Significance of manipulating tumour hypoxia and radiation dose rate in terms of local tumour response and lung metastatic potential, referring to the response of quiescent cell populations

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ABSTRACT. The purpose of this study was to evaluate the influence of manipulating intratumour oxygenation status and radiation dose rate on local tumour response and lung metastases following radiotherapy, referring to the response of quiescent cell populations within irradiated tumours. B16-BL6 melanoma tumour-bearing C57BL/6 mice were continuously given 5-bromo-2'-deoxyuridine (BrdU) to label all proliferating (P) cells. They received γ -ray irradiation at high dose rate (HDR) or reduced dose rate (RDR) following treatment with the acute hypoxia-releasing agent nicotinamide or local hyperthermia at mild temperatures (MTH). Immediately after the irradiation, cells from some tumours were isolated and incubated with a cytokinesis blocker. The responses of the quiescent (Q) and total (proliferating + Q) cell populations were assessed based on the frequency of micronuclei using immunofluorescence staining for BrdU. In other tumour-bearing mice, 17 days after irradiation, macroscopic lung metastases were enumerated. Following HDR irradiation, nicotinamide and MTH enhanced the sensitivity of the total and Q-cell populations, respectively. The decrease in sensitivity at RDR irradiation compared with HDR irradiation was slightly inhibited by MTH, especially in Q cells. Without γ -ray irradiation, nicotinamide treatment tended to reduce the number of lung metastases. With γ -rays, in combination with nicotinamide or MTH, especially the former, HDR irradiation decreased the number of metastases more remarkably than RDR irradiation. Manipulating both tumour hypoxia and irradiation dose rate have the potential to influence lung metastasis. The combination with the acute hypoxia-releasing agent nicotinamide may be more promising in HDR than RDR irradiation in terms of reducing the number of lung metastases.

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Many cells in solid tumours are quiescent *in situ* but still clonogenic [1]. Quiescent (Q) tumour cells are thought to be more resistant to low LET radiation because of their larger hypoxic fraction and greater capacity to recover from radiation-induced DNA damage than proliferating (P) tumour cells [1]. Actually, our original method for selectively detecting the response of intratumour Q cells [1] verified those characteristics of Q-cell population [1] and made it possible to evaluate the usefulness of various modalities for cancer therapy in terms of effectiveness against Q-cell populations within local tumours. Based on the characteristics of the response of intratumour Q cells to various DNA-damaging treatments obtained so far [1], more effective and useful treatment modalities for local tumour control can be developed.

Metastasis is a leading cause of cancer deaths and involves a complex, multistep process by which tumour cells disseminate to distant sites to establish discontin-

uous secondary colonies [2, 3]. It was reported that acute and cyclic, but not chronic, hypoxia significantly increased the number of spontaneous lung metastases in mice by a factor of about 2, and that this effect was due to the influence of the acute hypoxia treatment on the primary tumour and not to other potential effects of the treatment such as damage to the lung epithelium [4, 5]. Based on this report, we recently reported the significance of injection of an acute hypoxia-releasing agent, nicotinamide, into tumour-bearing mice as a combined treatment with high dose rate (HDR) γ -ray irradiation in terms of repressing lung metastasis [6]. However, when combined with reduced dose rate (RDR) γ -ray irradiation, the significance of manipulating hypoxia within local solid tumours has not yet been clarified in terms of lung metastasis. Meanwhile, concerning local tumour control, it was already reported that manipulating hypoxia in solid tumours during RDR as well as HDR γ -ray irradiation influences the radiosensitivity of local tumours, especially with γ -rays [7].

Thus, the aim of the current *in vivo* study is to elucidate the significance of the nicotinamide treatment as a combined treatment with RDR γ -ray irradiation in terms

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of lung metastases compared with the combination with mild temperature hyperthermia (MTH), which had already been shown to have the potential to manipulate intratumour hypoxia [8], and preferentially release diffusion-limited chronic hypoxia according to our previous reports [6, 9, 10]. In addition, concerning the local tumour response to γ -ray irradiation with or without nicotinamide or MTH, the effect not only on the total (P + Q) tumour cell population but also on the Q-cell population was also evaluated using an original method of detecting the response of Q cells in solid tumours [1].

