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Carbon-ion beams effectively induce growth inhibition and apoptosis in human neural stem cells compared with glioblastoma A172 cells

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ABSTRACT

Carbon-ion radiotherapy (CIRT) holds promise in the treatment of glioblastoma, an aggressive X-ray–resistant brain tumor. However, since glioblastoma cells show a highly invasive nature, carbon-ion (C-ion) irradiation of normal tissues surrounding the tumor is inevitable. Recent studies have revealed the existence of neural stem cells in the adult brain. Therefore, the damaging effect of C-ion beams on the neural stem cells has to be carefully considered in the treatment planning of CIRT. Here, we investigated the growth and death mode of human neural stem cells (hNSCs) and glioblastoma A172 cells after X-ray or C-ion beam irradiation. The X-ray dose resulting in a 50% growth rate (D_{50}) was 0.8 Gy in hNSCs and 3.0 Gy in A172 cells, while the D_{50} for C-ion beams was 0.4 Gy in hNSCs and 1.6 Gy in A172 cells; the relative biological effectiveness value of C-ion beams was 2.0 in hNSCs and 1.9 in A172 cells. Importantly, both X-rays and C-ion beams preferentially induced apoptosis, not necrosis, in hNSCs; however, radiation-induced apoptosis was less evident in A172 cells. The apoptosis-susceptible nature of the irradiated hNSCs was associated with prolonged upregulation of phosphorylated p53, whereas the apoptosis-resistant nature of A172 cells was associated with a high basal level of nuclear factor kappa B expression. Taken together, these data indicate that apoptosis is the major cell death pathway in hNSCs after irradiation. The high sensitivity of hNSCs to C-ion beams underscores the importance of careful target volume delineation in the treatment planning of CIRT for glioblastoma.

KEYWORDS: carbon-ion (C-ion) beams, neural stem cells, glioblastoma, radiosensitivity, apoptosis

INTRODUCTION

Glioblastoma is the most aggressive type of primary brain tumor and accounts for ~50% of all primary brain tumor cases [1]. Standard therapy for glioblastoma consists of maximum feasible surgical resection followed by concurrent chemoradiotherapy [2]. However, the clinical outcome of this therapy is unsatisfactory and many patients die within 2 years [1]. The X-ray–resistant behavior of glioblastoma observed in the clinic is also supported by the basic research [3, 4]. These data suggest that enhancement of radiotherapy intensity for glioblastoma is urgently needed.

Carbon-ion radiotherapy (CIRT) has generated a great deal of interest as a highly intensive radiotherapy compared with conventional X-ray radiotherapy, owing to the intensive energy deposition and the high cell-killing effect. Several clinical studies showing the excellent results of CIRT for X-ray–resistant tumors [5] highlights the potential of CIRT as a curative treatment modality for glioblastoma. On the

other hand, glioblastoma has a highly invasive nature that allows infiltration of tumor cells to adjacent normal tissues. Therefore, in radiotherapy for glioblastoma, irradiation to normal cells surrounding the tumor is inevitable, even using carbon-ion (C-ion) beams [6].

Recent research has indicated that the subventricular zone (SVZ), a frequent predisposition site of glioblastoma, is vulnerable to X-ray irradiation; irradiation of the SVZ can lead to late toxicity such as neurocognitive disorders [7]. Importantly, it is known that the SVZ harbors neural stem cells (NSCs) playing pivotal roles in self-renewal during neurogenesis after brain damage [8]. Although several previous studies have investigated the sensitivity of NSCs to X-rays and γ-rays [9–14], the effect of C-ion beams on NSCs is not well understood. The present study aimed to determine the biological basis for optimal treatment planning strategy in CIRT for glioblastoma. To this end, we evaluated the sensitivities of human NSCs (hNSCs) and glioblastoma cells to C-ion beams and X-rays, and analyzed the mode of cell death.

