LONG-LASTING CHANGES IN THE ANATOMY OF THE OLFACTORY BULB AFTER IONIZING IRRADIATION AND BONE MARROW TRANSPLANTATION

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Abstract—The adult brain is considered to be a radioresistant organ since it is mainly composed of non-dividing cells. However, in adult animals there are a few neurogenic brain areas that are affected by ionizing radiation whose plasticity and capacity for recovery are still unclear. Here, mice were irradiated with a minimal lethal dose of radiation in order to determine its effects on the subventricular zone (SVZ), the rostral migratory stream (RMS), and the olfactory bulb (OB). These regions underwent a dramatic reduction in cell proliferation and ensuing morphological alterations, accompanied by a patent reactive gliosis. Bone marrow stem cell (BMSC) transplants were also performed after the radiation treatment to allow the mouse survival with a view to analyzing long-term effects. Normal proliferation rates were not recovered over time and although bone marrow-derived cells reached the brain, they were not incorporated into the SVZ-RMS-OB pathway in an attempt to rescue the damaged regions. Since neurogenesis produces new interneurones in the OB, thus feeding cell turnover, the volume and lamination of the OB were analyzed. The volume of the OB proved to be dramatically reduced at postnatal day 300 (P300), and this shrinkage affected the peripherypendal white matter, the granule cell layer, the external plexiform layer, and the glomerular layer. These results should be taken into account in cell therapies employing BMSC, since such cells reach the encephalon, although they cannot restore the damage produced in neurogenic areas. This study thus provides new insight into the long-term effects of ionizing radiation, widely employed in animal experimentation and even in clinical therapies for human beings. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cell transplantation, irradiation, olfactory bulb, rostral migratory stream, stem cells, subventricular zone.

The olfactory bulb (OB) is a laminated structure with a well-organized distribution of known cell types and synaptic circuits. This structure is the first synaptic relay for the processing of olfactory signals (Petreanu and Álvarez-Buylla, 2002; Carleton et al., 2003; Baltanás et al., 2007). Olfactory information is refined and modulated by interneurons; mainly periglomerular cells and granule cells (Petreanu and Álvarez-Buylla, 2002; Carleton et al., 2003; Baltanás et al., 2007). New neuroblasts reach the OB to replace the interneurons that die along the lifespan of mammals (Carleton et al., 2003; Imayoshi et al., 2008) and this continuous cell turnover is necessary for the maintenance of cellular organization and the function of the OB (Imayoshi et al., 2008). Thus, this structure is one of the two main targets of adult neurogenesis in the mammalian brain (Altman, 1969; Carleton et al., 2003; González-Granero et al., 2006; Lois and Álvarez-Buylla, 1993), together with the dentate gyrus (DG) of the hippocampal formation (Altman and Das, 1965; Eriksson et al., 1998; van Praag et al., 2002).

The neurogenic process starts in the subventricular zone (SVZ) (Altman, 1969; Lois and Álvarez-Buylla, 1993; Carleton et al., 2003; González-Granero et al., 2006). Lining the lateral ventricles, the SVZ is a tissue layer formed by several types of cells; some of them are mitotically active throughout the lifespan of rodents, acting as stem cells (Lois and Álvarez-Buylla, 1993; Doetsch et al., 1997). The new neuroblasts generated in the SVZ migrate towards the OB through the pathway called the “rostral migratory stream” (RMS; Lois and Álvarez-Buylla, 1994; Lois et al., 1996; Carleton et al., 2003). This tangential migration is carried out through hollow structures named “glial tubes,” formed by astrocytes (Doetsch et al., 1997; Lois et al., 1996; Peretto et al., 1997). Along this corridor, migrating cells divide again (Lois and Álvarez-Buylla, 1994; Lois et al., 1996; Carleton et al., 2003). Once neuroblasts have reached their destination, they migrate radially from the extension of the RMS in the OB (RMS-OB) to outer layers. Finally, neuroblasts differentiate, mainly into either new granule cells or periglomerular cells, in a process of continuous turnover (Imayoshi et al., 2008; Mouret et al., 2009).

Previous experiments have demonstrated that brain injury, usually characterized by reactive gliosis (Garden and Moller, 2006; Milligan and Watkins, 2009), can modify the cell proliferation and migration patterns of neurogenic encephalic sites in an attempt to repair the damage suffered (Gould and Tanapat, 1997; Takagi et al., 1999; Jin et al., 2001; Goings et al., 2004; Sundholm-Peters et al., 2005). Under pathological conditions, new neurogenesis has also been detected in brain regions other than the SVZ.
and DG, although such findings are controversial (Zhao et al., 2003; Frielingsdorf et al., 2004). Moreover, it has been demonstrated that bone marrow stem cells (BMSC) can be harboured by different organs, including skeletal muscle (Bittner et al., 1999; Gussoni et al., 1999), cardiac muscle (Orlic et al., 2001), the liver (Petersen et al., 1999; Theise et al., 2000a,b), vascular endothelium (Jackson et al., 2001), and the central nervous system (Brazelton et al., 2000; Mezey et al., 2000; Mezey, 2005). BMSC can reach the brain and fuse with pre-established neurons or else transdifferentiate into new nerve cells (Brazelton et al., 2000; Mezey et al., 2000; Blau et al., 2001; Álvarez-Dolado et al., 2003; Álvarez-Dolado, 2007). It has also been demonstrated that brain damage increases the number of BMSC reaching this organ (Bae et al., 2005; Johansson et al., 2008), especially when accompanied by inflammation (Johansson et al., 2008), in close relationship with glial processes (Garden and Moller, 2006; Milligan and Watkins, 2009). This suggests the use of BMSC as a possible therapeutic strategy.

