Gamma-Ray Susceptibility of Immature and Mature Hippocampal Cultured Cells

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ABSTRACT. Ionizing radiation suppresses neurogenesis in the mammalian brain. This *in vitro* study compared the detrimental effect of acute gamma-irradiation on immature hippocampal cells with mature cells. Both rat immature (0.5 day *in vitro* (DIV)) and mature hippocampal cells (14 DIV) were irradiated with 0–4 Gy gamma-rays. Cell viability was analyzed by using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. DNA fragmentation study was performed by extracting intracellular DNA. Morphological features of apoptosis were characterized by 4',6-diamidino-2'-phenylindole, dihydrochloride (DAPI) staining. MTT assay revealed that the survival rate of immature hippocampal cells declined in a dose-dependent manner within the range of irradiation applied, but was not changed in mature cells. Intranucleosomal DNA fragmentation in a ladder like pattern was dose-dependently increased in immature cells, but not in mature cells. The number of apoptotic nuclei in immature cells increased significantly in a dose-dependent manner within the range of irradiation applied. Active caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP) expressions in immature hippocampal cells at 6 hr after 2 Gy exposure were markedly higher than control levels. The significantly greater radiosensitivity of immature hippocampal cells than that of the mature cells, indicates that the susceptibility of such hippocampal cells depends on their maturation. In addition, gamma-irradiation may induce caspase-dependent apoptosis in immature hippocampal cells.

KEY WORDS: apoptosis, cell viability, gamma-ray, hippocampal cell, radiosensitivity.

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Ionizing radiation is one of the most effective therapies for treatment of tumors in the central nervous system (CNS). Although it is effective in treating malignancies, application of radiotherapy is limited due to potential damage of normal CNS tissue [11]. Irradiation has a variety of effects on both the developing and adult brain, even though the adult brain is less severe morphological changes to irradiation than other organs. Especially, under very low doses of radiation, the developing brain is one of the most radiosensitive organs [9, 7, 16]. Even very low doses of irradiation to the brain of an infant below 18-months-of-age can affect the patient's cognitive abilities in adulthood [6].

Exposure of mammalian brains to ionizing radiation induces apoptosis of neural immature progenitor cells in the subventricular zone of the lateral ventricles and in the subgranular zone of the hippocampal dentate gyrus [3, 4, 10, 12, 17]. The phenomenon in adult mouse hippocampus may lead to hippocampal-dependent learning and memory impairment [10], although there is no radiation-induced apoptotic appearance in other hippocampal regions, including cornu ammonis (CA) 1 and CA3. Therefore, it is important to clarify the degree of radiosensitivity in the stages of neural development at which they are most vulnerable.

In addition, studies of radiation-induced effects on cognitive function related to adult hippocampus are very difficult because they rely on complicated *in vivo* models [2]. How-

ever, it may possible to address specific mechanisms associated with radiation effects on neural immature and mature hippocampal cells by using *in vitro* approaches. Thus, an *in vitro* system needs to be established to screen radioprotectants and study various brain perturbation factors caused by radiation exposure.

This study compared the detrimental effect of acute gamma-irradiation on 0.5 day *in vitro* (DIV) immature hippocampal cells with that on the 14 DIV mature cells to elucidate the differential effect of ionizing radiation on immature and mature hippocampal cells in an *in vitro* system.

MATERIALS AND METHODS

Primary hippocampal cell culture: Pregnant Sprague-Dawley rats were obtained from a specific pathogen-free colony at Oriental Inc. (Seoul, Korea). Hippocampi were dissected from postnatal day 0 rats, and prepared for culturing. After dissection, tissues were chopped into 1 mm³ dices, and digested with 10 units/ml papain (Worthington, Freehold, NJ, U.S.A.) and 100 units/ml DNase I (Roche, Basel, Switzerland) in dissociation buffer (82 mM Na₂SO₄, 30 mM K₂SO₄, 5.8 mM MgCl₂, 0.25 mM CaCl₂, 20 mM glucose, 0.001% phenol red, 0.45 mg/ml cysteine, and 1.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6) at 37°C for 30 min. The digestion was washed with dissociation buffer and triturated with Neurobasal A medium (Invitrogen, Carlsbad, CA, U.S.A.). The cells were seeded at a density of 0.4×10^6 cells/well and 2×10^6 cells/dish on poly-D-lysine hydrobromide (150 µg/ml; Sigma-

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Aldrich, St. Louis, MO, U.S.A.) coated 4-well plates and 35 mm dishes (NUNC™; Thermo Fisher Scientific, Pittsburgh, PA, U.S.A.), respectively. Two hours after plating, Neurobasal A was replaced with growth media including Neurobasal A, 1 × B27 supplement (Invitrogen), 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.5 mM glutamine and all cultures were kept at 37°C, 5% CO₂.

