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Differences in Linear Energy Transfer Affect Cell-killing and Radiosensitizing Effects of Spread-out Carbon-ion Beams

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Abstract. *Background/Aim: The cell-killing and radiosensitizing effects of carbon-ion (C-ion) beams with low linear energy transfer (LET) are underexplored. We aimed to demonstrate the cell-killing effects of ^{60}Co gamma rays and C-ion beams at various LET values and the radiosensitizing effect of C-ion beams at various LET and cisplatin levels. Materials and Methods: Human uterine cervical cancer cells were irradiated with ^{60}Co gamma rays and C-ion beams at different levels of LET, with and without cisplatin treatment. Results: Low-LET C-ion beams had a superior cell-killing effect compared to ^{60}Co gamma rays. Survival curves under low-LET C-ion beams were more similar to that of ^{60}Co gamma rays than that of high-LET C-ion beams. Cisplatin significantly reduced cell survival after 1, 2, and 3 Gy C-ion beam irradiations at LET values of 13/30/70 keV/μm, 13/30 keV/μm, and 13 keV/μm, respectively. Conclusion: Low-LET C-ion beams combined with cisplatin have higher radiosensitizing effects than high-LET C-ion beams.*

Carbon-ion radiotherapy (C-ion RT) was initiated in 1994 to treat various cancer types, and favorable treatment outcomes have been reported (1-10). These outcomes are due to its localizing properties and more significant biological advantages than X-ray RT, including distal tail-off due to the Bragg peak, a sharp lateral penumbra, and higher relative biological effectiveness (RBE) due to high linear energy transfer (LET) in the Bragg peak (1, 11, 12). Additionally, C-ion beams with high LET have a superior cell-killing effect against radioresistant and hypoxic cells (13).

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Recently, in photon therapy, concurrent chemoradiotherapy has become standard treatment for several types of cancer in expectation of a sensitizing effect of RT and preventing micrometastasis throughout the body (14-16). RT with C-ions, combined with anticancer drugs has been performed for uterine cervical cancer, pancreatic cancer, and malignant melanoma of head and neck (2, 8, 17-19) for example. The radiosensitizing effect of C-ion beams combined with anticancer drugs has also been reported *in vitro*, with high LET (70 keV/μm or higher) C-ion beams (20, 21).

In the clinical use of C-ion RT, the narrow radiation peaks are swept over an extended region by a ridge filter to create the spread-out Bragg peak (SOBP) corresponding to the size of the target volume; an SOBP has an LET of between 10 keV/μm and 120 keV/μm (11, 22, 23) (Figure 1). Although high-LET C-ion beams have shown higher RBE and superior cell-killing effect compared to X-ray and ^{60}Co gamma-ray irradiation *in vitro*, there are not many studies that compared the cell-killing effects of X-ray or ^{60}Co gamma-ray and C-ion beam irradiation with lower LET (24). We hypothesized that anticancer drugs would have a higher sensitizing effect when combined with C-ion beam irradiation with a lower LET than a higher one. Furthermore, to date, there are no reports on the radiosensitizing effect of low-LET C-ion beams and anticancer drugs in combination. Here, we aimed to compare, *via in vitro* experiments, the cell-killing effect of ^{60}Co gamma-ray and C-ion beam irradiation at different LET levels, and the radiosensitizing effect of C-ion beams at various LET values when combined with cisplatin.

Materials and Methods

Cell line. The human cervical adenocarcinoma cell line, HeLa, was obtained from the American Type Culture Collection (Manassas, VA, USA). This cell line possesses wild-type P53 gene. Cells were maintained in 25-cm^2 culture flasks at 37˚C in a humidified atmosphere with 5% CO_2 in Eagle's Minimum Essential Medium containing 20% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. The medium and serum were purchased from Sigma Chemical Co. (St. Louis, MO,

