Biological Gain of Carbon-ion Radiotherapy for the Early Response of Tumor Growth Delay and against Early Response of Skin Reaction in Mice

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LET/Intra-track damage/Inter-track damage/Repair.

The biological effectiveness of carbon ions relative to γ rays (RBE) was compared between the tumor growth delay and an early skin reaction of syngeneic mice. The RBE was larger for a tumor than skin when irradiated with large doses of high-LET (linear energy transfer) carbon ions. The intra-track damage (α term of a linear quadratic model) of a tumor and skin increased equally with an increase of the LET, while the inter-track damage (β term) of skin alone increased with the LET. These data provide evidence that high-LET radiotherapy could achieve therapeutic gain by minimizing the difference in response to fractionated irradiation between the tumor and normal tissue.

INTRODUCTION

An accelerated high-energy charged particle loses energy through interactions with atoms in the irradiated volume, and releases a large amount of energy at the end of the track, forming the Bragg peak. The linear energy transfer, or LET, which is the energy loss per unit length of particle track traveling in water, well describes biological effects. LET depends on the energy and particle species, such that photons, including X rays and gamma rays, possess LET values of 0.1-10 keV/μm, while a LET exceeding 10,000 keV/μm is deposited by uranium.1) Because the largest biological effectiveness is achieved by an intermediate LET value of 150 keV/μm,2) charged particles possessing an intermediate LET can be more effective against deep-seated malignant tumors. Starting as early as 1975 at Lawrence Berkeley Laboratory,3) charged-particle radiotherapy including protons and carbon ions are being currently conducted at more than 10 facilities around the world. Carbon-ion radiotherapy started at the National Institute of Radiological Sciences (NIRS) using the HIMAC synchrotron accelerator in 1994. So far, HIMAC at NIRS has treated over 1600 patients, including those with soft tissue sarcomas.4) Proton radiotherapy at Loma Linda University in the U.S.A. has treated more than 7,500 patients (http://www.proton-therapy.org/facts.htm). While high-LET radiotherapy results in better tumor control, it is anticipated that the side effects would also be increased by high-LET particle radiotherapy. Because the difference in the response of normal and tumor cells is minimum at an intermediate LET of 150 keV/μm, it is always questioned whether a therapeutic gain can be achieved by using high-LET carbon ions.5) We previously reported that the RBE of carbon ions in the response of tumors depends on the dose per fraction,6) and that fractionated irradiation with high-LET carbon ions delayed tumor growth more effectively than causing skin reactions.7) We here report that carbon ions kill tumor cells more efficiently than skin cells in vivo.

MATERIALS AND METHODS

Mice and tumour

C3H/HeMsNrsf mice aged 12–18 weeks were used: males for the tumor study and females for the skin study. The animals were produced and maintained in the specific pathogen-free (SPF) facilities. The tumor was a syngeneic NFSa fibrosarcoma, and its 16 through 18th generations were transplanted intramuscularly into the right hind legs of mice 7 days before the first irradiation.

Hairs on the right hind leg of female mice were removed by applying a depilatory agent (Shiseido, Tokyo) 7 to 8 days before the first irradiation.

A total of 881 male mice for the tumor experiment and of 2,323 female mice for the skin reaction experiment were used with 5 mice for each irradiation dose point. All of the

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Irradiation

Carbon-12 ions were accelerated by the HIMAC synchrotron up to 290 MeV/u. The desired LET was obtained by inserting a given thickness of polymethyl methacrylate (PMMA) upstream of the mice. Carbon beams with 14 and 20 keV/μm LET were obtained at the entrance of the plateau, while those with 40 through 100 keV/μm LET were within the SOBP. 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 × 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 ± 0.5 mm (mean ± range). Cs-137 γ-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. Daily fractionation was given with equal daily doses using an interfractional interval of 24 ± 1 hours. Several graded doses were used to determine an isoeffect dose, and animals assigned to a given dose group received equal daily doses. As the γ-ray energy produced by Cs-137 is lower than that by Co-60, and as the track average LET of Co-60 γ-rays is 0.22 keV/μm while that for 200 kV X-rays is 1.7 keV/μm, we assumed that LET of Cs-137 γ-rays as 1 keV/μm.