Methods and materials

Mice and tumours

B16-BL6 murine melanoma cells (Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University) derived from C57BL/6 mice were maintained *in vitro* in RPMI-1640 medium supplemented with 10% fetal bovine serum. Tumour cells (1.25×10^5) were inoculated subcutaneously into the left hind leg of 8-week-old syngeneic female C57BL/6 mice (Japan Animal, Osaka, Japan). 18 days later, the tumours, approximately 7 mm in diameter, were employed for the cytotoxic treatment, and the body weight of the tumour-bearing mice was 21.1 ± 2.3 g (mean $\pm$ SD). 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. p53 of B16-BL6 tumour cells is the wild type [11].

Labelling with 5-bromo-2'-deoxyuridine

12 days after inoculation, mini-osmotic pumps (Durect Corporation, Cupertino, CA) containing 5-bromo-2'-deoxyuridine (BrdU) dissolved in physiological saline (250 mg ml^{-1}) were implanted subcutaneously to label all proliferating (P) cells for 6 days. The percentage of labelled cells after continuous labelling with BrdU was $54.2 \pm 6.0\%$, and reached a plateau at this stage. Therefore, tumour cells not incorporating BrdU after continuous labelling were regarded as Q cells.

Cytotoxic treatment

After labelling with BrdU (18 days after inoculation), solid tumours grown in the left hind legs of mice were irradiated with a cobalt-60 γ -ray irradiator at 2.5 Gy min^{-1} as a HDR or $0.039 \text{ Gy min}^{-1}$ as a RDR. Lead blocks were used to avoid irradiating other body parts. For irradiation, the animal was held in a specially designed device made of acrylic resin with the tail firmly fixed with adhesive tape under no anaesthetic. RDR irradiation was performed by maintaining an appropriate distance between the cobalt-60 and the mouse fixed within the device.

Some tumour-bearing mice received an intraperitoneal administration of nicotinamide (1000 mg kg^{-1} of mouse

weight) dissolved in physiological saline 1 h before the irradiation. Others were subjected to local MTH at 40°C for 60 min by immersing the tumour-bearing left hind leg in a water bath immediately before the irradiation. Temperatures at the tumour centre equilibrated within 3–4 min after immersion in the water bath and remained $0.2\text{--}0.3^\circ\text{C}$ below the bath's temperature. The water bath's temperature was maintained at 0.3°C above the desired tumour temperature [12].

Each treatment group also included mice not pre-treated with BrdU.

Immunofluorescence staining of BrdU-labelled cells and micronucleus assay

Immediately after irradiation, some tumours excised from the mice given BrdU were minced and trypsinised (0.05% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA) in phosphate-buffered saline (PBS), 37°C , 15 min). Tumour cell suspensions were incubated for 72 h in tissue culture dishes containing complete medium and $1.0 \mu\text{g ml}^{-1}$ of cytochalasin-B to inhibit cytokinesis while allowing nuclear division; the cultures were then trypsinised 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 neutralise the acid.

BrdU-labelled tumour cells were detected by indirect immunofluorescence staining using a monoclonal anti-BrdU antibody (Becton Dickinson, San Jose, CA) and a fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody (Sigma, St Louis, MO). To observe the double staining of tumour cells with green-emitting FITC and red-emitting propidium iodide (PI), cells on the slides were treated with PI ($2 \mu\text{g ml}^{-1}$ in PBS) and monitored under a fluorescence microscope.

The micronucleus (MN) frequency in cells not labelled 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 [1]. A photomicrograph showing the double staining of tumour cells with PI and FITC was published in our previous report [13].

The ratios obtained in tumours not pre-treated with BrdU indicated the MN frequency at all phases in the total tumour cell population. More than 300 binuclear cells were counted to determine the MN frequency.

