Reducing intratumour acute hypoxia through bevacizumab treatment, referring to the response of quiescent tumour cells and metastatic potential

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Objectives: The aim was to evaluate the influence of bevacizumab on intratumour oxygenation status and lung metastasis following radiotherapy, with specific reference to the response of quiescent (Q) cell populations within irradiated tumours.

Methods: 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 following treatment with the acute hypoxia-releasing agent nicotinamide or local mild temperature hyperthermia (MTH) with or without the administration of bevacizumab under aerobic conditions or totally hypoxic conditions, achieved by clamping the proximal end of the tumours. Immediately after the irradiation, cells from some tumours were isolated and incubated with a cytokinesis blocker. The responses of the Q and total (P + Q) cell populations were assessed based on the frequency of micronuclei using immunofluorescence staining for BrdU. In the other tumour-bearing mice, macroscopic lung metastases were enumerated 17 days after irradiation.

Results: 3 days after bevacizumab administration, acute hypoxia-rich total cell population in the tumour showed a remarkably enhanced radiosensitivity to γ -rays, and the hypoxic fraction (HF) was reduced, even after MTH treatment. However, the hypoxic fraction was not reduced after nicotinamide treatment. With or without γ -ray irradiation, bevacizumab administration showed some potential to reduce the number of lung metastases as well as nicotinamide treatment.

Conclusion: Bevacizumab has the potential to reduce perfusion-limited acute hypoxia and some potential to cause a decrease in the number of lung metastases as well as nicotinamide.

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It was believed that antiangiogenic therapy prevents tumour vascular growth and proliferation and deprives the tumour of oxygen and nutrients necessary for survival [1]. However, subsequent study has suggested that antiangiogenic therapy may also “normalise” the tumour vasculature for a short period of time, thereby providing a window of opportunity for improved drug delivery and enhanced sensitivity to radiation [1, 2]. Tumour hypoxia results from either limited oxygen diffusion (chronic hypoxia) or limited perfusion (acute hypoxia) [3]. Furthermore, it has been reported that acute and cyclic, but not chronic, hypoxia significantly increases the number of spontaneous lung metastases, and that this effect is due to the influence of acute hypoxia treatment on the primary tumour [4, 5].

In this study, we attempted to analyse hypoxia in solid tumours after the administration of the vascular endothelial growth factor (VEGF) inhibitor, bevacizumab, using the acute hypoxia-releasing agent nicotinamide combined

with γ -ray irradiation in terms of both local tumour response and lung metastasis compared with irradiation combined with mild temperature hyperthermia (MTH), which has already been shown to have the potential to release tumour cells from diffusion-limited chronic hypoxia [6, 7]. In addition, for the local tumour response, the effect on the total (proliferating (P)+quiescent (Q)) tumour cell population and on the Q cell population was evaluated using our original method for detecting the response of Q cells in solid tumours [8].

Methods and materials

Mice and tumours

B16-BL6 murine melanoma cells (Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan) derived from C57BL/6 mice were maintained *in vitro* in RPMI-1640 medium supplemented with 10% foetal 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 Co. Ltd., Osaka, Japan). 18 days later, the tumours, approximately

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7 mm in diameter, were employed for cytotoxic treatment. The body weight of the tumour-bearing mice was 20.1 ± 2.1 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 at our university. The p53 of B16-BL6 tumour cells is the wild type [9].

Labelling with 5-bromo-2-deoxyuridine

12 days after inoculation, mini-osmotic pumps (Durct Corporation, Cupertino, CA) containing 5-bromo-2-deoxyuridine (BrdU) dissolved in physiological saline (250 mg ml^{-1}) were implanted subcutaneously into the animals' backs for 6 days to label all P cells. The percentage of labelled cells after continuous treatment with BrdU reached a plateau at this stage. Therefore, tumour cells not incorporating BrdU after continuous labelling were regarded as Q cells.

Treatment

15 days after tumour cell inoculation, bevacizumab dissolved in physiological saline was intravenously administered at a dose of 10 mg kg^{-1} through a tail vein in a single injection. Bevacizumab has already been shown to induce the period of vascular normalisation in B16-F10 murine melanoma tumours originating from B16-F1 murine melanoma cells [10]. Thus, in B16-BL6 tumours, also originating from B16-F1 murine melanoma cells, bevacizumab was thought to work in the same way. 3 days later (18 days after inoculation), the percentage of labelled cells after 6 days of continuous administration of BrdU were $60.1 \pm 6.8\%$ and $54.3 \pm 6.0\%$ treated with bevacizumab and those not, respectively. 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} on day 18. 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 with no anaesthetic.

