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Alterations in the rat forebrain apoptosis following exposure to ionizing radiation

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Abstract: Ionizing radiation commonly used in the radiotherapy of brain tumours can cause adverse side effects to surrounding normal brain tissue. The most significant response of adult brain to radiation damage is induction of apoptosis. The adult mammalian subventricular zone (SVZ) of the brain lateral ventricles (LV) and their subsequent lateral ventricular extension, the rostral migratory stream (RMS), is one of the few areas, which retains the ability to generate new neurons and glial cells throughout life. Taking into account the fact, that ionizing radiation is one of the strongest exogenous factors affecting cell proliferation, the aim of the present study was to investigate the occurrence of radiation-induced apoptosis in this neurogenic region. Adult male Wistar rats were investigated 1, 5 or 10 days after single whole-body gamma irradiation with the dose of 3 Gy. Apoptotic cell death was determined by in situ labelling of DNA nick ends (TUNEL) and fluorescence microscopy evaluation of TUNEL-positive cells. Considerable increase of apoptotic TUNEL-positive cells was observed 24 hrs after irradiation in caudal parts of RMS; i.e. in the vertical arm and elbow of RMS. Initial increase was followed by strong reduction of apoptosis in the RMS and by secondary over-accumulation of apoptotic cells in the animals that survived ten days after exposure. Results showed, that the proliferating population of cells, arisen in SVZ are highly sensitive to radiation-induced apoptosis. This observation should have implications for clinical radiotherapy to avoid complications in therapeutic brain irradiation.

Key words: ionizing radiation; rat brain; rostral migratory stream; apoptosis; TUNEL

Introduction

The forebrain subventricular zone (SVZ) of the brain lateral ventricles (LV) is one of the few areas which retain the proliferating activity and give rise both new neuronal and glial cells during adulthood. Progenitor cells of the SVZ migrate along the well defined pathway, called the rostral migratory stream (RMS) to the olfactory bulb (OB), which can establish some functional connections (Carleton et al. 2003; Abrous et al. 2005; Lledo et al. 2006). Under physiological conditions, this process is closely associated with simultaneous reduction in the number of stem or precursor cells via apoptosis (Brunjes & Armstrong 1996; Thomaidou et al. 1997). The effects of ionizing radiation on the rat brain have been studied primarily in prenatal and neonatal animals (Shinohara et al. 1997; Amano et al. 2002) as well as in adults (Peissner et al. 1999; Martončíková et al. 2006). Acute low dose irradiation (0.5 Gy) has effect on various metabolic pathways that do not appear to be directly involved in cell death (Silasi et al. 2004). Whole-brain irradiation of juvenile rats with the single doses of 1–3 Gy by gamma rays caused expressive reduction in the number of neural stem and progenitor cells in the SVZ and increased manifestations of apoptosis, persisted several months after initial exposure (Amano et al. 2002). There were performed many studies, which provides evidence for a radiosensitivity of proliferating cells in the adult rodent brain (Peissner et al. 1999; Mizumatsu et al. 2003; Rola et al. 2004). Early acute radiation-induced consequence of ionizing radiation represented by clonogenic cell death is not the only mode of apoptosis in the CNS after exposure. Late effects, occurring after recovery from the early syndromes, are irreversible and most devastating and thus are the most clinically important. The influence of dose, fractionation treatment, time of irradiation on late functional and histopathological changes have been derived from studies in rodents. Münter et al. (1999) demonstrated, that single high-dose of irradiation (30–50 Gy) of frontal lobe of rats results in increased vascular permeability founded up to nineteen months after exposure. In clinical radiotherapy, limited radiation tolerance of patients with intracra-

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Table 1. Total numbers of apoptotic TUNEL-positive cells in individual parts of RMS.

Group parts of RMS	C	Irr-1	Irr-5	Irr-10
Vertical arm	no cells	25.58 ± 2.11	6.17 ± 1.42	55.26 ± 6.38
Elbow	no cells	26.84 ± 3.14	14.35 ± 2.70	28.83 ± 1.78

Explanations: Distribution of apoptotic TUNEL-positive cells in individual parts of RMS (total numbers of cells × $10^3/mm^3$) of control (C) and experimental groups of rats, investigated 1, 5 or 10 days after irradiation (Irr-1, Irr-5, Irr-10, respectively). Data are presented as mean ± standard error (SEM).

nial lesions results in developing late delayed effects on surrounding brain parenchyma manifesting as cognitive impairments, which include demyelization, glial atrophy, necrosis of white matter and varying degrees of vascular changes in both white and grey matter (Wong & Van der Kogel 2004). The underlying histopathological mechanisms and their relevance to the neurocognitive dysfunction observed after irradiation are poorly understood.