Proliferating cells are highly susceptible to ionizing radiation, in contrast to mature, non-dividing neurons (Tada et al., 1999; Monje et al., 2002; Mizumatsu et al., 2003; Balentova et al., 2006; McGinn et al., 2008). Indeed, this is the basis of radiation therapy, widely used to treat several kinds of brain tumours (Strother et al., 2002; Hellström et al., 2009). Different studies have attempted to determine the true effects of ionizing radiation on proliferating areas of the encephalon. Most of them have focused on the damage undergone by the DG of the hippocampal formation, characterized by an impairment of hippocampal neurogenesis and cognitive deficits (Monje et al., 2002; Mizumatsu et al., 2003; Monje and Palmer, 2003; Rola et al., 2004; Manda et al., 2009). By contrast, fewer studies have analyzed the effect of ionizing radiation in the SVZ, and even fewer have studied the RMS or the OB (Hellström et al., 2009; Lazarini et al., 2009) with low (sublethal) doses of radiation (Balentova et al., 2006, 2007). In addition, those studies were performed with different doses of radiation (some of them very high), focused exclusively on the heads of the animals, and addressed a broad range of maximum survival times (from 30 to 180 days). The results of such studies regarding cell proliferation vary, reporting a lack of recovery, complete restoration, and even an increase in neurogenesis (Shinohara et al., 1997; Tada et al., 1999; Balentova et al., 2006; Hellström et al., 2009; Lazarini et al., 2009; Manda et al., 2009).

The purpose of the present study was to analyze the alterations occurring in the SVZ-RMS-OB pathway after a minimal lethal irradiation. We focused on (a) the immediate effects on cell proliferation, cell death, and the ensuing glial response, and (b) on the long-term effects of such radiation, determining whether that neurogenic pathway can be restored after the injury over time. Regarding the latter, BMSC transplants were performed to allow the survival of mice injured. Secondly, the incorporation of bone marrow-derived cells into this neurogenic region was also addressed to determine whether these cells reaching the brain might have an effect on the regions affected by the radiation. The results provide new insight into our understanding of the extent of the effects of ionizing radiation -widely employed in research and even in clinical therapy- and the capacity of damaged neurogenic regions to recover.

**EXPERIMENTAL PROCEDURES**

**Animals**

Mice of the C57BL/DBA strain (Mus musculus, L. 1758) were used. The animals were housed at the Animal Facilities of the University of Salamanca at constant temperature and humidity, with a 12/12 h photoperiod, and were fed ad libitum with water and special rodent chow (Rodent toxicity diet, B&K Universal G.J., S.L. Molins de Rei, Barcelona). Nine groups of animals were employed. One of them was used to determine the correct dose of radiation (see below), and the other eight (n=5 each one) were divided among irradiated or non-irradiated mice for four different survival ages: 20 days (P20), P60, P150 and P300. Only male mice were used to determine the effect of radiation on cell proliferation, since dimorphic hormonal stages can affect neurogenesis in the forebrain (Díaz et al., 2009). Mice analyzed at P20 were also employed to analyze apoptosis, cell proliferation, and gliosis. These P20 animals did not receive any transplantation since they were sacrificed 24 h after the irradiation to determine its direct effects, with no other extrinsic factor. Mice that were allowed to survive up to P60, P150 and P300 were employed to analyze the proliferation, tissue damage and possible recovery of neurogenic regions at mid- and long-term survival times.

Transgenic mice of the BALBc strain (Hadjantonakis et al., 1998) with constitutive green fluorescent protein (GFP) were also used as bone marrow donors to perform transplants into the irradiated mice. All donors were adult mice of about P60 age. These animals were also housed under the same conditions mentioned above.

All animals were housed, manipulated and sacrificed in accordance with current European (86/609/ECC and directive 2003/65/CE) and Spanish legislation (RD 1201/2005 and law 32/2007) and the experiments were approved by the Bioethical Committee of the University of Salamanca.

**Irradiation of mice**

Whole animals were irradiated with a gamma irradiation device with a 137Cs source for mice, model GammaCell 1000 Elite (MDS Nordion, Ottawa, Canada). This device provides a radiation rate of 243 cGy/min, with energy of 0.662 MeV.

The most suitable dose of irradiation (minimal lethal irradiation) was established by irradiating six subgroups of P19 mice (four animals each) in the 6.5 and 10 Gy range. These mice did not receive any transplant and were kept in standard, ventilated individual cages to determine the direct effect of the irradiation on their survival.

Once the minimal lethal level of irradiation had been established, four groups of P19 mice (five animals each) were irradiated with 7.5 Gy (see “Results”) and received a bone marrow transplant from a GFP animal donor 24 h later (Fig. 1A). After the irradiation, the mice were housed separately in an insulated rack specific for immunodepressed animals at the Animal Facilities of the University of Salamanca for 2 weeks and thereafter under standard conditions.

**Bone marrow transplantation**

Three GFP transgenic BALBc mice were used as donors; one for each transplanted group. In these animals, GFP is expressed constitutively and the hence incorporation of the transplant could be determined using flow cytometry analysis of peripheral blood (see below) and the fate of its cells could be followed because of their distinctive green fluorescent labeling.
Donors were sacrificed by cervical dislocation. Then, their hind paws were removed and the femurs and tibias dissected. Bone marrow extraction was performed by injection of IMDM medium (Iscove’s Modified Dulbecco’s Medium; Invitrogen; Carlsbad, CA, USA) with an insulin syringe at both ends of the bone. The bone marrow wash was filtered through a 70-μm pore size filter (BD Falcon; Bedford, MA, USA) and centrifuged at 1500 rpm for 5 min. The supernatant was removed and the pellet was re-suspended in lysis buffer (140 mM NH₄Cl, 17 mM Tris-base, pH 7.4) for 5 min to break up erythrocytes. Following this, the reaction was stopped by adding 45 ml of 0.1 M phosphate-buffered saline, pH 7.4 (PBS), to each collection tube, and an aliquot was collected to estimate the number of BMSC extracted using a Thoma chamber. The cell suspension was centrifuged again at 1500 rpm for 5 min and the pellet was resuspended in PBS. 7.5 × 10⁶ cells were transplanted in each animal in a maximum volume of 150 μl. Thus, the volume of cell suspension injected into each mouse depended on the concentration of cells. The injection of BMSC was performed at P20 in the tail vein. The irradiated mice sacrificed at ages of P60, P150 and P300 received this transplant (Fig. 1A).

