Radiation-induced Long-term Alterations in Hippocampus under Experimental Conditions

Ionizujúcim žiarením indukované dlhodobé zmeny v hipokampe za experimentálnych podmienok

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Summary

Backgrounds: The aim of the present study was to investigate the effect of ionizing radiation on the cell population that co-forms hippocampal formation in an adult rat brain. ***Materials and Methods:*** Adult male Wistar rats were exposed to whole-body irradiation with fractionated doses of gamma rays (the total dose of 4 Gy). Thirty, 60 and 90 days after irradiation the cell-specific types housed in the CA1, CA3 subregions and adjacent layers were labelled using immunohistochemistry for specific cell phenotypes; Ki-67 marker was used for proliferating cells and GFAP for detection of astrocytes. ***Results:*** During the 30th day post-exposure, a considerable increase in the numbers of Ki-67-positive cells was seen. Moreover, significant decline in the density of neurons, mostly in the CA1 subregion, was observed on the 60th day. Slight overaccumulation of Ki-67-positive cells was seen in CA1 area 90 days after radiation treatment. Temporary decrease of GFAP-positive astrocytes was seen thirty days after irradiation, followed by their subsequent increase 60 days after exposure. Secondary decrease of GFAP-positive cells in both of regions was found in the group surviving 90 days post-irradiation. ***Conclusion:*** Results showed that radiation response of neurons and astrocytes that form the adult hippocampus may play contributory role in the development of prognostically unfavourable adverse radiation-induced late effect.

Key words

ionizing radiation – radiation dosage – rat – hippocampus – Ki-67 antigen – GFAP

Súhrn

Východiska: Cieľom práce je študovať účinok ionizujúceho žiarenia na populáciu buniek, ktorá sa podieľa na zložení hipokampálnej formácie v mozgu dospelého potkana. ***Materiál a metodika:*** V experimente sme ožiarili dospelé samce potkanov kmeňa Wistar celotelovou frakcionovanou dávkou gama žiarenia (celková dávka 4 Gy). Po uplynutí zvolených časových intervalov (30, 60 a 90 dní po ožiarení) sme pomocou imunohistochemického farbenia identifikovali bunkové typy, ktoré sa nachádzajú v oblastiach CA1, CA3 hipokampu a v priľahlých vrstvách. Pomocou markera Ki-67 sme identifikovali proliferujúce bunky a na detekciu astrocytov sme použili marker GFAP. ***Výsledky:*** Pozoruhodný nárast v počte Ki-67-pozitívnych buniek sme zaznamenali tridsať dní po ožiarení, ktorý bol následne vystriedaný ich výrazným poklesom do 60. dňa po expozícii, najmä v oblasti CA1 a opätovným miernym nárastom do 90. dňa po radiačnom zásahu. V počte astrocytov sme zaznamenali ich dočasný úbytok tridsať dní po ožiarení a následné zvýšenie do 60. dňa. V poslednej skupine, ktorá prežívala deväťdesiat dní po expozícii, sme zistili sekundárny pokles GFAP-pozitívnych buniek v obidvoch sledovaných oblastiach. ***Záver:*** Výsledky poukazujú na to, že postradiačná odpoveď neurónov a astrocytov, ktoré sa podieľajú na zložení hippokampu, môže zohrávať určitú úlohu vo vývoji neskorých postradiačných prejavov, ktoré sú z hľadiska prognózy nepriaznivejšie.

Kľúčové slová

ionizujúce žiarenie – radiačná dávka – potkan – hipokampus – Ki-67 antigen – GFAP

The authors declare they have no potential conflicts of interest concerning drugs, products, or services used in the study.

Autoři deklarují, že v souvislosti s předmětem studie nemají žádné komerční zájmy.

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Backgrounds

The hippocampus is a brain structure which belongs to the limbic system and lies under the medial temporal lobe, one on each side of the brain. It is sometimes grouped with other adjacent structures including the dentate gyrus, subiculum and entorhinal cortex forming a structure so-called the hippocampal formation. The hippocampus is crucial for the formation of new autobiographical and fact memory traces. It may function as a memory gateway through which new memories must pass before entering permanent storage in the brain. Bilateral hippocampal damage can result anterograde amnesia i.e. loss of ability to form new memories, although older memories may be safe.

