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The effect of postirradiation tumor oxygenation status on recovery from radiation-induced damage *in vivo*: with reference to that in quiescent cell populations

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Post-irradiation oxygenation status and recovery potential

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Abstract

Purpose: To elucidate the effect of tumor oxygenation status on recovery from damage following γ-ray or accelerated carbon ion irradiation *in vivo*, including in quiescent (Q) cells.

Methods: SCC VII tumor-bearing mice were continuously given 5-bromo-2′-deoxyuridine (BrdU) to label all proliferating (P) cells. They received γ-ray or accelerated carbon ion irradiation with or without tumor clamping for inducing hypoxia. Immediately after irradiation, cells from some tumors were isolated, or acute hypoxia-releasing nicotinamide was loaded to the tumor-bearing mice. For 9 hours after irradiation, some tumors were kept aerobic or hypoxic. Then isolated tumor cells were incubated with a cytokinesis blocker. The response of Q cells was assessed in terms of the micronucleus frequency using immunofluorescence staining for BrdU. That of the total (= P + Q) tumor cells was determined from BrdU non-treated tumors.

Results: Clearer recovery in Q cells than total cells and after aerobic than hypoxic γ-ray irradiation was efficiently suppressed with carbon ion beams. Inhibition of recovery through keeping irradiated tumors hypoxic after irradiation and promotion of recovery by nicotinamide loading were observed more clearly with γ-rays, after aerobic irradiation and in total cells than with carbon ion beams, after hypoxic irradiation and in Q cells, respectively.

Conclusions: Tumor oxygenation status following irradiation can manipulate recovery from radiation-induced damage, especially after aerobic γ-ray irradiation in total cells. Carbon ion beams are promising because of their efficient suppression of the recovery.

Key words:

Recovery from radiation-induced damage; Tumor oxygenation status; Quiescent cell; Carbon ion beam; γ-Ray

The authors declare no conflicts of interest concerning this study.

Introduction

Charged particle beams can offer an improved dose conformation to the target volume as compared with photon radiotherapy, with better sparing of normal tissue structures close to the target (Torikoshi et al. 2007). Further, beams of ions heavier than helium exhibit a strong increase in linear energy transfer (LET) in the Bragg peak as compared with the entrance region (Torikoshi et al. 2007). High-LET radiation gives a higher relative biological effectiveness (RBE) value for cell killing, a reduced oxygen effect, and a reduced dependence on the cell-cycle (Hada and Georgakilas 2008), making it potentially superior to low-LET radiation in the treatment of malignant solid tumors. However, almost all these biological advantages of charged particle beams were identified based on the effects on total tumor cells using cell cultures *in vitro* or solid tumors *in vivo* (Masunaga and Ono 2002).

Many cells in solid tumors are quiescent *in situ* but are still clonogenic (Masunaga and Ono 2002). The quiescent (Q) tumor cells are more resistant to low-LET radiation because of their larger hypoxic fraction and greater capacity to recover from potentially lethal damage (PLD) than the proliferating (P) tumor cells. PLD is the component of radiation damage that can be modified by post-irradiation conditions. Under ordinary circumstances, PLD causes cell death. Changing cellular growth conditions or the microenvironment of cells influences the expression of PLD or its repair, and thereby influences sensitivity to radiation (Hall 2006).

Some recent reports showed that oxygenation status of irradiated cultured tumor cells influences sensitivity to radiation

in vitro, probably through suppressing homologous recombination (HR) under hypoxic conditions, one of the two major pathways for the repair of DNA double-stranded breaks (dsbs) (Sprong et al. 2006; Chan et al. 2008).

Thus, we examined the effect of post-irradiation oxygenation status on recovery from radiation-induced damage in the total (= P + Q) and Q cell populations in solid tumors *in vivo* after low-LET γ-ray or high-LET 290 MeV/u accelerated carbon ion beam irradiation, using our original method for selectively detecting the response of Q cells within solid tumors (Masunaga and Ono 2002). This is the first attempt to detect the effect of post-irradiation oxygenation status on recovery from radiation-induced damage *in vivo*.

Materials and Methods

Mice and tumors

SCC VII squamous cell carcinomas (Department of Radiotherapy, Kyoto University) derived from C3H/He mice were maintained *in vitro* in Eagle's minimum essential medium supplemented with 12.5 % fetal bovine serum. Tumor cells (1.0×10^5) were inoculated subcutaneously into left hind leg of 9-week-old syngeneic female C3H/He mice (Japan Animal Co., Ltd., Osaka, Japan). Fourteen days later, the tumors, approximately 1 cm in diameter, were employed for experimental treatment, and the body weight of the tumor-bearing mice was 22.1 ± 2.3 (Mean ± SD) g. Mice were handled according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety Handling Regulations for Laboratory Animal Experiments, Kyoto University and National Institute of Radiological Sciences (NIRS). The p53 of SCC VII tumor cells is the wild type (Masunaga et al. 2001).