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. The tumor volume was plotted against days after irradiation, and the growth delay was calculated by subtracting the days for a non-irradiated control tumor to reach 5-times the initial volume from the days for an irradiated tumor to reach 5-times the initial volume of irradiation. The tumor growth (TG) time, i.e., the time required for each tumor to become 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.

Irradiated legs were observed for skin reaction scoring every other day up to 5 weeks. The five highest scores in an individual mouse were averaged, and this averaged score was designated as the averaged peak reaction.

To analyze the effectiveness of various fractionation schemes, a dose-response curve was constructed by plotting either the TGD time or a skin reaction score of 3.0 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 either a TGD time of 15 days or a skin reaction score of 3.0. 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 (TGD time of 15 days and skin reaction score of 3.0) was calculated using the Maharanobis distance.

The Fe-plot proposed by Douglas and Fowler was used as a multifraction linear quadratic model. A plot between the reciprocal of the isoeffect dose and the dose per fraction resulted in a straight line with a slope of β/E, and a y-axis
Fig. 3. Biological effectiveness of carbon ions relative to γ rays. The ratio of the isoeffect dose for γ rays to that for carbon ions at a given number of fractions, i.e., RBE, is plotted against the number of fractions (Fig. 3A) and the dose per fraction (Fig. 3C). The symbols and bars are the same as those in Fig. 2. The therapeutic gain is the ratio of the tumor RBE to the normal tissue (skin) RBE, and means positive when the RBE ratio exceeds 1.0 (Fig. 3B).
Fig. 4. Intra-track and inter-track damage in the tumor and skin. The reciprocal of the isoeffect dose is plotted against the dose per fraction (Fig. 4A). The symbols in the left panel represent tumors receiving (◇) γ rays and carbon ions of 14 (■), 20 (□), 44(○) and 74 (○) keV/μm, respectively. The symbols in the right panel represent skin receiving (◇) γ rays and carbon ions of 14 (■), 20 (□), 40 (○), 50 (△), 60 (▲), 80 (○) and 100(▽) keV/μm, respectively. The intercept gives the intra-track damage (α term), while the slope gives the inter-track damage (β term). The α and β terms are plotted against LET (Fig. 4B). The symbols and bars are the means and 95% confidence limits for the tumor growth delay (●) and the skin reaction (◇). Quasi-survival curves of tumor cells and skin cells (Fig. 4C) were constructed by using the α and β terms obtained in Fig. 4B. The symbols are calculated values, and represent the tumor cells receiving γ rays (●), 77 keV/μm carbon ions (■), skin cells receiving γ rays (◇) and 77 keV/μm carbon ions (△).
intercept of $\alpha/E$, where $E$ is the isoeffect, which is the negative natural logarithm of the surviving fraction at a given isoeffect, i.e., a TGD time of 15 days and a skin reaction score of 3.0. The RBE value (mean ± 95% confidence limits) was obtained by using

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

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

**RESULTS**

Fig. 1 shows the tumor growth delay and skin reactions against the total dose for $\gamma$ rays or carbon ions of 14-77 keV/\(\mu\)m. As the LET increased, the dose response curves for both tumor growth delay and skin reactions shifted to the left. We obtained all dose-responses for 1 through 6-fractionation irradiation, and calculated the isoeffect doses of the tumor growth delay and the skin reaction (Fig. 2). The isoeffect dose of $\gamma$ rays after a single dose was 50 and 60 Gy for skin reaction and tumor growth delay, respectively. Overall, the isoeffect total dose was smaller for carbon ions compared with that for $\gamma$ rays. This was LET related, and the higher was the LET, the smaller was the total isoeffect dose. The isoeffect dose progressively increased with an increase in the number of fractions for both the skin reaction and tumor growth delay until it became less prominent, or plateaued, when the number of fractions exceeded 4. This was true for $\gamma$ rays as well as low-LET carbon ions, such as 14 and 20 keV/\(\mu\)m. For high-LET carbon ions of 42 and 77 keV/\(\mu\)m, not only was the isoeffect dose further reduced, but the difference between the skin reaction and of the tumor growth delay also diminished. No fractionation effect was observed for 77 keV/\(\mu\)m carbon ions. The RBE values of low-LET carbons (14 and 20 keV/\(\mu\)m) ranged from 1.2 to 1.7, and did not show any apparent dependence on the number of fractions (Fig. 3a). When the LET of carbon ions increased to 42 keV/\(\mu\)m, the RBE values not only became large, but also increased with an increase in the number of fractions. The RBE values for tumor growth delay at 2 and 4 fractions were significantly ($P < 0.05$) larger than those for the skin reaction. When we compared the RBE values of the growth delay to the RBE values of the skin reaction, the resulting ratio or therapeutic gain of carbon ions was $1.16 ± 0.09$ (95% confidence limits) and $1.31 ± 0.09$ at 2 and 4 fractions, respectively (Fig. 3b). The therapeutic gain was also larger than 1.0 at 3 and 5 fractions with 77 keV/\(\mu\)m. The RBE values of carbon ions decreased with an increase in dose per fraction (Fig. 3c). The RBE values of low-LET carbon ions (14 and 20 keV/\(\mu\)m) were not different between the tumor growth delay and the skin reaction. Although an apparent difference between the tumor growth delay and the skin reaction was observed, it was for the RBE values of high-LET carbon ions (42 and 77 keV/\(\mu\)m) with a large dose per fraction.