Exogenous agents, such as ionizing radiation could have impact on the rate of hippocampal cell population and ultimately on cognitive functions associated with hippocampus. Ionizing radiation currently used in the radiotherapy can result in significant injury to normal brain structures. Adult CNS neurons are posmitotic therefore they have been regarded as not having a primary role in the pathogenesis of radiation. Research over the past fifty years demonstrating that adult mammalian brain contain at least two discrete brain regions with persistent capacity to generate new neurons and glial cells. The first regions is known as the subventricular zone (SVZ) lining the brain lateral ventricles (LV) and the second one is represented by the subgranular zone (SGZ) in the dentate gyrus of hippocampal formation [1,2]. Progenitor cells of the hippocampal formation migrate into adjacent granular cell layer (GCL) where they can establish mature morphological and functional characteristics. They develop granule cell morphology and connect with their target area, cornu ammonis region 3 (CA3). Research into irradiation effects in normal brain tissue has been mostly focused to studies of single-dose irradiation [3–6]. In animal models, hippocampal formation with vulnerable neural precursor cells from SGZ has been widely used as successful model for studying of radiation-induced reduction of neurogenesis and following deficits in hippocampal-dependent functions of learning, memory and spatial information processing. 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. Although the extent of apoptosis later decreased, remained unchanged for 1–9 months after initial exposure [5–10]. However, there are extensive experiences with single doses application, the leading mode of radiation delivery in clinic is fractionated radiotherapy. There has been reported that fractionated treatment led to development of cascade radiation-induced acute and late changes. Initiate clonogenic cell death of endothelial cells is followed by breakdown of the blood-brain barrier result in vasogenic edema, ischemia, hypoxia and hypoxia-induced expression of several proteins such as vascular endothelial growth factor (VEGF), intercellular adhesion molecule-1 (ICAM-1) with subsequent demyelinisation and ultimately led to tissue necrosis [11].

In the present study we investigated the effect of fractionated irradiation (total dose of 4 Gy) on distribution of cell-specific types housed the hippocampal formation in forebrain of rats, survived 30, 60 and 90 days after exposure.

Materials and Methods

Animals. Adult male rats of the Wistar strain (SAV Dobrá Voda, SR) 7–8 months old at the beginning of experiment and weighing approximately 380 g were used in this study. The animals were kept under standard conditions (temperature of 22–24 °C, light-controlled enviroment with 12/12 h light/dark cycle) and provided with food and water *ad libitum*. All animal procedures were performed in accordance with the requirements for ethical standards of welfare and treatment of animals.

Irradiation. For 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.). The rats were whole-body irradiated using a ^{60}Co radiation source (apparatus TERAGAM 02 UJP Prague). The total radiation dose administered was 4 Gy of gamma rays (1 Gy × 4) given at seven days intervals and the animals survived 30, 60 or 90 days after the last exposure (three animals at each time interval). Control animals were killed 30 days (n = 2), 60 days or 90 days (n = 2) after sham irradiation.

Immunohistochemistry. Thirty to ninety days after irradiation, the animals were overdosed by inhalation of mixture 3% sevoflurane, 68% N_20 and 30% 0_2 and transcardially perfused with saline followed by fixative 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were immediately removed from the skull, postfixed overnight in the same fixative at 4 °C and cryoprotected in 30% sucrose for 18 hours. Serial sagittal 30 µm sections were cut on cryostat, collected on lysine coated slides and air-dried. To minimize nonspecific binding of the secondary antibody, sections were incubated for 1 hour at RT in goat blocking solution (10% goat serum, 1% BSA, 0.5% Tween 20 in PBS) and then covered overnight at 4 °C by rabbit anti-Ki-67 (Abcam, Cambridge, UK), a nuclear antigen that is expressed during the entire cell cycle except G_0 stage and rabbit anti-glial fibrillary acid protein (GFAP; 1 : 50, Chemicon, Temecula, CA), selective marker for astrocytes. After rinsing the sections were incubated for 2 h at RT with goat anti-rabbit secondary antibody labeled with Alexa Fluor 488 (1 : 100, diluted in 0.3% Triton X-100 and 1% BSA in PBS, Molecular Probes, Eugene, OR) and finally coverslipped with Fluoromount (Serva, Germany). The slides were viewed with an confocal laser scanning microscope Olympus FluoView FV10i (Olympus, Japan) with 10× objective, equipped with Alexa Fluor 488 (excitation: 499 nm; emission: 520 nm) or Alexa Fluor 594 (excitation: 590 nm; emission: 618 nm). The image capturing was performed with an Olympus Fluoview FV10–ASW software, version 02.01 (Olympus).

