Relative Biological Effectiveness of 290 MeV/u Carbon Ions for the Growth Delay of a Radioresistant Murine Fibrosarcoma

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The relative biological effectiveness (RBE) for animal tumors treated with fractionated doses of 290 MeV/u carbon ions was studied. The growth delay of NFSa fibrosarcoma in mice was investigated following various daily doses given with carbon ions or those given with cesium $\gamma$-rays, and the RBE was determined. Animal tumors were irradiated with carbon ions of various LET (linear energy transfer) in a 6-cm SOBP (spread-out Bragg peak), and the isoeffect doses; i.e. the dose necessary to induce a tumor growth delay of 15 days were studied. The isoeffect dose for carbon ions of 14 and 20 keV/µm increased with an increase in the number of fractions up to 4 fractions. The increase in the isoeffect dose with the fraction number was small for carbon ions of 44 keV/µm, and was not observed for 74 keV/µm. The $\alpha$ and $\beta$ values of the linear-quadratic model for the radiation dose-cell survival relationship were calculated by the Fe-plot analysis method. The $\alpha$ values increased linearly with an increase in the LET, while the $\beta$ values were independent of the LET. The $\alpha/\beta$ ratio was 129 ± 10 Gy for $\gamma$-rays, and increased with an increase in the LET, reaching 475 ± 168 Gy for 74 keV/µm carbon ions. The RBE for carbon ions relative to Cs-137 $\gamma$-rays increased with the LET. The RBE values for 14 and 20 keV/µm carbon ions were 1.4 and independent of the number of fractions, while those for 44 and 74 keV/µm increased from 1.8 to 2.3 and from 2.4 to 3.0, respectively, when the number of fractions increased from 1 to 4. Increasing the number of fractions further from 4 to 6 was not associated with an increase in the RBE. These results together with our earlier study on the skin reaction support the use of an RBE of 3.0 in clinical trials of 80 keV/µm carbon beams. The RBE values for low doses of carbon beams were also considered.

INTRODUCTION

A heavy-ion accelerator has been installed at the National Institute of Radiological Sciences (NIRS) for the radiotherapy of malignant tumors. Carbon-ion beam radiotherapy using this accelerator (Heavy Ion Medical Accelerator in Chiba or HIMAC) was initiated in 1994, and more than 1000 patients suffering from malignant diseases have been treated1,2. Recently, carbon ion beams are also being used in radiotherapy at Gesellschaft fur Schwerionenforschung (GSI), Germany3,4. It is known that high-LET (linear energy transfer) radiation, such as carbon ions, forms a Bragg peak at a given depth, depending on the beam energy. To give a uniform dose distribution in a tumor, the peak must be spread out by using a ridge filter. Deep-seated tumors have been irradiated in this spread-out...
Bragg peak (SOBP) volume that contains high LET radiation. In the SOBP, the LET increases with the beam path, resulting in a non-uniform distribution of the LET within a tumor. This non-uniformity of the LET distribution requires designing the ridge filter to deliver a radiobiologically equivalent dose throughout the SOBP, since the relative biological effectiveness (RBE) depends on the LET. We have investigated the dependence of the RBE on the carbon ion LET for \textit{in vitro} cell lethality\(^5,^6,^7\) and an \textit{in vivo} skin reaction\(^8\). These biological data have been used to design ridge filters that provide a uniform distribution of biological effectiveness within the SOBP\(^9\).

In the clinic, fractionated radiotherapy has been widely used. The RBE depends not only on the LET, but also on the fraction size or the dose per fraction, indicating the dependence of the RBE on the number of fractions. We have studied the RBE for the fractionated irradiation of various LET carbon beams using an experimental mouse tumor. The radioresistance of NFSa fibrosarcoma is probably due to that the number of tumor cells required to recur after \(\gamma\)-ray irradiation is less than\(^10\). The significance of the fraction size and the LET on the RBE for various LET carbon beams is discussed.

