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ORIGINAL ARTICLE

Responses of total and quiescent cell populations in solid tumors to carbon ion beam irradiation (290 MeV/u) in vivo

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Abstract
Purpose. The aim of this study was to clarify the radiosensitivity of intratumor total cells and quiescent (Q) cells in vivo to accelerated carbon ion beams compared with γ-ray irradiation.
Materials and methods. Squamous cell carcinoma (SCC) VII tumor-bearing mice received continuous administration of 5-bromo-2′-deoxyuridine (BrdU) to label all intratumor proliferating (P) cells. They then were exposed to carbon ions (290 MeV/u) or γ-rays. Immediately after and 12 h after irradiation, immunofluorescence staining for BrdU was used to assess the response of Q cells in terms of micronucleus frequency. The response of the total (P + Q) tumor cells was determined from the tumors not treated with BrdU.
Results. The apparent difference in radiosensitivity between total and Q cell populations under γ-ray irradiation was markedly reduced with carbon ion beams, especially with a higher linear energy transfer (LET) value. Clearer recovery in Q cells than in total cells through delayed assay under γ-ray irradiation was efficiently inhibited by carbon ion beams, especially those with a higher LET.
Conclusion. In terms of the tumor cell-killing effect as a whole, including intratumor Q cells, carbon ion beams, especially with higher LET values, were extremely useful for suppressing the dependence on the heterogeneity within solid tumors as well as depositing the radiation dose precisely.

Key words Quiescent cell · Carbon ion beam · γ-Ray · Tumor heterogeneity

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Introduction

It is thought that charged particle beams can offer better dose conformation to the target volume than photon radiotherapy, with more adequate sparing of normal tissue structures close to the target.[1] In addition, beams of ions heavier than helium exhibit a strong increase in linear energy transfer (LET) in the Bragg peak compared with the entrance region.[1] These physical properties are much more favorable than those of photon radiotherapy.

Meanwhile, high-LET radiation is more effective[2] than low-LET X- or γ-radiation at inducing biological damage. High-LET radiation gives a higher relative biological effectiveness (RBE) value for cell killing,[3] a reduced oxygen effect,[3] and reduced dependence on the cell cycle,[4,5] making it potentially superior to low-LET radiation in the treatment of cancer. Thus, particle radiation therapy with protons or heavy ions has gained increasing interest worldwide, and many clinical centers are considering introducing the use of radiation therapy with charged particles. However, almost all these radiobiological characteristics of high-LET charged particle beams were based on the response of total tumor cell

populations as a whole using in vitro cell cultures or in vivo solid tumors.[1]

Many cells in solid tumors are quiescent in situ but are still clonogenic.[6] These quiescent (Q) tumor cell populations have been thought to be more resistant than proliferating (P) tumor cells to low-LET radiation because of their much larger hypoxic fractions and greater recovery capacities from potentially lethal damage, mainly based on the characteristics of plateau-phase cultured cells in vitro.[6] So far, employing our method for selectively detecting the response of intratumor Q cell populations in vivo, we have shown that all these characteristics could be applied to Q state cells in solid tumors in vivo.[7]

In this study, we examined the characteristics of radiosensitivity and recovery capacity in the total (P + Q) and Q cell populations in solid tumors irradiated with carbon ion beams (290 MeV/u) of various LET values in a-6-cm spread-out Bragg peak (SOBP), compared with those irradiated with cobalt-60 γ-ray beams, using our method for selectively detecting the response of Q cells in solid tumors.[7] The carbon ion beams were produced by a heavy-ion accelerator installed at the National Institute of Radiological Sciences in Chiba, Japan. The accelerator was originally set up for radiation therapy for refractory malignant solid tumors.[8]

