

Fig. 1. Photomicrographs depicting specific cellular responses in mouse dentate gyrus before irradiation (A, C, and E) and either 12 h (B) or 48 h after 10 Gy (D and F). Panels include apoptosis (A and B), proliferating cells (Ki-67, C, and D), and immature neurons (Dcx, E, and F). The SGZ is a narrow band of cells between the hilus (H) and GCL. Apoptotic nuclei are characterized by TUNEL labeling (arrows in A and B) or dense chromatin/nuclear fragmentation (arrowhead, B). Although an occasional apoptotic nucleus was seen in tissues from unirradiated mice (A), a significant increase in apoptosis was seen in the SGZ 12 h after irradiation (B). Proliferating Ki-67-positive cells (arrows, C) are spread out within the SGZ in tissues from unirradiated animals; only an occasional Ki-67-positive cell was found after 10 Gy (D). Dcx-positive cells are highly concentrated in the SGZ and lower regions of the GCL of unirradiated mice (arrows, E). After 10 Gy, there are substantially fewer Dcx-positive cells (F). All micrographs are $\times 40$; the scale bar in F represents 50 μ m. The inset in each panel is a low power image ($\times 10$) of the dentate gyrus; black boxes, the areas photographed at $\times 40$.



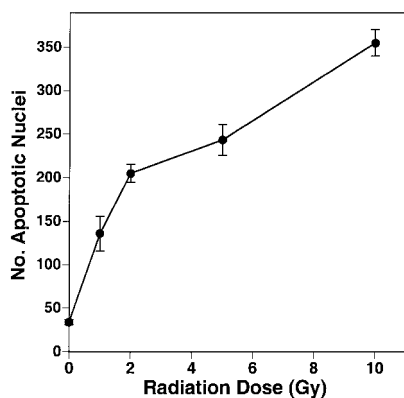


Fig. 2. Cells in the dentate SGZ undergo dose-dependent apoptosis after low to moderate doses of X-rays. Apoptosis was quantified based on TUNEL labeling and morphological changes in irradiated cells. The steepest part of the response was dominated by loss of actively proliferating cells, whereas the shallower slope, >2 Gy, largely represented the response of immature neurons. Each datum point represents a mean of 4–7 mice; error bars, SE.

Dcx. After deparaffinization and quenching, sections were microwave treated in citrate buffer as described above. After washing with PBS and blocking for 30 min using 5% normal horse serum, sections were incubated overnight at 4°C with primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in PBS with 5% normal horse serum. Sections were washed and incubated for 60 min at room temperature in biotinylated anti-goat IgG (Vector) diluted 1:500 in 5% normal horse serum. Sections were finally incubated with ABC reagent, developed with DAB, and counterstained.

The number of cells showing specific characteristics of apoptosis, along with the numbers of proliferating cells and immature neurons, were scored blind using a histomorphometric approach (27, 28, 34). A standardized counting area was used that involved 6- μ m-thick coronal sections from three different brain levels representing the rostral/mid hippocampus (27, 28). The brain levels were \sim 50 μ m apart, and the most rostral brain level corresponded to a point \sim 2.5 mm behind the bregma. For each mouse, three nonoverlapping sections were analyzed, one each from the three regions of the hippocampus. Quantification was made of all positively labeled cells within the SGZ of the suprapyramidal and infrapyramidal blades of the dentate gyrus. The total number of positively labeled cells was determined by summing the values from both hemispheres in all three tissue sections.

Neurogenesis. To determine the effects of irradiation on the production of new cells in the SGZ (*i.e.*, neurogenesis), groups of mice were given whole brain doses of 0, 2, 5, or 10 Gy and allowed to recover from anesthesia. Four weeks after irradiation, each mouse received a single i.p. injection (50 mg/kg) of BrdUrd (Sigma) daily for 7 days. Three weeks after the last BrdUrd injection, mice were anesthetized and perfused with cold saline followed by cold 4% paraformaldehyde made up the day of perfusion. The brain was removed and postfixed in paraformaldehyde overnight and then equilibrated in phosphate-buffered 30% sucrose. Free floating 50- μ m-thick sections were cut on a freezing microtome and stored in cryoprotectant. Sections were immunostained as described (29, 40) using the following primary antibodies and working concentrations: (a) rat anti-BrdUrd (1:10; Oxford Biotechnology, Kidlington, Oxford, United Kingdom); (b) mouse anti-NeuN (1:200; Chemicon, Temecula, CA); (c) rabbit anti-NG2 (1:200; Chemicon); (d) guinea pig anti-GFAP (1:800; Advanced Immunochemical, Inc., Long Beach, CA); and (e) rat anti-CD68 (FA11; 1:20; Serotec, Inc., Raleigh, NC).