Labeling with 5-bromo-2'-deoxyuridine (BrdU)

Nine days after inoculation, mini-osmotic pumps (Durect Corporation, Cupertino, CA) containing BrdU dissolved in physiological saline (250 mg/ml) were implanted subcutaneously to label all P cells for 5 days. The labeling index after continuous labeling with BrdU was 55.3 ± 4.5 %, and reached a plateau at this stage. Therefore, tumor cells not incorporating BrdU after continuous labeling were regarded as Q cells.

Treatment

After labeling with BrdU, tumor-bearing mice received γ-ray or accelerated carbon ion whole-body irradiation, with the animal held

in a specially designed device made of acrylic resin with the tail or all four legs firmly fixed with adhesive tape with no anesthetic. Some Tumors were made totally hypoxic by clamping the proximal end 15 min before irradiation (Masunaga and Ono 2002). This clamping for 15 min did not influence clonogenic cell survival or the level of micronucleation.

γ-Rays were delivered with a cobalt-60 γ-ray irradiator at a dose rate of 2.5 Gy/min.

Carbon-12 ions were accelerated up to 290 MeV/u by the synchrotron of the Heavy Ion Medical Accelerator in Chiba (HIMAC) installed at NIRS, Chiba, Japan. The accelerator was originally set up to treat malignant solid tumors refractory to conventional cancer therapy (Torikoshi et al. 2007). The dose rate was regulated through a beam attenuation system, and irradiation was conducted using horizontal carbon beams with a dose rate of 1.0 Gy/min. The LET of the carbon ion beam with the 6-cm spread-out Bragg peak (SOBP) ranges from 14 keV/μm to greater than 200 keV/μm, depending on depth. A desired LET beam was obtained by selecting the depth along the beam path using a Lucite range shifter. The LET of 50 keV/μm at the middle of the SOBP was employed (Torikoshi et al. 2007).

Irradiated tumor-bearing mice were divided into 4 groups. **I**) Tumors were excised immediately after irradiation. **II**) The tumor-bearing mice were kept under aerated conditions for 9 h after irradiation, then the tumors were excised. If necessary, the clamping was released immediately after irradiation. **III**) The tumors were kept totally hypoxic for 9 h after irradiation, then excised. If necessary, tumors were made totally hypoxic by clamping the proximal end immediately after irradiation. **IV**) The

tumor-bearing mice received an intraperitoneal administration of nicotinamide (1000 mg/kg of mouse weight) dissolved in physiological saline immediately after irradiation, and were kept under aerated conditions for 9 h before the tumors were excised. If necessary, the clamping was released immediately after irradiation.

Each treatment group also included mice that did not receive BrdU pretreatment.

Immunofluorescence staining of BrdU-labeled cells and micronucleus (MN) 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 were incubated for 72 h in tissue culture dishes containing complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while allowing nuclear division, and the cultures were then trypsinized and cell suspensions were fixed. After the 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) and fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody (Sigma,

St. Louis, MO). 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 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 (Masunaga and Ono 2002). The MN frequency has also been shown to be a tool for detecting radio-sensitivity to carbon ion beams (Masunaga et al. 2008).

The ratios obtained in tumors not pretreated with BrdU indicated the MN frequency at all phases in the total tumor cells. More than 400 binuclear cells were counted to determine the MN frequency.

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 ± 1.1) x 10^7/g tumor weight.

As stated above, the MN frequencies for Q cells were obtained from BrdU non-labeled tumor cells after continuous BrdU labeling. The MN frequencies and surviving fractions for total cells were obtained from cells in tumors not pretreated with BrdU. Thus, there was no effect of interaction between BrdU and irradiation on the values of MN frequency and SF. More than 3 tumor-bearing mice with a tumor on left hind leg were used to assess each set of conditions and each experiment was repeated at least twice. Namely, more than

6 mice were used for each set of conditions. 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. *p*-Values are from two-sided tests.

Results

The plating efficiency and MN frequency at 0 Gy are shown in **Table 1**. The values after hypoxia for 9 h were significantly smaller and higher than those for other conditions ($P < 0.05$), respectively, in both the total and Q cells. The MN frequencies were significantly higher in Q cell than total tumor cells ($P < 0.05$), except after hypoxia for 9 h.