Radiation damage to tissues is primarily caused by energy deposit to the critical target in cells, i.e., DNA. Because high-LET radiation produces dense energy deposit, single-hit or inter-track damage caused by high-LET radiation is more prominent than that caused by low-LET radiation, which produces dual-hit or inter-track damage to DNA. Using an Fe-plot, we analyzed the isoeffect dose to evaluate the dependence of the intra-track damage (\(\alpha\) term) and of inter-track damage (\(\beta\) term) on LET (Fig. 4a). The reciprocal total dose for tumor growth delay and skin reaction also increased with an increase of the dose per fraction. When LET increased, the regression lines moved upward, which was more prominent for a higher LET. The slope of the line fitted to skin reaction was steeper for 80 keV/\(\mu\)m carbon ions $\{5.235 ± 1.205\} \times 10^4$ Gy$^{-1}$; mean and 95% confidence limit} than $\gamma$ rays $\{1.376 ± 0.308\} \times 10^4$ Gy$^{-1}$. The \(\alpha\) and \(\beta\) terms of each regression line were calculated for all of the LET values shown in Fig. 4a as well as for the skin reaction data of LET 50, 60 and 100 keV/\(\mu\)m. The \(\alpha\) terms of the tumor growth delay and skin reaction also apparently increased with an increase in the LET (Fig. 4b); the increase in the \(\alpha\) terms was slightly larger for the tumor growth delay than for the skin reaction, even though no statistical difference was detectable between the two tissues. On the other hand, the \(\beta\) terms of the two tissues depended differently on the LET; the \(\beta\) term of the skin reaction significantly ($r = 0.807, P = 0.015, F = 11.19$) increased with LET, while that of the tumor growth delay was independent of LET. Using the \(\alpha\) and \(\beta\) terms calculated from the regression lines shown in Fig. 4b, quasi-survival curves for tumor cells and skin cells were reconstructed. The \(\alpha\) and \(\beta\) terms contain an isoeffect surviving fraction (E) that produces a given magnitude of tumor growth delay and skin reaction, namely, $\alpha/E$ amu and $\beta/E$. We used here $10^{5}$ for tumor cells which was experimentally obtained by a transplantation assay, while it was empirically determined $10^{6}$ for skin cells. The quasi-surviving fractions of skin cells after $\gamma$ rays were lower than those of tumor cells (Fig. 4c). Carbon ions of 77 keV/\(\mu\)m moved both the quasi-survival curves of the skin and tumor cells to the left. Because the move is more prominent for tumor cells than skin cells, the two survival curves become indistinguishable.