Confocal microscopy was performed using a Zeiss 510 confocal microscope (Thornwood, NY), using techniques described previously (29, 40). The primary confocal end point was the proportion of BrdUrd-positive cells that coexpressed each lineage-specific phenotype. Cell counts were limited to the dentate GCL and a 50- μ m border along the hilar margin that included the SGZ. When possible, \sim 100 BrdUrd-positive cells were scored for each marker per animal. Each cell was manually examined in its full “z” dimension, and only those cells for which the BrdUrd-positive nucleus was unambiguously associated with the lineage-specific marker were scored as positive.

Statistics. For immunohistochemical end points, values for all animals in a given treatment group were averaged, and SE were calculated. A two-sided

Jonckhere-Terpstra test was used to determine whether cellular changes in radiation response were monotonic, *i.e.*, either increasing or decreasing with increasing treatment dose.

RESULTS

The SGZ is an area of active cell proliferation in young adult mice, and proliferating Ki-67-positive cells occur along both blades of the dentate gyrus (Fig. 1C). Proliferating cells were observed occasionally in the hilus, but no Ki-67-positive cells were observed in the dentate GCL. In our standardized counting area, the number of Ki-67-positive cells averaged 137.2 ± 7 ($n = 7$) in sham-irradiated mice. Immature neurons (Dcx positive) were observed in large numbers in the SGZ (Fig. 1E), averaging 480.3 ± 19.9 ($n = 4$) in sham-irradiated animals. Dcx-positive cells were also observed in the GCL, averaging 135.8 ± 9.6 ($n = 4$) in the first 25 μ m from the SGZ, 27.3 ± 3.1 in the next 25 μ m, and 2 ± 0.8 in the rest of the GCL.

In sham-irradiated mice from our dose response study, the total number of apoptotic nuclei in our standardized counting area averaged 33.8 ± 2.9 ($n = 5$); apoptotic nuclei occurred in both blades of the dentate gyrus and usually appeared alone. Apoptotic nuclei were observed in the GCL of sham-irradiated mice rarely, and only an occasional apoptotic body was observed in the hilus. After irradiation, apoptotic nuclei occurred singly or in small groups and were detected in the SGZ of both blades of the dentate gyrus (Fig. 1B). Apoptotic nuclei were seen in the GCL and hilus after irradiation but at much lower levels than in the SGZ. On the basis of morphological identification of the microvasculature, there were few apoptotic endothelial cells seen after irradiation.

The time course for SGZ apoptosis was determined to select a time for tissue collection in our dose response study. Six h after irradiation, the number of apoptotic nuclei was not substantially different from that seen in sham-irradiated mice; apoptosis peaked 12 h after irradiation and then decreased to near control levels by 48 h (data not shown). The dose response curve for SGZ apoptosis was then determined at the time of peak apoptosis, 12 h after irradiation. There was a significant increase in apoptosis with radiation dose ($P < 0.001$), and the dose response curve had two components: (a) a steep portion from 0 to 2 Gy; and (b) a shallower slope after higher doses (Fig. 2). Relative to sham-irradiated controls, the numbers of proliferating cells observed at 12 h were reduced (Fig. 1D) by 75% after 1 Gy and \sim 90% after doses of 2–10 Gy. At that time, the numbers of immature neurons in the SGZ were reduced (Fig. 1F) by 36, 51, 56, and 67% after 1, 2, 5, and 10 Gy, respectively.