In the present study, we investigated the occurrence of apoptosis in the forebrain of adult rats exposed to whole-body dose (3 Gy) of gamma rays at various intervals after post-irradiation survival.

Material and methods

Animals

The experiment was performed with 15 adult male rats of the Wistar strain (SAV Dobrá Voda) 2–3 months old at the beginning of experiment and weighing 200–260 g. Controlled group survived 1 day ($n = 2$), 5 days ($n = 2$) or 10 days ($n = 2$) after sham exposure. Moreover, experimental rats ($n = 9$) were killed 1, 5 or 10 days after exposure (three animals at each time interval). The animals were kept under standard conditions (temperature of 22–24 °C, light-controlled environment with 12/12h light/dark cycle) and provided with food and water *ad libitum*. The methods for animals use were approved by the Animal Care and Use Committee, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovakia (approval number EK 165/2005 for animal experiments).

Irradiation procedure

The animals were anaesthetized by i.p. injection of ketamine (1–2 ml/kg body weight) and a s.c. injection of xylasine (0.1–0.2 ml/kg b.w.) and were fixed in animal holder to obtain the reproducible body position. The irradiation was performed with a single whole-body dose of 3 Gy by gamma rays from ^{60}Co source (apparatus TERAGAM 02 UJP Prague) at a dose rate of 0.84 Gy min^{-1}.

In situ labelling of DNA strand breaks

After deep anaesthesia and decapitation the brains were immediately removed from the skull and immersed in the 4% formalin solution for 3 days until the paraffin embedding. Serial sagittal 10 μm thick sections were cut on microtome and after deparaffinization and pretreatment with Proteinase K, the tissue sections were processed using by In Situ Cell Death Detection Kit, POD (Roche, Germany). This assay allow to labelling DNA strand breaks by terminal deoxynukleotidyl transferase (TdT), which catalyses polymerization of fluorescein labelled nucleotides to free 3'-OH DNA ends in template-independent manner. Incorporated fluorescein isothiocyanate (FITC) was detected by anti-FITC antibody Fab fragments, conjugated with horse-radish peroxidase (POD). After substrate reaction, the slides with FITC-labelled DNA fragments (TUNEL-labelled cells) were viewed with an epifluorescence microscope Olympus BX51 (Olympus, Japan) equipped with FITC (excitation: 450–480 nm; emission: 500–515 nm). Serial digital images were captured by digital camera (DP50) connected with DP Imaging software (version 3.0) and displayed on computer. A standardized counting area was used and included 10 μm thick sagittal sections from two different forebrain areas, i.e., the vertical arm and elbow, representing the individual parts of RMS. The vertical arm of the RMS begins in anterior horn of brain lateral ventricles (LV) and curved ventrally between the corpus callosum and striatum. Then the RMS turned in a prominent angle, the elbow and continued along the ventral forebrain surface until reaching the OB. The numbers of all TUNEL labelled cells (green fluorescent nuclei) were counted only in sections with entire extent of the RMS (4–5 sections per animal). Quantitative analysis was performed using the Dissector program, version 2.0 (Tomori et al. 2001) allowing the cell counting in three-dimensional space browsing through the stack of serial optical sections. Data were analyzed using ANOVA one-way analysis followed by a Tukey–Kramer test comparison and presented as mean ± standard error (SEM). Statistical significance was set at $P \leq 0.05$.

Results

Fluorescence microscopy examination of sagittal sections through the forebrain of controlled and irradiated rats displayed different patterns of density of apoptotic cells (Table 1). Quantitative analysis in non-irradiated control animals did not show any incidence of apoptotic TUNEL-positive ($TUNEL^+$) cells within caudal parts of RMS (Figs 1A, B). After irradiation, the total numbers of apoptotic nuclei in our standardized counting area showed different time-related changes after irradiation and spatio-temporal distribution pattern in individual parts of adult rat forebrain. Considerable increase of apoptotic $TUNEL^+$ cells was observed 24 h after irradiation predominantly in caudal parts of RMS, represented by vertical arm ($25.58 \pm 2.11 \times 10^3/mm^3$; $P \leq 0.001$) and caudal border of elbow (the place, where vertical arm and elbow come together; 26.84 ± 3.14; $P \leq 0.001$) (Figs 1C, D). Enhanced apoptosis has been gradually decreased and until reaching the olfactory bulb (OB), apoptotic cells were seen only rarely. Opposite to these values, five days after irradiation there was a strong decrease in the number of $TUNEL^+$ cells (vertical arm: 6.17 ± 1.42; $P \leq 0.05$, elbow: 14.35 ± 2.70; $P \leq 0.01$) and apoptotic nuclei occurred simply or in

