Neuroscience Research 73 (2012) 263–268

Contents lists available at SciVerse ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Rapid communication

Proliferation and differentiation of neural stem cells irradiated with X-rays in logarithmic growth phase

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ARTICLE INFO

Article history:
Received 26 October 2011
Received in revised form 17 March 2012
Accepted 14 April 2012
Available online 24 April 2012

Keywords:
Neural stem cells
Embryonic stem cells
X-irradiation
Capabilities for proliferation and differentiation

ABSTRACT

Exposure of the fetal brain to ionizing radiation causes congenital brain abnormalities. Normal brain formation requires regionally and temporally appropriate proliferation and differentiation of neural stem cells (NSCs) into neurons and glia. Here, we investigated the effects of X-irradiation on proliferating homogenous NSCs prepared from mouse ES cells. Cells irradiated with X-rays at a dose of 1 Gy maintained the capabilities for proliferation and differentiation but stopped proliferation temporarily. In contrast, the cells ceased proliferation following irradiation at a dose of >5 Gy. These results suggest that irradiation of the fetal brain at relatively low doses may cause congenital brain abnormalities as with relatively high doses.

Radiation damage to the brain is a major concern in the field of radiation therapy and the assessment of radiation risk because such damage can cause cognitive dysfunction, encephalopathy, and vascular abnormalities (Crossen et al., 1994; Abayomi, 1996; Tofilon and Fike, 2000). Especially, radiation exposure during the developmental stage of the fetal human brain is known to cause severe damage, resulting in mental retardation and microcephaly (Kimler, 1998; Otake and Schull, 1998). In the adult brain, radiation is also known to cause cognitive dysfunction in radiation therapy (Welzel et al., 2008). Previous *in vivo* studies in mice and rats have demonstrated that exposure of the brain to ionizing radiation results in blocked neurogenesis, microcephaly, and induction of apoptosis in the dentate gyrus of the hippocampus (Jensh et al., 1995; Peissner et al., 1999; Inouye et al., 2000; Raber et al., 2004). For example, Rola et al. (2004) reported that radiation induced cognitive impairment due to changes in hippocampal neurogenesis in mice.

Neurogenesis occurs throughout the whole of the developing central nervous system (CNS), while it occurs only in the subventricular zone (SVZ) or the hippocampus in the adult CNS (Altman and Das, 1965). Neural stem cells (NSCs), which play an important role in organization of the brain, are responsible for neurogenesis. NSCs proliferate and increase in number, and then differentiate into neurons, astrocytes, and oligodendrocytes (Gage, 2000; Temple, 2001). Therefore, radiation-induced damage of NSCs may cause brain structural and functional abnormalities. However, the cellular mechanisms underlying these abnormalities are not yet understood. In the present study, to investigate the cellular mechanisms involved in these brain structural and functional abnormalities, we investigated the effects of irradiation on homogeneous NSCs derived from mouse embryonic stem (ES) cells by the Neural Stem Sphere (NSS) method (Nakayama et al., 2003, 2004).

NSCs were prepared from mouse ES cells (HK cell line) by the NSS method, as reported previously (Nakayama et al., 2003, 2004). The cells were cultured in proliferation medium (PM), Neurobasal™ Medium (Invitrogen, Carlsbad, CA) supplemented with 2% B-27 (Invitrogen) and 20 ng/mL FGF-2 (recombinant human basic FGF 157 aa; R&D Systems, Minneapolis, MN). The culture media were replaced every 48 h.

First, we examined the effects of X-irradiation on proliferation of NSCs. The NSCs were seeded in PM on Matrigel (BD Matrigel™ Basement Membrane Matrix Growth Factor Reduced; Invitrogen)-coated dishes at 2×10^4 cells/cm^2. The cells were irradiated at various doses with a 6 MV X-ray source (CLINAC-21EX; Varian

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http://dx.doi.org/10.1016/j.neures.2012.04.005

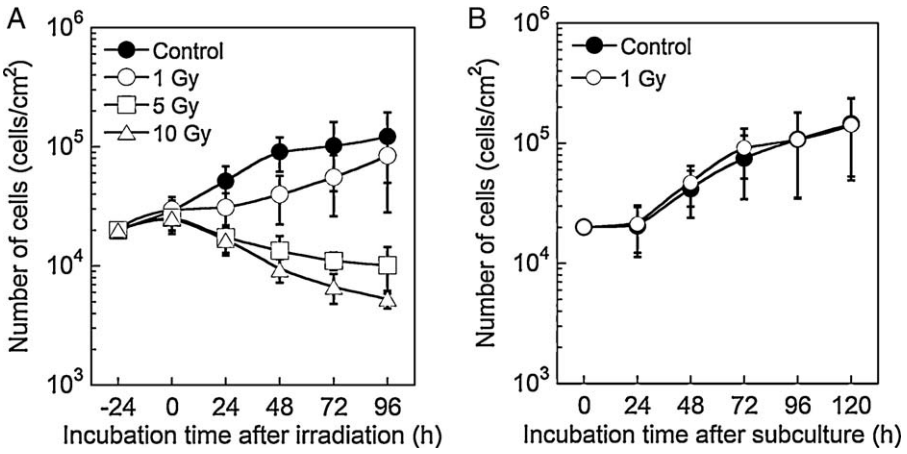

Fig. 1. Growth curves of NSCs after X-irradiation and subculture in PM. (A) NSCs were irradiated at 0 h and cultured in proliferation medium (PM) for 96 h. (B) The cells irradiated at 1 Gy and controls were collected at 96 h after irradiation. The cells were replated and cultured in PM for 120 h. Values represent the means of four experiments ± SD.

