Effectiveness of Carbon-ion Beams for Apoptosis Induction in Rat Primary Immature Hippocampal Neurons

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Carbon-ion beams/Apoptosis/Neuron.

The direct biological effects of radiation, particularly accelerated heavy particle ions, on neurons are not fully known. Hence, the direct effect of carbon-ion beams on immature neurons was investigated by comparing to the effect of X-rays in vitro using primary hippocampal neurons. Primary neurons were prepared from hippocampi of fetal rats at embryonic day 18 from timed pregnant Wistar rats and cultured with Banker’s methods. At 7 Days In Vitro (DIV), the cells were irradiated with 140 kV X-ray and 18.3 MeV/amu carbon-ion beams (LET = 108 keV/µm). The cells were fixed with 4% paraformaldehyde at 12 hours after irradiation. Then, the cells were treated with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and DAPI staining for measuring the percentage of apoptosis (apoptotic index: AI). AI in sham-irradiated hippocampal neurons was 18%. The value of AI (AIs) of the cells irradiated with X-rays at 10 or 30 Gy were 15% or 23%, respectively. AI in cells irradiated with carbon-ion beams at 1 Gy, 3 Gy, 5 Gy and 10 Gy were 22%, 23%, 24% and 33%, respectively. AI was significantly increased by carbon-ion beams at 10 Gy (p < 0.001). The apoptosis of hippocampal neurons increased in a dose-dependent manner following both X-ray and carbon-ion beams irradiation. Carbon-ion beams were about 10-fold more effective than X-rays for apoptosis induction in immature hippocampal neurons.

INTRODUCTION

Radiation therapy plays a major role in the treatment of both adult and pediatric patients with various malignant brain tumors.1−4) Since the radiation therapy technique has rapidly developed recently, it has become possible to irradiate tumors with a higher dose, while sparing the surrounding normal tissues with minimum exposure;5) however, severe chronic adverse effects following brain irradiation cannot completely be avoided.6−10) Because radiotherapy to pediatric patient is more harmful than to adult from clinical results,7)

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doi:10.1269/jrr.10050

it is generally believed that young brain is basically more radiosensitive than adult brain. X-ray irradiation to the brain tissue, in vivo, induced the apoptosis of neuronal precursor cells in the SVZ and in the subgranular zone (SGZ) of the dentate gyrus, suggesting that this enhanced apoptosis led to later neuropsychological impairment.10) High linear energy transfer (LET) heavy-ion beams, such as carbon-ion beams, have beneficial physical and biological aspects that might improve radiotherapy.11,12) The biological advantages of carbon-ion beams compared with X-rays are summarized as a decreased oxygen enhancement ratio,13) diminished capacity for sublethal and potentially lethal damage repair, and diminished cell cycle-dependent radiosensitivity.14) By those characteristics, carbon-ion beam therapy is considered to have a therapeutic gain over conventional photon therapy, to improves tumor control and the survival of radioresistant brain tumors. Hence, therefore, it is important to understand the effect of carbon-ion beams on normal neural tissue, such as neurons. Takahashi et al. reported histological changes in the rat brain after irradiation with carbon-ion beams.15,16) In the report, necrotic tissue damage, hemorrhage, and vasodilatation occurred only by high-dose irradiation with carbon-ion beams, but necrotic changes by
carbon-ion beams occurred earlier than by X-rays or gamma-rays. One of the possible reasons for this difference was the different biological effectiveness between carbon-ion beams and gamma-rays; however, little is known about the direct biological effect of carbon-ion beams on neurons. In *in vivo* study, it is difficult to exclude secondary effects on neurons via injuries to blood vessels and glial cells from the total effects of irradiation on neurons. We previously reported by using Bunker’s neuronal cell culturing method that 30 Gy X-rays caused apoptosis on immature hippocampal neurons.\(^{17}\) In the current study, the direct effect of carbon-ion beams on immature neurons was examined by comparing with the effect of X-rays utilizing primary hippocampal immature neuronal culture regardless the effect of glial cells on neurons *in vitro*.