Materials and methods

Mice and tumors

SCC VII squamous cell carcinomas (Department of Radiology, Kyoto University) derived from C3H/He mice were maintained in vitro in Eagle's minimum essential medium supplemented with 12.5 % fetal bovine serum. Cells were collected from exponentially growing cultures, and 1.0×10^5 cells were inoculated subcutaneously into the left hind legs of 8- to 11-week-old syngeneic female C3H/He mice (Japan Animal, Osaka, Japan). Fourteen days after the inoculation, each tumor approximately 1 cm in diameter was employed for experimental treatment. At the time of treatment, the mean ± SD body weight of the tumor-bearing mice was 22.1 ± 2.3 g. Mice were handled according to the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University. All experimental procedures mentioned here were in accordance with institutional guidelines for the care and use of laboratory animals in research. Incidentally, the p53 of the SCC VII tumor cells is the wild type.[9]

Labeling with 5-bromo-2′-deoxyuridine

Nine days after the tumor cell inoculation, mini-osmotic pumps (Durect, Cupertino, CA, USA) containing 5-bromo-2′-deoxyuridine (BrdU) dissolved in physiological saline (250 mg/ml) were implanted subcutaneously to label all P cells for 5 days. Administration of BrdU did not change the tumor growth rate. The tumors were 1 cm in diameter on treatment. The labeling index after continuous labeling with BrdU was 55.3% ± 4.5%, and reached a plateau at this stage. Therefore, we regarded tumor cells that did not incorporate BrdU after continuous labeling as Q cells.

Irradiation

After labeling with BrdU, tumor-bearing mice underwent γ-ray irradiation or accelerated carbon ion beam irradiation. Irradiation was carried out with tumor-bearing mice held in a specially designed device made of acrylic resin with the tail and both the arms and legs firmly fixed with an adhesive tape. γ-Ray irradiation was performed with a cobalt-60 γ-ray irradiator at a dose rate of 2.75 Gy/min. Carbon-12 ions were accelerated up to 290 MeV/u by the synchrotron of the Heavy Ion Medical Accelerator in Chiba (HIMAC). Irradiation was conducted using horizontal carbon beams with a dose rate of approximately 1 Gy/min. The LET of the 290 MeV/u carbon beam with the SOBP ranges from 14 keV/μm to more than 200 keV/μm, depending on depth. The SOBP employed here is composed of various LET components with different weighting factors at each depth that were obtained from penetration of monoenergetic charged particles through a different thickness of bar-ridge filters.[10] Desired LET beams were obtained by selecting the depth along the beam path using a Lucite range shifter. Carbon beams with 18, 43, 50, and 74 keV/μm LET were obtained at the middle of the plateau, upstream within the SOBP, at the middle of 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.

According to ICRU Report 58 concerning dosimetry in intracavitary brachytherapy for uterine cancer, high-, middle-, and low-dose-rate irradiation is defined as >0.2 Gy/min (12 Gy/h), 0.033–0.200 Gy/min (2–12 Gy/h), and <0.033 Gy/min (2 Gy/h), respectively.[11] Thus, whichever beam was used, the dose rate employed fits with high-dose-rate irradiation.

Each irradiation group also included mice that were not pretreated with BrdU. The tumors were then excised immediately and 12 h after irradiation.

Immunofluorescence staining of BrdU-labeled cells and micronucleus assay

Tumors excised from the mice given BrdU were minced and trypsinized [0.05% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA) in phosphate-buffered saline (PBS), 37°C, 15 min]. Tumor cell suspensions thus obtained were incubated for 72 h in tissue culture dishes containing complete medium and cytochalasin-B (1.0 μg/ml) to inhibit cytokinesis while allowing nuclear division; the cultures were then trypsinized, and cell suspensions were fixed. After centrifugation of fixed cell suspensions, the cell pellet was resuspended with cold Carnoy's fixative (ethanol/acetic acid 3 : 1 in volume). The suspension was then placed on a glass microscope slide, and the sample was dried at room temperature. The slides were treated with 2 M hydrochloric acid for 60 min at room temperature to dissociate the histones and partially denature the DNA. The slides were then immersed in borax-borate buffer (pH 8.5) to neutralize the acid. BrdU-labeled tumor cells were detected by indirect immunofluorescence staining using monoclonal anti-BrdU antibody (Becton Dickinson, San Jose, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) antibody (Sigma, St. Louis, MO, USA). To observe the double staining of tumor cells with green-emitting FITC and red-emitting propidium iodide (PI), cells on the slides were treated with PI (2 μg/ml in PBS) and monitored under a fluorescence microscope.