Graft monitoring

The percentage of GFP-positive cells in peripheral blood was measured to check the correct assimilation of the transplant.

Blood was extracted by means of a small prick in the supraorbital plexus 2 weeks after transplantation, once the period of isolation of the immunodepressed animals in the rack had ended. Blood samples (approximately 50 μl) were collected in 100 μl of heparinized saline (1000 U/ml in 0.9% p/v NaCl). Erythrocytes were destroyed with 6 ml of a specific lysis buffer (0.83% w/v NH₄Cl, 0.1% w/v KHCO₃, 0.372% w/v EDTA, pH 7.3) for 20 min Then, the cell suspension was centrifuged at 1500 rpm for 5 min and the supernatant was removed. The pellet was resuspended in PBS and centrifuged again at 1500 rpm for 5 min, and the resulting pellet was again resuspended in 1 ml of PBS. The percentage of GFP-positive cells was determined by flow cytometry (FACSCalibur, Becton Dickinson; NJ, USA). These procedures were repeated four and six weeks after transplantation and at the time of sacrifice.

Tissue preparation

Animals were deeply anaesthetized with 1 μl/g b.w. of a mixture containing ketamine hydrochloride (Ketolar; Parke-Davis, Barcelona, Spain) and xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany) at a proportion of 3:4. Then, the animals were perfused intracardially with PBS, followed by 5 ml/g b.w. of Zamboni’s fixative (4% w/v of depolymerized paraformaldehyde and 15% v/v saturated picric acid in PBS). After perfusion, the brains were dissected and rinsed for 2 h with PBS.

Tissue blocks were cryoprotected by immersing them in 30% (w/v) sucrose in PBS. When the blocks had sunk, they were frozen with liquid nitrogen and stored at −80 °C until sectioning. Forty-μm thick slices were obtained using a freezing-sliding microtome (Jung SM 2000, Leica Instruments, Nussloch, Germany) attached
to a freezing unit (Frigomobil, Leica Instruments) and rinsed in PBS (3×10 min).

**Immunofluorescence**

A double immunofluorescence technique was carried out in order to study gliosis in proliferative regions after the irradiation. Tissue slices were washed in PBS and incubated overnight at room temperature under continuous rotary shaking in a medium containing 0.2% (w/v) Triton X-100, 5% (v/v) normal goat serum, and the primary antibodies in PBS. An anti-glial fibrillary acidic protein (GFAP) polyclonal mouse IgG (1:1,000; Sigma-Aldrich, St Louis, MO, USA) was employed to label astrocytes, and an anti-Iba1 polyclonal rabbit IgG (1:1,000; Wako, Osaka, Japan) was used to label microglia. Then, the sections were washed in PBS (3×10 min) and incubated in a second medium for 2 h at room temperature under continuous rotary shaking. This second medium contained 0.2% (w/v) Triton X-100, secondary Cy2-conjugated goat anti-mouse fluorescent antibody (1:500; Jackson, West Grove, PA, USA), and Cy3-conjugated goat anti-rabbit fluorescent antibody (1:500; Jackson) in PBS. Thirty min before the end of the incubation DAPI, 1:2,000 v/v, was added to the medium to counterstain the cell nuclei.

To determine the localization of bone marrow-derived cells in the encephalon, an immunofluorescence technique was developed. In this case, the first incubation medium contained 0.2% (w/v) Triton X-100, 5% (v/v) normal donkey serum, and the primary anti-GFP polyclonal goat antibody (1:2,000; Abcam, Cambridge, UK) in PBS. The second medium contained 0.2% (w/v) Triton X-100 and the secondary Cy2-conjugated donkey anti-goat fluorescent antibody, (1:500; Jackson) in PBS. Thirty min before the end of the incubation DAPI, 1:2,000 v/v, was added to the medium to counterstain the cell nuclei.

Finally, and in both techniques, sections were rinsed in the darkness in PBS and mounted with coverslips using a freshly prepared anti-fade medium made of 0.42% (w/v) glycine, 0.021% (w/v) sodium hydroxide, 0.51% (w/v) sodium chloride, 5% (w/v) N-propyl-gallate, 70% (v/v) glycerol, and 0.002% (w/v) thymersol in distilled water. Specifically controls were carried out in parallel with the immunofluorescence technique, using the same procedures without the first or the second antibodies. No labeling was detected in these controls.

**PCNA immunofluorescence**

The immunolabeling technique against proliferating cell nuclear antigen (PCNA) was employed to detect dividing cells. The tissues required a special treatment to obtain an adequate PCNA staining (Valero et al., 2005). Tissue slices were incubated in Bouin-4% fixative (4% w/v of depolymerized paraffomaldehyde, 75% v/v saturated picric acid and 5% v/v acetic acid in distilled water) for 1 h and then washed thoroughly in PBS (Valero et al., 2005). Following this, the sections were subjected to an antigen recovery procedure, as previously described (Shi et al., 1991; Dover and Patel, 1994). First, they were boiled twice in citrate buffer (0.1 M citric acid and 0.1 M sodium citrate in distilled water) for 4 min. Then, the tissue slices were rinsed in PBS (1×10 min) and incubated for 24 h at room temperature under continuous shaking in a medium containing 0.2% (w/v) Triton X-100, 5% (v/v) normal goat serum, and the primary anti-PCNA antibody (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS. Following this, the sections were washed in PBS (3×10 min) to remove unbound antibody and were then incubated in a second medium for 2 h at room temperature under continuous rotary shaking. This second medium contained the secondary Cy2-conjugated goat anti-mouse fluorescent antibody, 1:500 (Jackson) and 0.2% (w/v) Triton X-100 in PBS. Thirty min before the end of the incubation, propidium iodide (PI; Sigma) at 1:2,000 (v/v) was added to the medium in order to provide a nuclear counterstain. Finally, sections were rinsed in the darkness in PBS and coverslipped, applying anti-fade medium. The specificity controls were the same as above, with identical results.