Cell survival curves for total tumor cells as a function of radiation dose immediately and 9 h after irradiation are shown in **Figure 1**. The data for γ-ray and carbon ion irradiation are shown in the left and right panels, respectively. The data for normoxic and hypoxic irradiations are shown in the upper and lower panels, respectively. Apparently, the SFs were much smaller for carbon ions than for γ-rays. The increase in the SF for γ-ray irradiation caused by the 9 h interval between the end of irradiation and the beginning of the colony forming assay and the rise in the SF caused by the change from aerobic to hypoxic conditions for irradiation with γ-rays was efficiently suppressed by the carbon ion beam.

For baseline correction, we used the normalized MN frequency to exclude the MN frequency in non-irradiated tumors. The normalized MN frequency was the MN frequency in the irradiated tumors minus that in the non-irradiated tumors. The dose response curves of the normalized MN frequency for total and Q cells as a function of radiation dose are shown in **Figures 2** and **3**, respectively. The data for γ-ray and carbon ion irradiation are shown in the left and right panels, respectively. The data for aerobic and hypoxic irradiation are shown in the upper and lower panels, respectively. Overall, the normalized MN frequencies were lower for Q cells than for the

total cells under all conditions, especially for γ-ray irradiation under aerobic conditions. In both total and Q cells, under all conditions, the normalized MN frequencies were much larger for carbon ion beams than for γ-rays. In both cell populations, carbon ion irradiation efficiently suppressed the decrease in sensitivity caused by leaving an interval between irradiation and the assay and by changing aerobic into hypoxic irradiation.

To evaluate the RBE of the carbon ion beam in both total and Q cells compared with γ-rays, the data for immediately after and for keeping the tumor aerobic after irradiation were used (**Table 2**). Under both sets of conditions, the values were significantly larger for Q cells than for total cells ($P < 0.05$). Those for 9 h after irradiation and under hypoxic irradiation were also significantly larger than the values for immediately after irradiation and under aerobic irradiation ($P < 0.05$), respectively.

To estimate the effect of aerobic irradiation in both total and Q cells compared with hypoxic irradiation, the data for immediately after and for keeping the tumor aerobic after irradiation were used (**Table 3**). Following γ-ray irradiation, the values were significantly smaller for Q cells than total cells ($P < 0.05$), and in both total and Q cells, the values for tumors kept aerobic for 9 h following irradiation were significantly smaller than those for immediately after irradiation ($P < 0.05$). In both total and Q cells, carbon ion beams produced significantly smaller values than γ-rays ($P < 0.05$) and the values were almost equal to 1.0.

To assess the degree of the recovery from damage produced by γ-ray or carbon ion irradiation in total and Q cells, for the tumors kept aerobic, the tumors kept hypoxic, and the nicotinamide-loaded

tumors kept aerobic during the 9 h interval between irradiation and the assay, dose-modifying factors were calculated using the data given in **Figures 1** through **3** (**Table 4**). On the whole, the values after hypoxic irradiation, in total cells, and after carbon ion irradiation were smaller than those after aerobic irradiation, in Q cells, and after γ-ray irradiation, respectively. The values for tumors kept hypoxic were decreased compared with those for tumors kept aerobic, especially in total cells after aerobic γ-ray irradiation. Conversely, the values for nicotinamide-loaded tumors kept aerobic were increased, especially in total cells after aerobic γ-ray irradiation. Carbon ion irradiation markedly reduced the values, causing them to approach 1.0, even for tumors loaded with and without nicotinamide kept aerobic after aerobic irradiation.

To examine the difference in sensitivity between the total and Q cells, the dose-modifying factors, which compare the radiation doses necessary to obtain a normalized MN frequency of 0.3 in Q cells with those in total cells, were calculated using the data in **Figures 2** and **3** (**Table 5**). In total cells, for each treatment group, the value of the irradiated dose showing the SF of 0.04 in **Figure 1** was almost the same as that showing the normalized MN frequency of 0.3 in **Figure 2**. Thus, as an end point, 0.04 was used for SF and 0.3 was used for normalized MN frequency. The values for tumors kept aerobic during the interval increased compared with those for immediately after irradiation. They increased further when the tumors were kept hypoxic during the interval. In contrast, the values for keeping the nicotinamide-loaded tumors aerobic did not increase as remarkably as those for keeping the nicotinamide non-loaded tumors aerobic. These changes were typically observed

after aerobic γ-ray irradiation. Carbon ion beams significantly reduced the difference in sensitivity observed under γ-ray irradiation ($P < 0.05$), causing the values approach 1.0.