To estimate the full acute impact of irradiation on the SGZ, we

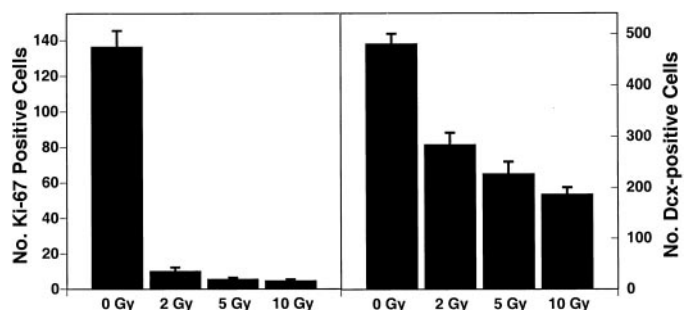


Fig. 3. Numbers of proliferating cells (left panel) and immature neurons (right panel) in the dentate SGZ are significantly decreased 48 h after irradiation. Antibodies against Ki-67 and Dcx were used to detect proliferating cells and immature neurons, respectively. All doses substantially reduced the numbers of proliferating cells, and the dose response from 2 to 10 Gy was significant ($P < 0.05$). Immature neurons were also reduced in a dose-dependent fashion ($P < 0.001$). Each bar represents an average of 4 animals; error bars, SE.