\section*{MATERIALS AND METHODS}

\textbf{Mice and tumor}

C3H/HeMsNrsf male mice aged 12–18 week-old were used for this study. The animals were produced and maintained in specific pathogen-free (SPF) facilities in our Institute, and were housed in groups. The tumor was a syngeneic NFSa fibrosarcoma, and generations 16th through 18th were used in these studies. Single-cell suspensions were prepared by enzymatically digesting tumors and transplanted subcutaneously into the right hind leg\(^11\). Mice irradiated with \(\gamma\)-rays were kept in SPF facilities, while mice selected for irradiation with carbon ions were transported to the accelerator facility shortly before the first irradiation, and maintained in an associated conventional facility. All experiments were repeated at least once and all data were combined for analysis. A total of 920 mice were used for the present experiments, with at least 25 mice in each irradiation dose group.

\textbf{Irradiation}

Carbon-12 ions were accelerated by the HIMAC synchrotron up to 290 MeV/u. Irradiation was conducted using horizontal carbon beams with a dose rate of approximately 3 Gy/min\(^7\). The physical dose decreased to approximately 50% when the irradiation position was changed from the proximal edge of a 6-cm SOBP to the distal edge of the SOBP\(^9\). The LET of the 290 MeV/u carbon beams ranged from 14 keV/\(\mu\)m to greater than 200 keV/\(\mu\)m, depending on the depth. Desired LET beams were obtained by selecting the depth along the beam path using a Lucite range shifter. Carbon beams with 14, 20, 44 and 74 keV/\(\mu\)m LET were obtained at the entrance of the plateau, at the middle of the plateau, at the upstream within the SOBP and downstream within the SOBP, respectively.

A desired irradiation field was obtained by the simultaneous use of an iron collimator and a brass collimator. With pentobarbital anesthesia (50 mg per kg) and taping, five mice were immobilized on a Lucite plate to place their right hind legs in a rectangular field of 28 \(\times\) 100 mm, and received either a single dose or daily-fractionated doses. The foot was excluded from the irradiation field. The tumor diameter at the first irradiation time was 7.5 \(\pm\) 0.5 mm (mean \(\pm\) range). Cs-137 \(\gamma\)-rays with a dose rate of 1.6 Gy/min at an FSD of 21 cm were used as a reference beam for determining the RBE. For \(\gamma\)-ray irradiation, a doughnut-shaped radiation field with a 30 mm rim was used to collimate the vertical beam. Daily fractionation was given with equal daily doses using an interfractional interval of 24 \(\pm\) 1 hours. Several graded doses were used to determine an isoeffect dose, and animals assigned to a given dose group received equal daily doses. The LET of Cs-137 \(\gamma\)-rays was assumed to be 1 keV/\(\mu\)m, since the \(\gamma\)-ray energy produced by Cs-137 is lower than that by Co-60 and the track average LET values for Co-60 \(\gamma\)-rays and 200 kV X-rays are 0.22 and 1.7 keV/\(\mu\)m, respectively (ICRU 1970).
Endpoints and data analysis

Tumors were transplanted into the hind legs of the animals 7 days before the first irradiation, and a tumor volume measurement was used for a tumor growth delay assay. Three diameters of a tumor were measured by a caliper every other day for up to 2 months after irradiation. The tumor volumes were plotted on a semi-logarithmic graph using a computer. The tumor growth (TG) time, i.e., the time required for each tumor to reach 5 times as large as the initial volume, was calculated from the first irradiation day, and the TG times obtained for all animals were averaged per each dose group. The difference between the TG time of an experimental group and that of an unirradiated control was defined as the tumor growth delay (TGD) time.