**MATERIALS AND METHODS**

**Neural cell culture**

Primary neurons were prepared from the hippocampi of fetal rats at embryonic day 18 from timed pregnant Wistar rats. Details of the methods have been reported elsewhere (Fig. 1).\(^{18,21}\) Briefly, dissected hippocampi were dissociated by trypsin treatment, triturated by pipetting with Pasteur pipette. Suspensions of cells were plated at a density of 5000 cells/cm\(^2\) on glass coverslips coated with poly-L-lysine (Sigma, St. Louis, MO, USA) and were incubated in Minimum Essential Medium (MEM; Invitrogen, San Diego, CA, USA) supplemented with 10% fetal bovine serum, 0.6% glucose and 1 mM sodium pyruvate for 3 hours. Coverslips were transferred into a culture dish containing a glial monolayer sheet and maintained in serum-free MEM with 2% B27 supplement (Invitrogen), 0.6% glucose and 1 mM sodium pyruvate. The cells were maintained in a humidified incubator with 5% CO\(_2\). Cytosine β-D-arabinofuranoside (Sigma) (10 μM) was added to the culture at 3 days *in vitro* (DIV) to inhibit glial proliferation. Glial cells were prepared from the cerebral cortex of newborn rats (postnatal day 1). All animal experiments were performed according to the Animal Care and Experimentation Committee (Gunma University, Showa Campus, Maebashi, Japan).

**X-ray and carbon-ion beam irradiation**

At 7 DIV, cells were irradiated with 140 kv X-rays at 10 Gy and 30 Gy using a Hitachi (MBR-1505R) X-ray machine at a dose rate of 1.11 Gy/min at Gunma University and 18.3 MeV/amu carbon-ion beams at 1 Gy, 3 Gy, 5 Gy and 10 Gy (LET = 108 keV/μm) at Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) at the Japan Atomic Energy Agency (Takasaki, Japan).\(^{22}\) Irradiation was performed at room temperature. When the cells were irradiated, the coverslips transferred into another dishes with MEM. Following the irradiation, the coverslips were re-transferred into the culture dishes. Absorbed dose [Gy] was calculated according to the following formula: Dose [Gy] = fluence (number of ion particles/cm\(^2\)) × LET (keV/μm) = 1.602 × 10\(^{-9}\).\(^{22}\) Ten coverslips were prepared for each dose. Control cells were sham-irradiated and handled in parallel with the test cells.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining**

The cells were fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) at 12 hours after irradiation. The TUNEL assay was then performed using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Cemicon International, Temecula, CA, USA) according to the manufacturer’s instructions. Fixed cells were permeabilized with ethanol: acetic acid, in a 2:1 ratio for 15 minutes at –20°C. Cells were then washed twice in PBS for 5 min and incubated with Apoptag equilibration buffer for 5 minutes prior to terminal deoxynucleotidyl transferase enzyme linkage of dUTP-digoxigenin to the 3'-OH DNA ends at 37°C for 60 minutes. The reaction was then terminated in stop/wash buffer at 37°C for 30 minutes. After washing the cells, the slides were treated with anti-digoxigenin-peroxidase for 30 min, and mounted with Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA).

**Fig. 1.** Banker’s Method. Primary neurons were prepared from hippocampi of E18 Wistar rats and cultured with Banker’s methods.
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Cell count

All coverslips were counted by the blind test method. All fluorescent images of cells were obtained on a Keyence BZ-9000 microscope (Keyence, Osaka, Japan) equipped with a 20 x objective lens and a cooled CCD camera. Sixteen to eighteen fields in each coverslip were randomly selected and counted. TUNEL-positive cells were considered to be undergoing apoptosis. The percentage of apoptosis (apoptotic index: AI) was determined above the total cell number with DAPI staining. All fluorescent images were analyzed with the BZ-II analysis application (Keyence). Both TUNEL-positive cells and DAPI-positive cells were counted automatically by the BZ-II analysis application after modification of the application by haze reduction (Fig. 2). All data were reviewed by human observation.

Statistical analyses

Simple linear regression analyses of the results were performed using the statistical package R version 2.9.0 (http://www.r-project.org/) and the relative biological effectiveness (RBE) value was calculated as the rate of gradient of each linear line. Statistical analysis was performed by the unpaired t-test or one-way analyses of variance (ANOVA: Fisher’s test for post-hoc multiple comparisons). Statistical significance was assigned at the p < 0.05 level. The average apoptotic index was plotted using Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

RESULTS

The median number of cells in each coverslip was 548 (range: 248–1006). The AI of sham-irradiated hippocampal neurons was 18 ± 9% (n = 20) (Fig. 3). The AI of cells irradiated with X-rays at 10 Gy and 30 Gy were 15 ± 13% and 22 ± 5%, respectively (n = 10). The AI was not significantly increased with X-rays at 30 Gy (p = 0.19). The AI in cells irradiated with carbon-ion beams at 1 Gy, 3 Gy, 5 Gy and 10 Gy were 21 ± 9%, 23 ± 6%, 24 ± 6% and 33 ± 7%, respectively (n = 10). The AI with carbon-ion beam at 10 Gy irradiation was significantly increased compared with the sham control (p < 0.01). In simple regression analysis, the gradients of the linear line of carbon-ion beams and X-rays were 1.42 and 0.138, respectively. Hence, the relative biological effectiveness (RBE) of carbon-ion beams to X-rays on immature hippocampal neurons was calculated as 10.2 by simple regression analysis (p = 0.02).