**TUNEL technique**

The TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin Nick End Labeling) technique was employed to detect apoptotic cells the sections obtained from P20 mice. Tissue slices were washed with PBS (3×10 min) and incubated for 20 min in 0.5% sodium borohydride solution (w/v) in PBS to prevent the fluorescent staining background (Wuruga-Prieto et al., 1996). They were then washed with PBS (3×10 min) and treated for 15 min with 0.2% (v/v) Triton X-100 and 0.1% (v/v) sodium citrate in distilled water. Following this, they were washed again with PBS (2×10 min) and incubated with TUNEL buffer for 30 min. This buffer comprised 30 mM Tris–HCl, 140 mM sodium cacodylate, 1 mM CoCl2 and 0.3% Triton X-100 (v/v). Then, the tissue slices were incubated in a medium with terminal transferase (800 U/ml; Roche Diagnostics, Mannheim, Germany) and biotinylated dUTP (1 μM; Roche Diagnostics) in TUNEL buffer for 2 h. The reaction was terminated by the addition of saline sodium citrate buffer (0.15 M sodium chloride and 0.015 M sodium citrate). Finally, the slices were washed again with PBS (3×10 min) and developed with a medium containing Cy2-conjugated streptavidin (1:200) in PBS. Both PI staining and slide mounting were performed similarly to the procedures used in the PCNA immunofluorescence technique.

In some sections, the TUNEL technique was developed with immunoperoxidase. Once the reaction of the terminal transferase had ended and the sections had been washed, they were incubated with the avidin-biotin-peroxidase complex (1:200 v/v) in PBS for 2 h. The sections were then rinsed in PBS (3×10 min), and the immunoreaction was revealed with 0.65% 3,3′-diaminobenzidine (DAB) and 0.03% (v/v) H2O2 in PBS. The reaction was controlled under the microscope and stopped with cold PBS.

Sections were dehydrated in increasing ethanol series and xylene, after which they were mounted with Entellan (Merck, Darmstadt, Germany) and coverslipped. Specificity controls for the TUNEL technique were performed without the addition of terminal transferase, dUTP, streptavidin or the avidin-biotin-peroxidase complex. No labeling was detected in these control tests.

**Analyses**

Three large proliferating regions were analyzed: the SVZ, the RMS and the RMS-OB. For a better comparison of the experimental groups, five equidistant and comparative rostro-caudal levels were selected, as previously described (Wuruga et al., 1999; Murias et al., 2007; Díaz et al., 2009). These were designated SVZ I (bregma: 0.5 mm, according to the atlas of Hof et al., 2000), SVZ II (bregma: 1.3 mm), RMS I (bregma: 2.1 mm), RMS II (bregma: 2.9 mm) and RMS-OB (bregma: 3.7 mm). The regions of interest in each level were those showing proliferation: the SVZ with its dorsal horn, the RMS itself, and the rostralmost extension of the RMS in the OB. To determine neural proliferation, the PCNA-positive cells in the areas of interest of each level were counted unilaterally on focal planes obtained with a confocal microscope (Leica TCS SP2, Leica Instruments). As expected, no differences were observed between either brain hemispheres. Accordingly, the side of each section was chosen at random. The percentages of PCNA-positive cells in each area were calculated and referred to the total number of cells of these areas (labeled with PI). Animals with ages of P20, P60 and P150 were analyzed for proliferation studies.

P20-irradiated mice were used to determine the extent of cell death caused directly by radiation at the time of transplant in comparison with P20 control animals. Apoptotic cells were scarce in the non-irradiated animals. Thus, all TUNEL-stained cells were...
counted not only at the five representative levels, but also throughout the three regions analyzed, as previously described (Díaz et al., 2009). Each brain was sectioned in six consecutive series and one of them was used to perform this quantitative analysis. Thus, a one-in-six series was studied in each animal. Photographs of the areas of interest in each slice were taken with an Olympus DP70 digital camera (Olympus Optical, Tokyo, Japan) attached to an Olympus Provis AX70 photomicroscope (Olympus Optical) and measured using the Image J program (Wayne Rasband, National Institutes of Health, USA). Estimation of the cell death rate was referred to as the number of apoptotic cells per μm² of the area analyzed. Thus, the density of apoptotic cells in the three regions of the proliferation pathway -SVZ, RMS and RMS-OB- was obtained as a whole. All counts were performed by the same person (D.D.), following the same criteria and in a double blind study (F.C.B. and J. S. R.).

Once homoscedasticity (quality of samples with normal distribution and equal variances) had been checked with the Kolmogorov–Smirnov test, Student’s t-test was employed to analyze possible differences between the irradiated and non-irradiated animals in each level at the different survival ages (SPSS 17.0 for Windows, SPSS, Chicago, IL, USA).

Nissl staining with thionine was performed in P300 OB sections to measure the areas of the OB layers. As mentioned above, a one-in-six series was studied in each animal. Photographs of complete OB sections were taken with an Olympus DP70 digital camera (Olympus Optical, Tokyo, Japan) attached to an Olympus Provis AX70 photomicroscope (Olympus Optical). The areas of the different OB layers and the total area of each section were measured using the ImageJ program (Wayne Rasband, National Institutes of Health, USA). With knowledge of the number of sections of the series employed and the areas of each section, estimation of the volume of these OB and their layers was performed using the TableCurve 2D program, version 5.01 (SYSTAT Software, Chicago, IL, USA).

**RESULTS**

**Determination of minimum lethal dose of radiation**

All animals treated with radiation higher than 6.5 Gy died at different time-points, their survival time being inversely correlated with the dose used (Fig. 1B). Regarding the group irradiated with 6.5 Gy, only one mouse died 10 days after the irradiation. Four weeks later, the remaining animals of the same group were sacrificed and their bone marrow extracted (as with donor mice; see Experimental procedures), obtaining a similar number of cells as in the non-irradiated mice. Thus, a 6.5 Gy dose was not considered sufficient to ablate the bone marrow in these mice. In a control experiment, mice irradiated with 7.5 and 8.5 Gy were transplanted with healthy bone marrow from a donor. In both groups, all animals survived for a long time (more than 5 months). Therefore, the death of the mice irradiated with 7.5 and 8.5 Gy was mainly due to ablation of the bone marrow. In light of the foregoing, a dose of 7.5 Gy was chosen as the minimal lethal dose of radiation.