To analyze the effectiveness of various fractionation schemes, a dose-response curve was constructed by plotting the TGD time as a function of the radiation dose for each scheme. This dose-response curve was used to obtain an isoeffect dose, that was defined as the radiation dose necessary to produce a TGD time of 15 days. Namely, the data for each dose response curve were fitted to a cubic polynomial function using a least-squares method. The 95% confidence limit around the isoeffect dose (the TGD time of 15 days) was calculated using the Maharanobis distance8).

The Fe-plot proposed by Douglas and Fowler12) was used as a multifraction linear quadratic model. A plot between the reciprocal of the isoeffect dose and the dose per fraction results in a straight line with a slope of $\beta/E$, and a y-axis intercept of $\alpha/E$, where E is the isoeffect that is the negative natural logarithm of the surviving fraction at a TGD time of 15 days, i.e. – $\ln(SF_{TGD15})$. The $\alpha/\beta$ ratio can be calculated from the slope and the intercept. The RBE value (mean ± 95% confidence limits) was obtained by using

$$RBE(A / B) = (A / B) \pm (A / B) \times \sqrt{\{(a / A)^2 + (b / B)^2\}}$$

where $A$ and $B$ are the mean RBE for $\gamma$-rays and carbon ions, respectively, and $a$ and $b$ are the 95% confidence limits for $\gamma$-rays and carbon ions, respectively.

RESULTS

Growth Curves

A tumor-growth curve was drawn for each tumor. The NFSa tumor grew from 200 to 1000 mm$^3$ in ~5 days and the growth was retarded after irradiation. Figure 1 shows a typical example of a series of growth curves for tumors that received a single dose of 74 keV/µm carbon ions. The symbols and bars are the mean and the 95% confidence limits for unirradiated control (□), 5 Gy (▲), 10 Gy (▲), 15 Gy (▲), 20 Gy (▲), 25 Gy (▲) and 30 Gy (▲).

Fig. 1. Tumor regrowth after various single doses given with 74 keV/µm carbon ions. The symbols and bars are the mean and the 95% confidence limits for unirradiated control (□), 5 Gy (▲), 10 Gy (▲), 15 Gy (▲), 20 Gy (▲), 25 Gy (▲) and 30 Gy (▲).

Dose Response

The TGD times were obtained as described above, and the dose response relationship between the TGD time and the total radiation dose was obtained for a single dose and 2, 3, 4, 5 and 6 fractions (Fig. 2). The shape of the dose-response curve depended on the LET. A linear dose response was characteristic of the highest LET (74 keV/µm) carbon ions regardless of the number of fractions, and the dose-response curve for $\gamma$-rays, the lowest LET radiation, was best fit.
with a quadratic curve. In the figure, the dose-response curve for \( \gamma \)-rays was located on the very right of each plot, and those for carbon ions shifted to the left with an increasing LET. When the number of fractions increased, the dose-response curve for \( \gamma \)-rays was displaced toward the right, while that of 74 keV/\( \mu \)m carbon ions showed an insignificant displacement (Fig. 2a and f).

**Isoeffect dose**

To analyze the fractionation effect, the isoeffect dose was defined as the radiation dose necessary to induce a TGD time of 15 days. The isoeffect dose was obtained from the dose-response curve shown in the Fig. 2 and plotted as a function of the number of fractions (Fig. 3). In general, the isoeffect dose increased with an increase in the number of fractions up to four fractions. The magnitude of this increase depended on the LET of radiation. The radiation dose for \( \gamma \)-rays to induce a TGD time of 15 days was 61.2 Gy for the single dose case. The isoeffect dose for fractionated \( \gamma \)-ray doses increased with an increase in the number of fractions, and reached a maximum of 79.7 Gy at 4 fractions. A further increase in the number of fractions

**Fig. 2.** Relationship between the radiation dose and the TGD time. Dose response curves were obtained for single dose (a), 2 fractions (b), 3 fractions (c), 4 fractions (d), 5 fractions (e) and 6 fractions (f). Symbols and bars are mean and 95% confidence limits for \( \gamma \) rays (□) and carbon ions of 14 keV/\( \mu \)m (●), 20 keV/\( \mu \)m (▲), 44 keV/\( \mu \)m (□) and 74 keV/\( \mu \)m (●), respectively.