Discussion

RBE depends on radiation quality (LET), radiation dose, number of dose fractions, dose rate and biologic system or end-point, including the kind of irradiated cells, tumors and tissues (Hada and Georgakilas 2008). In SCC VII tumors, 9 hours were long enough to repair the damage from γ-ray irradiation, and the capacity to recover from PLD was greater in Q cells than in total cells (Masunaga et al. 1992; Masunaga and Ono 2002). Here, RBE of carbon ion beams was significantly larger in Q cells than total cells (**Table 2**), reflecting the finding that Q cells were significantly and relatively less sensitive than total cells under γ-ray irradiation and under carbon ion irradiation, respectively (Masunaga et al. 2008). In both total and Q cells, carbon ion irradiation efficiently suppressed the decrease in sensitivity caused by leaving an interval between γ-ray irradiation and the assay (Masunaga et al. 2008) and by changing aerobic into hypoxic irradiation. This is probably why the RBE values after keeping tumors aerobic following irradiation and for hypoxic irradiation were significantly larger than those for immediately after irradiation and for aerobic irradiation, respectively, in both total and Q cells (**Table 2**).

In addition, this is also probably why the effect of aerobic irradiation in both total and Q cells compared with hypoxic irradiation were significantly more markedly decreased by carbon ion irradiation than by γ-ray irradiation ($P < 0.05$) and the values approached 1.0 (**Table 3**). This was probably because the frequency of closely spaced DNA lesions forming a cluster of DNA damage produced by high LET carbon ion irradiation is much less dependent

on oxygenation status at the time of irradiation than that of DNA damage produced by low LET γ-ray irradiation (Hada and Georgakilas 2008). Meanwhile, the degrees of recovery from radiation-induced damage after aerobic irradiation, in Q cells, and after γ-ray irradiation were larger than those after hypoxic irradiation, in total cells, and after carbon ion irradiation, respectively (**Table 4**). This is probably why, following γ-ray irradiation, the effects of aerobic irradiation compared with hypoxic irradiation, after keeping tumors aerobic following irradiation and for Q cells were significantly smaller than those for immediately after irradiation and for total cells, respectively (**Table 3**).

Clear recovery from γ-ray irradiation damage was efficiently suppressed by keeping irradiated tumors hypoxic, especially in total cells. Conversely, the recovery from γ-ray irradiation damage was facilitated by keeping the tumors aerobic after loading an acute hypoxia-releasing agent, nicotinamide, immediately after irradiation, especially in total cells. Needless to say, it is totally true that intratumor oxygenation status immediately before and during γ-ray irradiation has a great effect on tumor radio-sensitivity (Hall 2006). But, even *in vivo*, it was clarified here that post-irradiation oxygenation status also has an impact on the tumor radio-sensitivity. Meanwhile, the damage from carbon ion beam damage was very difficult to recover. Clustered DNA damage produced by high LET accelerated carbon ion beams is much less repairable than the DNA damage from low LET γ-rays (Hada and Georgakilas 2008).

Two major pathways for the repair of potentially lethal DNA double-stranded breaks (dsbs) exist in mammalian cells. The

non-homologous end-joining (NHEJ) pathway is imprecise, error-prone, and mutagenic. This pathway is the predominant repair process for cells in G_0, G_1 or early-S phase. HR is a more precise (error-free) repair mechanism and is more important for the repair of dsbs in late-S and G2 when a sister chromatid is available (Hada and Georgakilas 2008). Meanwhile, some recent *in vitro* studies reported that hypoxia-induced translational repression can explain the decreased HR under hypoxia (Chan et al. 2008), and that HR plays a greater role in determining hypoxic radiosensitivity than normoxic radiosensitivity (Sprong et al. 2006). Here, the post-irradiation hypoxia-induced repression of HR could be more clearly observed in the total cells including late-S and G2 phase cells than in the Q cells. However, as shown in our previous study employing caffeine (Masunaga et al. 2007), known to inhibit HR by targeting ataxia telangiectasia mutated protein kinase (ATM) and ATM- and Rad3-related protein kinase, not only in total cells but also in Q cells, HR process is also certainly caused to some extent. The significant decrease in plating efficiency and the significant increase in MN frequency in total cells after keeping the tumor hypoxic for 9 h without irradiation (**Table 1**) might also be partly due to the hypoxia-induced repression of HR. Similarly, the post-irradiation oxygenation-induced HR might also be more clearly observed in the total cells including late-S and G2 phase cells where HR can play a substantial role in the repair of dsbs, since nicotinamide can release the perfusion-limited acutely hypoxic fraction through a decrease in intratumor interstitial pressure and the total cells include a larger acutely hypoxic fraction than the Q cells in SCC VII tumor (Masunaga and Ono 2002). Although there

is the report that the effect of nicotinamide can be lost with as little as a 3-hour interval (Chaplin et al. 1990), the drug was thought to show the enough effect during the only first 3 hours after administration.