The micronucleus (MN) frequency in cells not labeled with BrdU could be examined by counting the micronuclei in the binuclear cells that showed only red fluorescence. The MN frequency was defined as the ratio of the number of micronuclei in the binuclear cells to the total number of binuclear cells observed.[7] The MN frequency was also been shown to be a tool for detecting the radiosensitivity to carbon ion beams.[7,12]

The ratios obtained in tumors not pretreated with BrdU indicated the MN frequency at all phases in the total (P + Q) tumor cell population. More than 400 binuclear cells were counted to determine the MN frequency. The respective MN frequencies in the nonirradiated condition were 0.056 ± 0.005 and 0.081 ± 0.011 for total tumor and Q cell populations.

Clonogenic cell survival assay

The clonogenic cell survival assay was also performed in the mice given no BrdU using an in vivo–in vitro assay method. Tumors were disaggregated by stirring for 20 min at 37°C in PBS containing 0.05% trypsin and 0.02% EDTA. The cell yield was $4.5 \pm 1.1 \times 10^7$/g tumor weight. Appropriate numbers of viable tumor cells from the single cell suspension were plated on 60- or 100-mm tissue culture dishes; 12 days later colonies were fixed with ethanol, stained with Giemsa, and counted. The plating efficiency at 0 Gy was 52.0% ± 4.5%.

Three mice were used to assess each set of conditions, and each experiment was repeated twice. To examine the differences between pairs of values, Student's *t*-test was used when variances of the two groups could be assumed to be equal; otherwise the Welch *t*-test was used. The *P* values are from two-sided tests.

Results

The clonogenic cell survival curves for total tumor cell populations immediately after and 12 h after γ-ray irradiation or carbon ion beam irradiation with various LET values are shown in the left and right panels of Fig. 1, respectively. Apparently, whether immediately or 12 h after irradiation, the surviving fractions (SFs) were much smaller for carbon ions than for γ-rays. Whether immediately after or 12 h after carbon ion beam irradiation, as the LET value increased the SFs decreased. Furthermore, the increase in the SF caused by the 12-h interval between the end of irradiation and the beginning of the colony-forming assay under γ-ray irradiation was efficiently suppressed by the irradiation with carbon ions.

For baseline correction, we used the normalized MN frequency to exclude the MN frequency in nonirradiated control tumors. The normalized MN frequency was the MN frequency in the irradiated tumors minus that in the nonirradiated tumors. The normalized MN frequencies of total or Q cell populations immediately and 12 h after γ-ray irradiation or carbon ion beam irradiation with various LET values are shown in Figs. 2 and 3, respectively. In both Figs. 2 and 3, the data for total and Q cells are shown in the left and right panels, respectively. Overall, the normalized MN frequencies were lower for Q cells than for the total cell population under all irradiation conditions. In both total and Q cell populations, whether immediately after or 12 h after irradiation, the normalized MN frequencies were significantly larger for carbon ions than for γ-rays ($P < 0.05$). In both cell populations, carbon ion irradiation efficiently inhibited the decrease in the normalized MN frequency when there was a 12-h interval between the end of irradiation and the start of the MN assay.