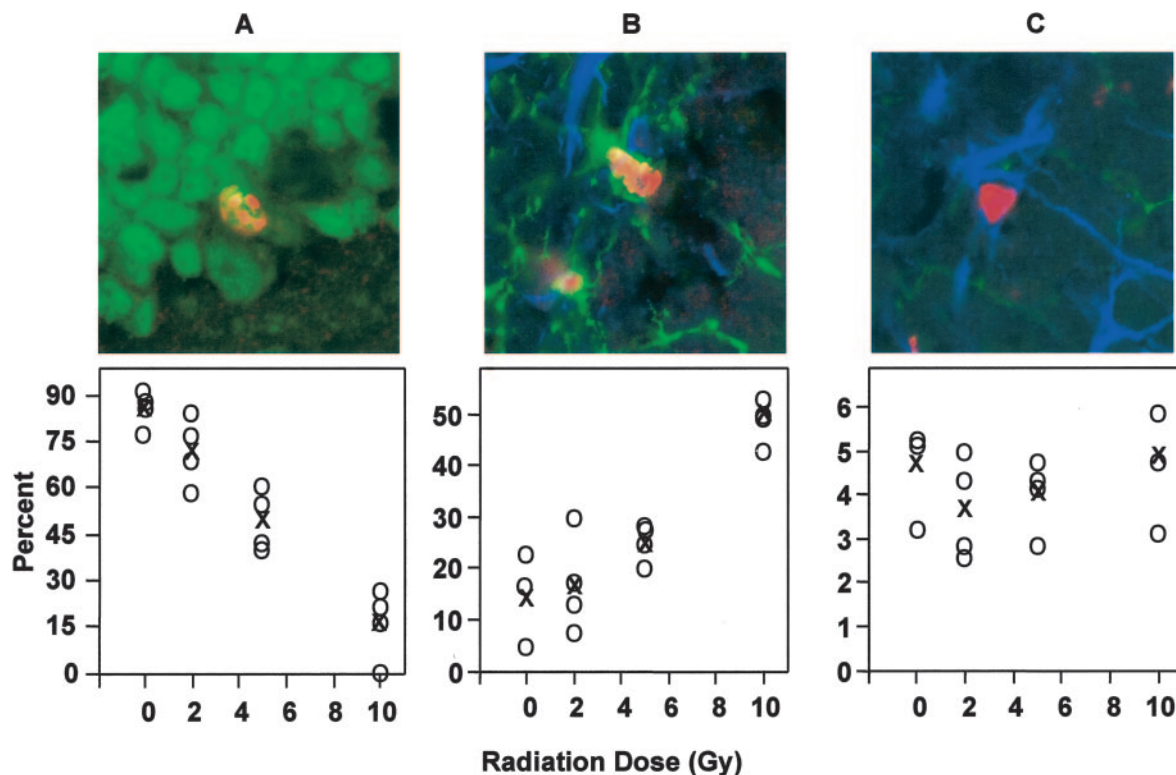


Fig. 4. Two months after irradiation, cell fate in the dentate gyrus is altered by low to moderate doses of X-rays. Confocal images (top) were used to quantify the percentage of BrdUrd-positive cells that coexpressed mature cell markers. Proliferating cells were labeled with BrdUrd (red/orange stain in confocal images), and 3 weeks later, the relative proportion of cells adopting a recognized cell fate was determined as a function of radiation dose (bottom). Neurons (green cells in A, top), oligodendrocytes (green cells in B, top), and astrocytes (blue cells in C, top) were labeled with antibodies against NeuN, NG2, and GFAP, respectively. Each of the confocal images shows a double-labeled cell. The production of new neurons (A, bottom) was reduced in a dose-dependent fashion ($P < 0.001$), whereas there was no apparent change in the production of GFAP with dose (C, bottom). In contrast, the percentage of BrdUrd-positive cells adopting an oligodendrocyte fate (B, bottom) appeared to increase, particularly after 10 Gy. In the graphs, each circle represents the value from an individual animal; each X represents the mean value for a given dose group.

quantified the numbers of proliferating cells and immature neurons remaining after apoptosis was complete. Forty-eight h after exposure, there was a substantial reduction in the number of proliferating cells (Fig. 3); the dose response in Ki-67 labeling from 2 to 10 Gy was significant ($P < 0.05$). Dcx-positive cells were reduced by all doses, and the dose response relationship was highly significant ($P < 0.001$; Fig. 3). Because immature neurons generally move into the GCL as they differentiate, we quantified the numbers of Dcx-positive cells in the GCL to determine whether the sensitivity of immature neurons changed as they moved away from the SGZ. In the SGZ, the percentage decrease in cell number relative to controls was 41, 53, and 61% in the SGZ after 2, 5, and 10 Gy. The numbers of Dcx-positive cells in the GCL were decreased ~ 19.8 , 26, and 52.7% after 2, 5, and 10 Gy, respectively.

To determine the fate of new cells produced by surviving precursor cells, we gave multiple injections of BrdUrd and 3 weeks later used cell-specific antibodies to assess the phenotype of BrdUrd-positive cells. Overall, BrdUrd labeling was reduced by all of the doses used here, with 2 and 5 Gy reducing the number of BrdUrd-positive cells by 40–50% and 10 Gy by $\sim 75\%$. In sham-irradiated controls, $85.3 \pm 5.1\%$ ($n = 4$) of BrdUrd-positive cells coexpressed the neuronal marker NeuN. After irradiation, there was a significant dose-dependent decrease in the percentage of BrdUrd-positive cells coexpressing NeuN ($P < 0.001$; Fig. 4A), and after the highest dose used here (10 Gy), the fraction of double-labeled cells was $\sim 19\%$ of control.

In contrast to our results for new neuron production, there was no apparent effect of irradiation on the percentage of BrdUrd-labeled cells that coexpressed GFAP (Fig. 4C). With respect to newly pro-

duced immature oligodendrocytes, there appeared to be a significant ($P < 0.001$) dose-related increase in the percentage of BrdUrd-labeled cells colabeling with NG2; however, that increase was dominated by the response seen after 10 Gy (Fig. 4B). Subsequent studies by us indicated that after 10 Gy, many BrdUrd-NG2 double-labeled cells also colabeled with the monocyte marker CD11B and represent infiltrating peripheral monocytes.⁴

On the basis of our earlier rat study showing that decreased neurogenesis after irradiation was attributable in part to an altered microenvironment (29), we were interested in determining if the dose-related changes we observed in neurogenesis were accompanied by changes in the local inflammatory response. Forty-eight h after 10 Gy, there were no activated microglia detected in or around the SGZ (data not shown). However, there was a significant dose-related increase in the number of activated microglia ($P < 0.001$) observed 2 months after irradiation (Fig. 5).

