Original Article
Comparisons between bio-radiation effects of X-rays and carbon-ion irradiation on glioma stem cells

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Abstract: Heavy-ion therapy has demonstrated significant benefits such as well-defined range, small entrance dose and high relative biological effectiveness, but the characteristics of heavy-ion induced radio-biological effects on cancer stem cells is not completely clear. We used human glioma cancer stem cells and glioma virus-induced mice model irradiated by carbon ions or X-rays to investigate whether heavy ions offering a radio-biological advantage, by effectively eliminate cancer stem cells, in comparison with conventional X-rays. In vitro experiments, the repair rate of DNA damage generated by 2 Gy carbon ions was lower than that by X-rays in glioma stem cells. Micronucleus assay showed the micronuclei frequency induced by carbon ions was higher than that by X-rays. MTT assay showed the cellular viability of glioma stem cells irradiated by 1-4 Gy carbon ions was lower than that by X-rays. Results of in vivo experiment demonstrated carbon-ion radiation could significantly reduce the radio of CD133+ glioma stem-like cells compared with X-rays. In conclusion, carbon ions showed radio-biological advantage over X-rays by effectively eliminate glioma cancer stem cells both in vitro and in vivo, which have significant importance in understanding the high relative biological effectiveness related to heavy-ion therapy.

Keywords: Heavy-ions, X-rays, glioma, cancer stem cells, irradiation

Introduction

The charged heavy-ion (HI) beams allow for a precise localization of the radiation dosage to the tumor region because of the inverted depth-dose profile and the sharp dose fall-off behind the Bragg peak [1]. Due to this high precision, the surrounding healthy tissues receive a much lower dose compared to conventional radiotherapy with photons, such as X-rays. The heavy-ion therapy has the advantage both in physics and in biology. The high linear energy transfer (LET) of carbon ion radiation has shown a higher relative biological effectiveness (RBE) compared to conventional low-LET photon radiation. Therefore, the efficiency of carbon ions inducing DNA damage, cell cycle arrest and cell death in tumor cells is higher than X-rays [2-4]. Even on radio-resistant (with respect to X-rays) tumors, carbon ions still have high lethality [5-7].

Glioma is one of the most common primary tumors of intracranial central nervous systems [8]. About 28% of all primary intracranial tumors and 80% of malignant tumors are gliomas [9]. Gliomas are known for invasive growth, high recurrence rate, special location, being difficult to resect completely by surgery, even in combination with conventional radiotherapy and chemotherapy, it’s still hard to be eradicated [10-13].

Recently, the discovery, isolation, and existence confirmation of glioma stem cells (GSCs) are significant breakthroughs in the study of gliomas. GSCs are small numbers of stem cell like cells in solid tumors with potential of unlimited growth.
X-rays and carbon-ion irradiation effects on GSCs

proliferation, self-renewal, multiple differentiation and high tumorigenicity [14]. GSCs play a decisive role in the formation, growth, invasion, metastasis, recurrence, and treatment sensitivity of gliomas [15]. Cancer stem cells (CSCs) maintain tumor growth by keeping the balance between self-renewal, proliferation and differentiation. Though CSCs occupy a tiny minority of gliomas, they are the fundamental reason for the formation and recurrence of gliomas [16]. CSCs hypothesis suggests that CSCs populate an original tumor, which is resistant to treatments, and repopulate the recurrent tumor after most of the tumor has been removed [17]. CSCs research is one of the most thriving and competitive areas in oncology research because it has the potential to become a predictive factor in radiotherapy [18].

Currently, carbon-ion radiotherapy has been approved for treatment of specific types of cancers including melanoma, chordoma and glioma [19]. According to results of heavy-ion irradiation therapies from LBL (Lawrence Berkeley Laboratories), NIRS (National Institute of Radiological Sciences), GSI (Gesellschaft für Schwerionenforschung) and IMP (Institute of Modern Physics), this kind of therapy does indeed appear to have clinical advantages over other modalities such as photon irradiation [20-22]. The use of heavy ion therapy is becoming more and more extensive. We speculate that there's a relationship between heavy-ion irradiation therapy's excellent therapeutic effects and its prominent lethality to CSCs. However, relevant studies have not been reported.

In the present study, human glioma CSCs and murine models of virus induced glioma were used to investigate whether heavy ions offered a biological advantage over conventional X-rays in glioma. Our work may provide a useful support of heavy-ion tumor therapy against glioma for the future applications.

Materials and methods

Cell culture

The GSC-3 (No. 3 glioma cancer stem cells) cells were a gift from Kunming Institute of Zoology (KIZ, Chinese Academy of Science, Kunming, China).