**Graft monitoring**

The analysis of peripheral blood performed with flow cytometry revealed an increase in the percentage of GFP-positive blood cells in all transplanted animals from 2 weeks post-transplantation to 6 weeks (Fig. 1C). These results point to a progressive replacement of the original blood cells by those derived from the new GFP bone marrow. In addition, the percentages of GFP-positive blood cells in the transplanted animals at the time of sacrifice (P150) were not statistically different from the percentages of GFP-positive blood cells of the donors (donors: 77.25±1.74% SEM; transplanted: 74.51±1.69% SEM; P>0.05). Thus, the replacement of host bone marrow by grafted BMSC and restoration of the hematopoietic system were complete, allowing the survival of the transplanted animals.

**Increase in cell death after irradiation**

TUNEL-labeled apoptotic cells were observed in the three regions (SVZ, RMS and RMS-OB) of the P20 animals studied. This technique counterstained with PI allowed us to visualize the typical chromatin condensation, as previously described (Gavrieli et al., 1992; Charriaut-Marlangue et al., 1996; Sairanen et al., 2006). The number of apoptotic cells in the control mice was very scarce in all regions studied. Indeed, analysis with Student’s t-test revealed significant global differences between the irradiated and control animals (Fig. 2A; P<0.05), with a higher density of apoptotic cells in the former, as expected. These differences were also found separately in all three zones of study: the SVZ (Fig. 2B; P<0.01), RMS (Fig. 2B and Fig. 3; P<0.01) and OB (Fig. 2B; P<0.01). In addition, the density of apoptotic cells increased towards the rostralmost regions (Fig. 2B). No differences in cell death were observed in adjacent regions between the irradiated animals and the control mice.

In light of these results, it may be concluded that irradiation increases apoptotic cell death in the neurogenic sites analyzed. The direction of neuroblast migration and the higher density of apoptotic cells in the rostralmost region point to a displacement of these apoptotic cells towards the OB.

**Reactive gliosis in the proliferating regions**

Microglial cells were detected throughout the brain, with no special distribution in the control mice (Fig. 4A, E, I). By contrast, an extremely dense staining for microglia appeared in the RMS and RMS-OB levels of the irradiated mice (Fig. 4B, F, J). Astrocytes were also detected in different regions of the brain, with a clear concentration in the proliferating zones in both control and irradiated mice (Fig. 4C, D, G, H, K, L), since astrocytes carry out specific support functions for the proliferation and migration of neuroblasts in neurogenic brain sites (Lois and Alvarez-Buylla, 1993; Lois et al., 1996; Doetsch et al., 1997; Peretto et al., 1997, 1999; Doetsch, 2003). Moreover, the staining for astrocytes in the RMS and the RMS-OB was much more widespread and denser in the irradiated than in the control mice (Fig. 4G, H, K, L). However, only a very slight increase in these glial markers was observed in the SVZ levels of the irradiated mice in comparison with the controls (Fig. 4A–D).

In sum, an evident reactive gliosis appeared in the irradiated mice at P20 in the rostral regions of the SVZ-RMS-OB pathway, where the density of apoptotic cells...
was higher. Thus, dying cells and activated glial cells had concordant distributions. No differences in the distribution of glial cells were observed at P60, P150 or P300 (data not shown), suggesting that the reactive gliosis had appeared soon after irradiation, when apoptotic events were taking place.

Decreases in cell proliferation

Most of the PCNA-positive cells were restricted to the areas of proliferation and migration (SVZ, RMS, RMS-OB; Fig. 5). In P20 animals, some PCNA-positive nuclei were also detected in neighboring areas of the SVZ and RMS, as previously described (Martoncikova et al., 2006). This presence of dividing cells outside the proliferating regions could be due to the migration of neuroblasts towards the accessory olfactory bulb (Bonfanti et al., 1997), and/or because forebrain development in these young mice had not yet been completed (He and Crews, 2007) and because their proliferation rate is higher than in older animals (Tropepe et al., 1997). In P60 and P150 animals, some of these “uncommon” PCNA-positive nuclei were also detected, but only near the RMS, because of neuroblast migration to the accessory olfactory bulb. In agreement with previous findings, the percentage of proliferating cells along the RMS was not uniform, and it tended to be higher towards the caudal-most part (Martoncikova et al., 2006).

On P20, that is 24 h after irradiation, Student’s t-test revealed significant differences between the irradiated and control mice as regards the global percentage of proliferating cells (control: 27.45±3.801% SEM; irradiated: 5.81±0.83% SEM; P<0.01; Fig. 6A), with fewer proliferating cells in the irradiated animals. Sorted rostrocaudally, significant differences persisted in the five levels: SVZ I (Figs. 5A, B and 6B; P<0.01), SVZ II (Fig. 6B; P<0.05), RMS I (Figs. 5C, D and 6B; P<0.01), RMS II (Fig. 6B; P<0.05) and RMS-OB (Fig. 6B; P<0.05). In addition, morphological alterations were observed in the SVZ region (Fig. 5B) and in the RMS (Fig. 5D), especially in the size and cell density of these regions. Thus, an irradiation with 7.5 Gy dramatically decreases cell proliferation in the SVZ-RMS-OB pathway, also affecting the structure of rostral proliferating regions.