Clonogenic cell survival assay

The clonogenic cell survival assay was also performed for the implanted tumours in mice given no BrdU using an *in vivo-in vitro* assay method immediately after irradiation. Tumours were excised, weighed, minced

and disaggregated by stirring for 20 min at 37°C in PBS containing 0.05% trypsin and 0.02% EDTA. The cell yield was $1.2 \pm 0.4 \times 10^7 \text{ g}^{-1}$ tumour weight. Appropriate numbers of viable tumour cells from the single cell suspension were plated on 60- or 100-mm tissue culture dishes, and, 14 days later, colonies were fixed with ethanol, stained with Giemsa, and counted.

As stated above, the MN frequencies for Q cells were obtained from non-labelled tumour cells after continuous BrdU labelling. The MN frequencies and surviving fractions (SFs) for total cell populations were obtained from cells in tumours not pre-treated with BrdU. Thus, there was no effect of interaction between BrdU and irradiation on the values of MN frequency and SF.

Growth of B16-BL6 tumours

After irradiation with γ -rays at a dose of 0 or 16 Gy on the 18th day after inoculation with or without nicotinamide treatment or MTH, the size of the tumours implanted in the left hind legs of some tumour-bearing mice was checked two or three times a week for about 20 days. Tumour volume was calculated using the formula: $V = \pi/6 \times a \times b^2$, where a and b are respectively the longest and shortest diameters of the tumour measured with callipers.

Metastasis assessment

As the lungs are the primary sites of metastatic spread from B16-BL6 tumours in the legs, the development of pulmonary metastases was also assessed.

After 17 days of irradiation (35 days after the inoculation of B16-BL6 melanoma cells), the tumour-bearing mice were killed by cervical dislocation, and their lungs were removed, briefly washed with distilled water, cleaned of extraneous tissue, fixed in Bouin's solution overnight (Sigma), and stored in buffered formalin 10 % (Sigma) until metastases were counted. Macroscopically visible metastases (lung tumour colonies) were counted under a dissection microscope [14]. 18 days after the inoculation and immediately before γ -ray irradiation with or without nicotinamide treatment or MTH, the numbers of macroscopic lung metastases were also counted as background data. The number obtained was 8.0 ± 2.2 .

More than three mice with a tumour in the left hind leg were used to assess each set of conditions and each experiment was repeated at least twice. Namely, more than six 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

Table 1 shows the plating efficiencies for the total tumour cell population and the MN frequencies without γ -ray irradiation for the total and Q-cell populations. Nicotinamide and MTH caused slightly lower plating

Table 1. Plating efficiency and micronucleus frequency at 0 Gy

	Total cell population	Quiescent cells
Plating efficiency (%)		
Control	84.4 ± 8.2^a	–
Nicotinamide only	81.4 ± 7.3	–
MTH only	83.5 ± 8.7	–
Micronucleus frequency		
Control	0.050 ± 0.008	0.077 ± 0.009
Nicotinamide only	0.057 ± 0.006	0.084 ± 0.009
MTH only	0.054 ± 0.005	0.081 ± 0.009

MTH, mild temperature hyperthermia.

^aMean $\pm$ standard deviation

efficiencies and higher MN frequencies for both populations, although not significantly. Q tumour cells showed significantly higher MN frequencies than the total tumour cell population under each set of conditions.

Figure 1 shows clonogenic cell survival curves for the total cell population as a function of the dose of γ -rays at HDR or RDR with or without nicotinamide or MTH. Figure 2 shows normalised MN frequencies as a function of irradiated dose with or without nicotinamide or MTH in the total (left panel) and Q (right panel) tumour cell populations. The normalised MN frequency was the MN frequency in tumours that received γ -ray irradiation minus that in tumours that did not. Overall, the normalised MN frequencies were significantly smaller in Q cells than the total cell population. The SFs and the normalised MN frequencies under all conditions increased and decreased respectively as the dose rate of radiation decreased, especially after nicotinamide treatment. In both cell populations, at HDR irradiation, the SFs and the normalised MN frequencies decreased and increased respectively in the following order: HDR irradiation only, HDR irradiation after MTH and HDR irradiation after nicotinamide treatment. In contrast, at RDR irradiation, in both cell populations, the SFs and the normalised MN frequencies decreased and increased respectively in the following order: HDR irradiation only, HDR irradiation after nicotinamide treatment and HDR irradiation after MTH.