Image analysis. Quantitative assessment was performed in standardized counting area which included 30 µm thick serial sagittal sections about 0.90–1.40 mm laterally to bregma, from different areas of the hippocampus according to localisation of appropriate

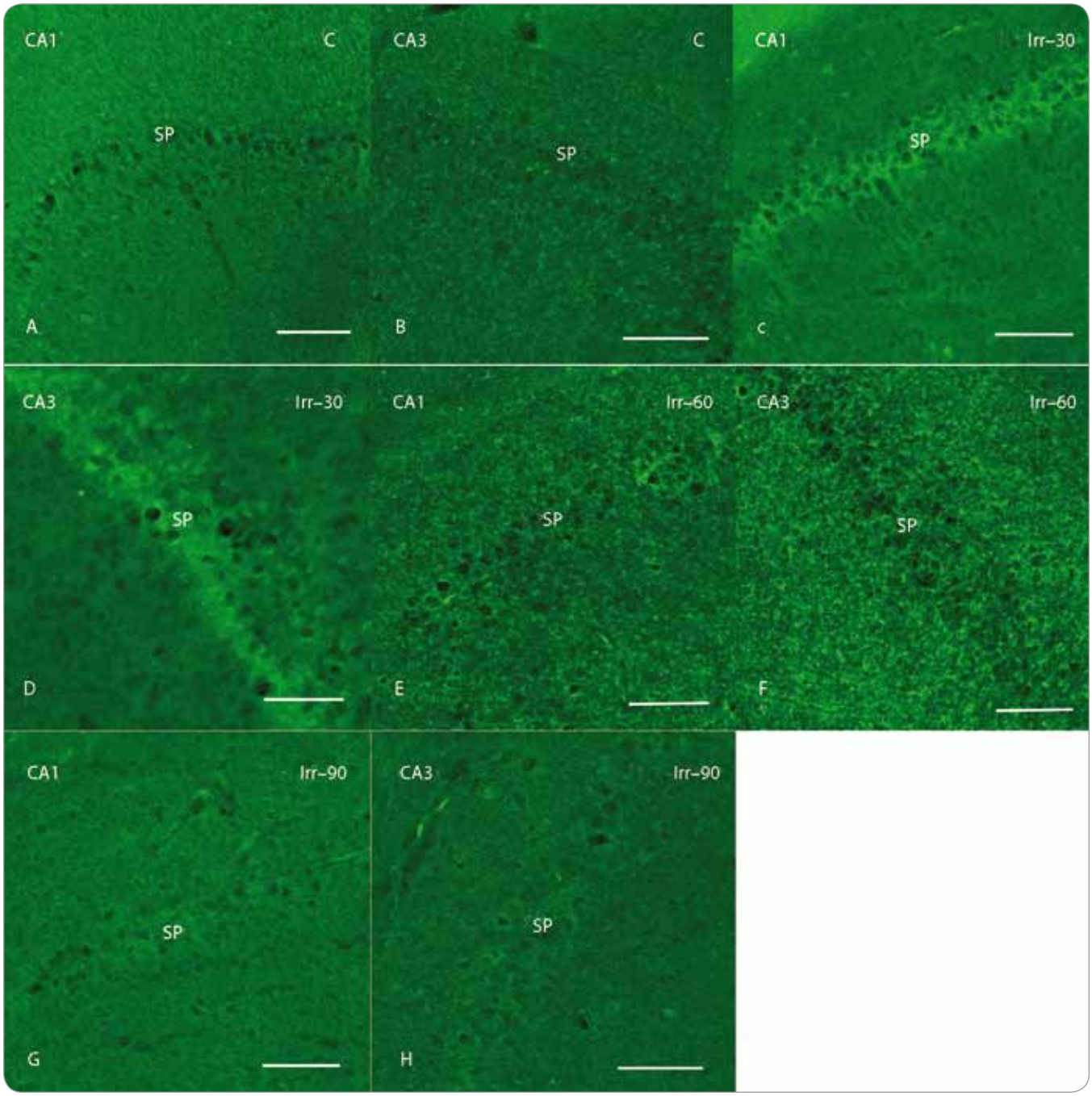


Fig. 1. Photomicrographs of the sagittal sections through the hippocampus of adult male rats showing cornu ammonis region 1 (CA1), cornu ammonis region 3 (CA3) and adjacent layers labeled with immunofluorescent staining for detection of Ki-67 a nuclear antigen that is expressed during the entire cell cycle except G_0 stage (green fluorescence) in control group (A,B) and in the forebrain of rats, investigated thirty, sixty and ninety days after fractionated irradiation (Irr–30, Irr–60, Irr–90) (C–H) with the total dose of 4 Gy of gamma rays. SP – stratum pyramidale. Calibration bars: A–G = 100 µm.

cell-specific types in the stratum pyramidale of the cornu ammonis region 1 (CA1), cornu ammonis region 3 (CA3) and in the adjacent layers, i.e. stratum oriens, str. lucidum, str. radiatum, str. lacunosum and str. moleculare. The numbers of Ki-67-positive (Ki-67$^+$) and GFAP-positive (GFAP$^+$) cells (green fluorescent nuclei and cytoplasm, respectively) were counted in 10–15 randomly selected sections per animal. Values of (GFAP$^+$) cells counted in layers adjacent to CA1 and CA3 subregions were summarised and averaged. The counting was performed by ImageJ (NIH, Bethesda, MD) a public domain image processing and analysis program. The density of fluorescent labelled cells was quantified automatically at a magnification of 10×. The total number of positively labeled cells