Some tumour-bearing mice received an intraperitoneal administration of nicotinamide (1000 mg kg^{-1}) dissolved in physiological saline 1 h before the irradiation. Others were subjected to local mild temperature hyperthermia (MTH) at 40°C for 60 min by immersing the implanted tumour in a water bath immediately before the irradiation [11]. Temperatures at the tumour centre were equilibrated within 3 to 4 min of immersion in the water bath, and remained at $0.2\text{--}0.3^\circ\text{C}$ below the bath temperature. The water bath temperature was maintained at 0.3°C above the desired tumour temperature [11]. Some tumours implanted in other tumour-bearing mice were made totally hypoxic by clamping the proximal end of the tumours 5 min before irradiation, as reported previously [12]. The tumours were kept entirely hypoxic during the irradiation. This clamping method did not influence cell survival or the levels of micronuclei. Immediately after the irradiation, the clamp was released. 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 trypsinised, and cell suspensions were fixed and resuspended with cold Carnoy's fixative (ethanol:acetic acid=3:1). The suspension was placed on a glass microscope slide, dried at room temperature and treated with 2 M hydrochloric acid for 60 min at room temperature to dissociate the histones and partially denature the DNA. The slides were 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 distinguish between the tumour cells stained with and those without green-emitting FITC and observe them separately, cells on the slides were treated with red-emitting propidium iodide (PI, $2 \mu\text{g ml}^{-1}$ in PBS) as a background staining 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 [8].

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 not given 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, 12 days later, colonies were fixed with ethanol, stained with Giemsa and counted. For the tumours that received no irradiation, the plating efficiencies for the total tumour cell populations and the MN frequencies for the total and Q cell populations are shown in Table 1. The plating efficiency indicates the percentage of cells seeded that grow into colonies when the tumours received no irradiation. The fraction of cells surviving a given dose is determined by counting the number of macroscopic colonies as a fraction of the number of cells seeded, followed by allowance; that is, dividing by the plating efficiency.

Table 1. Plating efficiency and micronucleus frequency at 0 Gy

	Total cell population	Quiescent cells
Plating efficiency (%)		
Absolutely control	84.4 ± 8.2	–
Totally hypoxic	73.9 ± 7.3	–
Nicotinamide only	81.4 ± 7.3	–
MTH only	83.5 ± 8.7	–
BV only	80.1 ± 8.0	–
BV+nicotinamide	76.3 ± 7.8	–
BV+MTH	78.4 ± 7.9	–
Micronucleus frequency		
Absolutely control	0.050 ± 0.008	0.077 ± 0.009
Totally hypoxic	0.069 ± 0.008	0.087 ± 0.007
Nicotinamide only	0.057 ± 0.006	0.084 ± 0.009
MTH only	0.054 ± 0.005	0.081 ± 0.009
BV only	0.058 ± 0.007	0.083 ± 0.009
BV+nicotinamide	0.068 ± 0.008	0.086 ± 0.010
BV+MTH	0.069 ± 0.008	0.087 ± 0.010

Values are presented as mean ± standard error (*n*=9). MTH, mild temperature hyperthermia; BV, bevacizumab.

As stated above, the MN frequencies for Q cells were obtained from unlabeled tumour cells after continuous BrdU labelling. The MN frequencies and surviving fractions (SFs) for total cell populations were obtained from cells in tumours not pretreated with BrdU. Thus, no interaction between BrdU and γ -ray irradiation could be observed on the values of MN frequency and SF.

Measurement of the hypoxic fraction in B16-BL6 tumours

The MN frequency in tumour cells not labelled with BrdU (=Q cells) were translated to the surviving fraction (SF) using the regression line for the relationship between the normalised MN frequency and the SF determined for the total cell populations in tumours from mice that were not pre-treated with BrdU [6, 8].

To determine the hypoxic fraction (HF) of the tumours, the paired survival curve method was employed using

the values of the SFs for more than 16 Gy [5, 13]. The best linear parallel lines were fitted to the dose-survival curves under both aerobic and hypoxic conditions by least squares regression, and the HF was determined from the vertical displacement of the parallel lines [6–8].