Culture irradiation: Cells at 0.5 and 14 DIV were irradiated with 0, 0.5, 2, or 4 Gy of ¹³⁷CS-generated gamma rays using a Gamma-cell Elan 3000 (Nordion International, Kanata, ON, Canada). The mean dose rate of the gamma-rays was 3.1 Gy/min. The irradiation doses were measured at the cultured plate exposure position with an IC-17 ionization chamber (Far West Technology, Goleta, CA, U.S.A.) and an RDM 2A electrometer (Therados-Scanditronix, Uppsala, Sweden). Control samples were sham-irradiated with 0 Gy. The cells were assayed at 6, 12, 24 and 48 hr post-irradiation (pi.).

Cell viability evaluation: Cell viability was evaluated by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The assay was based on the reduction of MTT by living cells to yield a soluble formazan product that could be detected colorimetrically. MTT was added to cells in 4-well plates (NUNC™; Thermo Fisher Scientific) to 0.5 mg/ml in culture medium without phenol red and serum. After 2 hr incubation at 37°C with 5% CO₂, the resulting formazan crystals were dissolved in MTT solubilization solution (Sigma-Aldrich). The solubilized formazan products were quantified by an Emax spectrophotometer (Molecular Devices, Sunnyvale, CA, U.S.A.) for absorbance at a wavelength of 570 nm. The background absorbance was subtracted by spectrophotometric measurements at a wavelength of 690 nm.

DNA fragmentation analysis: For the DNA analysis, 2 × 10⁶ control cells/dish and the irradiated ones with 0–4 Gy were collected at 12 hr pi. The cells were scraped and collected by centrifugation, washed with phosphate buffered saline (PBS) and resuspended in lysis buffer (1% sodium dodecyl sulfate (SDS), 0.2 mg/ml proteinase K, 100 mM NaCl, 20 mM Tris-HCl, pH 8.0) and incubated for 4 hr at 37°C. Cells were centrifuged at 16,000 × g for 15 min at 4°C to separate the fragmented DNA (supernatant) from intact chromatin DNA (pellet). Supernatant DNA was extracted with an established phenol/chloroform/isoamylalcohol method. Ten microliters of extracted DNA, was used for 1.2% agarose electrophoresis.

4',6-Diamidino-2'-phenylindole, dihydrochloride (DAPI) staining for counting of apoptotic cells: Morphological features of apoptosis were characterized by condensed and fragmented nuclei after staining with DAPI (Thermo Fisher Scientific). Cell cultures were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 20 min at room temperature (RT). After three PBS washes, 1 µg/ml DAPI in PBS was added and the cells were incubated at RT for 5 min. After two washes, the number of stained nuclei with apoptotic morphology was determined with a ProgRes® CFscan fluorescence digital camera (Jenoptik, Jena, Germany) and

enumerated using ProgRes® CapturePro 2.5 software (Jenoptik) in four random fields of quadruplicate samples at 400 × magnifications.

Western blotting: Medium was removed and a SDS sample buffer (4×) was added to each culture. The cells scraped and sonicated for 10 sec. The samples were heated to 100°C for 10 min. The samples were then separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and blocked with 1% normal goat serum and 0.5% bovine serum albumin in PBS-T (PBS, 0.1% tween 20) for 30 min at RT. The membranes were then incubated with a 1:1,000 dilution of either rabbit anti-cleaved (active) caspase-3 antibody (Cell Signaling Technology, Beverly, MA, U.S.A.) or rabbit anti-poly (ADP-ribose) polymerase (PARP) antibody (Cell Signaling Technology) in PBS-T overnight at 4°C. After extensive washing and incubation with a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.) for 1.5 hr at RT, the signals were visualized using chemiluminescence methods

Fig. 1. Gamma-ray decline in cell viability in immature hippocampal cells. MTT assay showing cytotoxicity of hippocampal cells induced by gamma irradiation at 0.5 (A) and 14 DIV (B). MTT was added to each well 12 hr after irradiation, and absorbance was measured using a microplate reader. At 0.5 DIV, the cell viability significantly declined in dose-dependent manner within the range of irradiation applied (0–4 Gy). In contrast, at 14 DIV, cell viability was not changed. The data represent the mean ± SE, n=8 cultures per condition.