**Fig. 3.** Isoeffect dose as a function of the number of fractions. The isoeffect doses necessary to produce a TGD time of 15 days with 95% confidence limits are shown as \( \gamma \)rays (□) and carbon ions of 14 keV/\( \mu \)m (●), 20 keV/\( \mu \)m (▲), 44 keV/\( \mu \)m (□) and 74 keV/\( \mu \)m (●), respectively.
was not associated with an increase in the isoeffect dose. The isoeffect dose for the lower LET (14 and 20 keV/µm) carbon ions was 44.9 Gy for a single dose, and increased with an increase in the number of fractions up to 4 fractions. Similar to γ-rays, a further increase in the number of fractions did not increase the isoeffect dose. Intermediate LET carbon ions of 44 keV/µm showed smaller isoeffect doses than the lower LET carbon ions. The isoeffect dose was 34.3 Gy for single dose, and was less sensitive to the number of fractions.

Fig. 4. Relationship between the reciprocal total doses and dose per fraction (4a) and the two constants in the linear-quadratic model, α and β, and the α/β values obtained by Fe-Plot. (4b, c and d). The symbols are γ-rays ( □ ) and carbon ions of 14 keV/µm ( □ ), 20 keV/µm ( □ ), 44 keV/µm ( □ ) and 74 keV/µm ( □ ), respectively. The surviving fraction of $1.81 \times 10^{-5}$, which produces a TGD time of 15 days (see text) was applied to calculate $\alpha$ (b), $\beta$ (c) and $\alpha/\beta$ ratio (d). The symbols ( □ ) and bars are the mean and standard deviation, respectively. The horizontally dotted line in (4c) represents the averaged value of all data points in the figure.
fractions than the lower LET carbon ions. The smallest isoeffect dose of 25.9 Gy was obtained for a single dose of the highest LET (74 keV/µm) carbon ions; this isoeffect dose was independent of the number of fractions up to 6 fractions.

To investigate the Fe-plot, the reciprocal of the total dose was plotted as a function of the dose per fraction for different LET carbon beams and for γ-rays (Fig. 4a). The linear-regression lines were separated depending on the LET, with the lowest LET in the bottom and the highest LET in the top of the figure. The surviving fraction at the isoeffect dose (E) following a single γ-ray dose was calculated from an equation, 

\[ E = \exp(-\alpha D - \beta D^2), \]

where \( D = 61.17 \text{ Gy} \), i.e., the single γ-ray dose that led to the TGD time of 15 days (Fig. 3). The \( \alpha \) and \( \beta \) values of \( 7.51 \times 10^{-2} \text{ Gy}^{-1} \) and \( 1.69 \times 10^{-3} \text{ Gy}^{-2} \), respectively, obtained for NFSa tumor cells using the TD50 assay (10) were used. The resulting surviving fraction was \( 1.81 \times 10^{-5} \). Using this surviving fraction and the isoeffect, the \( \alpha \) and \( \beta \) values were calculated for each LET beam together with the \( \alpha/\beta \) ratios. These values are plotted as a function of the LET in the figures 4b, 4c and 4d. The \( \alpha \) values increased linearly with an increase in the LET (Fig. 4b), while the \( \beta \) values was appeared to be independent of the LET (Fig. 4c). The \( \alpha/\beta \) ratio was 129 ± 10 Gy for γ-rays, and increased with an increase in the LET to a maximum value of 475 ± 168 Gy for the highest LET carbon ions of 74 keV/µm (Fig. 4d).