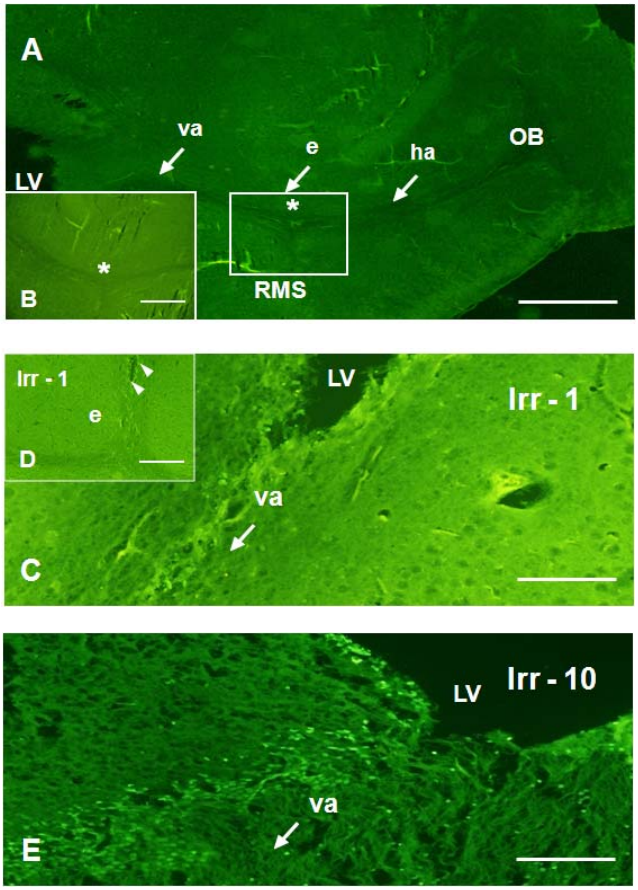

Fig. 1. Distribution of apoptotic cells in the adult rat forebrain stained by fluorescence in situ labeling of DNA strand breaks (TUNEL technique). A – photomicrographs of the sagittal sections through the forebrain of adult male control rat showing whole extent of the rostral migratory stream (RMS) and the RMS elbow (B; asterisks). Details of labeled apoptotic nuclei of TUNEL-positive cells (green fluorescence) in the RMS (C, E) vertical arm and (D) caudal border of elbow (arrowheads) of exposed rats 1 and 10 days after irradiation (Irr-1 and Irr-10, respectively) with the dose of 3 Gy of gamma rays. In course of ten days after irradiation the appearance of apoptosis increased following caudo-rostral gradient. LV – lateral ventricle; va – vertical arm; e – elbow; ha – horizontal arm of RMS; RMS – rostral migratory stream; OB – olfactory bulb. Calibration bars: A – 500 μm; B, D, E – 200 μm; C – 100 μm.

small groups. The secondary steep increase of apoptotic cells was observed ten days after exposure, most visible in the anterior horn of LV and caudal part of vertical arm (55.26 ± 6.38; $P \leq 0.001$) (Fig. 1E). Large numbers of apoptotic cells also resides the OB, the rostral termination of RMS (unpublished results).

Discussion

The presented results showed that alterations in apoptosis induced by the single dose of 3 Gy of gamma rays were determined on the basis of the density of apoptotic TUNEL$^+$ cells quantified within the vertical arm and elbow of the RMS at different time intervals after exposure. The time course changes were similar in investigated areas, indicate similar radiosensitivity in the caudal part of migratory pathway. It was characterized by steep increase of the numbers of TUNEL$^+$ cells until 24 hours after irradiation followed by transient sharp decrease until 5 days after exposure. The highest, secondary increase of apoptosis was seen in the caudal parts ten days after irradiation but apoptotic cells also occurs in rostral termination of the RMS, represented by horizontal arm (data not presented in results). This finding suggests how ionizing radiation influences the spatio-temporal distribution of cells in the rat forebrain. Our previous published results (Bálentová et al. 2006, 2007) partially confirm well-known finding, that density of cell division decreases following a caudorostral gradient, with a maximum around the LV and minimum in the OB (Carleton et al. 2003). Comparison of values in both experiments ten days after irradiation showed, that the numbers of proliferating cells surpassed the numbers of dying cells. This is consistent with findings of Uberti et al. (2001), who detected a biphasic response to irradiation in dentate gyrus in mice, characterized by an early inhibition and delayed stimulation of cell proliferation. Later effects of ionizing radiation may involve the population of quiescent stem or precursor cells that appear to be responsible for repopulating of damaged SVZ (Rola et al. 2004; Martončíková et al. 2006).