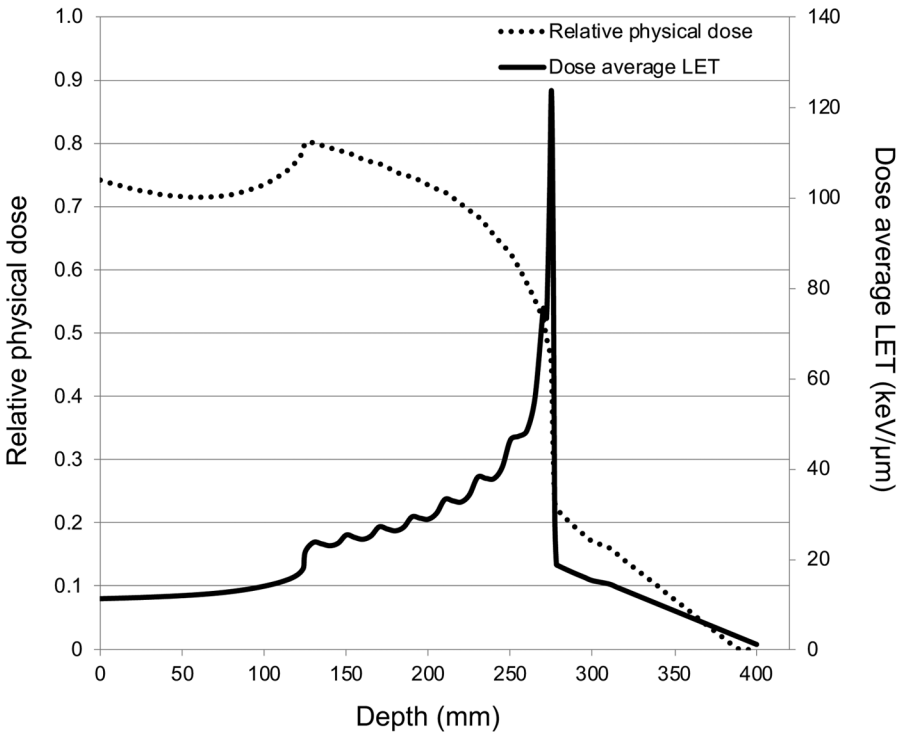

Figure 1. *Relative physical dose and dose average linear energy transfer (LET) in 15-cm spread-out Bragg peak for carbon-ion beam.*

USA). Cells were passaged before confluence and at passage 5 were used for all experiments.

^{60}Co gamma-ray and C-ion beam irradiation. Irradiation was performed at the National Institute of Radiological Sciences. The dose of ^{60}Co gamma-ray irradiation was 2.5 Gy/min. The cells were irradiated with different doses (1, 2, 3, and 4 Gy). C-Ions were accelerated at 290 MeV/nucleon using a Heavy-ion Medical Accelerator at Chiba synchrotron. The irradiation system and biophysical characteristics were reported previously (22-24). The cells were irradiated at the above doses with LET of 13 keV/μm, 30 keV/μm, and 70 keV/μm of C-ion beams.

Anticancer drug. Cisplatin was purchased from Nippon Kayaku Ltd. (Tokyo, Japan). Cells were exposed to 5 μM cisplatin 1 hour before irradiation for the combination group, the dose of which was approximately the median effective dose (half-maximal inhibitory concentration). The drug-containing medium was replaced with drug-free medium immediately after irradiation.

Clonogenic cell-survival assay. The effect of treatment on cell survival was evaluated using the clonogenic cell-survival assay. Cells were grown under standard conditions. Three weeks after irradiation, cells were fixed with methanol and stained with crystal violet. Colonies consisting of at least 50 cells were counted. Survival fractions were calculated as the ratio of surviving colonies per number of plated cells. Cell survival fractions were normalized to the survival fraction in the absence of irradiation. For the radiosensitizing effect, data for cells exposed to C-ion beams and cisplatin were normalized to the survival fraction in cisplatin alone. We also compared the survival fractions between cells exposed or unexposed to cisplatin in combination with C-ion beams at different doses and LET levels. The sensitizer enhancement ratio (SER), as an indicator of the radiosensitizing effect of cisplatin, was calculated as the ratio of cell survival with irradiation alone to that with irradiation and cisplatin. Each experiment was performed in quadruple and repeated on three different days.

Statistical analysis. The data from three independent experiments are expressed as the mean±standard deviation. Statistical significance was determined using Student's *t*-test. We determined the strength of associations of SER with LET, and with dose of C-ion RT using Pearson correlation coefficient. All statistical analyses were performed using the Statistical Package of the Social Sciences software, version 25.0 (IBM Inc., Armonk, NY, USA). A value of $p<0.05$ was defined as statistically significant.