The correlation between the normalised MN frequency and the SF of the total tumour cell population under each set of conditions was examined. For each set of irradiation conditions, the regression lines were calculated and found to be statistically identical. Thus, the regression line was calculated from pooled data for all sets of conditions, and had a significant positive correlation ($p < 0.001$) (Figure 3).

To estimate the radio-enhancing effect of nicotinamide or MTH in both the total and Q-cell populations compared with irradiation only, the data shown in Figures 1 and 2 were used (Table 2). The enhancing effect of nicotinamide was more marked in the total cell population, especially with HDR irradiation. The effect was little observed for RDR irradiation. In contrast, the enhancing effect of MTH was marked in the Q cells, again especially with HDR irradiation.

To investigate the reduction in radiosensitivity caused by a decrease in radiation dose rate, dose-modifying factors were calculated using the data for all irradiation

Figure 1. Cell survival curves for the total cell population from B16-BL6 tumours irradiated with γ -rays at a high dose rate (HDR) or reduced dose rate (RDR) with or without nicotinamide treatment or mild temperature hyperthermia (MTH) on day 18 after tumour cell inoculation. Circles, triangles, and squares represent the surviving fractions after irradiation only under aerobic conditions, after irradiation under aerobic conditions following MTH and after irradiation under aerobic conditions following nicotinamide treatment, respectively. Open and solid symbols represent the surviving fractions after HDR and RDR irradiation, respectively. Bars represent standard errors. The differences between HDR and RDR irradiation for each combination condition were significant ($p < 0.05$). The differences between nicotinamide combination and radiation only and between MTH and nicotinamide combination were also significant at HDR irradiation ($p < 0.05$).

conditions given in Figures 1 and 2 (Table 3). On the whole, the reduction in radiosensitivity was more marked in the Q-cell than the total cell population. However, MTH repressed the reduction. In the total cell population, the degree of the reduction in radiosensitivity was decreased in the following order: irradiation with nicotinamide > irradiation only > irradiation with MTH. In Q cells, the degree was decreased in the following order: irradiation with nicotinamide = irradiation only > irradiation with MTH.

To examine the difference in radiosensitivity between the total and Q-cell populations, dose-modifying factors, which allow a comparison of the dose of radiation necessary to obtain a normalised MN frequency of 0.3 in Q cells with that in the total cell population, were calculated using the data in Figures 1 and 2 (Table 4). Overall, the difference in radiosensitivity was greater at RDR than HDR irradiation. At HDR, the difference in

radiosensitivity increased in the following order: irradiation with MTH < irradiation only < irradiation with nicotinamide. At RDR, the difference in radiosensitivity increased in the following order: irradiation with MTH < irradiation only = irradiation with nicotinamide.

Figure 4 shows tumour growth curves after irradiation at HDR or RDR with or without nicotinamide treatment or MTH 18 days after the tumour cell inoculation. To evaluate tumour growth after irradiation with or without nicotinamide treatment or MTH, the period required for each tumour to become 3 times as large as on day 18 after the inoculation was obtained using the data shown in Figure 4 (Table 5). Without irradiation, there were no significant differences in the periods without nicotinamide or MTH, with nicotinamide and with MTH. With HDR or RDR irradiation at a dose of 16 Gy, the period required was significantly prolonged compared with no irradiation, and the period was significantly longer for HDR irradiation than RDR. At HDR, the period required increased in the following order: irradiation at 16 Gy alone < irradiation at 16 Gy with MTH < irradiation at 16 Gy with nicotinamide. At RDR, the period increased in the following order: irradiation only = irradiation with nicotinamide < irradiation with MTH.