**RBE**

The RBE values for the high LET carbon ion radiation were obtained as a function of the number of fractions, the dose per fraction (dose size), or the LET. In this study, the RBE values for carbon ions relative to γ-rays were calculated as a function of the number of fractions and as a function of the dose per fraction. First, the RBE values are shown as a function of the number of fractions in Fig. 5a. Carbon ions of 74 keV/µm showed the largest RBE among all of the LET beams examined. The RBE increased from 2.4 to 3.0 when the number of fractions was increased from single dose to 4 fractions, and further increases in the number of fraction were not associated with an increase in the RBE. The RBE for 44 keV/µm carbon ions was 1.8 at a single dose, and increased up to 2.3
when the number of fractions was increased to 6. Carbon ions of either 14 or 20 keV/µm showed the smallest RBE value of 1.4 at a single dose. An increase in the number of fractions minimally affected the RBE values.

Second, the RBE values are shown as a function of the dose per fraction, where the dose per fraction is calculated by dividing the isoeffect dose by the corresponding number of fractions (Fig. 5b). The RBE for 74 keV/µm carbon ions showed a prominent dose dependence. An increase in the dose per fraction from 6 to 14 Gy resulted in a decrease in the RBE from 2.9 to 2.7. The RBE for 44 keV/µm carbon ions decreased slightly with an increase in the dose per fraction. A dose increase from 6 to 14 Gy decreased the RBE values from 2.2 to 2.1. Carbon ions of 14 and 20 keV/µm showed no dependence of the RBE on the dose per fraction.

DISCUSSION

The RBE for high-LET radiation depends on several factors, including the LET, dose per fraction and biological system. In the present study, we studied the RBE values for fractionated irradiation with 290 MeV carbon ions accelerated by the HIMAC synchrotron at NIRS using an experimental animal tumor system. $^{137}$Cs γ-rays were used as the reference radiation for the RBE determination, and the study endpoint was the determination of the isoeffect dose to induce a TGD time of 15 days. In a clinical trial of carbon ions at NIRS, the biological doses range from 2.2 Gy/f (uterus cervix) to 5.3 Gy/f (liver). An RBE value of 3.0 has been used for carbon beams of 80 keV/µm LET or at 8 mm up-stream of the distal fall-off within the SOBP. This value is based on the results of both in vitro cell culture studies and the in vivo normal tissue response. No previous study measured the RBE of implanted the tumors treated with HIMAC synchrotron-produced carbon ions.

The shape of the isoeffect curve, i.e., the relationship between the isoeffect dose and the number of fractions, depended on the LET. For low-LET γ-rays, the total isoeffect dose increased with an increase in the number of fractions up to 4 fractions, and a further increase in the number of fractions to six doses was not associated with an increase in the total dose (Fig. 3). For high-LET carbon beams of 74 keV/µm, the relationship was independent of the number of fractions; i.e., the isoeffect doses for one to six fractions were all identical. For intermediate LET beams, the isoeffect curves were found to be between these two curves. Because of these differences in the isoeffect curve, the RBE values for the various LET carbon ions showed a different relationship with the number of fractions. The RBE values for the carbon ions of the highest LET of 74 keV/µm depended on the number of fractions and, accordingly, on the dose per fraction (Fig. 5a and b). The RBE increased from 2.4 for a single dose to 3.0 for the 4 fractions, and became constant for the 4–6 fractions. In terms of the dose per fraction, the RBE increased with a decrease in the dose per fraction from 25.9 Gy of single dose to 6.6 Gy of 4 fractionated doses and became constant. Namely, the RBE values for 4, 5 and 6 daily doses, i.e., for the dose per fraction of 6.6, 5.5 or 4.5 Gy, were 3.0, 3.0 and 2.9, respectively. The RBE values obtained for the tumor are similar to the RBE value of 3.0 ± 0.15 (5% of 3.0) that is used in the clinical trials.

The maximum number of fractions tested in the present study was six due to a manpower limitation, i.e., the smallest fraction size was a highest LET carbon dose of 4.5 Gy/fraction or a γ-ray dose of 13.3 Gy/fraction given in the regime of six fractions. We need further data and consideration to determine how the RBE changes if the number of fractions increases, or the dose per fraction becomes smaller than a carbon dose of 4.5 Gy.