A similar study was performed at P60 and P150 in order to determine both the mid- and long-term effects of ionizing irradiation and the possible recovery of cell proliferation. An initial qualitative analysis revealed that the
morphological alterations of the regions studied not only persisted, but were increased at the P60 and P150 survival ages (Fig. 5). The density of cells in the RMS was especially affected, with very few surviving cells (Fig. 5H, L). In fact, the identification of the RMS I regions was not easy owing to the scarce cell population in comparison with the normal, easily distinguishable morphology of this structure. The quantitative study revealed differences in global proliferation at both survival ages: the irradiated animals had a lower percentage of proliferating cells than the control groups (P60 control: 18.21 ± 2.39% SEM; P60 irradiated: 5.37 ± 0.94% SEM; P150 control: 14.17 ± 1.94% SEM; P150 irradiated: 4.55 ± 0.92% SEM; P<0.01; Fig. 6C, E). However, the differences in each separate level of study did not always match the global result. At P60, significant differences were observed in the SVZ I (Figs. 5E, F and 6D; P<0.01), SVZ II (Fig. 6D; P<0.01), RMS I (Figs. 5G, H and 6D; P<0.01), and RMS-OB (Fig. 6D; P<0.01) levels, but not at the RMS II level (Fig. 6D; P>0.05), between the irradiated and control animals. At P150 the differences appeared at the SVZ I (Figs. 5I, J and 6F; P<0.05), SVZ II (Fig. 6F; P<0.01), RMS I (Figs. 5K, L and 6F; P<0.01) and RMS II (Fig. 6F; P<0.01) levels, but not in the RMS-OB (Fig. 6F; P>0.05). These differences can be explained in terms of the decrease in proliferation with age (Troppe et al., 1997) or the morphological changes in these regions due to irradiation (see Discussion). Regarding the irradiated mice, no statistically significant differences were detected in the global percentages of proliferating cells of the P20, P60 and P150 animals (P>0.05). Therefore, no recovery in cell proliferation occurred along these survival times.

In conclusion, a dose of 7.5 Gy (minimal lethal dose) significantly diminishes the proliferation rate in the forebrain and alters the morphology of the neurogenic areas of the olfactory system 24 h after exposure. Our results demonstrate that the normal morphology and proliferation rate are not recovered by 40 and 130 days after transplantation (P60 and P150, respectively), either by endogenous repopulation or by cells derived from a bone marrow transplant.

**Long-term survival studies**

Mice allowed to survive up to P300 were used to determine the effects of cell radiation over a very long-term survival period. An initial macroscopic study revealed a dramatic decrease in OB size in the irradiated mice (Fig. 4).
7A–C). The brains of these mice were sectioned and Nissl staining with thionine was performed to compare them with the brains of control animals of the same age. The morphology of the SVZ was still altered, with the observation of a smaller size and a lower cell density than the control animals, as at P20, P60 and P150. The RMS was almost completely absent, whereas in the age-matched control animals it was always readily distinguishable (Fig. 8A, B, arrows). The total volume of the OB was reduced in the irradiated animals (Fig. 7D; reduction to 66.14%; $P<0.01$). The volume of the different layers of the olfactory bulb was also altered, with a decrease in the thickness of the granule cell layer (GCL; Figs. 7D and 8C–F, arrows), the external plexiform layer (EPL; Figs. 7D and 8C–F, arrowheads) and, to a lesser extent, the glomerular layer (GL; Fig. 7D). By contrast, the volume of the periependymal white matter (PWM) did not change (Fig. 8C–F), probably due to the reduction in GCL cell numbers (see Discussion).

The above results demonstrated that the structural and functional damage to the SVZ-RMS-OB pathway still persists at P300, including severe alterations in the structure of the OB.

Arrival of GFP cells in the brain

To determine whether bone marrow-derived cells had arrived at the encephalon, the cerebella of transplanted mice were also examined to look for fusion events between such cells and Purkinje cells (Álvarez-Dolado et al., 2003; Weimann et al., 2003; Johansson et al., 2008; Recio et al., in press). Scarce GFP-positive Purkinje cells were observed in the cerebella of the P150 animals (Fig. 9A) but not in the P60 mice, indicating the slowness of the fusion process. All of them had two nuclei, suggesting fusion phenomena. Bone marrow-derived cells also reached other brain regions of the transplanted mice. The most common cell type was microglial, characterized by a double labeling for Iba1 and GFP (Fig. 9B, C). Both activated and latent microglial cells were observed. These cells were identified throughout the brains of all the host animals, mainly located at the periphery of the OB or in the cortex, in close contact with the meninges. In the SVZ-RMS-OB pathway, some small, round GFP-positive and Iba1-negative cells were detected (Fig. 9B–E). They were also identified in other brain locations, with no special distribution (Fig. 9B, C).
and they did not exhibit bipolar processes, characteristic of migrating neuroblasts (Nam et al., 2007). In addition, no differences in the number of these GFP-positive cells were observed between the P60 and P150 transplanted mice.

According to these observations, bone marrow-derived cells reached the brain but they did not integrate in neurogenic regions.

**DISCUSSION**

In light of our results, it may be concluded that ionizing radiation can severely affect well-defined brain structures such as the OB by means of an impairment of its cell turnover mechanism. This turnover of interneurones was disrupted, and hence these cells died without being replaced. The loss of neural elements generated a decrease in the volume of the OB and alterations in its layering. A minimum lethal irradiation was strong enough to induce a dramatic increase in cell death—mainly of proliferating cells—accompanied by a reactive gliosis in all the proliferating regions of the rostral neurogenic pathway. The subsequent marked decrease in cell proliferation was not recovered over time either by endogenous cell proliferation or by repopulation derived from the grafted BMSC.

**Irradiation with 7.5 Gy is the minimal lethal irradiation for mice**

Several studies have demonstrated a dose-dependent effect of the damage due to ionizing radiation in the
brain (Shinohara et al., 1997; Tada et al., 1999; Mizumatsu et al., 2003; Rola et al., 2004; McGinn et al., 2008). However, in those works very broad radiation ranges were studied: 2–10 Gy (Rola et al., 2004), 5–15 Gy (McGinn et al., 2008), 2–15 Gy (Tada et al., 1999) or even 3–30 Gy (Shinohara et al., 1997) and, in addition, no specific criteria were described to justify the choice of those radiation ranges. In the present work a range of radiation between 6.5 and 10 Gy was tested, with values lying within those previously described in cell transplant studies (Stewart and Przyborski, 2002; Álvarez-Dolado et al., 2003; Weimann et al., 2003; Massengale et al., 2005; Moore et al., 2005; Magrassi et al., 2007), to find the minimal lethal dose of radiation that would allow BMSC transplantation. Doses lower than 6.5 were considered too weak to ablate the bone marrow and injure the SVZ-RMS-OB pathway, even causing increases in cell proliferation (Tada et al., 1999; Balentova et al., 2006, 2007). Our results demonstrated that a dose of 6.5 Gy was insufficient to ablate the bone marrow of the host animals, allowing their survival and the recovery of a normal number of BMSC. All the animals irradiated with doses between 7.5 and 10 Gy died if they were not transplanted with new BMSC. Thus, the dose of 7.5 Gy was chosen as the minimal lethal dose.