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 treatment with bevacizumab, nicotinamide or MTH, the size of the tumours implanted in the left hind legs of some tumour-bearing mice was checked 2 to 3 times a week for approximately 20 days. Tumour volume was calculated using the formula:

$$V = \pi/6 \times a \times b^2$$

where *a* and *b* are the longest and shortest diameters of the tumour measured with callipers, respectively. There was no significant difference in tumour growth between the non-irradiated tumours treated with intravenous administration of bevacizumab on day 15 and those not.

Metastasis assessment

17 days after 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 were counted under a dissection microscope [14]. 18 days after the inoculation and immediately before γ -ray irradiation with or without bevacizumab, nicotinamide or MTH, the numbers of macroscopic lung metastases were also counted as background data. The numbers obtained were 5.5 ± 1.8 and 7.5 ± 2.2 with and without the intravenous administration of bevacizumab on day 15, respectively.

Figure 1. Cell survival curves for the total cell population from B16-BL6 tumours irradiated with γ -rays with or without treatment with bevacizumab (BV), nicotinamide or mild temperature hyperthermia (MTH) on day 18 after tumour cell inoculation. The clonogenic surviving fractions for irradiation under totally hypoxic conditions achieved by clamping the proximal end of the tumours are also shown. ○ and ●, irradiation under aerobic conditions; □ and ■, irradiation under aerobic conditions following nicotinamide treatment; △ and ▲, irradiation under aerobic conditions following MTH; ○, □ and △, irradiation without BV; ●, ■ and ▲, irradiation with BV; ▼, irradiation under totally hypoxic conditions. Bars represent standard error (*n*=9).

Figure 2. Dose response curves of the normalised micronucleus frequency for total (left panels) and quiescent (right panels) cell populations from B16-BL6 tumours irradiated with or without bevacizumab (BV) in combination with nicotinamide (upper panels) or mild temperature hyperthermia (MTH) (lower panels) on day 18 after inoculation. ○ and ●, irradiation under aerobic conditions; □ and ■, irradiation under aerobic conditions following nicotinamide treatment; △ and ▲, irradiation under aerobic conditions following MTH; ○, □ and △, irradiation without BV; ●, ■ and ▲, irradiation with BV; ▼, irradiation under totally hypoxic conditions. Bars represent standard errors ($n=9$).

Data analysis and statistics

Three mice with a tumour in the left hind leg were used to assess each set of conditions and each experiment was repeated three times; i. e., nine mice were used for each set of conditions. To examine the difference between pairs of values, Student's *t*-test was used where 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. The data of cell survival and MN frequencies were fitted to the linear quadratic dose relationship [15].

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. The Q cell population showed significantly higher MN frequencies than did the total cell population under each set of conditions.

Figure 1 shows cell survival curves for the total cell population as a function of the dose of γ -rays with or

Table 2. Enhancement ratios^h owing to combined treatment with bevacizumab, nicotinamide or mild temperature hyperthermia

	Total cell population	Quiescent cells
Surviving fraction=0.05		
+Nicotinamide	1.3 ± 0.1	–
+MTH	1.1 ± 0.05^a	–
+BV only	1.2 ± 0.1	–
+BV+Nicotinamide	1.25 ± 0.1	–
+BV+MTH	1.35 ± 0.1^a	–
Normalised micronucleus frequency=0.3		
+Nicotinamide	1.2 ± 0.1^b	1.05 ± 0.05^b
+MTH	$1.05 \pm 0.05^{c,d}$	1.25 ± 0.05^c
+BV only	1.15 ± 0.05^e	$1.05 \pm 0.05^{e,f}$
+BV+Nicotinamide	1.15 ± 0.05^g	1.05 ± 0.05^g
+BV+MTH	1.25 ± 0.1^d	1.3 ± 0.1^f

Values are presented as mean $\pm$ standard error ($n=9$).

Letters ^{a–g} represent the significant differences between two values ($p<0.05$).

^hThe 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.

MTH, mild temperature hyperthermia; BV, bevacizumab.

without bevacizumab, nicotinamide or MTH. Figure 2 shows normalised MN frequencies as a function of irradiated dose with or without bevacizumab, nicotinamide or MTH in the total (left panels) and Q (right panels) 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 in the total cell population ($p<0.05$).