MATERIALS AND METHODS

Cell culture

hNSCs derived from NIH-approved H9 (WA09) human embryonic stem cells (*TP53*-wild-type) [15] were cultured in KnockOut™ DMEM/F-12 supplemented with 2% StemPro® Neural Supplement, 2 mM GlutaMAX, 20 ng/ml basic fibroblast growth factor, and 20 ng/ml epidermal growth factor. The cell culture dishes were coated with CTS™ CELLstart™ substrate in Dulbecco's Phosphate-Buffered Saline CTS™ supplemented with calcium chloride and magnesium chloride. Human glioblastoma A172 cells (*TP53*-wild-type; ATCC, VA, USA) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS; Biowest, Nuaille, France), 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, 4 mM L-glutamine, and 1% penicillin–streptomycin. In the cell growth and death assays, the culture media for both cell lines was replaced with fresh media, or fresh media was added every 48 h after cell seeding. All reagents except for FBS were purchased from Gibco (Carlsbad, CA, USA).

Irradiation

Cells were irradiated with X-rays or C-ion beams. X-ray irradiation (150-kVp, ~1 Gy/min) was performed using MBR-1520R-4 (Hitachi Power Solutions, Ibaraki, Japan). C-ion beam irradiation (290 MeV/nucleon; an average linear energy transfer at the center of a 6-cm spread-out Bragg peak, 50 keV/μm) was performed at Gunma University Heavy Ion Medical Center (GHMC; Gunma, Japan) [16].

Cell growth assay

hNSCs and A172 cells were collected using TrypLE™ Express (Gibco) and trypsin-EDTA solution (Immuno-Biological Laboratories, Gunma, Japan), respectively, and suspended in culture medium. The cells were counted using a hemocytometer just before irradiation (0 h) and every 24 h after irradiation. The relative growth rate of cells receiving a given dose was calculated by dividing the difference in the number of cells between 0 h and 72 h by that of non-irradiated cells (n.b. the non-irradiated cells showed exponential growth within 72 h). The dose–response curves were generated by fitting the data plots to the exponential approximation, from which D_{50} (a dose giving 50% growth rate) was calculated. Finally the relative biological effectiveness (RBE) values of C-ion beams were calculated by dividing the D_{50} for X-rays by that for C-ion beams [17, 18].

Cell death assay

Radiation-induced apoptosis and necrosis were determined by fluorescence staining with acridine orange (Dojindo laboratories, Kumamoto, Japan) and ethidium bromide (Wako, Osaka, Japan) (Supplementary Fig. 1) as previously described [19, 20]. The percentages of apoptotic or necrotic cells were calculated with normalization to the total number of cells. The dose–response curves were generated using the polynomial approximation by KaleidaGraph software (Hulinks, Tokyo, Japan) (Supplementary Fig. 2) as previously described [21]. The RBE values were calculated using the coefficients of the linear components of the curve-fitting–formulae (Supplementary Table 1). At least 400 cells were counted per sample.

Immunoblot analysis

Immunoblot analysis was performed as previously described [22]. The antibodies used were listed in Supplementary Table 2.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) of three independent experiments. Statistical significance was assessed by a one-way ANOVA followed by Tukey's *post-hoc* test. A *P*-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

First we examined the sensitivity of hNSCs and A172 cells to X-rays or C-ion beams. The radiosensitivity was assessed by counting the total number of adherent cells on culture dishes because hNSCs lack colony-forming ability [23]. In the absence of irradiation, the cell growth speed was comparable between hNSCs and A172 cells (Fig. 1); the population doubling time was 28.2 ± 1.5 h in hNSCs and 23.9 ± 1.7 h in A172 cells. X-ray irradiation for 2 Gy and 10 Gy significantly suppressed the growth of hSNCs (Fig. 1A). The X-ray irradiation also suppressed the growth of A172 cells. However, the effect was much smaller than that observed in hNSCs; A172 cells kept proliferating after irradiation for 10 Gy (Fig. 1C). C-ion beams showed a greater growth-suppression effect in both cell lines than that induced by X-rays (Fig. 1B and D). Notably, 10 Gy C-ion beams completely suppressed the growth of A172 cells over 96 h post-irradiation (Fig. 1D).