The GSC-3 cells were maintained at 37°C with 5% CO$_2$ in serum-free DMEM/F12 medium (Gibco, Life Technologies, NY, USA) with B-27 Supplement (Gibco, Life Technologies, NY, USA), 20 ng/ml epidermal growth factor (EGF, Pepro Tech, Rocky hill, NJ, USA), 20 ng/ml basic fibroblast growth factor (bFGF, Pepro Tech, Rocky hill, NJ, USA).

The GSC-3 cells were digested with TrypLE Express (Gibco, Life Technologies, NY, USA) instead of conventional trypsin enzyme. Before the cells were seeded, in order to make the cells adhered, all the flasks and plates and dishes needed to be disposed by Laminin (Sigma, St. Louis, MO, USA).

In vivo experiment

All the animal experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health, and approved by the Ethics Committee of Kunming Institute of Zoology, Chinese Academy of Sciences.
The mice were provided and pre-treated in Kunming Institute of Zoology. Briefly, 6-week old male Kunming mice were selected for the experiment. The weight of each mouse was 18-22 grams. The virus (ptomo-ras-sip53) was injected into the brain as the position showed in Figure 1A and 1B to induce tumor formation. Each mouse was weighed and was administrated with different dosage of ketamine by intraperitoneal injection. The titer of virus was 1.06*10^10/mL. The total injection volume was 1 μL and injection velocity was 0.1 μL/min for each mouse. Three days after the injection, the mice were transferred to Lanzhou IMP (Chinese Academy of Science, Lanzhou, China) by air. Finally, 20 mice, in good condition, were separated into 3 groups (6 for control, 7 for carbon ions and 7 for X-rays). The tumor of each mouse was irradiated by carbon ions or X-rays 3 weeks after virus injection. Each mouse was dissected with the brains being taken out immediately after it’s sacrificed.

**X-ray irradiation**

The GSC-3 cells or mice were irradiated with conventional X-rays generated by Faxitron RX-650 (Faxitron Bioptics, Lincolnshire, IL, USA), which was operated with 60 kVp 5 mA at room temperature. The calculated dose rate was 0.46 Gy/min. GSC-3 cells were irradiated with 0, 1, 2, 3, 4 Gy of X-rays, and the mice were irradiated with 3.16 Gy of X-rays. For mice irradiation, X-rays struck on the tumor area (injection hole as the center) vertically through an 8*8 mm hole in a 3 mm thick lead plate.

**Carbon ion irradiation**

Cells and mice were irradiated with 0, 1, 2, 3, 4 and 3.16 Gy of carbon ions at the cancer treatment room of HIRFL-CSR (Heavy Ion Research Facility in Lanzhou-Cooler Storage Ring) facility at the IMP. The initial energy of the carbon ion beams was 165 MeV/u. For mice irradiation, we used plateau region of the beam (LET was 17.96 KeV/μm) to get a penetrate irradiation for the brain tissues and the beam struck on the tumor area horizontally through an 8*8 mm channel structured by 4 lead bricks.

**53BP1 & XRCC1 foci**

Irradiated cells were fixed for 10 minutes in 4% paraformaldehyde, permeabilized for 5 minutes in methanol at -20°C, blocked for 1 hour with 5% skim milk, and stained with rabbit anti-53BP1 antibody (Upstate Biotechnology, Lake Placid, NY, USA) or mouse anti-XRCC1 antibody (Upstate Biotechnology, Lake Placid, NY, USA) for 2 hours. The bound antibody was visualized using Alexa Fluor® 594 anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) or Alexa Fluor® 488 anti-mouse antibody (Molecular Probes, Eugene, OR, USA) and cell nuclei were counter-stained with DAPI (PharMingen, San Jose, CA, USA). Slides were observed under a Zeiss LSM700 confocal laser scanning microscope (Zeiss, Jena, Germany). At least 100 cells were scored for each sample and the average number of foci per cell was calculated.

**Micronucleus assay**

Right after irradiation, cells were collected and reseeded in 12-well plates. Simultaneously, 2.5 μg/mL of cytochalasin B (Sigma, St. Louis, MO, USA) was added into each well. Thirty-six hours later, cells were washed with PBS and fixed with methanol-glacial acetic acid (3:1, V/V). After being stained with 150 μg/mL acridine orange, at least 500 of binucleated cells for each sample were counted.

**MTT assay**

Cells were collected and reseeded in 96-well plates, and incubate till 70% of well filled with single layer of cells. Irradiate plates with dosage gradient of 0, 1, 2, 3, 4 Gy carbon ions and X-rays, 10 duplications for each group. After irradiation, keep incubating plates, and observe cell growth under microscope. At the time when cells of control group almost fill the plate, add 5 mg/mL MTT solution into each well and incubate 4 hours, aspirate media, add 150 μL DMSO (set blank group with only DMSO), and vibrate by table concentrator for 10 minutes to dissolve crystal substance completely. The values of OD490 and OD630 were detected by Infinite m200-pro microplate reader (TECAN, Mannedorf, Switzerland). For all groups, subtract the OD630 values from the OD490 values, then calibrated by subtract blank group values. Finally, the results were normalized dividing by control group.