**DISCUSSION**

The inter-track damage of the skin reaction, but not of tumor growth delay, increases with an increase of the carbon LET up to 100 keV/\(\mu\)m (Fig. 4b). As for the skin reaction, whether high-LET irradiation increases the inter-track damage is controversial. Fractionated irradiation with 400 MeV/amu carbon ions to hamster skin seems to increase both the \(\alpha\) and \(\beta\) terms compared to photons, even though a statistical significance is not reached due to the small number of data.
points. Joiner et al. report that 3 MeV fast neutrons increase the α and β terms of mouse skin reaction by a factor of 7.2 and 0.83, respectively using doses ranging from 20 to 30 Gy of X rays, which are close to those in our experiments. Simultaneously, by irradiating mouse foot with a mixture of 3 MeV neutrons and X rays at various dose ratios, the authors can also report that an increase of LET increases the α term by a factor of 4.8 without affecting the β term. Comparing 14 MeV fast neutrons with 290 MeV/u carbon ions, we found that the LET of carbon ions isoeffective to fast neutrons is 80 keV/μm in our mouse skin reaction. The LET of 3 MeV neutrons is higher than that of 14 MeV neutrons. It is probable that the range of LET in Joiner’s mixed neutron experiments is, at least partly, similar to that of the carbon LET in the present study. This implies that skin damage caused by fractionated irradiation is different between carbon ions and fast neutrons. We previously reported that the α term of gut crypt survival increases with LET, while the β term did not show any LET dependence. High-LET fast neutrons also increase the α term of in vitro cell kill, but not the β term. The doses we used in the present study were much larger than those for in vitro cell survival and gut crypt survivals. Because it is the α and not the β term that dominates cell kill at small doses, a change of β might not have been detected with the small doses that were used for in vitro cell kill and in vivo crypt survival.

Concerning physical aspects, the projectile particles are fragmented to produce light ions, such as hydrogen and helium, when the projectile particles pass through PMMA. These light fragments contribute 20% of the total dose after the projectile particles pass a 90 mm thickness of PMMA. The light fragments may account for the increase in the inter-track damage of skin at high LET values (Fig. 4b).

The mechanisms to repair inter-track damage caused by high-LET radiation could differ between skin and a tumor. DNA damage caused by high-LET radiation is less repairable than that elicited by low LET. A prediction is that 30% of the DSBs induced by low-LET radiation is of the complex type, and are repaired slowly, while 70% of the DSBs is from high-LET radiation. Complex lesions are produced within 1 through 20 base pair regions of the DNA, and high-LET alpha particles induce more base damage than do X rays. Because fewer strand breaks in DNA irradiated with high LET than those with γ rays are converted by endonuclease III from oxidized pyrimidines in DNA, base damage seems to be important for DNA repair after high-LET radiation. Photon irradiation activates the bindings of NF-kappa B to DNA. Increased DNA binding activity after photon radiation is noted with oligonucleotides containing the CREB, NF-kappa B and Sp1 consensus sites in human melanoma cells. Promoters containing p53-binding sites show a dramatic transcriptional response to DNA damage caused by photon or ultraviolet irradiation. DNA-PK, a protein involved in repairing DSBs caused by photons, does not account for repairing DSBs caused by high-LET neutrons, while CDKN1A (p21) localizes at a DNA site immediately after a single high-LET lead particle hits the site. Also, chromatin compaction could be critical for high-LET damage if the accessibility of repair enzymes differs between fast and slow DSB repair. It is not yet known, however, whether chromatin or the DNA configuration of the NFSa tumor cells differ from that of skin cells.

The fraction size is important for high-LET radiotherapy. The α term dominates the low-dose radiation damage, while the β term becomes important for high-dose irradiation. As the α term or the intra-track, the non-reparable damage increased with an increase in the LET, irrespective of the tissue type (Fig. 4a), the difference between the skin reaction and the tumor growth delay should be smaller for high-LET carbon ions than photons. This is true for the quasi-survival curves shown in Fig. 4c. One of the factors different between skin and tumor is hypoxia. The NFSa tumor used in the present study contained 10% hypoxic cells. When a large dose was delivered, hypoxic cells dominated the radiosponsesiveness. A slight return observed in the relationship between the dose/fraction and the RBE in Fig. 3c could be due to hypoxic cells in the NFSa tumor. This does not, however, account for the slight return also observed for the skin reaction, because hypoxic cells in skin are very few and merely detectable by 30 Gy or over after x-ray irradiation. As RBE of high LET radiation for hypoxic cells is generally larger than that for oxic cells, the larger move to the left of tumor cells in Fig. 4c could be due to a small oxygen enhancement ratio (OER) of carbon ions.

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