Figure 5 shows the counted numbers of lung metastases on day 35 after inoculation as a function of the dose of γ -rays at HDR or RDR irradiation with or without nicotinamide or MTH. Without irradiation, nicotinamide treatment and MTH tended to decrease and increase the numbers of macroscopic metastases, respectively. As the dose of γ -rays increased, the numbers decreased, especially at HDR. Further, with HDR irradiation, the numbers decreased in the following order: irradiation only > irradiation with MTH > irradiation with nicotinamide treatment. With RDR irradiation, no apparent difference in the number of metastases was found among these three irradiation conditions.

The numbers of lung metastases from the local tumours that received γ -ray irradiation with or without nicotinamide or MTH, which produced an identical SF of 0.05 as an initial effect on the local tumour, were estimated using the data shown in Figure 5 (Table 6). At HDR, the combination with nicotinamide resulted in smaller numbers than any other irradiation condition. At RDR, the numbers were apparently larger than at HDR, and no difference in the number of metastases was found among the three conditions.

Discussion

Tumour hypoxia is a direct consequence of structural abnormalities of the microvasculature and functional abnormalities of the microcirculation in solid tumours, and results from either limited oxygen diffusion (chronic hypoxia) or limited perfusion (acute hypoxia, transient hypoxia or ischaemic hypoxia). Large intercapillary distances resulting from rapid tumour cell proliferation led to chronically hypoxic cells existing at the rim of the oxygen diffusion distance [15]. Factors such as vessel plugging by blood cells or circulating tumour cells, the collapse of vessels in regions of high tumour interstitial pressure, or spontaneous vasomotor activity in normal tissue vessels incorporated into the tumour which

Figure 2. Dose-response curves of normalised micronucleus frequency for total (left) and quiescent (right) cell populations from B16-BL6 tumours irradiated at a high dose rate (HDR) or reduced dose rate (RDR) with or without nicotinamide treatment or mild temperature hyperthermia (MTH) on day 18 after inoculation. Symbols are as in Figure 1. Bars represent standard errors. The differences between total and quiescent cells for each combination condition were significant ($p < 0.05$). The difference between nicotinamide combination and radiation only at HDR irradiation in total cells was significant ($p < 0.05$). The differences between MTH combination and radiation only at both HDR and RDR irradiation were significant in both total and quiescent cells ($p < 0.05$). The differences between MTH and nicotinamide combination at HDR irradiation were also significant in both total and quiescent cells ($p < 0.05$).

Figure 3. The correlation between the normalised micronucleus frequency and the surviving fraction of the total tumour cell population for each tumour under each set of treatment conditions. The regression line had a significant positive correlation: $\ln Y = -5.24 X$ ($r = -0.99$, $p < 0.001$). Symbols are as in Figure 1. Bars represent standard errors. HDR, high dose rate; MTH, mild temperature hyperthermia; RDR, reduced dose rate.

subsequently affects flow in downstream tumour microvessels cause intermittent blood flow in tumours, which results in acute hypoxia [15]. Thus, acute hypoxic areas are distributed throughout the tumour depending on these causative factors and can occur sporadically in large areas of a solid tumour. Nicotinamide, a vitamin B₃ analogue, is known to prevent these transient fluctuations in tumour blood flow that lead to the development of acute hypoxia [16]. MTH was shown to increase the tumour response to radiation by improving tumour oxygenation through an increase in tumour blood flow [17], thereby overcoming chronic hypoxia based on our previous report employing SCC VII mouse squamous cell carcinoma tumours [7, 18].