**Immunohistochemistry**

All the fresh brain samples were fixed immediately by 4% paraformaldehyde for 72 hours,
Figure 2. Repair rate of DNA damage generated by carbon-ion was lower than that generated by X-rays in CSC. A. 53BP1 foci per cell of the #3 GSCs after irradiated with 2 Gy X-rays or carbon ions. B. 53BP1 foci (DSBs) repair rates
of the #3 GSCs after irradiated with 2 Gy X-rays or carbon ions. C. XRCC1 foci per cell of the #3 GSCs after irradiated with 2 Gy X-rays or carbon ions. D. XRCC1 foci (SSBs) repair rates of the #3 GSCs after irradiated with 2 Gy X-rays or carbon ions. E. 53BP1 and XRCC1 immunofluorescence assay of the #3 GSCs after irradiated with 2 Gy carbon ions. F. 53BP1 and XRCC1 immunofluorescence assay of the #3 GSCs after irradiated with 2 Gy X-rays. The graphs show the mean, standard error and p value, they were calculated from at least 3 independent experiments.

Figure 3. Carbon-ion irradiation induced micronuclei more efficiently than X-rays. A. The frequency of binucleate (BN) cell with micronucleus (MN) of the #3 GSCs after irradiated with 0-3 Gy X-rays or carbon ions. B. Micronucleus frequency (MNF) of the #3 GSCs after irradiated with 0-3 Gy X-rays or carbon ions. The graphs show the mean, standard error and p value, they were calculated from at least 3 independent experiments.

Figure 4. Cell viability of #3 GSCs irradiated by 0-4 Gy carbon-ion and X-rays.

Statistics
All experiments were independently repeated at least three times and all data were presented as the means ± standard error. Student’s t-tests were used for statistical analysis. Probability (p) values less than 0.05 were considered to be statistically significant.

Results
The repair rate of DNA damage generated by carbon-ion was lower than that generated by X-rays in CSCs

After cells were irradiated by 2 Gy of X-rays or carbon ions, CSCs samples were taken and stained by immunofluorescence assay at different times points to observe the differences of...
induced DNA single strand breaks (SSB, XRCC1 foci) and double strand breaks (DSB, 53BP1 foci) between the CSCs samples irradiated by X-rays or carbon ions. As could be seen from the results (Figure 2A, 2E and 2F), the initial yield of CSCs DSBs induced by X-rays was more effective than by carbon ions. But as time went on the difference became slight. As the breaks of double strands were repaired, the number of DSBs was almost equal at 24 hours post irradiation. We further calculated the DSBs repair rate (Figure 2B) and found that the repair rate of carbon ions was lower than the rate of X-rays, but there was no significant difference between them. As for SSBs, the absolute foci yield showed that X-rays still induced more initial foci than carbon ions (Figure 2C, 2E and 2F), but the damage generated by X-rays was repaired faster over time. The repair rate of carbon ions was significantly lower than the repair rate of carbon ions.

**Figure 5.** Compared with X-rays, carbon-ion could significantly kill the glioma stem cells in the mouse brains. A. The example of circled tumor area and CD133+ highlighted area in three different groups. B. Ratio of tumor area CD133+ cells in total cells in three different groups. C. The photos of mice’s brains in three different groups. The brains were taken out immediately after mice’s sacrifice. The graphs show the mean, standard error and p value, they were calculated from at least 3 independent experiments.
X-rays, and the difference became larger and larger over time (Figure 2D).

**Carbon-ion irradiation induced micronuclei more efficiently than X-rays**

In micronucleus assay, the frequency of binucleated cells with micronucleus (Figure 3A) and micronucleus frequency (Figure 3B) were collected. By analyzing two slopes of the trend line given by the frequency of binucleated cells with micronucleus versus 0-3 Gy and micronucleus frequency versus 0-3 Gy, both two frequencies of CSCs irradiated by carbon ions are significantly (P=0.017 & P=0.041) higher than the frequencies of CSCs irradiated by X-rays.

**In MTT assay, compared with X-rays, heavy-ion could significantly reduce GSCs viability**

The viability of GSC-3 cells was detected by MTT assay after carbon ion and X-ray irradiation from 0 to 4 Gy and then graphed separately (Figure 4). From the results, it can be seen that the GSCs viability irradiated by carbon ions are significant lower than that irradiated by X-rays for all doses.