The transplants guaranteed the survival of irradiated mice, with a progressive enrichment of peripheral blood in GFP-positive cells, signaling complete substitution of the host hematopoietic system by a new GFP-positive one, due to the “homing effect” (Whetton and Graham, 1999; Fuchs et al., 2004).

Apoptotic cell death is increased by irradiation and is displaced towards the OB

The damage produced by radiation in proliferating cells is dependent on their proliferation rate (Romanko et al., 2004a,b; Hellström et al., 2009), cells that divide faster being more susceptible. Therefore, the transit-amplifying neurogenic precursors (type C cells) would be those most severely affected by radiation since their proliferation rate is the highest, followed by neuroblasts (type A cells) and the stem cells of the SVZ (type B2 cells; Doetsch et al., 1997, 2002; García-Verdugo et al., 1998; Hellström et al., 2009). Therefore, the highest apoptotic cell density should be found in the SVZ, diminishing towards the OB. However, the results obtained with the TUNEL technique revealed that irradiation caused apoptotic cell death in the regions of study, with a progressive increase in the number of apoptotic cells from the SVZ to the OB. Our experiments also revealed a patent reactive gliosis throughout the RMS and RMS-OB regions, whereas it was almost absent in the SVZ. Thus, this gliosis appeared displaced to the rostralmost regions, as did the apoptotic events. There is a close relationship between apoptosis and glial activation since damaged neuronal cells may induce a rapid reactive gliosis (Garden and Moller, 2006; Milligan and Watkins, 2009). Following irradiation, differences in apoptosis can be detected 3 h after exposure and these differences reach a maximum at 6 h (Shinohara et al., 1997). 24 hours after irradiation, the rate of apoptosis decreases but is still detectable (Shinohara et al., 1997). By contrast, the response of glial cells to neural injury is
extremely fast (Garden and Moller, 2006; Milligan and Watkins, 2009), indirectly indicating where neural cells have died recently. Accordingly, a substantial fraction of the apoptotic cells detected could correspond to cells that died not at the moment of irradiation but later. Neuroblasts are migrating cells that continue to divide in their trajectory towards the OB (Lois and Álvarez-Buylla, 1994; Lois et al., 1996; Carleton et al., 2003), and therefore they may be affected by radiation, although not as severely as type C cells (Romanko et al., 2004a,b; Hellström et al., 2009). Thus, these injured cells could continue their migration along the RMS, but in a subsequent phase in which they stop to divide (Lois and Álvarez-Buylla, 1994; Peretto et al., 1999) they die as a consequence of the damage they have undergone owing to the radiation treatment, since radiation can induce not only acute but also delayed cell toxicity and death (Haydont et al., 2007). Therefore, an accumulation of apoptotic cells in the rostral-most regions would exist, coinciding with the distribution of the reactive glia.

**Irradiation with 7.5 Gy affects the morphology and proliferation of neurogenic regions, with no recovery over time**

Regarding the direct effects of radiation, our studies demonstrate the existence of morphological changes in the regions analyzed. The changes observed in the SVZ are in agreement with previous data (McGinn et al., 2008), but we also detected damage throughout the RMS at 24 h after irradiation, and especially at longer survival times. This region, characterized by densely packed cells (Lois and Álvarez-Buylla, 1994; Lois et al., 1996; Doetsch et al., 1997; Peretto et al., 1997; Carleton et al., 2003), is readily
distinguishable in tissue sections. However, in the irradiated mice it was difficult to identify the boundaries of the RMS, especially at the ages of P150 and P300, owing to the reduction in neuroblast density.

Our results reveal a marked decrease in cell proliferation 24 h after exposure to radiation throughout the complete SVZ-RMS-OB pathway. They also demonstrated that the reduction in the percentage of proliferating cells observed at P20 was still present in the animals sacrificed at P60 and P150, with no statistical differences among the three ages; therefore, the SVZ-RMS-OB pathway remains injured and no recovery occurs over time. Previous studies have reported an initial decrease in cell proliferation in the SVZ after irradiation followed by an increase, suggesting

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Fig. 9. Microphotographs of bone marrow-derived GFP cells in the brain of transplanted mice (green). Purkinje cells in the cerebellum derived from the BMSC transplant were detected as a control of the engraftment efficacy (A). GFP-positive microglial cells, also positive for Iba1 immunostaining (red), were the predominant cell type (B, C arrows). Some GFP-positive but Iba1-negative cells (arrowheads) were detected in different regions, including the RMS (D, E). Cell nuclei are counterstained with DAPI (blue). LV, lateral ventricle. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
an attempt to restore the proliferating regions (Shinohara et al., 1997; Tada et al., 1999). Nevertheless, this increase was delayed (depending on the dose applied) and temporary, the proliferation rate thereafter falling again (Tada et al., 1999). In the same study, no variations in the morphology of the RMS or the OB were described either. However, that work only addressed the study of the SVZ, and the RMS pathway was not analyzed in detail. In fact, the authors did not specify the doses chosen in the RMS analysis and hence, their results would match the use of a low dose. Hellström et al. (2009) reported a partial recovery of cell proliferation in the SVZ but not in the DG of the hippocampal formation. The authors explained this difference in terms of the cellular composition of both structures: the SVZ has more “true” stem cells than the DG, and since stem cells are less vulnerable than neural progenitor cells to different injuries, including radiation (Romanko et al., 2004a,b), the SVZ would have a better ability to recover after a moderate degree of injury. The animals of that study were irradiated with a dose of 6 Gy (Hellström et al., 2009). Our results show that a dose of 6.5 Gy is not enough to ablate the bone marrow and then destroy the BMSC and possibly other stem cells such as neural cells. A dose of 7.5 Gy would damage the “true” stem cell population of the SVZ severely and would be strong enough to prevent the recovery of the SVZ-RMS-OB pathway. Other studies focused on the RMS have reported an increase in cell proliferation in this region after irradiation (Balentova et al., 2006, 2007). Nevertheless, this difference could be explained in terms of the low doses of radiation employed in those studies (3 Gy), which was insufficient to induce permanent damage.