Fig. 2. Gamma-ray induces DNA fragmentation in immature hippocampal cells. Intranucleosomal DNA fragmentation indicated by a ladder like pattern was dose-dependently increased 12 hr after irradiation at 0.5 DIV (A). However, the mature cells (B) did not show the DNA fragmentation after irradiation at 14 DIV.

(SuperSignal® West Pico; Thermo Fisher Scientific). For normalization purposes, the membranes were reprobed with a 1:20,000 dilution of beta-actin antibody (Sigma-Aldrich). Several exposure times were used to obtain the signals in the linear range. The bands were quantified using Scion Image Beta 4.0.2 for Windows XP software (Scion, Frederick, ME, U.S.A.).

Statistical analysis: The data is reported as the mean $\pm$ SE. The data was analyzed using one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test for multiple comparisons. In all cases, a p value <0.05 was considered significant.

RESULTS

Effect of gamma-ray on viability of hippocampal cells: We first analyzed the cell viability of cultured hippocampal cells induced 12 hr after irradiation of 0–4 Gy by the MTT assay and determined whether there was a difference of cell viability between 0.5 DIV immature and 14 DIV mature cells. At 0.5 DIV, cell viability decreased in a dose-dependent pattern within the range of irradiation applied (0–4 Gy) (Fig. 1A). At 14 DIV, however, irradiation did not decrease cell viability (Fig. 1B).

DNA fragmentation in cultured hippocampal cells with gamma-ray exposure: Neural DNA was isolated and subjected to conventional electrophoresis. At 0.5 DIV, a clear ladder-like pattern in the DNA from the irradiated hippocampal cells indicated that radiation induced internucleosomal DNA fragmentation, a common characteristic of apoptosis. The DNA laddering obviously increased 12 hr after irradiation with increasing irradiation dose (Fig. 2A), whereas it was hardly detected in irradiated cells at 14 DIV (Fig. 2B).

Morphological appearance of apoptosis in immature hippocampal cells with gamma-ray exposure: Differential interference contrast images showed that cellular shrinkage and loss in irradiated cells (Fig. 3C) was increased compared with sham-irradiated controls at 0.5 DIV (Fig. 3A).

DAPI fluorescence images also showed that nuclear pyknosis increased 12 hr after irradiation (Fig. 3D), compared with sham-irradiated controls (Fig. 3B). At 14 DIV, however, irradiation did not increase cellular shrinkage and nuclear pyknosis (data not shown). To characterize the time- and dose-dependent changes of the apoptotic response in 0.5 DIV cells, nuclear pyknotic cells were scored 6, 12, 24 or 48 hr after 0–4 Gy irradiation. As shown in Fig. 3E, there was an increase in apoptotic cells as early as 6 hr after irradiation, with the peak incidence occurring at 12 and 24 hr, but at 48 hr the fraction of cells displaying nuclear fragmentation had diminished.

Active caspase-3 and cleaved PARP are significantly increased by gamma-ray exposure: To determine whether radiation-induced apoptosis in immature hippocampal cells was associated with the caspase-dependent pathway, caspase-3 activation and caspase-specific PARP cleavage were assessed by Western blot (Fig. 4). At 0.5 DIV, active caspase-3 and cleaved PARP expressions were significantly increased 6 hr after 2 Gy irradiation, compared to sham-irradiated control levels (active caspase-3: relative optical density (OD), 10.86 ± 1.1 -fold increase, $p < 0.001$; cleaved PARP: relative OD 15.45 ± 1.94 -fold increase, $p < 0.001$).

DISCUSSION

In this study, viability of immature hippocampal cells, but not mature cells, significantly declined upon exposure of gamma-rays, indicating that neuronal resistance to gamma-irradiation was associated with neuronal differentiation. In addition, gamma-irradiation induced apoptosis in immature hippocampal cells through the caspase-dependent pathway.