The difference in radiosensitivity between total and Q cells after γ-ray irradiation increased compared with immediately after irradiation (**Table 5**) again because of more remarkable recovery of Q cells than total cells after keeping the irradiated tumors aerobic after irradiation, although the difference after hypoxic irradiation was smaller than after aerobic irradiation. The difference became greater because of stronger inhibition of the recovery of the radiosensitive total cells than radioresistant Q cells after keeping the tumors hypoxic after irradiation. In contrast, the difference after keeping the nicotinnamide-loaded tumors aerobic after irradiation was not as great as after keeping the non-loaded tumors aerobic because of the greater recovery in total cells loaded with nicotinamide than without nicotinamide. Meanwhile, after carbon ion irradiation, whether after aerobic or hypoxic irradiation, the repair-resistant clustered DNA damage was little dependent on post-irradiation oxygenation status in tumors, leading to an almost constant difference in radiosensitivity between total and Q cells under any conditions.

Solid tumors, especially human tumors, are thought to contain a high proportion of Q cells (Vaupel 2004). The presence of Q cells is probably due, in part, to hypoxia and the depletion of nutrients in the tumor core, another consequence of poor vascular supply (Masunaga and Ono 2002; Vaupel 2004). This might promote the formation of micronuclei at 0 Gy in Q tumor cells (**Table 1**). Here,

Q cells showed less sensitivity and a greater capacity to recover from PLD. This means more Q cells can survive radiation therapy than P cells. However, it should also 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. During fractionated irradiation, the Q cell compartment substantially decreases because of recruitment from Q to P status due to preferential death of P cells (Masunaga et al. 1993). Nevertheless, even after fractionated radiotherapy, Q cell populations still remain as long as solid tumors cannot fully regress to an extent where Q status cannot exist. Thus, the control of Q cells has a great impact on the outcome of radiation therapy. In both total and Q cells, carbon ion irradiation was less dependent on oxygen condition at irradiation with little or no recovery from radiation-induced DNA damage (Masunaga et al. 2008), additionally without dependency on post-irradiation intratumor oxygenation status, leading to high RBE values compared with γ-ray irradiation. In terms of the tumor cell-killing effect as a whole, including intratumor Q cell control, carbon ion beam radiotherapy can be a promising treatment for refractory tumors because of its very efficient cytotoxic effect on intratumor Q cells, taking the potential to deposit radiation very precisely using SOBP into account.

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Figure legends

Fig. 1. Cell survival curves for total tumor cells as a function of radiation dose immediately and 9 h after irradiation. The data after γ-ray and accelerated carbon ion irradiation are shown in the left and right panels, respectively. The data after irradiation under aerobic and hypoxic conditions are shown in the upper and lower panels, respectively. Open circles, open triangles, solid triangles, and open squares represent the surviving fractions immediately after irradiation, after keeping tumors aerobic for 9 h following irradiation, after keeping tumors hypoxic for 9 h following irradiation, and after keeping tumors aerobic for 9 h following the administration of nicotinamide (NA) immediately after irradiation, respectively. Bars represent standard errors.

Fig. 2. Dose response curves of normalized micronucleus frequency for total tumor cells as a function of radiation dose immediately and 9 h after irradiation. The data after γ-ray and accelerated carbon ion irradiation are shown in the left and right panels, respectively. The data after irradiation under aerobic and hypoxic condition are shown in the upper and lower panels, respectively. Symbols are as in Figure 1. Bars represent standard errors.

Fig. 3. Dose response curves of normalized micronucleus frequency for quiescent tumor cells as a function of radiation dose immediately and 9 h after irradiation. The data after γ-ray

and accelerated carbon ion irradiation are shown in the left and right panels, respectively. The data after irradiation under aerobic and hypoxic condition are shown in the upper and lower panels, respectively. Symbols are as in Figure 1. Bars represent standard errors.

Table 1.

Plating efficiency and micronucleus frequency at 0 Gy

	Total tumor cells	Quiescent cells
<Plating efficiency>		
Under aerobic conditions	35.0 ± 6.2[a]	----
After hypoxia for 9 h	8.9 ± 1.7	----
After aerobic conditions for 9 h following nicotinamide administration	30.4 ± 4.0	----
<Micronucleus frequency>		
Under aerobic conditions	0.045 ± 0.005	0.066 ± 0.009
After hypoxia for 9 h	0.103 ± 0.014	0.11 ± 0.015
After aerobic conditions for 9 h following nicotinamide administration	0.067 ± 0.009	0.093 ± 0.012

[a]; Mean ± standard deviation

Table 2.