**Compared with X-rays, carbon-ion could significantly (P=0.0004) kill the glioma stem cells in the mouse brains**

After irradiation and the mice were sacrificed, we took out the brains to make paraffin sections through the injection holes for the immunohistochemical assay. After stained with CD-133, the whole section was photographed under microscope one field after another and then pieced together by Adobe Photoshop. The tumor area was circled cautiously and cut out manually, the CD133-stained area within was highlighted according to how red it is (Figure 5A). We repeated these steps for each section under the same settings and calculated the pixels of total highlight area and tumor area of each section separately. The value of the pixels of total highlight area divided by pixels of total tumor area was used to get the percentage of CD133⁺ cells in the tumor area.

According to the above method, we could get the ratio of CD133⁺ cells in total cells (Figure 5B). As could be seen, the ratio of CD133⁺ cells irradiated by carbon ions was significantly (P=0.0076) lower than the ratio of CD133⁺ cells irradiated by X-rays. However, there was no significant difference between the X-ray treatment and control group.

From the photos of the brains (Figure 5C), we could observe that Control group had the densest blood vessels at the tumor region. At the same time, X-rays irradiated brain had a better condition and the carbon-ion irradiated brain had the best condition.

**Discussion**

In this study, we used both in vivo and in vitro GSC models to investigate whether carbon ions offered a radio-biological advantage over conventional X-rays. We observed that carbon ions showed a significantly higher lethality than X-rays in GSCs.

In cellular experiments, compared with sorted GSCs by surface marker, our use of GSCs as a cell model was more direct and persuasive [5, 23-25]. In the murine models of virus induced glioma, compared with injection of tumor cells into the groin to form glioma, the method to form glioma in the brain was more authentic.

Ionizing radiation causes both DSBs (53PB1 foci) and SSBs (XRCC1 foci) in CSCs. The initial yield of foci generated by X-rays was significantly higher than the yield of foci generated by carbon ions at the same dose. That is because the average energy deposited by a single photon of X-ray generator is much lower than the energy deposited by a single carbon-ion particle. The absolute flux of photons is much more than carbon-ion particles at the same dose [26, 27]. However, many studies in this area have examined that a single ion-generated focus (DNA damage site) may form clustered DNA damages which contain more than one DNA strand break and even multiple DNA damages, such as DSBs, SSBs, base damages, and high LET radiation is more efficient to induce cluster DNA damages [28]. Although the initial yield of DNA damage induced by X-rays was significantly higher than that induced by carbon ions, the clustered damages induced by carbon ions were much more difficult to repaired, and the difference between the repair rates of DNA damage induced by X-rays and carbon ions became larger as time went on. This result is more significant in SSB repair, which is also consistent with other researches [29].
higher residual level is the major response for the higher biological effectiveness of high LET radiation. According to the data of micronuclei frequency and binucleated cells with micronuclei, the GSC-3 cells were more resistant to X-rays than carbon ions, which is consistent with other experiment results on normal tumor cells [26, 30]. So, the complexity of DNA damages may plays a crucial role to influence the biology effects.

In the experiment, MTT was used to detect cell viability of GSC-3 cells which irradiated by X-rays and carbon ions. According to our results, from 1 Gy to 4 Gy of GSC-3 cells, the cell viability irradiated by carbon ions are all significant lower than that irradiated by X-rays. In the MTT assay section, at first we planned to use cell clonogenic survival experiment to get dose-survival curves of GSC-3 cells irradiated by 2 Gy X-rays and carbon ions. However, majority single cells could not form effective cloning in 20 days, cell survival experiment cannot be implement, so we used MTT assay instead of clonogenic survival method. There are intercommunities between MTT method and clonogenic survival method, as well as differences. The proliferation curve simulated by MTT method is gentler than the curve graphed in the clonogenic survival experiment, but both of them have the same general trend. Because the cell proliferation capacity tested by clonogenic survival is testing single cells which could proliferate among living cells, but MTT method tests the ratio of living cells, regardless of apoptosis, necrosis or cycle arrest. MTT detects all living cells therein. There were researches discussed the correlations between those two methods as early as 1988, they thought these two methods have a preferable correlation in studying radio-sensitivity of murine solid tumors [31-34].

CD133 was used as a surface marker of brain tumor stem cells for a long time. In 2004, Singh and the others isolated brain tumor stem cells through brain tumor stem cell surface marker CD133 [35]. The existence of tumor stem cells with CD133 surface marker has been proved in some cell lines, such as GL261, U251 and U87 [36-38]. In mice experiment, it can be seen that the tumor area of CD133+ highlighted cells of the carbon ions group was significantly smaller than the X-rays group. Altogether, it can be concluded that under the same dose, carbon-ion irradiation was more effective to induce radio-biological effects than X-rays both in vitro and in vivo. Carbon-ion irradiation could induce lethality in the GSCs more efficiently than X-rays. Carbon-ion irradiation provide a significant performance over X-rays in targeting and inducing lethality in GSCs. Based on these findings, we conclude that carbon-ion therapy is worthy to popularize to achieve better outcomes despite the high expense.

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Disclosure of conflict of interest

None.

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