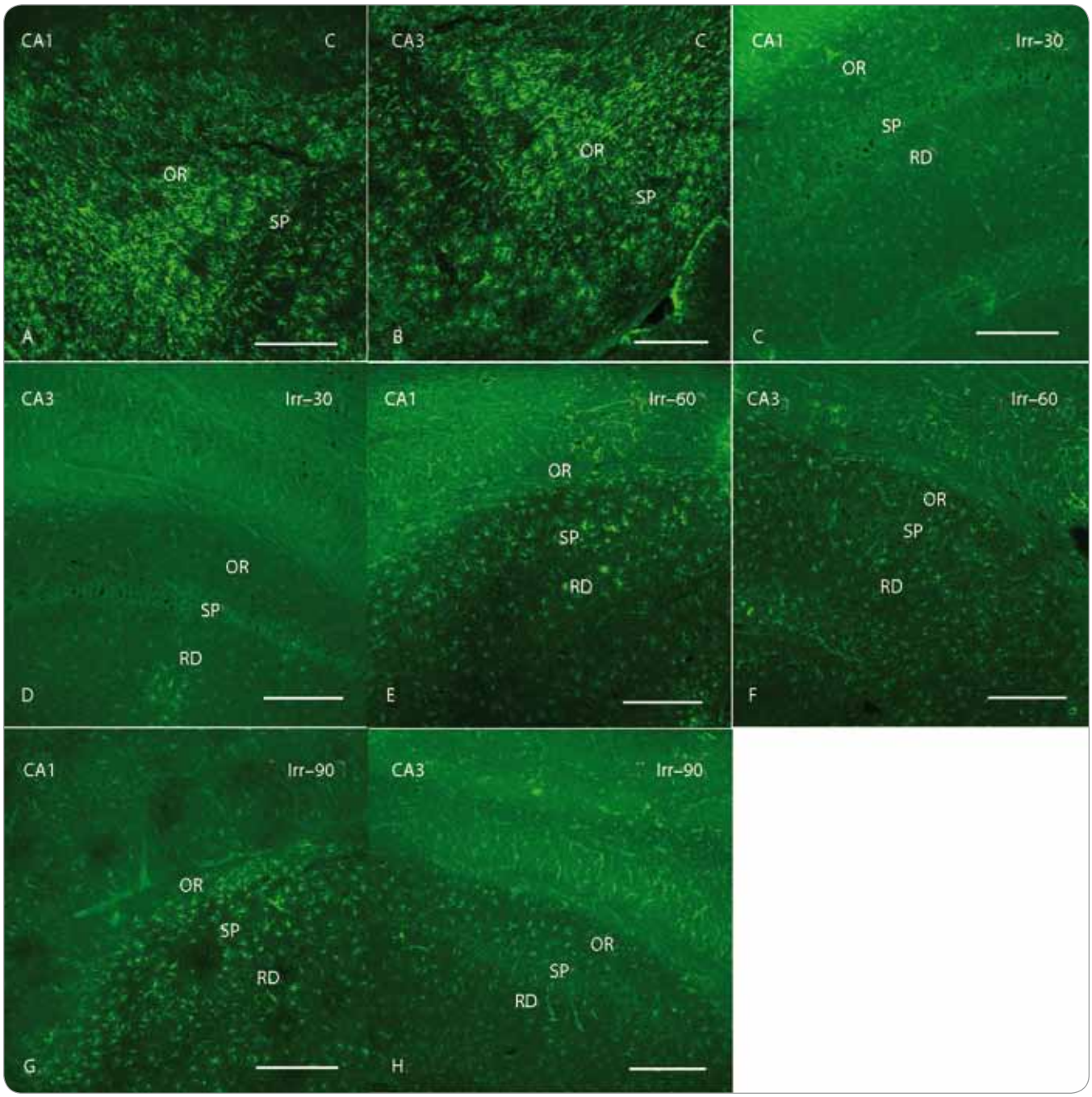


Fig. 2. Photomicrographs of the sagittal sections through the hippocampus of adult male rats showing cornu ammonis region 1 (CA1), cornu ammonis region 3 (CA3) and adjacent layers labeled with immunofluorescent staining for detection of glial fibrillary acid protein (GFAP) (green fluorescence), marker for mature astrocytes in control group (A,B) and in the forebrain of rats, investigated thirty, sixty and ninety days after fractionated irradiation (Irr–30, Irr–60, Irr–90) (C–H) with the total dose of 4 Gy of gamma rays. OR – stratum oriens, RD – stratum radiatum. Calibration bars: A–G = 200 µm.

was determined by summing the values from all sections per individual experimental group. Data were analyzed using ANOVA one-way analysis followed by a Tukey-Kramer test comparison and presented as mean ± standard error (S.E.M). Statistical significance was set at $P \leq 0.05$.

Results

Quantitative image analysis showed difference in spatio-temporal distribution of selected cell population which resides hippocampal formation of the brains of control group and animals, investigated 30, 60 and 90 days after radiation treatment (Fig. 1,2). In control group, higher abundance of proliferating