Numerous studies have been performed on the dose-fractionation for human and animal tumors, and the involvement of various factors during the fractionation period, including repair, reoxygenation and repopulation, is well documented. Maciejewski et al. reported that the TCD-50 (50% tumor control dose) values for the human oral cavity and oropharynx cancers treated with low-LET radiation are identical if the total dose was given over 10 to 30 days. Exceeding
the treatment time of 35 days is associated with an increase in the TCD-50 that is most likely to be due to repopulation in the tumor. The reason why the isoeffect γ-ray doses for 4–6 fractions were identical in the present study may be due to a similar mechanism as reported for the human tumors. Namely, opposing factors of repair, reoxygenation and repopulation involved in the fractionated irradiation were most likely counteracting to the result in the constant isoeffect γ-ray doses for 4–6 fractions.

The present results showed that the isoeffect dose of the highest LET carbon beams was also identical for a single dose to 6 fractionated doses. The mechanisms for this may be due to a different process than that which occurred during the fractionated γ-ray treatments. The high-LET radiations including neutrons and charged heavy ions produce more non-reparable damage than the low-LET radiation. Reoxygenation of hypoxic cells following carbon-ion radiation occurs more rapidly than after a γ-ray dose, and the oxygen enhancement ratio for high-LET radiation is smaller than that for γ-rays. The induction of apoptosis is more sensitive to high-LET carbon ions than X rays. If these mechanisms also take place during the fractionated treatment, it is likely that the increase of dose required for fractionated irradiation is small for the highest LET carbon beams. As long as the fractionated treatment is given over a short treatment period when the isoeffect doses for γ-rays and carbon beams are independent of the number of fractions, the RBE for the carbon beams is likely to be constant.

If the fractionated irradiation is given with a small dose per fraction, such as 2 Gy, or less of γ-ray dose and within a short period of time when no repopulation occurs, an alternative hypothesis based on the linear-quadratic model could be considered. The dual radiation theory assumes that ionizing radiation induces two components of cell killing; i.e., non-reparable and reparable damages. The former is proportional to the dose and the latter is proportional to the square of the dose. The surviving fraction (S) following a dose of D is expressed as $S = \exp\left(- (\alpha D + \beta D^2)\right)$ where $\alpha$ and $\beta$ are constants. This forms a survival curve that is linear at a low dose, i.e. the α component and the quadratic at the higher dose, i.e. the β component. At the low dose where the α component dominates and the β component is negligibly small, the survival is proportional to the dose, or $S = \exp(-\alpha D)$; thus, the RBE for carbon ions can be expressed by the ratio of the α value for carbon ions to the α value for γ-rays. In the present study, the α value was related linearly with the LET up to 74 keV/µm, while the β value was independent of the LET (Fig. 4b and c). This suggests that the RBE for the carbon ions increases with an increase in the LET up to 74 keV/µm, where the RBE reaches 3.1. This RBE value is again similar to the RBE value of 3.0 that is used in clinical trials.

The present results indicated that the RBE value for the carbon beams of 74 keV/mm was between 2.9 and 3.1 if the carbon ion dose of this LET was smaller than 6.6 Gy per fraction and the treatment was given within a short period of time when no repopulation of surviving tumor cells occurred. The RBE value of 80 keV/µm carbon ions was studied for a skin reaction and was found to be similar to the present RBE value of 74 keV/µm carbon ions for the tumor growth delay. This result together with the present results supports the use of an RBE of 3.0 in clinical trials of high-LET carbon beams. The constant RBE value for low doses of 74 keV/µm carbon ions suggests that, in clinical studies, a dose escalation up to 6.6 Gy per fraction may not significantly affect the homogeneity of the biological effectiveness in the SOBP.

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