To evaluate the relative biological effectiveness (RBE) of the carbon ion beams with various LET values in both total and Q cell populations compared with that of γ-rays, the data given in Figs. 1–3 were used (Table 1). On

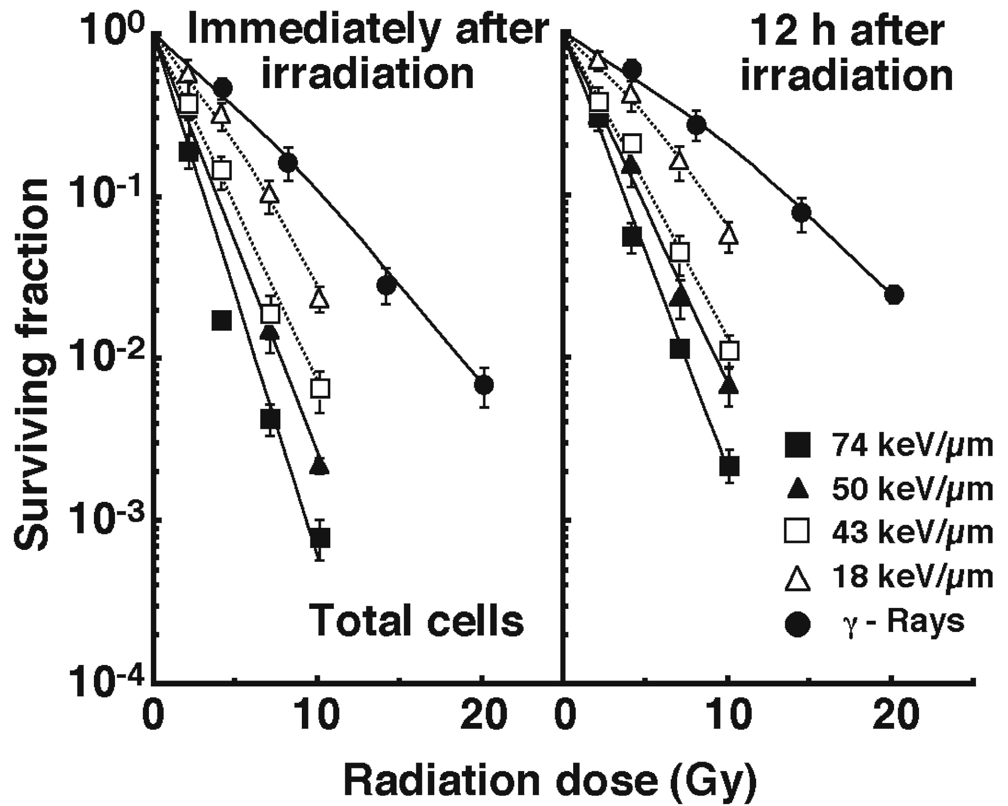

Fig. 1. Cell-survival curves for total tumor cell populations as a function of radiation dose immediately after and 12 h after irradiation are shown in the left and right panels, respectively. *Open triangles*, *open squares*, *solid triangles*, and *solid squares* represent the surviving fractions after irradiation with carbon ion beams having linear energy transfers (LETs) of 18, 43, 50, and 74 keV/μm, respectively. *Solid circles* represent the surviving fractions after γ-ray irradiation. *Bars* represent standard errors