Results of recent studies in rats indicated that moderate single doses of ionizing radiation induced time-dependent changes. It has been established that after single irradiation with various radiation doses (2–10 Gy) the numbers of apoptotic cells in hippocampal dentate gyrus dramatically increased 3–6 h after exposure and in the following 6–12 h reached the maximum. However, the extent of apoptosis later decreased, remained unchanged for 1–9 months after initial exposure (Peissner et al. 1999; Tada et al. 1999; Mizumatsu et al. 2003; Raber et al. 2004; Rola et al. 2004; Fan et al. 2007). Simultaneously, the numbers of proliferating cells decreased in greater extent in the following weeks (by 60–79% after three weeks) and months after irradiation (80–90% after three months) (Peissner et al. 1999; Kee et al. 2002; Mizumatsu et al. 2003; Raber et al. 2004; Winocur et al. 2006). Long-term decrease in proliferating activity lead to reduction of neurogenesis in dentate gyrus, and may followed by decline in spatial long-term memory and learning (Madsen et al. 2003; Raber et al. 2004; Rola et al. 2004). In humans, impaired hippocampal plasticity may contribute to chronic cognitive impairment and dementia (Abayomi 2002). Mitigating radiation damage by application of neuroprotectants preserving neurogenic potential might be a successful strategy for reducing of late delayed effects (Jenrow et al. 2010). Moreover, irradiation of mice with a single dose of 15 Gy by gamma rays decreased neurogenesis in the SVZ and subsequently long-term olfactory memory (Lazarini et al. 2009). However, the functional relevance of adult neurogenesis in olfaction remains unclear.

There are extensive experiences of using ionizing radiation for medical treatment of brain tumors. Doses used in clinical radiotherapy in brain cancer are often much larger than the levels needed to eliminate the new

neurons (Laack & Brown 2004). However, in medical treatment there have been applied fractionated doses, which are considered to be less damaging to the brain tissue than a single large dose. On the other side, long-term side effects of such large dose (totally more than 65 Gy) result in widespread late effects (i.e., neurocognitive changes, manifestation of radiation necrosis, vascular lesions) which are usually progressive and irreversible and occur in a distinct chronologic order several months or years after radiotherapy (Brandsma et al. 2008).

As mentioned above, the experimental studies confirmed that cellular response to fractionated and single dose of irradiation is different; the single dose-response is mostly rapid, within hours or weeks of treatment, whereas the fractionated response is delayed and persisted for months after exposure (Tada et al. 1999; Mizumatsu et al. 2003; Rola et al. 2004; Fan et al. 2007). Our experimental model may partially clarify different cellular response to irradiation. Findings of our further research concern with effects of fractionated doses should be valuable for comparing of different radiation-induced dynamics of proliferative activity and apoptosis. Therefore, they might be important for creation of prevention strategies in human radiotherapy.

Our data also provided indirect evidence that the cells affected by radiation-induced apoptosis in the RMS belong to the progenitor cells responsible for adult neurogenesis. We couldn't determine if the progenitor cells were permanently destroyed or only reversibly damaged. Several reports indicated, that in the radiation injured pathway, just neuronal differentiation was altered, therefore the majority of the newborn cells were neurons (Amano et al. 2002; Schmitz et al. 2005). Recently, the cellular composition of adult RMS is well-established (Doetsch et al. 1997; Law et al. 1999). The cells types forming the RMS are represented by migrating immature neurons or neuroblasts surrounded by astrocytes, which providing an astrocytic scaffold (Alvarez-Buylla & García-Verdugo 2002). For phenotypic identification of apoptotic cells are needed double labelled techniques for distinct visualization of these cell types. However, in ongoing experiment which is dealing with consequences of fractionated doses of gamma rays on adult RMS, we perform direct phenotypic identification. Given the recently reported evidence about continuing neurogenesis in adult human forebrain (Curtis et al. 2007), the observation that the progenitor cells are highly sensitive to radiation-induced apoptosis may have implications for radiotherapy. Further research should clarify whether depletion of progenitors from the SVZ could contribute to complications of therapeutic brain irradiation.

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