Relative biological effectiveness of carbon ion beams[a] compared with γ-rays

Aerobic irradiation	Hypoxic irradiation
<Surviving fraction = 0.04>	
Total cells	
Immediately after irradiation	
2.2 (2.1-2.3)[b]	3.7 (3.5-3.9)
After aerobic conditions for 9 h following irradiation	
2.6 (2.45-2.75)	4.1 (3.9-4.3)
<Normalized micronucleus frequency = 0.3>	
Total cells	
Immediately after irradiation	
2.4 (2.3-2.5)	2.8 (2.65-2.95)
After aerobic conditions for 9 h following irradiation	
3.3 (3.15-3.45)	4.0 (3.8-4.2)
Quiescent cells	
Immediately after irradiation	
3.1 (2.95-3.25)	3.5 (3.3-3.7)
After aerobic conditions for 9 h following irradiation	
3.9 (3.7-4.1)	4.4 (4.2-4.6)

[a]; The ratio of the dose of radiation necessary to obtain each end-point with γ-rays to that needed to obtain each end-point with carbon ion beams.

[b]; Values in parentheses are 95% confidence limits, determined using standard errors. If the ranges of 95 % confidence limits showed no overlap between any two values, the difference between the two values was considered significant ($p < 0.05$).

Table 3.

Irradiation under aerobic conditions compared with irradiation under hypoxic conditions[a]

	γ-Rays	Carbon beams
<Surviving fraction = 0.04>		
Total cells		
Immediately after irradiation	1.9 (1.8-2.0)[b]	1.1 (1.0-1.2)
After aerobic conditions for 9 h following irradiation	1.6 (1.5-1.7)	1.0 (0.95-1.05)
<Normalized micronucleus frequency = 0.3>		
Total cells		
Immediately after irradiation	1.7 (1.6-1.8)	1.2 (1.1-1.3)
After aerobic conditions for 9 h following irradiation	1.5 (1.4-1.6)	1.1 (1.0-1.2)
Quiescent cells		
Immediately after irradiation	1.3 (1.2-1.4)	1.1 (1.0-1.2)
After aerobic conditions for 9 h following irradiation	1.1 (1.0-1.2)	1.0 (0.95-1.05)

[a]; The ratio of the dose of radiation necessary to obtain each end-point under hypoxic conditions to that needed to obtain each end-point under aerobic conditions.

[b]; As in Table 2.

Table 4.

Dose-modifying factors due to recovery from radiation-induced damage caused by a 9-h interval between irradiation and the assay[a]

Normoxia	Hypoxia	Normoxia after nicotinamide loading
<Surviving fraction = 0.04>		
Total cells		
γ-Rays		
After aerobic irradiation		
1.4 (1.3-1.5)[b]	1.15 (1.05-1.25)	1.55 (1.45-1.65)
After hypoxic irradiation		
1.25 (1.15-1.35)	1.1 (1.05-1.15)	1.35 (1.25-1.45)
Carbon beams		
After aerobic irradiation		
1.2 (1.1-1.3)	1.15 (1.05-1.25)	1.25 (1.15-1.35)
After hypoxic irradiation		
1.1 (1.05-1.15)	1.1 (1.05-1.15)	1.15 (1.1-1.2)
<Normalized micronucleus frequency = 0.3>		
Total cells		
γ-Rays		
After aerobic irradiation		
1.35 (1.25-1.45)	1.15 (1.05-1.25)	1.5 (1.4-1.6)
After hypoxic irradiation		
1.2 (1.15-1.25)	1.1 (1.05-1.15)	1.3 (1.2-1.4)
Carbon beams		
After aerobic irradiation		
1.15 (1.1-1.2)	1.1 (1.05-1.15)	1.2 (1.15-1.25)
After hypoxic irradiation		
1.1 (1.05-1.15)	1.1 (1.05-1.15)	1.15 (1.1-1.2)
Quiescent cells		
γ-Rays		
After aerobic irradiation		
1.5 (1.4-1.5)	1.3 (1.2-1.4)	1.55 (1.45-1.65)
After hypoxic irradiation		
1.3 (1.2-1.4)	1.2 (1.15-1.25)	1.4 (1.3-1.5)
Carbon beams		
After aerobic irradiation		
1.25 (1.15-1.35)	1.15 (1.1-1.2)	1.3 (1.2-1.4)
After hypoxic irradiation		
1.15 (1.1-1.2)	1.1 (1.05-1.15)	1.2 (1.15-1.25)

[a]; The ratio of the dose of radiation necessary to obtain each end-point with recovery to that needed to obtain each end-point without recovery.