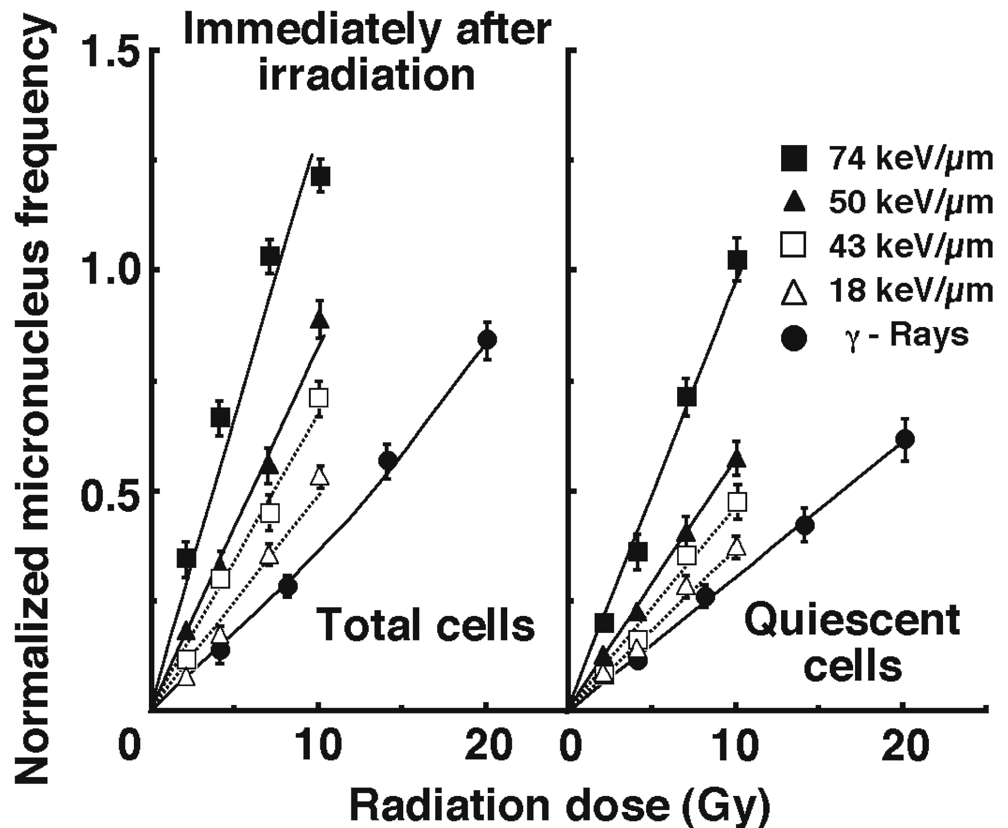

Fig. 2. Dose-response curves of normalized micronucleus (MN) frequency for total and quiescent cell populations as a function of radiation dose immediately after irradiation are shown in the left and right panels, respectively. *Open triangles*, *open squares*, *solid triangles*, and *solid squares* represent the normalized MN frequencies after irradiation with carbon ion beams having LETs of 18, 43, 50, and 74 keV/μm, respectively. *Solid circles* represent the normalized MN frequencies after γ-ray irradiation. *Bars* represent standard errors

