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Cell death induced by high-linear-energy transfer carbon beams in human glioblastoma cell lines

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Abstract The cytotoxic effect of high-linear-energy transfer (LET) carbon beams on two human glioblastoma cell lines (A172 and TK1) was analyzed, especially concerning cell death, including apoptosis. Gamma-ray radiation was used for comparison. The results of standard colony formation assay showed that the survival fraction of each cell line decreased in an LET-dependent manner. The results of other direct cytotoxic assays, dye exclusion test, and lactate dehydrogenase (LDH) release assay, also displayed a similar relationship between the cytotoxic effect of carbon beams and LET. The maximum values of the cell death index (CDI) were 50.2% in A172 and 37.5% in TK1, both obtained on day 7 after exposure to carbon beams of 80keV/μm. Apoptosis was observed only on days 4 and 7 after carbon beam irradiation, with maximum values of 7% in A172 and 4.5% in TK1, and the induction of apoptosis after high-LET radiation could be p53-independent. This indicated that a combination of multiple assays to detect cell death was important in evaluating the radiosensitivity of tumor cells, because this approach could more precisely reflect the clinical effectiveness of radiotherapy.

Key words Glioblastoma · High-LET · Cell death · Apoptosis · Charged particle

Introduction

The prognosis of patients with glioblastoma is still unsatisfactory in spite of the enormous efforts that have been made so far. One of the major reasons for its poor prognosis is its high radioresistance. Conventional radiotherapy using gamma rays or X-rays on glioblastoma is dose-limited because of the adverse effects on the surrounding normal brain tissue. Therefore, lethal doses for killing tumor cells may not be achieved with this low-linear-energy transfer (LET) photon radiation. In contrast, high-LET radiation, such as heavy charged particles, has advantages that may overcome the disadvantages of gamma rays or X-rays. Briefly, heavy charged particles can provide a greater localization of energy to the targeted volume as well as greater cell-killing potency. Tsujii et al. state that low-LET proton beams, which also have sharp Bragg peak ionization, may be more acceptable for the treatment of brain tumors.

However, their conclusions are based on the relative biological effectiveness (RBE) for the late effect on surrounding normal brain tissue, not for the cytotoxic effect on tumor cells. We believe it is important to evaluate the cytotoxic effects of high-LET radiation on glioblastoma cells, as well as to improve radiation techniques to achieve greater localization of energy to minimize damage to normal tissue as much as possible.

Although the clinical effectiveness of radiotherapy is usually determined by the visible volume reduction rate of the tumor caused by tumor cell death, the clonogenic survival assay in vitro has been used so far to measure the cytotoxic effects of ionizing radiation. This method demonstrates the reproductive cell inactivity calculated from the number of cells that still have the ability to form a colony after a certain dose of irradiation, not the actual death of irradiated cells.

We evaluated the cytotoxic effect of carbon beams on glioblastoma cells in vitro, using not only the standard colony formation assay but also three other kinds of cytotoxic assays: the dye exclusion test, lactate dehydrogenase (LDH) release assay, and detection of apoptosis, focusing...
on tumor cell death. These methods can evaluate the cytotoxicity of high-LET radiation on human glioblastoma cells more directly.

### Materials and methods

#### Cell lines and culture conditions

Human glioblastoma cell lines A172<sup>6</sup> and TK1<sup>7</sup> were used. The cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum and 100U/ml penicillin, 100μg/ml streptomycin in a 5% CO<sub>2</sub> atmosphere at 37°C. Then, 1 × 10<sup>5</sup> cells were plated in culture flasks (Nunc T25) for 40-48 h before irradiation. For the transfer and irradiation of cells, the culture flasks were filled with media, and the caps were closed and tightly sealed with parafilm.