Medical systems, Palo Alto, CA) the day after seeding of the cells. Fig. 1A shows the growth curves of the NSCs up to 96 h after X-irradiation at 1, 5, and 10 Gy. The curves were obtained from the phase-contrast images of the cells using an inverted microscope (ECLIPSE TE300; Nikon, Tokyo, Japan). Four images of 1154 μm × 769 μm per dish were obtained every 24 h, and the number of cells was counted on the images. The non-irradiated NSCs (controls) in this study showed an exponential increase up to 72 h, where the cell-doubling time was calculated to be 29.2 h, after which the cells reached confluence (Fig. 1A). The cells irradiated at 5 and 10 Gy did not show an increase in number, but a gradual decrease was observed reaching <50% of the plated number cells at 96 h after irradiation. In contrast, the cells irradiated at a dose of 1 Gy did not show a significant increase at 24 h after irradiation, although an exponential increase was observed from 24 h to 96 h after irradiation. The doubling time was calculated to be 46.6 h, which was approximately 1.6-fold longer than that of the controls.

To detect irradiation-induced cell death, cells were fixed in 4% paraformaldehyde (PFA) 24 h after irradiation and permeabilized with 0.05% Triton-X in PBS. Apoptotic cells were detected by TUNEL staining with an In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR). A small number of TUNEL$^+$ (green) nuclei were present in DAPI$^+$ (blue) nuclei of the controls (Fig. 2A and B). We observed a radiation dose-dependent decrease in the number of DAPI$^+$ nuclei (Fig. 2A, C, E and G) and a dose-dependent increase in TUNEL$^+$ apoptotic nuclei (Fig. 2B, D, F and H). Many small-sized TUNEL$^+$/DAPI$^+$ nuclei observed after irradiation with 5 and 10 Gy (Fig. 2E–H) localized with cell debris in phase-contrast images (data not shown), suggesting that they were apoptotic bodies. These results indicate that apoptosis was induced within 24 h after irradiation.

To investigate cell proliferation after irradiation, the cells were cultured with 10 μM 5-bromo-2′-deoxy-uridine (BrdU; Sigma, St. Louis, MO) in PM for 24 h after irradiation. The cells were fixed, permeabilized, and treated with 0.2 M HCl to denature DNA and with 0.1 M sodium borate to neutralize acid. The cells were immunostained with anti-BrdU primary antibody (1:200; Sigma) and Alexa Fluor® 488 rabbit anti-mouse IgG secondary antibody (1:200; Molecular Probes), and the nuclei were counterstained with DAPI. Almost all nuclei in the control cells were BrdU$^+$ (green) (Fig. 3A and B), indicating that these cells were actively proliferating, as described above. In contrast, the number of BrdU$^+$ nuclei decreased after irradiation (Fig. 3D, F and H), to a more marked extent than the decrease in DAPI$^+$ nuclei (Fig. 3I). Because BrdU can integrate into apoptotic cells (Magavi and Macklis, 2002), some of the BrdU$^+$ nuclei observed after irradiation may have originated from apoptotic cells and apoptotic bodies. These results indicated that irradiation induced cell arrest, or cytostasis, within 24 h. In addition, many irradiated cells had weakly stained nuclei (Fig. 3D, F and H), suggesting that some cells in cytostasis may have restarted proliferation. The results of our apoptosis and BrdU incorporation assays indicate that the gradual decrease in cell number after irradiation at 5 and 10 Gy (Fig. 1) was due to a loss of proliferative ability and to apoptosis, as described (Monje et al., 2002; Limoli et al., 2004; Kanzawa et al., 2006; Acharya et al., 2010). By contrast, the very slow increase in cell number after irradiation at 1 Gy for 24 h may be due to cell cycle arrest and retardation, as well as to apoptotic cell death, suggesting that 1 Gy-irradiation may not be lethal for these cells.

The irradiated cells were collected 96 h after irradiation and subcultured in PM for up to 120 h to check proliferation capability after irradiation at a dose of 1 Gy (Fig. 1B). The irradiated cells and controls proliferated exponentially with doubling times of 26.2 h and 29.9 h up to 72 h, respectively, and both reached confluence after 72 h. These results suggested that the cells irradiated at a dose of 1 Gy can repair the damage induced by irradiation and recover their growth capability, indicating that cells irradiated at 1 Gy retain the capability for proliferation. In contrast, subculture of cells irradiated with >5 Gy showed no evidence of proliferation, as well as a gradual decrease in cell number, indicating that these cells had lost their ability to proliferate (data not shown).

Next, gene expression analysis of cells irradiated at 1 Gy was performed by a quantitative real-time polymerase chain reaction (PCR) on an ABI PRISM 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (Applied Biosystems) and the primer pairs described previously (Otsu et al., 2011). Fig. 4A–D shows the relative expression levels of *Nestin*, *Musashi-1*, *MAP2*, and *GFAP*, which are representative marker genes for neural stem/precursor cells (Lendahl et al., 1990), neural precursor cells and protoplasmic astrocytes (Sakakibara and Okano, 1997), neurons (Dinsmore and Solomon, 1991), and astrocytes (Miller and Raff, 1984), respectively. These values were normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. For all four genes, the relative values of the cells irradiated at 1 Gy in PM showed no significant differences from those of the controls (Fig. 4A–D, left), and the irradiated cells were shown to express marker genes for neural stem/precursor cells.

To determine the ability of cells irradiated at 1 Gy to differentiate, these cells were cultured for an additional 120 h in PM (black