DISCUSSION

The main findings of the present study are: (a) cells of the dentate SGZ are extremely sensitive to X-rays; (b) hippocampal neurogenesis is altered by irradiation with the production of new neurons decreasing as a function of radiation dose; (c) a dose-dependent inflammatory reaction occurs in conjunction with altered neurogenesis; and (d) acute dose-related changes in SGZ precursor cells qualitatively cor-

⁴ M. L. Monje and T. D. Palmer, unpublished observations.

⁵ J. Raber, R. Rola, A. LeFevour, D. R. Morhardt, J. Curley, S. Mizumatsu, and J. R. Fike. Radiation-induced cognitive impairments are associated with changes in hippocampal neurogenesis, submitted for publication.

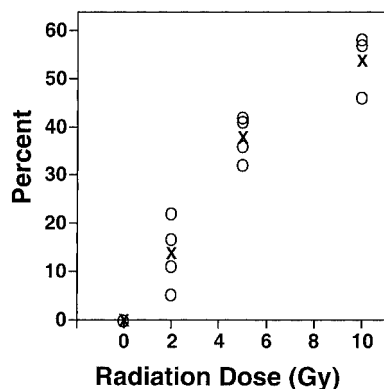


Fig. 5. Two months after irradiation, there is a substantial inflammatory response in the dentate SGZ. Proliferating cells were first labeled with BrdUrd, and 3 weeks later, the relative proportion of cells (*Percent*) adopting a microglial phenotype was determined as a function of radiation dose. Activated microglia were detected using an antibody against CD68. There were no activated microglia detected in unirradiated controls (*D*), but there was a significant ($P < 0.001$) dose-related increase in activated microglia after irradiation. Each *circle* represents the value from an individual animal; each *X* represents the mean value for a given dose group.

relate with later decreases in new neuron production. Given the potential of ionizing irradiation to induce significant cognitive effects in adults and children undergoing radiotherapy, and the role of the hippocampus in specific cognitive functions, our findings support the idea that changes in SGZ neurogenesis may play an important role in radiation-induced cognitive impairment.

The effects of ionizing irradiation on hippocampal structure and function have been addressed primarily in prenatal or neonatal animals (41–46) but also in adults (47–49). Although some investigators have proposed a link between radiation-induced hippocampal damage and cognitive deficits, it was not until recently that it was suggested that changes in the neural precursor population in the hippocampus might be involved (28, 29). Studies in rats (28, 31) indicated that proliferating SGZ cells were particularly sensitive to irradiation, a finding confirmed here in mice at both 12 and 48 h (Fig. 3) after irradiation. Our results, showing that ~90% of Ki-67-positive cells were already gone at the time of peak apoptosis, independent of dose > 1 Gy, suggested that the steep portion of the apoptosis dose response curve (Fig. 2) primarily reflected the response of actively proliferating cells. Given that the number of apoptotic nuclei measured here represented only a snapshot in time, the numbers of dying cells seen at 12 h far surpassed the number of proliferating cells in our standardized counting area. This difference was largely accounted for by the significant reduction in the number of immature neurons. The dose response relationship for Dcx-positive cells seen both 12 and 48 h (Fig. 3) after irradiation indicated that the death of immature neurons likely dominated the apoptosis dose response curve from 2 to 10 Gy. Thus, radiation not only affected the input of new cells in the SGZ (*i.e.*, proliferating Ki-67-positive cells) but also early differentiating neurons, and those effects were dose dependent. Our finding that irradiation affected Dcx-positive cells in the SGZ zone to a greater extent than those that had migrated into the GCL suggested that as cells migrated further away from the SGZ, they became less sensitive to irradiation. Whether this response represented different environmental factors or simply the fact that the cells were becoming more differentiated is not yet clear.