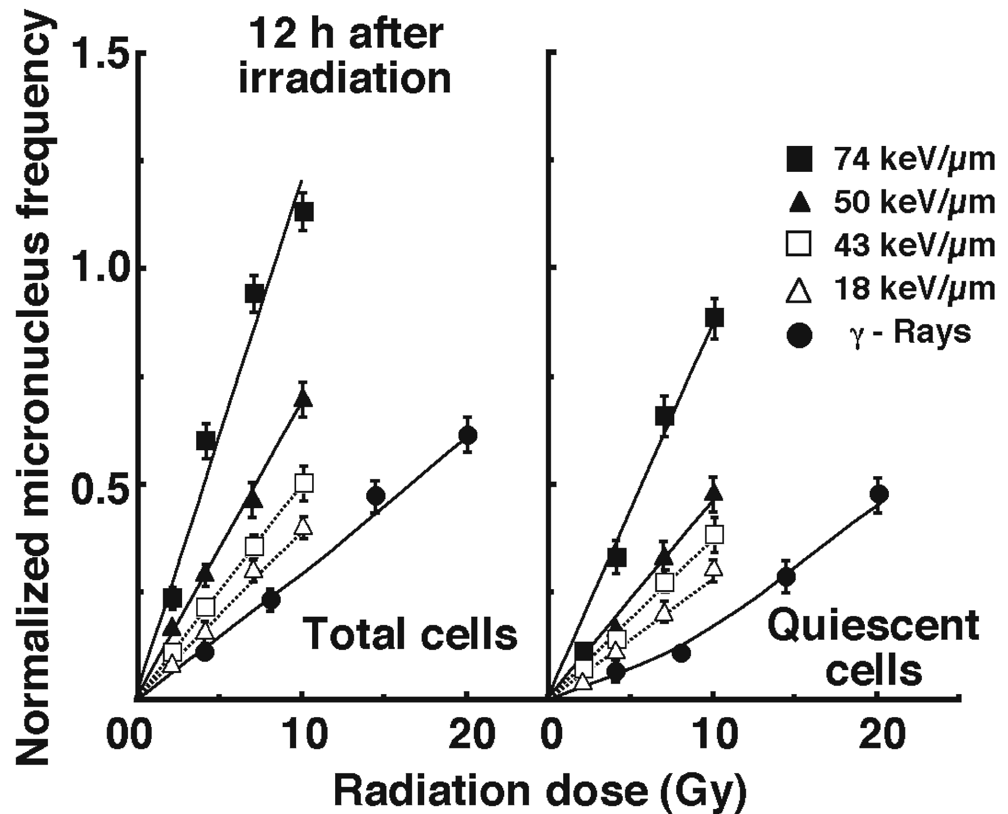

Fig. 3. Dose-response curves of normalized micronucleus (MN) frequency for total and quiescent cell populations as a function of radiation dose 12 h after irradiation are shown in the left and right panels, respectively. *Open triangles*, *open squares*, *solid triangles*, and *solid squares* represent the normalized MN frequencies after irradiation with carbon ion beams having LETs of 18, 43, 50, and 74 keV/µm, respectively. *Solid circles* represent the normalized MN frequencies after γ-ray irradiation. *Bars* represent standard errors

Table 1. Relative biological effectiveness of carbon ion beams[a] compared with γ-rays in total and quiescent populations

No. of tumor cells after irradiation	18 keV/µm	43 keV/µm	50 keV/µm	74 keV/µm
Surviving fraction = 0.05				
Total tumor cells				
Immediately after	1.45 (1.4–1.5)[b]	2.15 (2.1–2.2)	2.6 (2.5–2.7)	3.2 (3.1–3.3)
12 h after	1.55 (1.5–1.6)	2.3 (2.2–2.4)	2.75 (2.65–2.85)	3.3 (3.2–3.4)
Normalized micronucleus frequency = 0.25				
Total tumor cells				
Immediately after	1.25 (1.2–1.3)	1.5 (1.4–1.6)	1.9 (1.8–2.0)	3.1 (3.0–3.2)
12 h after	1.3 (1.25–1.35)	1.7 (1.6–1.8)	2.35 (2.25–2.45)	3.2 (3.1–4.5)
Quiescent tumor cells				
Immediately after	1.4 (1.35–1.45)	1.9 (1.8–2.0)	2.45 (2.35–2.55)	4.35 (4.2–4.5)
12 h after	1.45 (1.35–1.55)	1.95 (1.85–2.05)	2.60 (2.5–2.7)	4.5 (4.35–4.65)

[a] The ratio of the radiation dose necessary to obtain each endpoint with γ-rays and the radiation dose necessary to obtain each endpoint with carbon ion beams

[b] Numbers in parentheses are 95% confidence limits, determined using standard errors. When the ranges of 95% confidence limits have no overlapping between any two values, the difference between the two values was considered significant ($P < 0.05$)

the whole, the RBE values for Q cells and 12 h after irradiation were larger than those for the total cell population and immediately after irradiation, respectively. In both total and Q cell populations, as the LET value increased (i.e., as the irradiated point became deeper within the SOBP of the carbon ion beam) the RBE values also increased.