In this report, we examined the gamma-irradiation susceptibility of hippocampal neurogenesis using 0.5 DIV hippocampal cells and 14 DIV mature hippocampal cells. Exposure of mammalian brains to ionizing radiation induces apoptosis of neural immature progenitor cells *in vivo* [3, 4]. Cultured hippocampal cells form functional synapses and a network after 3–5 DIV [21]. Therefore, we chose to apply

Fig. 3. DAPI staining showing time- and dose-dependent apoptotic changes in immature hippocampal cells. (A-D) Apoptotic cells with condensed and fragmented nuclei were observed in immature hippocampal cells after acute gamma irradiation (a single dose of 2 Gy) at 0.5 DIV. Arrows indicate cells with apoptotic nuclei (B and D). (E) The number of apoptotic nuclei began to increase significantly 6 hr pi., reaching their highest level at 12 hr and 24 hr, respectively, and then subsequently declining at 48 hr pi. The data represents the mean $\pm$ SE, $n=4$ cultures per condition.

gamma-irradiation to 0.5 DIV-cultured hippocampal cells, which are comparable to neural immature progenitor cells before cell connection and synapse formation.

Many neuropathological conditions in CNS are characterized by neuronal apoptosis [1]. Exposure of the CNS to ionizing radiation induces neuronal apoptosis via DNA damage and oxidative stress [5]. The consensus view holds that noncycling cells such as neurons are more resistant to irradiation than cycling cells including astrocytes and vascular endothelial cells. However, an irradiation study using developing rat brains suggested that the radiosensitive population consists primarily of cells nearing cell division or cells that have recently completed mitosis and are beginning

Fig. 4. Western blot analysis showing active caspase-3 and cleaved PARP expression in immature hippocampal cells. (A) Representative immunoblots for detection of active caspase-3 (17 kDa), PARP (118 kDa) and cleaved PARP (89 kDa) at 6 hr after sham-irradiation and 2 Gy irradiation at 0.5 DIV hippocampal cell cultures. (B and C) The relative expression levels of active caspase-3 (B) and cleaved PARP (C) were determined by densitometric data analysis and normalized to the beta-actin signal from three different samples. The data represents the mean $\pm$ SE, $n=3$ cultures per condition.

to differentiate [8]. A previous *in vitro* study showed that non-cycling postmitotic neurons retain their susceptibility to X-irradiation [5]. Recently, 7 DIV neurons were reported as being susceptible to X-irradiation at a high dose (30 Gy), while 21 DIV neurons were resistant to this irradiation [18]. Gobbel *et al.* [5] reported that 2 Gy X-irradiation is the threshold irradiation dose at 7 DIV. Furthermore, X-irradiation of immature neurons causes structural deficits (*i.e.*, a loss of cell connection and reduction in the level of synapse formation) in the surviving neurons, which are thought to result in their dysfunction [14]. In this study, cell viability declined in a dose-dependent manner within the relative low-range of irradiation applied (0–4 Gy) at 0.5 DIV. Therefore, 0.5 DIV hippocampal cells are susceptible to irradiation of relative low doses (0–4 Gy), but 14 DIV hippocampal cells were resistant to this irradiation.

Additionally, cultures of 0.5 DIV hippocampal cells provide a useful tool for the analysis of critical mechanistic parameters associated with radiation response of the brain and to better understand the molecular changes in radiation-induced apoptosis of neural cells. During the caspase-

dependent apoptotic process, PARP is proteolytically cleaved and inactivated by caspase-3, a process that is widely used as an apoptotic marker [15]. Intriguingly, PARP is a 'death substrate' of caspase-3 [13, 20], and the cleavage of PARP abolishes its enzymatic activity and saves NAD⁺ and ATP, which drives the cell to apoptosis [19]. In this study, active caspase-3 and cleaved PARP expressions were significantly increased after exposure of 2 Gy gamma-rays, suggesting that radiation-induced apoptosis in immature hippocampal cells depends on pro-apoptotic caspase pathway.

In summary, this *in vitro* study has confirmed that the radiosensitivity of immature hippocampal cells is significantly higher than that of the mature cells, indicating that the susceptibility of such hippocampal cells depends on their differentiation. In addition, this study has established a beneficial *in vitro* system for screening drugs and studying various brain perturbation factors on related functions.

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