To further investigate the effect of X-ray and C-ion beam irradiation on the growth of hNSCs and A172 cells, we generated the dose–response curves (Fig. 2) and calculated the D_{50} and the RBE values (Supplementary Table 1). The D_{50} for X-rays was 0.8 Gy in hNSCs and 3.0 Gy in A172 cells. The D_{50} for C-ion beams was 0.4 Gy in hNSCs and 1.6 Gy in A172 cells. Consequently, the RBE value of C-ion beams at the D_{50} was 2.0 in hNSCs and 1.9 in A172 cells. The RBE value in A172 cells in the present study was comparable with the previously reported RBE value in glioblastoma cells assessed by clonogenic assay [24], indicating the validity of the cell-number counting method for radiosensitivity assessment. Together, these data indicate that hNSCs are ~4-fold more sensitive to X-rays and C-ion beams than A172 cells; the RBE values of C-ion beams over X-rays were comparable between hNSCs and A172 cells; C-ion

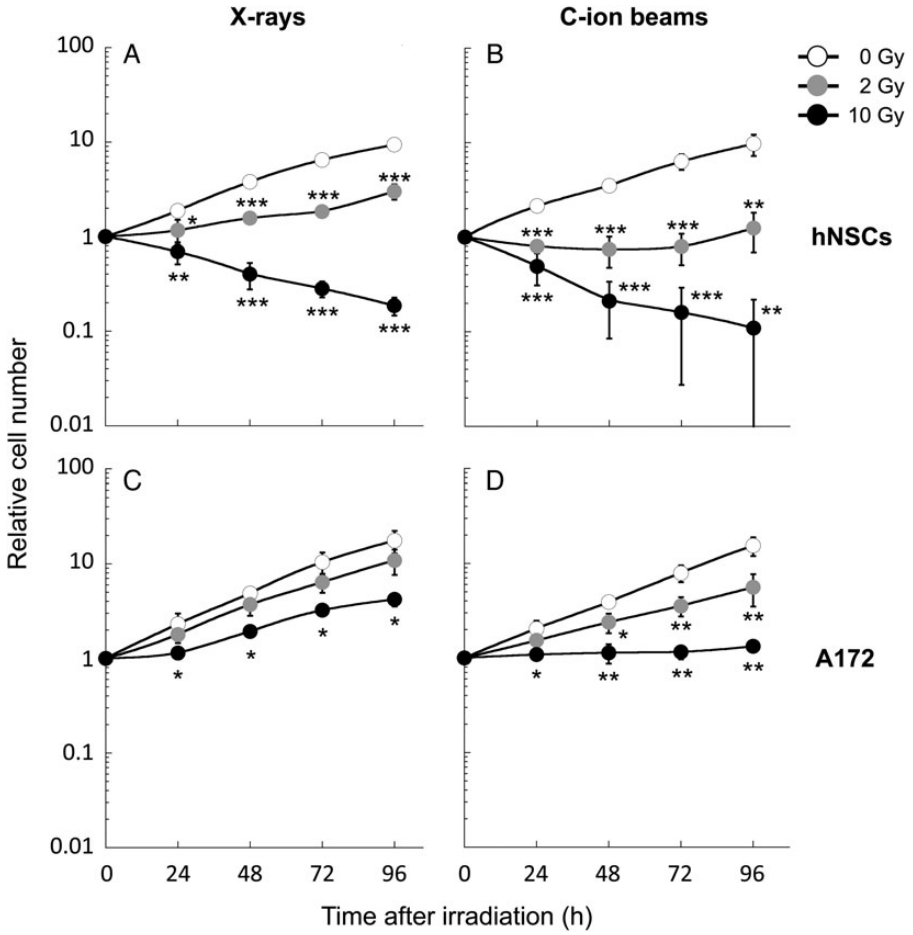

Fig. 1. Proliferation of hNSCs and A172 cells irradiated with X-rays or carbon-ion (C-ion) beams. The number of cells was counted every 24 h after irradiation and is shown after normalization to the number of non-irradiated controls at 0 h. (A) hNSCs irradiated with X-rays. (B) hNSCs irradiated with C-ion beams. (C) A172 cells irradiated with X-rays. (D) A172 cells irradiated with C-ion beams. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the corresponding control.