[b]; As in Table 2.

Table 5.

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

Immediately after irradiation	Delayed under aerobic	Delayed under hypoxic	Delayed with NA under aerobic
<Normalized micronucleus frequency = 0.3>			
γ-Rays			
After aerobic irradiation			
1.5 (1.4-1.6)[b]	1.75 (1.65-1.85)	2.0 (1.9-2.1)	1.65 (1.55-1.75)
After hypoxic irradiation			
1.2 (1.15-1.25)	1.25 (1.2-1.3)	1.35 (1.3-1.4)	1.25 (1.2-1.3)
Carbon beams			
After aerobic irradiation			
1.2 (1.1-1.3)	1.25 (1.15-1.35)	1.25 (1.15-1.35)	1.25 (1.15-1.35)
After hypoxic irradiation			
1.1 (1.05-1.15)	1.15 (1.1-1.2)	1.2 (1.15-1.25)	1.15 (1.1-1.2)

Abbreviation; NA = nicotinamide

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

[b]; As in Table 2.

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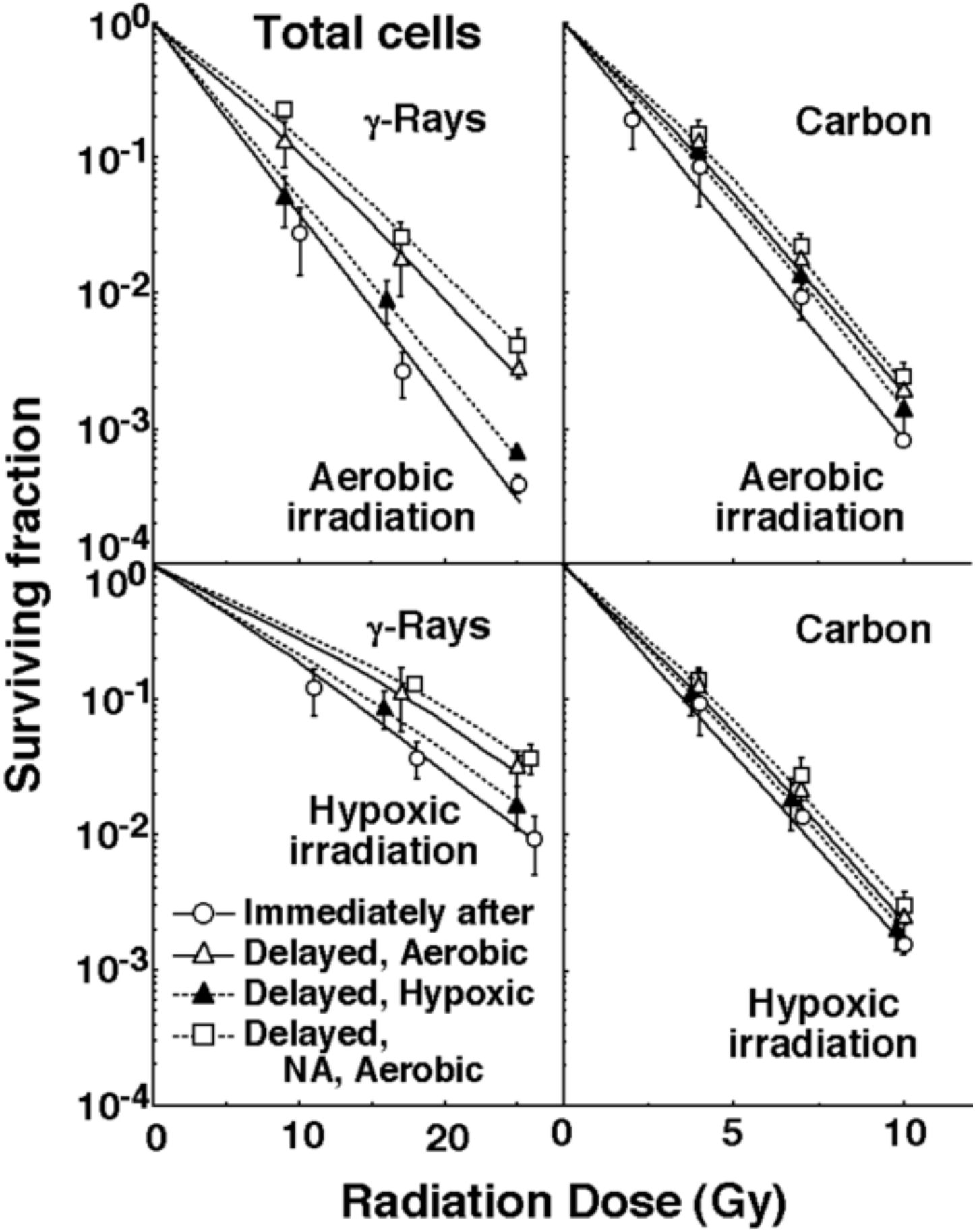