Results

Cell-survival curve. The survival of HeLa cells irradiated with ^{60}Co gamma rays and C-ion beams with various LET levels was assessed utilizing clonogenic survival assay. Figure 2 shows the survival curves under different irradiation schemes. C-Ion beams of 13 keV/μm had a superior cell-killing effect to ^{60}Co gamma rays. In contrast, RT with 13 keV/μm C-ion beams

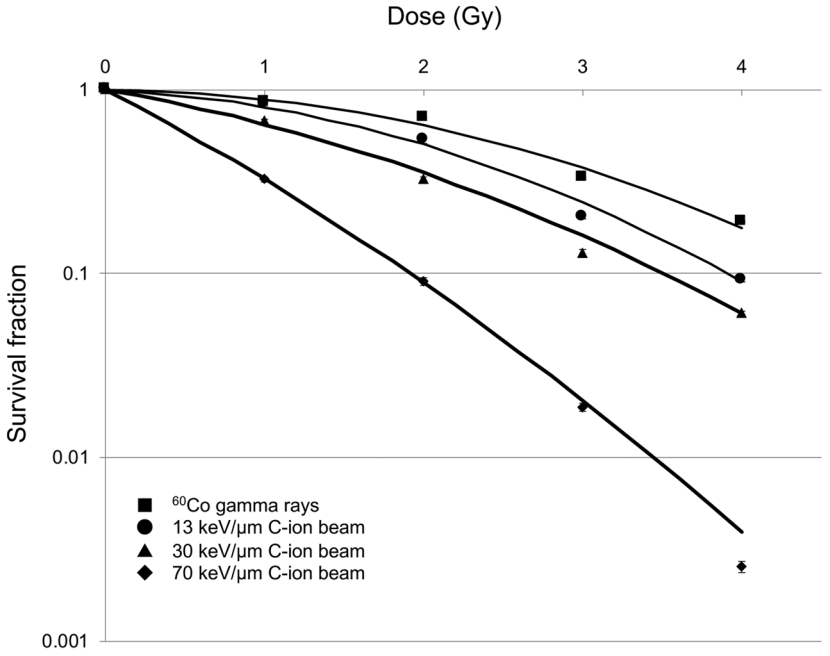

Figure 2. *Survival curves of HeLa cells after ^{60}Co gamma-ray or carbon-ion (C-ion) beam irradiation at different linear energy transfer levels. Data are presented as the mean±standard deviation, fitted to the linear-quadratic model.*

produced a survival curve more similar to that for ^{60}Co gamma rays than those with 30 keV/μm and 70 keV/μm C-ion beams.

Figure 3 shows the survival rate after each dose of C-ion RT and the comparison between RT with C-ion beams alone and at different LET values combined with cisplatin. A dose of 1 Gy C-ion beams in combination with cisplatin significantly reduced the cell survival fraction compared with C-ion RT alone at different LET levels (by 7-31%; $p<0.001$ for 13 keV/μm, $p<0.001$ for 30 keV/μm, and $p<0.05$ for 70 keV/μm). At a dose of 2 Gy, C-ion RT combined with cisplatin significantly reduced the cell survival fractions than C-ion RT alone at an LET of 13 and 30 keV/μm ($p<0.001$ and $p<0.05$, respectively); however, at 70 keV/μm, the difference was not significant. At a dose of 3 Gy C-ion RT, the combination again significantly reduced cell survival compared with C-ion RT alone at an LET of 13 keV/μm ($p<0.01$). In contrast, at 30 keV/μm and 70 keV/μm, the difference was not significant. At a dose of 4 Gy, there were no significant differences between the survival fractions after C-ion RT at different LET levels.

Sensitizer enhancement ratio. Table I shows the SER of cisplatin combined with C-ion RT at each LET and dose. These results showed that the radiosensitizing effect of C-ion RT were inversely related to LET and dose of C-ion RT. There were strong negative correlations between SER and LET (r=–0.79 for 1 Gy, r=–0.79 for 2 Gy, r=–0.79 for 3 Gy, and r=–0.97 for 4 Gy, respectively), and SER and dose of C-ion RT (r=–0.96 for 13 keV/μm, r=–0.93 for 30 keV/μm, and r=–0.99 for 70 keV/μm, respectively).

Discussion

We demonstrated the cell-killing effect of ^{60}Co gamma-ray and C-ion beam irradiation at various LET, and the *in vitro* radiosensitizing effect of C-ion beams at various LET levels when combined with cisplatin. Previous *in vitro* experiments revealed small radiosensitizing effects of high-LET C-ion beams (70 keV/μm or higher) combined with anticancer drugs (20, 21). Kubo *et al*. reported that radiosensitizing effects were observed with 50 keV/μm LET of C-ion beams combined with carboplatin and paclitaxel (25). However, our study is the first to report radiosensitizing effects of C-ion beams with lower LET combined in combination with cisplatin. Our results showed that differences in LET led to different radiosensitizing effects, especially for the low-LET C-ion beams combined with cisplatin; however, use of C-ion beams with higher LET combined with cisplatin had lower radiosensitizing effect.