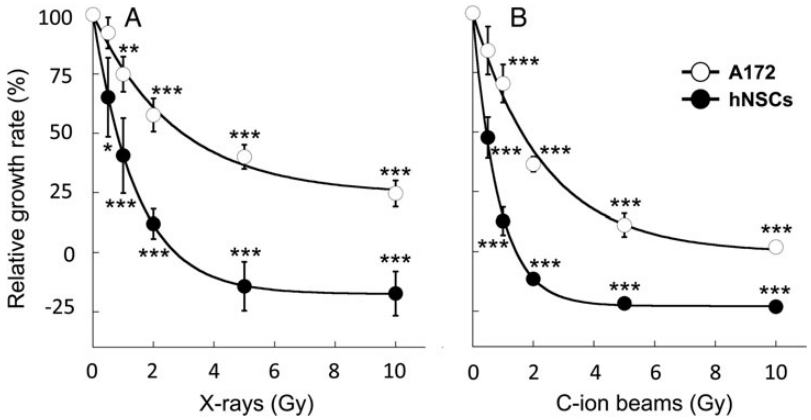

Fig. 2. Dose–response curves of hNSCs and A172 cells irradiated with X-rays or carbon-ion (C-ion) beams. The relative growth rate was calculated by dividing the difference in the number of cells irradiated with a given dose between 0 and 72 h by the difference in the number of non-irradiated cells between 0 and 72 h. The data plots of the relative growth rate were fitted to the exponential approximation to generate dose–response curves. (A) X-rays. (B) C-ion beams. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with the corresponding control.

beams more effectively suppress the growth of A172 cells than do X-rays.

To further investigate the effect of X-rays and C-ion beams on hNSCs and A172 cells, we next assessed the induction of apoptosis and necrosis after irradiation. hNSCs showed remarkably high sensitivity to apoptosis after X-ray irradiation; ~90% of X-ray–irradiated hNSCs underwent apoptosis (Fig. 3A). Efficient apoptosis induction in hNSCs was also observed after C-ion beam irradiation, and was slightly greater than that observed after X-ray irradiation (Fig. 3B). Necrosis was rarely observed in hNSCs after X-ray or C-ion beam irradiation (Fig. 3A and B). These results indicate that the high sensitivity to radiation-induced apoptosis underlies the high sensitivity to X-rays and C-ion beams in hNSCs. In contrast, A172 cells did not show evident induction of apoptosis or necrosis after X-ray irradiation (Fig. 3C). C-ion beams significantly induced apoptosis and necrosis in A172 cells, although the fractions were small (Fig. 3D). These results indicate the apoptosis-resistant nature of A172 cells.