To assess the degree of recovery from damage induced by γ-ray or carbon ion beam irradiation in total and Q cell populations during the 12 h between the end of irradiation and the beginning of the assay, dose-modifying factors (DMFs) were calculated in both cell populations at various endpoints using the data given in Figs. 1–3 (Table 2). In general, whichever form of radiation was

Table 2. Dose-modifying factors after recovery from potentially lethal damage[a]

No. of tumor cells	18 keV/μm	43 keV/μm	50 keV/μm	74 keV/μm	γ-Rays
Surviving fraction = 0.05					
Total tumor cells	1.15 (1.1–1.2)[b]	1.15 (1.1–1.2)	1.1 (1.05–1.15)	1.05 (1.0–1.1)	1.3 (1.2–1.4)
Normalized micronucleus frequency = 0.25					
Total tumor cells	1.25 (1.2–1.3)	1.2 (1.15–1.25)	1.15 (1.1–1.2)	1.05 (1.0–1.1)	1.35 (1.25–1.45)
Quiescent tumor cells	1.45 (1.35–1.55)	1.3 (1.2–1.4)	1.25 (1.15–1.35)	1.15 (1.1–1.2)	1.6 (1.45–1.75)

[a] The ratio of the radiation dose necessary to obtain each endpoint with recovery and the radiation dose necessary to obtain each endpoint without recovery

[b] Numbers in parentheses are 95% confidence limits, determined using standard errors. When the ranges of 95% confidence limits have no overlapping between any two values, the difference between the two values was considered significant ($P < 0.05$)

Table 3. Dose-modifying factors for quiescent cells relative to total tumor cells[a]

Normalized micronucleus frequency = 0.25	18 keV/μm	43 keV/μm	50 keV/μm	74 keV/μm	γ-Rays
Immediately after irradiation	1.5 (1.4–1.6)[b]	1.4 (1.3–1.5)	1.35 (1.25–1.45)	1.15 (1.1–1.2)	1.5 (1.4–1.6)
12 h after irradiation	1.55 (1.45–1.65)	1.5 (1.4–1.6)	1.4 (1.3–1.5)	1.35 (1.25–1.45)	1.7 (1.6–1.8)

[a] The ratio of the radiation dose necessary to obtain each normalized micronucleus frequency in quiescent cells and the radiation dose necessary to obtain each normalized micronucleus frequency in total tumor cells

[b] Numbers in parentheses are 95% confidence limits, determined using standard errors. When the ranges of 95% confidence limits have no overlapping between any two values, the difference between the two values was considered significant ($P < 0.05$)

employed, the recovery was more marked in Q cells than in the total cell population. However, carbon ion beams, especially with high LET values (i.e., deep within the SOBP), efficiently suppressed recovery in both total and Q cell populations.

To examine the difference in sensitivity between the total and Q cell populations, the DMFs under each set of irradiation conditions, which compare the radiation doses necessary to obtain a normalized MN frequency of 0.25 in Q cells with those in total cells, were calculated using the data in Figs. 2 and 3 (Table 3). Carbon ion beams, especially those with high LET values (i.e., deep within the SOBP), efficiently reduced the difference in sensitivity. This suppression of the difference was more marked at 12 h after irradiation than immediately afterward.

Discussion

It is true that the values of RBE may be substantially dependent on radiation quality (LET), radiation dose, number of dose fractions, dose rate, and biological system or endpoint, including the kind of irradiated cells, tumors, and tissues.[13] However, concerning the RBE values obtained in this study, as a whole those of carbon ion beams in Q cells were significantly larger than those in total cells (Table 1), reflecting the finding that Q cells showed significantly and relatively lower sensitivity than total cells under γ-ray irradiation and under carbon ion beams, respectively. Twelve hours had already been shown to be long enough to recover the initial radiation-induced damage after γ-ray irradiation, and the capacity for recovery was shown to be greater in Q cells than in the total cell population.[14] Therefore, in both cell populations, the RBE values of carbon ion beams at 12 h after irradiation were higher than those immediately after irradiation. Furthermore, not only in total cells but also in Q cells, because the sensitivity to carbon ion beams increased as the LET value increased (i.e., as the irradiated point became deeper in the SOBP of the carbon ion beam) (Figs. 1–3), the RBE values also became larger with the LET value. This means precise treatment planning for targeting tumors is essential. Otherwise, severe complications may be brought about in normal tissues around the target tumors.