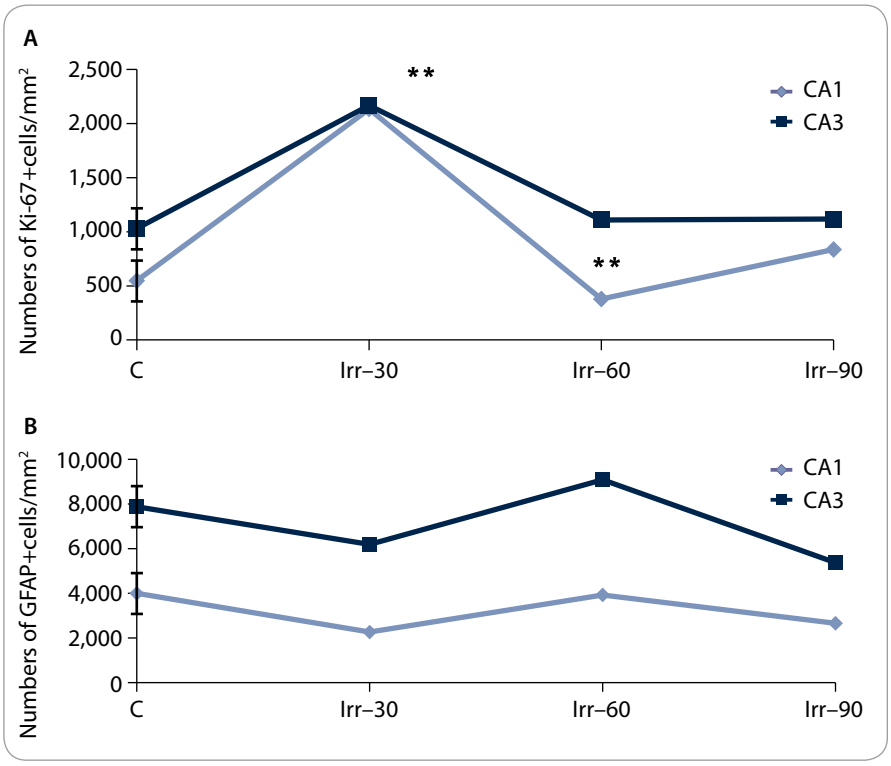
Ki-67$^+$ cells was detected in CA3 area (1,031 ± 157.97/cm^2). Investigation of animals, survived 30 days after irradiation showed strong enhancement in both of regions (CA1: 2,134 ± 420.26 versus 547 ± 190/cm^2 in control ones; ≤ 0.01; CA3: 2,168 ± 234.86). In the following group, examined 60 days after radiation treatment there was seen steep decrease in the numbers of proliferating cells more in CA1 subregion (378 ± 99.59 vs. Irr–30: 2 134 ± 420.26; P ≤ 0.01) then in CA3 (1,110 ± 300). Finally, ninety days after exposure was detected overaccumulation of Ki-67$^+$ cells in CA1 (836 ± 309.72); however, there was no change in density of neurons in CA3 (1,118 ± 222.17) when compared to previous post-radiation survival time. In the brain of control animals, higher density of GFAP-labeled astrocytes was observed rather in layers adjacent to CA1 subregion (3,997 ± 917.78) then in the neighboring layers of CA3 area (3,884 ± 1,919.6). In animals investigated 30 days post-irradiation numbers of astrocytes decreased in CA1 (2,264 ± 914.16) and in CA3 remained unchanged (3,927 ± 244.48). Moreover, the numbers of GFAP$^+$ cells partially increased 60 days after exposure mostly in CA3 region (5,155 ± 456.3; CA1: 3,931 ± 650.61). In brain of the animals, survived 90 days after irradiation, there was seen loss of astrocytes mostly in CA1 (2,658 ± 463.96) and less in CA3 (2,711 ± 774.14).

Discussion

In presented study we investigated the effect of radiation treatment with fractionated doses of gamma rays in the hippocampus of adult rats, survived 30, 60 or 90 days after exposure. Obtained data showed several alterations in spatio-temporal distribution of cell-specific types between the control and treated animals as well as among irradiated groups (Graph 