line figure 2
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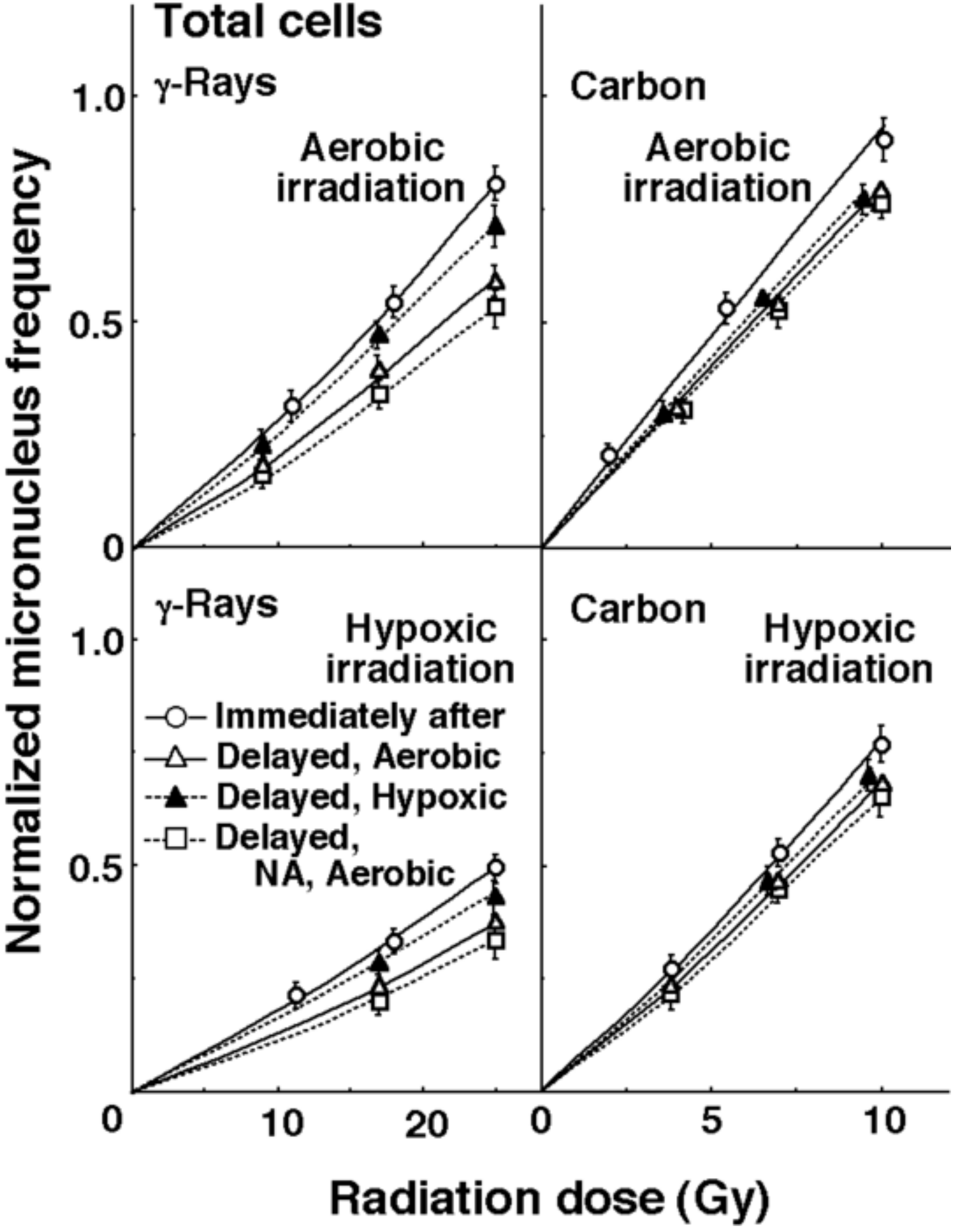

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