The current study showed that the difference in radiosensitivity between the total and Q cells populations was markedly reduced by using carbon ion beams, especially when high-LET beams were applied (Table 3). Although our previous report showed that Q cells have a much larger hypoxic fraction than the total tumor cell population,[7] the sensitivity to carbon ion beams of the total tumor cell population was similar to that of Q cells. Thus, it follows that oxygenated and hypoxic cells in SCC VII solid tumors have almost the same radiosensitivity to carbon ion beams. Furthermore, in this study the recovery capacity by Q cells was suppressed strongly with carbon ion beams, especially high-LET beams (Table 2). Because of less difference in radiation

sensitivity among cell cycles and a lesser recover capacity when carbon ion beams, especially high-LET beams, were used, the RBE values were largest for Q tumor cells with delayed excision (Table 1). These newly revealed radiobiological advantages concerning the responses of intratumor Q cell populations provided us with another rationale for high-LET radiation therapy. In addition to these biological advantages, because a precise dose of radiation can be deposited in deep-seated tumors using SOBP,[1] carbon ion beam radiotherapy has been used clinically since 1994 in Japan.[8] The clinical trials revealed that carbon ion radiotherapy provided definitive local control and offered a survival advantage without unacceptable morbidity for a variety of refractory tumors that were difficult to cure using conventional treatment modalities.[8]

In contrast, there is clearly heterogeneity in the response of solid tumor tissue to conventional low-LET radiation therapy.[6] Factors that cause the heterogeneity in laboratory systems include the distribution of inherent cellular radiation sensitivity; sensitivity of cells to irradiation in vivo as affected by the distribution of oxygen and other metabolites; cellular capacity to repair radiation damage; cell proliferation kinetics; immune-based rejection of the tumor by the host; extent of loss of tumor clonogens by exfoliation from the surface or into the vascular spaces, among others.[6] Furthermore, it is thought that the quiescent state of cells in solid tumors arises because of oxygen and nutrient deprivation caused by the heterogeneity in vivo within solid tumors.[6] In this study, irradiation with carbon ion beams markedly reduced the difference in radiosensitivity between total and Q cell populations in solid tumors. Hence, carbon ion beam radiotherapy has the possibility of overcoming the heterogeneity in solid tumors efficiently because of its biological advantages.

Solid tumors, especially human tumors, are thought to contain a large proportion of Q cells.[6] The presence of these cells is probably due, in part, to hypoxia and the depletion of nutrition in the tumor core, which is a consequence of poor vascular supply.[6] This might promote the formation of micronuclei in Q tumor cells. Because the Q cell population has less sensitivity, greater recovery capacity, and a larger hypoxic fraction than the P cell population in vivo,[7] more Q cells can survive after radiation therapy than P cells. Meanwhile, it should be noted that the role of Q cells as determinants of treatment success or failure is not fully established, and their impact may vary from tumor to tumor. It is thought that during fractionated radiotherapy the Q cell population substantially decreases because of recruitment from Q to P status in vivo due to preferential death of P cells.[15] However, even after fractionated radiotherapy, the Q cell population remains so long as solid tumors cannot fully regress to the extent where the Q cells cannot exist. Consequently, the control of Q cells has a great impact on the outcome of radiation therapy. As shown here, as the irradiation point became deeper in the SOBP (i.e., as the LET value became larger) the difference in sensitivity between the total and Q cell populations decreased, and the recovery capacity and RBE value in both total and Q cell populations decreased and increased, respectively. In terms of the tumor cell-killing effect as a whole, including intratumor Q cell control, carbon ion beam radiotherapy can be regarded as a promising treatment modality for deep-seated refractory tumors because of its highly efficient cytotoxic effect on intratumor Q cell populations, particularly at deeper points in the SOBP of the carbon beams and its advantageous potential physically to deposit a radiation dose precisely using the SOBP.

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