1A, B). However, observed changes were in most cases not very striking, there has been found several differences. Temporary decrease of astrocytes was observed between control group and animals survived 30 days after exposure replaced by mild increase during the 60th day after treatment. The secondary and more expressive decline was seen between sixty and ninety days. Loss or enhancement in density of glial cells such as astrocytes or oligodendrocytes are reported in many animal studies carried out with consequences of radiation treatment [5,12,13]. Regarding to astrocytes, studies in animals as well as clinical experiences showed that reactive astrogliosis is a characteristic response of astrocytes to inflammation and trauma of the adult CNS [11,13,14,15]. Prominent features of activation of astrocytes (reactive gliosis) are hypertrophy of astrocytic processes, upregulation of intermediate filaments, glial fibrillary acid protein (GFAP), expression. In the practice of radiation therapy has been used standard fractionation scheme and although increases in radiation dose improve survival in cancer patients, levels of radiation used for therapy are limited by the risks of radiation necrosis, brain atrophy and other post-irradiation complications in normal brain tissue surrounding the tumor.

There has been reported significant increase of astrocytes up to 180 days after fractionated radiation treatment with various doses per fraction [13,14]. Likewise, results of our previous simultaneously performed study which was carried out with consequences of fractionated delivery on forebrain's rostral migratory stream (represents lateral extension of the SVZ) showed increase of astrocytes along this migratory pathway two weeks after irradiation but these changes seems to be reversible [16].