Finally we investigated the mechanisms underlying the difference in the level of the radiation-induced apoptosis between hNSCs and A172 cells by immunoblot analysis (Fig. 4). In hNSCs, the levels of cleavage of poly (ADP-ribose) polymerase-1 (PARP-1), a marker for apoptosis [25], were increased after irradiation, and were greater for C-ion beams than for X-rays. In contrast, cleaved PARP-1 was undetectable in the X-ray or C-ion beam–irradiated A172 cells. These results were consistent with the results of the cell death assay, demonstrating the greater induction of apoptosis by C-ion beam irradiation than by X-ray irradiation in hNSCs, and the apoptosis-resistant nature of A172 cells after both X-ray and C-ion beam irradiation (Fig. 3). Interestingly, in hNSCs but not in A172 cells, phosphorylation of p53 at Ser15 was maintained at high levels after X-ray and C-ion beam irradiation over 24 h. Since phosphorylation of p53 at Ser15 plays a key role in inducing apoptosis after ionizing irradiation [26, 27], these data suggest that the prolonged upregulation of p53 phosphorylation contributes to the apoptosis-susceptible nature of hNSCs. Previous study demonstrated the involvement of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) signaling in irradiation-induced apoptosis in hNSCs [14]. Therefore, we checked the TRAIL expression; however, it was undetectable in our experimental systems (data not shown). On the other hand, we found that A172 cells, but not hNSCs, express high levels of the p65 subunit of nuclear factor kappa B (NF-κB) regardless of the presence or absence of X-ray or C-ion beam irradiation. It is known that activated NF-κB suppresses apoptosis induction by inhibiting caspase 3 and caspase 9 [28]. Moreover, the aberrant constitutive activation of NF-κB is known to be one of the major mechanisms underlying the treatment-resistant nature of glioblastoma [29]. With these facts taken together, these data suggest that the high basal level of NF-κB expression contributes to the

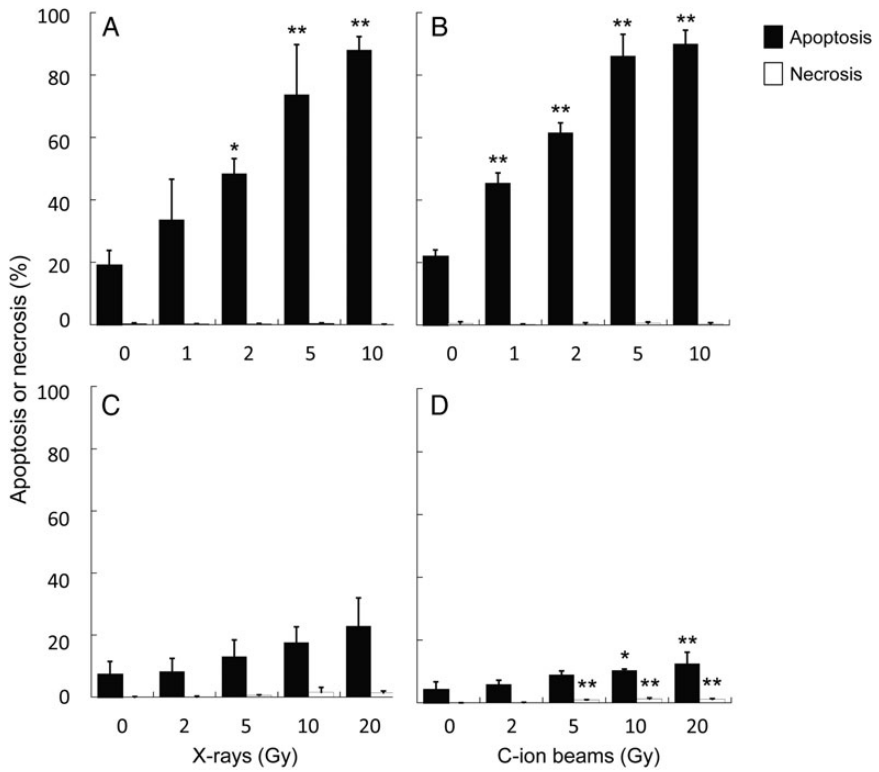

Fig. 3. Apoptosis and necrosis induction by X-ray or carbon-ion (C-ion) beam irradiation in hNSCs and A172 cells. Cells were simultaneously stained with acridine orange and ethidium bromide at 72 h after irradiation and analyzed for apoptosis and necrosis (see Materials and Methods and Supplementary Fig. 1 for definitions of apoptosis and necrosis). (A) hNSCs irradiated with X-rays. (B) hNSCs irradiated with C-ion beams. (C) A172 cells irradiated with X-rays. (D) A172 cells irradiated with C-ion beams. The percentages of apoptotic or necrotic cells were calculated following normalization to the total number of cells. At least 400 cells were counted per sample. *$P < 0.05$ and **$P < 0.01$ compared with the corresponding control.