Few reports compared the cell-killing effect of ^{60}Co gamma ray or X-ray and C-ion beam irradiation at various LET levels. A previous study reported survival fractions under ^{60}Co gamma-ray and low LET of C-ion irradiation were similar to those of ^{60}Co gamma-ray and high-LET of C-ion irradiation (24). In our experiments, similar results

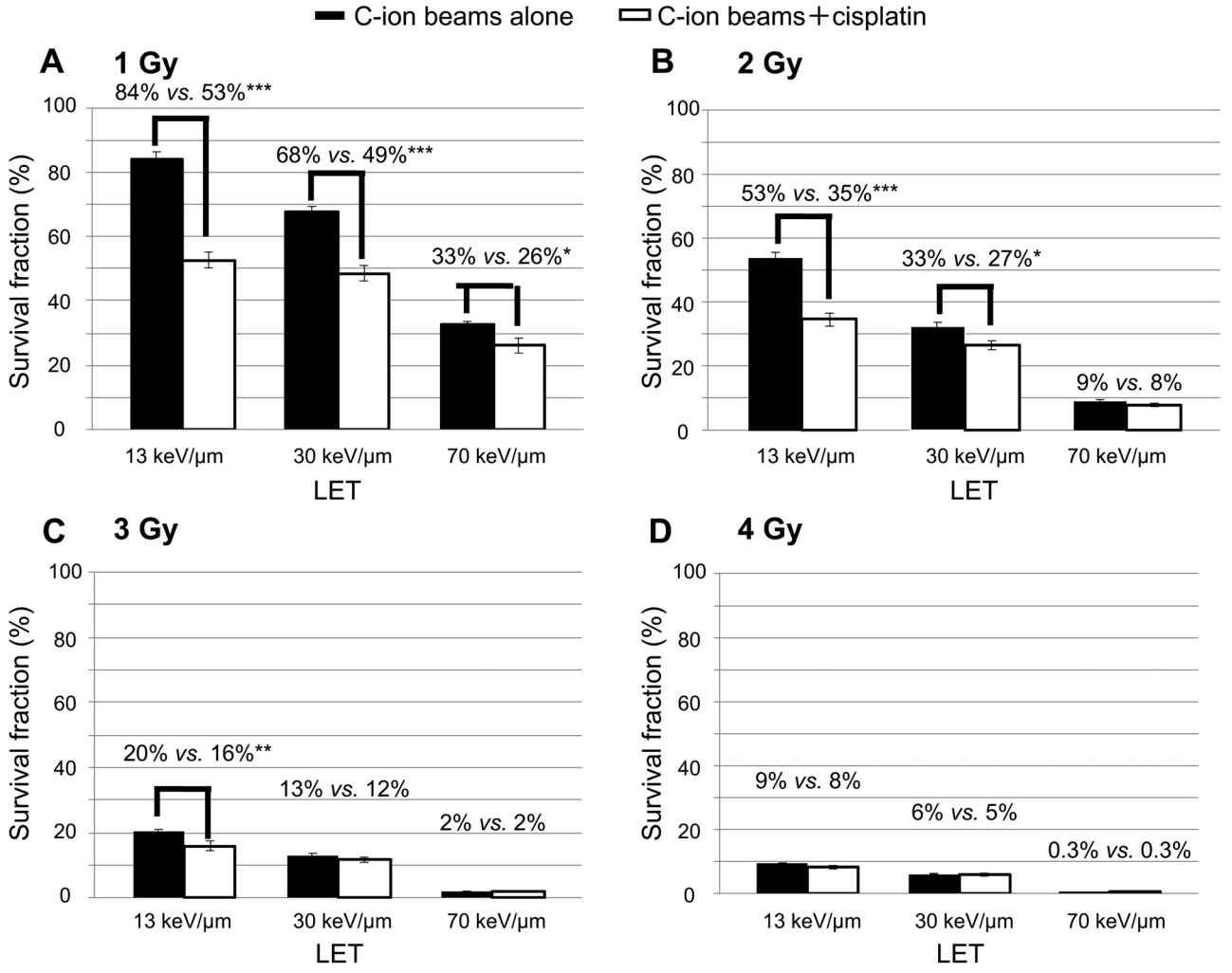

Figure 3. *The survival rate of HeLa cells after 1 Gy (A), 2 Gy (B), 3 Gy (C), and 4 Gy (D) of carbon-ion (C-ion) beam irradiation at different linear energy transfer levels alone and with cisplatin. Data are presented as the mean±standard deviation. Significantly different at *p<0.05, **p<0.01, and ***p<0.001.*