#### Irradiation

Gamma-ray irradiation was performed as a comparative study using 137Cs sources at a dose rate of approximately 1.2 Gy/min. A carbon beam of 290 MeV/u was generated by a Heavy Ion Medical Accelerator (HIMAC) at the National Institute of Radiological Science (NIRS) in Chiba, Japan. The LETs selected in this study were 20, 40, and 80 keV/μm. The cells in flasks were placed in a specially designed rack that automatically exposed the flasks in the track of carbon beams. In all radiation qualities, a single dose of 10 Gy was given to each flask at room temperature (approximately 26°C). Detailed procedures of dosimetry and LET measurement at biological cave in HIMAC have already been reported.<sup>8</sup>

#### Cytotoxic assays after irradiation

After exposure to gamma rays or carbon beams, three kinds of cytotoxic assays were carried out.

##### Colony-forming assay

Immediately after irradiation, the cells were trypsinized, counted, and plated in three 60-mm dishes. The dishes were incubated for 14-21 days before fixation and staining with methylene blue in methanol. The colonies with more than 50 cells were scored as survivors. The survival fractions were calculated as the ratio of plating efficiency of irradiated to nonirradiated cells. Three replicate experiments were carried out to calculate the standard deviations, and the resulting survival curves were fitted by a linear-quadratic (L-Q) model.

##### Dye exclusion test

On days 1, 4, and 7 following irradiation, all cells in the media as well as the cells adhering to the bottom were collected by trypsinization and washed twice with calcium- and magnesium-free phosphate-buffered saline. After staining with erythrosin B (courtesy of Dr. Ohyama at NIRS) at a final concentration of 0.05%, the total number of cells and the number of stained (dead) cells were counted under light microscopy, and the cell death index (CDI) was calculated as follows;

\[
CDI (%) = \frac{\text{number of stained cells}}{\text{total number of cells}} \times 100
\]

#### Lactate dehydrogenase release assay

On the same days, the LDH activity of each supernatant was measured using the LDH assay kit (Kyokuto Seiyaku, Tokyo, Japan), according to the manufacturer's protocol. Briefly, 50 μl of each supernatant was transferred into a 96-well microplate from the same flask and reacted with LDH assay reagent. After the absorbance at 540 nm had been measured with a microplate reader, the LDH activity of each sample was calculated.

#### Detection of apoptotic cells

The irradiated and nonirradiated cells were collected in the same way as in the dye exclusion assay. Then, the cells were washed in PBS and fixed with 2% glutaraldehyde in PBS at 4°C overnight. The fixed cells were stained by Hoechst 33342 (Sigma) at a final concentration of 1 mM. After about five minutes of incubation, the morphological change of the nucleus was observed, and the number of apoptotic cells was counted by fluorescent microscopy. The apoptotic index (AI) was calculated as follows after counting at least 1000 cells:

\[
AI (%) = \frac{\text{number of apoptotic cells}}{\text{total number of cells}} \times 100
\]

The same person counted the number of apoptotic cells throughout the experiment.

### Results

The results of the clonogenic survival assays are shown in Fig. 1. As compared to gamma rays, all cell lines were more sensitive to carbon beams in an LET-dependent manner. While typical shoulders were noticed in the survival curves in TK-1 after low-LET radiation, the high-LET carbon beams yielded an exponential dose-response. The RBE of carbon beams as compared with gamma rays calculated at 10% survival is shown in Table 1. The RBE was greater in

<table>
<thead>
<tr>
<th>Cell line</th>
<th>20 keV/μm</th>
<th>40 keV/μm</th>
<th>80 keV/μm</th>
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</thead>
<tbody>
<tr>
<td>A172</td>
<td>1.55</td>
<td>1.77</td>
<td>1.86</td>
</tr>
<tr>
<td>TK1</td>
<td>1.66</td>
<td>2.08</td>
<td>3.12</td>
</tr>
</tbody>
</table>

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<sup>6</sup> Human glioblastoma cell lines A172 and TK1 were used.

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<sup>8</sup> Gamma-ray irradiation was performed as a comparative study using 137Cs sources at a dose rate of approximately 1.2 Gy/min. A carbon beam of 290 MeV/u was generated by a Heavy Ion Medical Accelerator (HIMAC) at the National Institute of Radiological Science (NIRS) in Chiba, Japan.

<sup>9</sup> Detailed procedures of dosimetry and LET measurement at biological cave in HIMAC have already been reported.