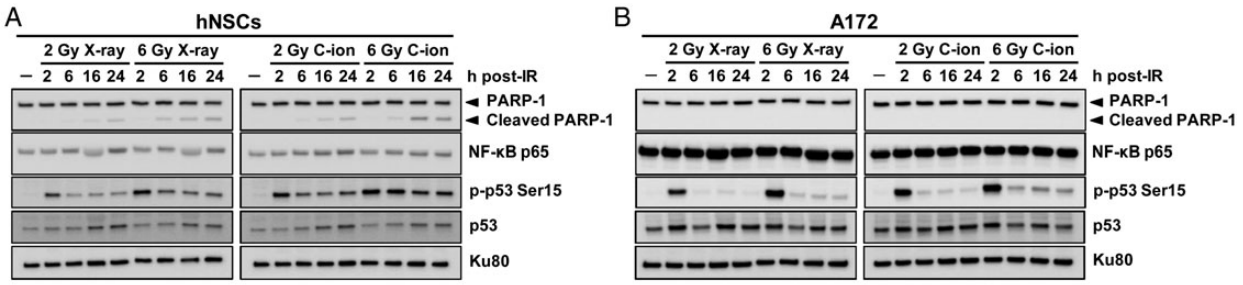

Fig. 4. Immunoblot analysis for the apoptosis-associated proteins in hNSCs and A172 cells after X-ray or carbon-ion (C-ion) beam irradiation. Cells were irradiated with 2 or 6 Gy of X-rays or C-ion beams. Levels of PARP-1 cleavage, p65 subunit of NF-κB (NF-κB p65), phosphorylated p53 at Ser15 (p-p53 Ser15) and p53 were detected by immunoblot analysis at 2, 6, 16 and 24 h post irradiation (IR). Ku80 was used as a loading control. (A) hNSCs. (B) A172 cells.

apoptosis-resistant nature of A172 cells after X-ray and C-ion beam irradiation.

The high sensitivity of hNSCs to radiation-induced apoptosis may partly be attributable to the fact that stem cells are programmed to preferentially undergo apoptosis to prevent the inheritance of damaged DNA, such as mutations, by daughter cells [30]. In accordance with this, hNSC showed a moderately high (~20%) background for apoptosis (Fig. 3A and B). In contrast, it is likely that modes for growth suppression other than apoptosis may occur in the irradiated A172 cells, because the RBE value calculated from the apoptosis data is much lower than that calculated from the cell growth data (0.7 vs 1.9; Supplementary Table 1 and Supplementary Fig. 2). Previous studies have demonstrated the induction of senescence and mitotic catastrophe in C-ion beam–irradiated glioblastoma cells [31, 32]. Such modes of cell arrest should be analyzed in the future.

The RBE value of 3.0 is used for CIRT in Japan. This value is based on the results of the clonogenic assay using human salivary gland tumor cells [16, 33]. However, recent studies suggest that the sensitivities of cells to X-rays and C-ion beams varies widely among cancer types and, more importantly, among normal tissues [34, 35]. In relation to this, the results of the present study will provide an insight into the RBE value of C-ion beams in NSCs.

In summary, the results of this study indicate that (i) hNSCs are highly sensitive to X-rays and C-ion beams, relative to glioblastoma cells; (ii) the RBE value of C-ion beams is comparable between hNSCs and glioblastoma cells; (iii) the high sensitivity of hNSCs to X-rays and C-ion beams may be partly due to their high susceptibility to apoptosis; and (iv) cellular outcomes other than apoptosis and necrosis may partly contribute to the efficient growth-suppression effect of C-ion beams in glioblastoma cells. To the best of our knowledge, this is the first report of the RBE value of C-ion beams in hNSCs. The high sensitivity of hNSCs to C-ion beams underscores the importance of careful target volume delineation in the treatment planning of CIRT for glioblastoma.

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