The process of neurogenesis consists of distinct developmental processes, including proliferation, survival, and differentiation (50). Many investigators use only proliferation as an indicator of neurogenesis, and immunostaining for BrdUrd is a well-established technique to detect cells in the S phase of the cell cycle. However, to measure migration and differentiation, post-BrdUrd survival time has

to be long enough to allow newly born cells to move away and begin to express mature cell markers. Our BrdUrd-labeling paradigm in conjunction with immunocytochemistry and confocal microscopy facilitated our assessment of all three criteria of neurogenesis. In our study, there was a clear dose response with respect to the percentage of BrdUrd-labeled cells that coexpressed neuron-specific NeuN; after 10 Gy, the reduction was 81% relative to sham-irradiated controls. In rats, a similar dose almost completely ablated neuronal production (29). Although this difference is probably species dependent, in both cases, there was a clear impact of X-rays on the production of new neurons, and in the present study, the impact was dose dependent and occurred after clinically relevant doses, *i.e.*, 2–5 Gy. Although the production of new neurons was substantially reduced relative to control, glial cell fate was unchanged by irradiation (astrocytes) or appeared to increase (immature oligodendrocytes). The relative increase in the percentage of cells adopting an oligodendrocyte phenotype was high relative to control but only after 10 Gy (Fig. 4). However, recent studies in rats show that after 10 Gy, about half of the cells double labeled with BrdUrd and NG2 are infiltrating monocytes.⁴ This agrees with recently published work that showed that after central nervous system injury, infiltrating monocytes were immunoreactive for the anti-NG2 antibody used here (51, 52). Thus, it is likely that the increase in immature oligodendrocytes seen here (Fig. 4B) did not really reflect the production of new oligodendrocytes but rather was a manifestation of a postirradiation inflammatory response. This is consistent with our previous work in rats (29) that showed that after irradiation, gliogenesis was relatively preserved compared with the production of new neurons. Whether this represents a relative resistance of glial progenitor cells, an aberrant regulation of differentiation, or alterations in the microenvironment that could adversely affect fate decisions is not known. Whatever the mechanism(s) involved, our data clearly showed that the production of new neurons was more sensitive to low doses of irradiation than the production of glia.

Neurogenesis depends on a complex microenvironment that involves signaling between multiple cell types, and irradiation could affect any or all of these cells or interactions (29). Although the precise nature of such effects has not yet been clarified, chronic inflammatory changes and disturbance of the normal association between precursor cells and the microvasculature have been suggested (29). We did not specifically address the microvasculature in this study, but we did see significant differences between irradiated animals and controls in the numbers of activated microglia (Fig. 5). Although our data indicated that activated microglia did not seem to be associated with the acute losses of proliferating cells and immature neurons, they did appear to be temporally related to changes in neurogenesis. Given the potential role of proinflammatory cytokines in radiation brain injury (53–55), and the impact of specific cytokines on neurogenesis (56), it is possible that the activation of microglia may constitute a critical factor in the radiation-induced depression of neuron production.

Low to moderate single doses of X-rays clearly induced early dose-related changes in the mouse SGZ, and cell proliferation was still reduced months after irradiation. Furthermore, although some proliferation still took place after irradiation, there were significant reductions in the production of new neurons. Although acute and later-developing changes in the SGZ were both dose dependent, there are some uncertainties about the relationship between these effects. Our earlier study in rats suggested that acute radiation toxicity in the SGZ primarily affected rapidly expanding yet committed precursor cell populations (29), and the present studies support that idea. However, later effects may involve another population: relatively quiescent stem/precursor cells that appear to be responsible for repopulating a

damaged SGZ (57). A loss of such a population could result in changes that are slower in evolving and that are dose dependent; our neurogenesis data constitute such a finding. Clearly, the relationships between acute and later-developing effects are complex, and the mechanism(s) linking cell loss, reduced neurogenesis, and other factors, such as inflammation, need to be determined.

Given the apparent relationship between hippocampal neurogenesis and associated memory formation (19), the responses seen here may have an important impact in our understanding of radiation-induced cognitive impairment. Although the present data do not directly link altered neurogenesis with cognitive function, in a separate study, we have been able to show that 3 months after a single dose of 10 Gy, there is a persistent and significant decrease in the numbers of proliferating cells and immature neurons in the SGZ and a concomitant impairment of hippocampal-dependent cognitive function.⁵ Although a cause-and-effect relationship has yet to be shown, the present data along with our unpublished cognitive studies are highly suggestive that there is a link between radiation-induced depression of neuron production and subsequent functional impairment. If true, and given the various factors that influence neurogenesis (24, 58–60), it may be possible to ameliorate or rescue individuals at risk for cognitive dysfunction after therapeutic irradiation involving the brain.

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