As shown in Figure 3, the normalised MN frequency can fully reflect radiosensitivity as precisely as clonogenic cell survival because of a statistically significant positive correlation with SF. In this study, in B16-BL6 tumours, the radiosensitisation was more marked with nicotinamide than MTH in the total cell population, and more remarkable with MTH than nicotinamide in Q cells (Figures 1 and 2, Table 2). This means that the hypoxic fractions in the total and Q-cell populations of B16-BL6 tumours, like SCC VII tumours [9], are predominantly composed of acute and chronic hypoxic fractions, respectively. At RDR, acutely hypoxic areas appear and disappear throughout the solid tumour during long periods of irradiation, and RDR irradiation even without nicotinamide could make it possible to irradiate all the acutely hypoxic areas in solid tumours during prolonged irradiation, thus leading to no radioenhancing effect of nicotinamide (Figures 1 and 2, Table 2) [7]. Meanwhile, the changes in tumour growth as a whole (Figure 4, Table 5) were reasonably consistent with and well supported the changes in radiosensitivity of the total tumour cell population in cell survival curves (Figure 1) and dose-response curves of the normalised MN frequency (Figure 2).

Table 2. Enhancement ratios^a due to nicotinamide or mild temperature hyperthermia

	High dose rate irradiation	Reduced dose rate irradiation
Surviving fraction=0.05		
Total cell population		
+ Nicotinamide	1.3 (1.2–1.4) ^{b,c}	1.05 (1.0–1.1) ^b
+ MTH	1.15 (1.1–1.2) ^c	1.15 (1.1–1.2)
Normalised micronucleus frequency =0.3		
Total cell population		
+ Nicotinamide	1.25 (1.15–1.35) ^{d,e}	1.05 (1.0–1.1) ^d
+ MTH	1.15 (1.1–1.2)	1.15 (1.1–1.2)
Quiescent cells		
+ Nicotinamide	1.15 (1.05–1.15) ^e	1.05 (1.0–1.1)
+ MTH	1.25 (1.15–1.35)	1.2 (1.1–1.3)

MTH, mild temperature hyperthermia.

Values in parentheses are 95% confidence interval, determined using standard errors.

^aThe ratio of the dose of radiation necessary to obtain each end-point without combined treatment to that needed to obtain each end-point with the combined treatment.

^{b,c,d,e}The differences between two values were significant based on a χ^2 -squared test ($p < 0.05$).

Table 3. Dose-modifying factors due to the reduction in radiosensitivity caused by a decrease in radiation dose rate^a

	Total cell population	Quiescent cells
Surviving fraction=0.05		
Radiation only	1.4 (1.3–1.5)	–
+ Nicotinamide	1.65 (1.5–1.8)	–
+ MTH	1.3 (1.2–1.4)	–
Normalised micronucleus frequency=0.3		
Radiation only	1.3 (1.2–1.4) ^b	1.6 (1.5–1.7) ^{b,c}
+ Nicotinamide	1.5 (1.4–1.6)	1.65 (1.5–1.8) ^d
+ MTH	1.2 (1.1–1.3)	1.3 (1.2–1.4) ^{c,d}

Values in parentheses are 95% confidence intervals, determined using standard errors.

MTH, mild temperature hyperthermia.

^aThe ratio of the dose of radiation necessary to obtain each end-point with a delayed assay or reduced dose rate irradiation to that needed to obtain each end-point with an assay immediately after high dose rate irradiation.

^{b,c,d}The differences between two values was significant based on a χ^2 -squared test ($p < 0.05$).

Enhancement of the irradiation dose rate effect on the normalised frequency of micronuclei by γ -ray irradiation in the presence of nicotinamide compared with γ -ray irradiation alone was observed in the acute hypoxia-rich total cells rather than the chronic hypoxia-rich Q cells (Table 3). A recent study *in vitro* showed that hypoxia-induced translational repression can explain decreased rates of homologous recombination (HR) [19], an important mechanism for the repair of radiation-induced DNA double-stranded breaks (dsbs) in late S and G2. Another study showed that HR plays a greater role in determining hypoxic radiosensitivity than normoxic radiosensitivity [20]. Thus, the use of nicotinamide influenced repair more in the total cell population including late S and G2 phase

cells than in Q cells. In contrast, the repression of the irradiation dose rate effect by irradiation with MTH compared with irradiation alone was observed in the chronic hypoxia-rich Q cells rather than the acute hypoxia-rich total cells (Table 3). MTH combined with RDR irradiation was reported to inhibit decreases in sensitivity through a reduction in the irradiation dose rate, especially in Q cells [21]. This effect was thought to be due to inhibition of non-homologous end-joining, the predominant repair process for dsbs in cells in G0, G1 or early S phase [21].