were obtained. Additionally, we hypothesized that anticancer drugs would sensitize tumor cells in combination with C-ion RT at a lower LET because ^{60}Co gamma-ray and X-ray irradiation have radiosensitizing effects (26) and we proved our hypothesis *via* several *in vitro* experiments. In clinical settings, a large SOBP (*i.e.* 15-cm) is used for large tumors, and in the prophylactic irradiation of uterine cervical cancer, a large SOBP includes low-LET components (2, 8) (Figure 1). To date, biological and physical characteristics of high-LET C-ion beams have been reported by several researchers (11, 20-24). However, higher LET components are included in the narrow area of the distal side on the C-ion beam pass and lower LET components are included in the premaximal side of the target on the C-ion beam pass (11, 24) (Figure 1). For example, the LET of the beam center in 15-cm SOBP C-ion beams is approximately 30 keV/μm, and that of the proximal side center with the same beams is lower (11, 24) (Figure 1). Our results suggested that these lower LET components might drive the radiosensitizing effect of C-ion beams in combination with anticancer drugs in a clinical setting and increase the therapeutic effect.

From our results, the radiosensitizing effect we observed might have been due to LET. Oike *et al.* reported the formation of complex DNA double-strand breaks (DSBs), which are more challenging to repair than single-strand breaks, is related to LET and that high LET C-ion RT induced complex DSBs (27). Cisplatin is a DNA-damaging and DNA-repair-inhibiting anticancer agent (28, 29). The SER was higher with low LET in our study, and cisplatin might have impaired the repair of single/DSBs induced by low-LET C-ion

Table I. *Sensitizer enhancement ratio (SER) of cisplatin at each linear energy transfer (LET) level and dose of carbon-ion (C-ion) irradiation.*

	SER at C-ion dose			
LET	1 Gy	2 Gy	3 Gy	4 Gy
13 keV/μm	1.59±0.11	1.56±0.13	1.35±0.20	1.26±0.24
30 keV/μm	1.40±0.09	1.23±0.11	1.08±0.07	1.09±0.11
70 keV/μm	1.38±0.24	1.19±0.17	1.05±0.15	0.94±0.08

Data are presented as the mean±standard deviation.

RT. Therefore, low-LET C-ion RT increased the SER. This is one of the hypotheses proposed for the mechanism of the radiosensitizing effect, and further research on the relationship between the radiosensitizing effect and DNA-damaging or DNA-repair-inhibitory effect is warranted.

Our study has certain limitations. Firstly, we performed ^{60}Co gamma-ray and C-ion beam irradiation only on a single cell line and used a single anticancer drug. Secondly, we assessed the effect of irradiation only on the survival of cancer cells, not of normal cells. The assessment of normal tissues is essential in clinical settings to test for side-effects. However, we believe our study is valuable as it is the first to evaluate the cell-killing effect of ^{60}Co gamma-ray and C-ion beam irradiation at various LET levels, and the radiosensitizing effects of C-ion beams at low LET in combination with cisplatin.

In conclusion, this study demonstrated the cell-killing effect of ^{60}Co gamma-ray and C-ion beam irradiation at different LET levels, as well as the radiosensitizing effect of C-ion RT at different LET levels combined with cisplatin. We also showed that low-LET C-ion beams have a superior cell-killing effect to that of ^{60}Co gamma rays, and survival curves similar to those under ^{60}Co gamma-ray RT. Additionally, C-ion beams at low LET levels in combination with cisplatin exerted a higher radiosensitizing effect. The results of our study have clinical applications for improving anticancer therapies using irradiation.

Conflicts of Interest

The Authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

Conceptualization: S.S., M.W.; methodology: S.S., M.W.; formal analysis: S.S., M.W.; investigation: S.S., M.W.; resources: S.S., M.W.; data curation: S.S., M.W.; writing–original draft preparation: S.S., M.W.; writing–review and editing: S.S., M.W., T.O.; visualization: S.S.; supervision: T.O., T.N.; project administration: M.W.; funding acquisition: T.N.

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