Radiation injury to the CNS results in glial activation accompanied by expression of proinflammatory cytokines, which have been associated with radiation-induced gliosis [17]. Recent researches have speculated the roles of inflammatory mediators such as cyclooxygenase (COX) and the role of microglia (a main regulator of brain inflammation) in radiation injury. COX-2 appears



Graph 1A, B. Distribution of Ki-67-positive (Ki-67+) and GFAP positive (GFAP+) cells in the cornu ammonis region 1 (CA1), cornu ammonis region 3 (CA3) and in the neighboring layers of hippocampus of control (C) and irradiated adult male rats investigated thirty, sixty and ninety days after fractionated irradiation (Irr–30, Irr–60, Irr–90; ± SEM). Statistical significance of differences between control and irradiated groups and between Irr–30 versus Irr–60 groups: ** P ≤ 0.01.

to play a significant role in brain prostaglandin production. Prostaglandins (PGs) are found elevated in brain following injury [18,19]. Therefore, it appears that prostaglandin E_2 (PGE2) released from irradiated microglia is a key mediator of irradiation-induced gliosis. There is well-known that cellular response to single exposure is rapid whereas fractionated reply is delayed and surpassed the post-radiation treatment. We can speculated, that observed changes seems to be associated rather with temporary neuroprotective response of glial cells than late radiation-induced effects resulting in reactive gliosis.

Population of neurons, labeled with Ki-67 marker showed more expressive changes in cell density during the experiment. Most prominent increase was found between control animals and group survived 30 days after irradiation in CA1 subfields and later was replaced by significant decline until 60 days after treatment. In CA3 subregion were seen milder changes during the whole experiment as in CA1 area revealed differential radiation sensitivity in these subregions. Wu [20] reported about reduction of postmitotic neurons in CA1 and CA3 area up to two months after exposure and these changes seemed to recover. Moreover, density of neurons in the hippocampus of senescent rats remained unchanged at least 1 year after fractionated irradiation [21]. The highest sensitivity to radiation injury in adult brain is related to restricted population of neural precursor/stem cells arisen either from anterior SVZ or from SGZ in the dentate gyrus. Experiments done before showed dramatic decrease of the numbers of Ki-67$^+$ cells in SGZ of dentate gyrus in animals survived from several hours to few months after single treatment [6,8]. Regarding a fact, that there is extensive research of consequences of radiation injuried neurogenic population in adult hippocampus, we didn't performed measurement of proliferating cells in SGZ. However, in our previous experiment performed with rats, survived 1-80 days after single moderate irradiation we observed time-dependent changes along the forebrain's RMS. There was found strong long-lasting increase in density of cells, labeled with proliferation marker bromodeoxyuridine (BrdU) in rostral parts of the RMS adjacent to neurogenic SVZ up to 80 days after exposure [22,23]. However, these data seems to be not consistent with recent results, identification of cells with inmature characteristic in CA1 and CA3 area evoked another neurogenic area, the forebrain's SVZ and subsequent migratory pathway terminate in olfactory bulb (OB). Earlier studies did confirm that the OB does project into the ventral part of the lateral entorhinal cortex, and field CA1 in the ventral hippocampus sends axons to the main OB, the anterior olfactory nucleus, and to the primary olfactory cortex. There continues to be some interest in hippocampal olfactory responses, particularly the role of the hippocampus in memory for odors [24–26].

Existing animal studies suggest that adult-born neurons contribute to some forms of hippocampal-dependent learning and these new cells have functional synaptic inputs similar to those found in mature neurons [8,27,28]. For more precise study of radiosensitivity of young synaptic plasticity are needed additional studies dealed with immunohistochemical expression of markers of axons and dendrites.

Conclusion

Obtained data confirm previous findings about prolonged effect of irradiation on density of proliferating cells with neuronal features resides adult hippocampus. Moreover, dynamic of changes in population of mature astrocytes suggested that these cells are more susceptible to irradiation that was previously thought. Take to account well-known potential risk of radiation exposure to development of late symptoms included reactive gliosis, there is necessary to create prevention strategies to avoid irreversible effects in clinical radiotherapy.

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