Finally, only in two of the five rostrocaudal levels were no differences detected between the experimental groups (RMS II at P60 and the RMS-OB at P150; Fig. 6D, F). The changes in morphology and cell density observed in the regions of the irradiated mice analyzed could explain this particular lack of differences. The proliferation rate was calculated by referring the number of proliferating cells to the total number of cells. Their irradiation decreased both the number of proliferating cells and the total number of cells in the regions of study and, therefore, the ratio of proliferating cells in fewer total cells could afford similar percentage values to those found for the control animals. In addition, previous studies have demonstrated a progressive age-related physiological decrease in cell proliferation (Tropepe et al., 1997). Thus, both phenomena could blur the differences in proliferation between the irradiated and control animals and, in some regions where the variations between the experimental groups were not so dramatic, the general differences were not apparent.

**Bone marrow-derived cells reach the brain but have no effects on the damaged regions**

GFP-positive cells (hence derived from the new bone marrow) reached the encephalon of the transplanted mice, in agreement with previous results (Priller et al., 2001; Corti et al., 2002; Álvarez-Dolado et al., 2003; Weimann et al., 2003; Johansson et al., 2008; Recio et al., in press). However, no GFP-positive cells with the features of astrocytes or neuroblasts were found in the damaged neurogenic regions, microglia and small, round GFP-positive and Iba-1-negative cells being the only cell types detected there. The latter were possibly non-neuronal hematopoietic-like cells (Massengale et al., 2005; Moore et al., 2005). In addition, no clear differences in the number of these cells were found in the neurogenic regions along the different survival times, and neither did the damaged proliferating regions recover. Taking these data into account, bone marrow-derived cells did not help towards a recovery of the neurogenic regions analyzed.

**Irradiation with 7.5 Gy severely reduces the OB volume**

The dramatic shrinkage of the OB and the morphological changes in its lamination observed at P300 demonstrated the final consequence of the damage produced by irradiation: the pathway of proliferation and migration remained injured and hence too few neuroblasts were able to reach the different layers of the OB to replace the interneurones, with the subsequent decrease in the volume of this structure. A recent study has demonstrated that a radiation dose of 15 Gy significantly reduces the rostral neurogenesis affecting OB layers (Lazarini et al., 2009). In agreement with our results, no recovery in neurogenesis over time was detected in that study, but no variation in the volume of the OB layers was observed, probably because the maximum survival time employed (131 days) was not long enough (Lazarini et al., 2009). Our results demonstrate changes in the GCL, the EPL and the GL, the final fate of the migrating neuroblasts (Lois and Álvarez-Buylla, 1994; Petreanu and Álvarez-Buylla, 2002; Carleton et al., 2003), but not in the PWM. New neuroblasts are mainly incorporated into the GCL (Valero et al., 2007), this being the most severely affected layer of the OB. The EPL is characterized by a low cell density and a large amount of neuropil, the dendrites of the granule cells being its main component (Shepherd, 1972; Kratskin and Belluzzi, 2003). Thus, the volume reduction of the EPL could be induced not only by the decrease in cell turnover in this layer (Lazarini et al., 2009) but also by the loss of granule cells, and consequently their dendrites. The PWM is the most internal layer of the OB reached by the RMS-OB (Lois and Álvarez-Buylla, 1994; Lois et al., 1996; Carleton et al., 2003). Afferent and efferent OB fibers travel through this layer, connecting the OB with the brain (Price and Sprich, 1975; Willey et al., 1983). The cell loss and shrinkage of the GCL could expose more of these fibers, increasing the extent of the white matter. Nevertheless, the general decrease of the OB led to volume compensation in this layer, which finally appeared without alterations (Fig. 10). In addition, granule cells do not send any neurites to the PWM, since they have no axon (Shepherd, 1972; Kratskin and Belluzzi, 2003). Thus, the loss of these would not further reduce the volume of the PWM. Moreover, it has been demonstrated that the loss of OB neurons with dendrites and an axon—such as mitral cells—affects the more external OB layers but not the internal ones, because axon...
size is not significant in comparison with that of the soma or the dendritic tree (Valero et al., 2007). Finally, it has been described that radiation can also affect the cell replacement of the GL (Lazarini et al., 2009). Our results revealed a volume decrease in this layer, but to a lesser extent than in the GCL or the EPL. Fewer neuroblasts reach the GL than the GCL (Valero et al., 2007), and therefore the volume of this layer is less affected by the impairment of cell turnover (Lazarini et al., 2009).

CONCLUSION

Interneuron turnover in the OB is necessary for the appropriate volume and structure to be maintained. Ionizing radiation seems to affect this turnover to a considerable extent and thus has more extensive and long-lasting effects in neurogenic brain regions than previously reported. Interestingly, the minimal lethal irradiation dose of 7.5 Gy affected not only proliferating cell populations but also other established neural systems, and the radiation doses employed in different lines of investigation are usually far above that dose. Taking these data into account, in future research the dose of radiation should be chosen carefully in order to avoid unexpected injuries that could lead to artificial results. This issue is especially important when radiation is employed to perform BMSC transplants, since bone marrow-derived cells do not repair injuries in neurogenic regions. Future research will clarify whether radiation should be substituted by other ablative procedures and whether the use of adjuvant strategies might complement BMSC engraftments to develop a more functional cell therapy.

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REFERENCES


bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A 98:10344–10349.


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