Human solid tumours are thought to contain moderately large fractions of Q tumour cells, but they are as viable as established experimental animal tumour lines

Table 4. Dose-modifying factors for quiescent cells relative to the total cell population^a

	High dose rate irradiation	Reduced dose rate irradiation
Normalised micronucleus frequency =0.3		
Radiation only	1.6 (1.5–1.7)	1.75 (1.6–1.9)
+ Nicotinamide	1.7 (1.6–1.8)	1.75 (1.6–1.9)
+ MTH	1.5 (1.4–1.6)	1.6 (1.5–1.7)

MTH, mild temperature hyperthermia.

Values in parentheses are 95% confidence intervals, determined using standard errors.

^aThe ratio of the dose of radiation necessary to obtain each end-point in the quiescent cell population to that needed to obtain each end-point in the total tumour cell population.

Figure 4. Tumour growth curves for B16-BL6 tumours after γ -ray irradiation at a high dose rate (HDR) or reduced dose rate (RDR) with or without nicotinamide treatment or mild temperature hyperthermia (MTH) on day 18 after tumour cell inoculation. Tumour growth was determined by comparing tumour volume with that at γ -ray irradiation. Circles, triangles and squares represent the rates after irradiation only under aerobic conditions, after irradiation under aerobic conditions following MTH, and after irradiation under aerobic conditions following nicotinamide treatment, respectively. Open and solid symbols represent the rates for the tumours without irradiation and after irradiation, respectively. Solid and dotted lines represent the rates for HDR and RDR irradiation, respectively. Bars represent standard errors.

that have been employed for various oncology studies. The presence of Q cells is probably due, at least in part, to hypoxia and the depletion of nutrition in the tumour core, a consequence of poor vascular supply [22]. As a result, Q cells are viable and clonogenic, but cell division has ceased. This might promote the formation of micronuclei

at 0 Gy in Q tumour cells (Table 1). Q cells were shown to have significantly less radiosensitivity than the total cell population (Figure 2, Table 4). This means that more Q cells survive radiation therapy than P cells. Thus, the control of Q cells has a great impact on the outcome of radiation therapy. Overall, the difference in radiosensitivity between the total and Q-cell populations was increased by reducing the dose rate because of the greater reduction in radiosensitivity in Q cells than in the total cell population (Table 4) [21]. Nicotinamide enhanced the radiosensitivity of the total cell population at HDR, leading to a widening of the difference in radiosensitivity. At RDR, however, its effect disappeared due to the characteristics of acute hypoxia in solid tumours, resulting in no change in the difference in radiosensitivity. MTH enhanced the radiosensitivity of the chronic hypoxia-rich Q cells more than that of the total cell population at both HDR and RDR, leading to a decrease in the difference in radiosensitivity. Thus, manipulating hypoxia during RDR irradiation influences tumour radiosensitivity as well as HDR in both total and Q-cell populations. Irradiation dose rate also should be considered when manipulating intratumour hypoxia in radiotherapy.

Hypoxia is suggested to enhance metastasis by increasing genetic instability in the tumour micro-environment, selecting for cells with a diminished apoptotic potential, and altering gene expression [23]. Acute but not chronic hypoxia increased the number of macroscopic metastases as well as spontaneous micro-metastases in mouse lungs *in vivo* [4, 5]. In the current study, with or without HDR irradiation, an acute hypoxia-releasing agent, nicotinamide, reduced the number of macroscopic lung metastases (Figure 5, Table 6), also supporting that releasing cells from acute hypoxia is more important in suppressing metastasis from the primary tumour *in vivo* than releasing cells from chronic hypoxia. Without irradiation, MTH increased the number of metastases slightly, implying that release from chronic hypoxia is not as important in repressing metastasis *in vivo* as release from acute hypoxia. At present, hyperthermia is not thought to induce metastasis in the clinical setting [24]. However, the effect of nicotinamide in combination with RDR irradiation on lung metastases disappeared because even without nicotinamide, γ -rays could be delivered to all acutely hypoxic areas in solid tumours during long periods of irradiation. Meanwhile, as the dose of γ -rays increased with both HDR and RDR irradiation, the number of macroscopic lung metastases decreased reflecting the decrease in the number of clonogenically viable tumour cells in the primary