DISCUSSION

It is generally believed that non-proliferating cells, such as neurons, are more radioresistant than proliferating cells, including co-existing cells in the brain (e.g. glial cells and vascular endothelial cells); however, Gobbel et al. reported that the response of postmitotic non-proliferating neurons to irradiation with X-rays was comparable to that of proliferating astrocytes, and further suggested that the slow repair of DNA damage played a role in the susceptibility of irradiated postmitotic neurons to apoptosis. Fukuda et al. showed that 4–12 Gy of X-rays to brain of juvenile rats and mice induced apoptosis in SVZ and SGZ of cerebrum where neural precursor cells and immature neurons exists abun-
mutated and null p53 gene.\(^3\) RBE values of wild-type, of three cell lines: human lung cancer cells with wild-type, in the dose and LET dependent manner.\(^2\) It was found that proliferating cells and immature neurons in the dentate SGZ in the part in which immature neurons and neuronal precursor cells exist is easy to be influenced by the irradiation, even if it is adult.

We showed that carbon-ion beams caused apoptosis in immature hippocampal neurons in a dose-dependent manner. In addition, although X-rays did not significantly cause apoptosis, the RBE value of carbon-ion beams to X-rays on immature hippocampal neurons was calculated to be 10.2. Because it was reported that the mean RBE value in the literature was 2.1,\(^7\) the RBE value in this report was more high. In general, the RBE values of carbon-ion beams vary by LET and cell lines, and also endpoints. Ando reported high. In general, the RBE values of carbon-ion beams to X-rays ranged from 1.1 to 1.8.\(^29\)–\(^31\) Aoki \(et\ al\) induced apoptosis. In these examinations, the RBE values of carbon-ion beams ranged from 1.1 to 1.8.\(^29\)–\(^31\) Aoki \(et\ al\) compared RBE values from apoptosis with those from the colony formation assay, and reported that the maximum RBE value from apoptosis was 1.6 and that from the colony formation assay was 5.5 when 110 keV/\(\mu\)m carbon-ion beams were used.\(^29\) Takahashi \(et\ al\) compared RBE values of three cell lines: human lung cancer cells with wild-type, mutated and null \(p53\) gene.\(^3\) RBE values of wild-type, mutated, and null genes were 1.34, 1.38, and 1.39, respectively, and were not significantly different. In in vivo study, Karger \(et\ al\) calculated the RBE value of carbon-ion beams in a rat brain using tolerance doses that changed the signal of magnetic resonance imaging (MRI), and they were 1.95 ± 0.20 and 1.88 ± 0.18 for T1- and T2-weighted MRI.\(^3\) We previously reported that the RBE value of carbon-ion beams for apoptosis induction in growing neurons is 2.5–2.9.\(^3\) In the report, two different chick neuronal tissues, dorsal root ganglion and sympathetic ganglion chain were employed. These RBE values were different from the RBE value in this paper because these neurons were growing neurons but not immature neurons, and these were not from rats. From the above findings, the RBE value in this report was high, and carbon-ion beams were effective for apoptosis induction in immature hippocampal neurons, although the examination conditions differed from the others.

Mizoe \(et\ al\) reported the results of treatment combining 50 Gy of X-ray, ACNU and 24.8 GyE carbon-ion beam radiotherapy for patients with malignant gliomas.\(^34\) They reported that the late adverse effects of the patients were Grade 2 or less with no evidence of Grade 3 or above, and concluded that the toxicity of carbon-ion beams was tolerable. Although the RBE of carbon-ion beams on immature neurons is higher, carbon-ion beam therapy for brain tumors may be possible with careful dose planning from the report; however, necrotic changes in the cortex and hippocampus after irradiation with carbon-ion beams were reportedly more severe than those in the lateral and posterior thalamus.\(^6\) This result may correlate with immature neurons existing in the hippocampus and around the anterior horn of lateral ventricles. It is therefore recommended to avoid irradiation of these areas as far as possible.

In conclusion, we showed that apoptosis of hippocampal neurons increased in a dose-dependent manner following carbon-ion beams irradiation. Additionally, carbon-ion beams were 10-fold more effective than X-rays for apoptosis induction in the immature hippocampal neurons. Carbon-ion beam therapy for brain tumors should be performed carefully.

**ACKNOWLEDGEMENTS**

This work was supported by the Gunma University 21\(^{\text{st}}\) Century COE Program funded by the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT).

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1160–1171.

Received on April 22, 2010
Revision received on June 28, 2010
Accepted on August 6, 2010
J-STAGE Advance Publication Date: October 6, 2010