Table 5. The period (days) required for each tumour to become three times as large as on day 18 after tumour cell inoculation

γ -Ray irradiation	0 Gy	16 Gy RDR	16 Gy HDR
Irradiation only	3.4 (2.8–4.0)	8.8 (7.8–9.8)	15.5 (13.0–17.0) ^{a,b}
Combined with nicotinamide	3.4 (2.8–4.0)	9.3 (8.3–10.3)	24.7 (22.7–26.7) ^{a,c}
Combined with MTH	4.3 (3.4–5.2)	10.7 (9.5–11.9)	20.7 (18.7–22.7) ^{b,c}

RDR, reduced dose rate; HDR, high dose rate; MTH, mild temperature hyperthermia.

Values in parentheses are 95% confidence intervals, determined using standard errors.

^{a,b,c}The differences between two values were significant based on a χ^2 -squared test ($p < 0.05$).

Whether at irradiation only, nicotinamide or MTH combination, the differences among the values for 0 Gy, 16 Gy (RDR) and 16 Gy (HDR) were significant ($p < 0.05$) each other based on a χ^2 -squared test.

Figure 5. Counted numbers of macroscopic metastases in the lung on day 35 after tumour cell inoculation as a function of the dose of γ -rays at a high dose rate (HDR) or reduced dose rate (RDR) with or without nicotinamide treatment or mild temperature hyperthermia (MTH) on day 18 after tumour cell inoculation. Circles, triangles, and squares represent the numbers after irradiation only under aerobic conditions, after irradiation under aerobic conditions following MTH, and after irradiation under aerobic conditions following nicotinamide treatment, respectively. Open and solid symbols represent the numbers after HDR and RDR irradiation, respectively. Bars represent standard errors. The differences between nicotinamide combination and radiation only and between nicotinamide and MTH combination were significant only at HDR irradiation ($p < 0.05$).

tumour (Figure 5). Further, the metastasis-repressing effect of the release from acute hypoxia without irradiation became less distinct by combining with irradiation. This may be partly because the metastasis-repressing effect achieved through a reduction in the number of clonogenic tumour cells by irradiation is much more remarkable than that achieved by releasing tumour cells from acute hypoxia.

Nicotinamide combined with HDR irradiation and HDR rather than RDR irradiation were thought to be promising for reducing numbers of lung metastases. Namely, control of the acute hypoxia-rich total cell population in the primary tumour in addition to control of the primary tumour as a whole with HDR, rather than RDR irradiation, has an impact on the potential to regulate lung metastasis. Manipulating hypoxia in solid

Table 6. The numbers of metastases from the irradiated tumours that received cytotoxic treatment producing a similar initial local effect^a

	High dose rate irradiation	Reduced dose rate irradiation
Surviving fraction=0.05		
Radiation only	15.4	18.8
+ Nicotinamide	14.5	18.8
+ MTH	15.3	18.2

MTH, mild temperature hyperthermia.

^aUsing the data shown in Figure 5, the numbers of lung metastases were estimated from local tumours that received γ -ray doses combined with or without nicotinamide or mild temperature hyperthermia, which produced an identical surviving fraction of 0.05 as a cell survival after *in vivo-in vitro* assay method for irradiated local tumours.

tumours has the potential to influence not only local tumour control but also lung metastasis. In the future, from the viewpoint of repressing lung metastasis, we plan to evaluate the usefulness of nicotinamide in combination with chemotherapy or high linear energy transfer radiation therapy at HDR and RDR, referring to its effect on the response of quiescent tumour cells *in vivo*.

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