To estimate the radio-enhancing effect of bevacizumab, 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). In both cell populations, each combined treatment significantly enhanced the radiosensitivity compared with irradiation only ($p<0.05$). Nicotinamide only, bevacizumab only and bevacizumab + nicotinamide significantly enhanced the radiosensitivity of total cell populations compared with Q cell populations ($p<0.05$). In contrast, MTH affected the Q cell populations more than total cell populations. In the total cell population, the addition of bevacizumab to MTH significantly increased the radio-enhancing effect ($p<0.05$). In the Q cell population, the addition of MTH to bevacizumab significantly increased the effect ($p<0.05$).

To examine the difference in radiosensitivity between the total and Q cell populations, dose-modifying factors, were calculated using the data in Figure 2 (Table 3). The difference in radiosensitivity, especially without bevacizumab, was widened with nicotinamide and reduced with MTH. However, the difference under irradiation after MTH was significantly enlarged with bevacizumab ($p<0.05$). By contrast, the difference under irradiation only and irradiation after nicotinamide administration did not change very much with bevacizumab.

For each set of irradiation conditions, the regression lines for the relationship between the normalised MN frequency and the clonogenic SF determined for the total

Table 3. Dose-modifying factors for quiescent cells relative to the total cell population^d

	Without bevacizumab	With bevacizumab
Normalised micronucleus frequency = 0.3		
Radiation only	1.7 ± 0.1 ^{a,b}	1.75 ± 0.1
+Nicotinamide	1.9 ± 0.1 ^a	1.85 ± 0.1
+MTH	1.5 ± 0.1 ^{b,c}	1.7 ± 0.1 ^c

Values are presented as mean ± standard error (*n*=9).

MTH, Mild temperature hyperthermia.

Letters ^{a-c} represent the significant differences between two values (*p*<0.05).

^dThe 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.

tumour cell population were found to be statistically identical. Thus, a regression line was calculated from pooled data for all sets of conditions and found to have a significant positive correlation (*p*<0.001) (Figure 3). The

Figure 3. The correlation between the normalised micronucleus frequency and the surviving fraction of the total cell population for each tumour under each set of treatment conditions. The regression line had a significant positive correlation: $\ln Y = -5.11 \times X$ ($r = -0.96$, $p < 0.001$). ○ and ●, irradiation under aerobic conditions; □ and ■, irradiation under aerobic conditions following nicotinamide treatment; △ and ▲, irradiation under aerobic conditions following MTH; ○, □ and △, irradiation without bevacizumab; ●, ■ and ▲ irradiation with bevacizumab; ▼, irradiation under totally hypoxic conditions. Bars represent standard errors (*n*=9).

normalised MN frequency of Q cells was translated to the clonogenic SF of Q cells using the regression line determined for the total tumour cell population.

Based on directly determined clonogenic SFs of the total tumour cell populations, the HF of total cells was determined for all conditioned tumours except totally hypoxic tumours (Table 4). Based on the clonogenic SFs of Q cell populations determined as shown above, the HF of Q cells was determined except for totally hypoxic tumours (Table 4). Overall, the values were significantly larger for Q cells than the total cells under each set of conditions (*p*<0.05). Without bevacizumab, the size of the HF was significantly reduced in the following order: without MTH or nicotinamide > with MTH > with nicotinamide in the total cell population, and without nicotinamide or MTH > with nicotinamide > with MTH in the Q cell population. With bevacizumab, in both populations, the further combination with MTH significantly decreased the size of the HF (*p*<0.05). Similarly, with MTH, in both populations, the further combination with bevacizumab significantly decreased the size of the HF (*p*<0.05). In the total cell population, bevacizumab significantly decreased the size of the HF (*p*<0.05) compared with no combination.

Figure 4 shows tumour growth curves after irradiation with or without treatment with bevacizumab, nicotinamide or MTH 18 days after the tumour cell inoculation. To evaluate tumour growth, the period required for each tumour to become three times as large as on day 18 was obtained using the data shown in Figure 4 (Table 5). Without irradiation, with or without bevacizumab, there was no significant difference in the period among without nicotinamide or MTH, with nicotinamide and with MTH. With irradiation at a dose of 16 Gy, the period required was significantly prolonged (*p*<0.05). Without bevacizumab, the period was significantly prolonged in combination with nicotinamide or MTH, especially with nicotinamide (*p*<0.05). With bevacizumab, the period was significantly extended with MTH

Table 4. The size of the hypoxic fractions (%) in total and quiescent tumour cell populations^f

	Without bevacizumab	With bevacizumab
Total tumour cell population		
Without nicotinamide or MTH	16.1 ± 2.8 ^{a,b,c}	8.4 ± 1.7 ^a
+Nicotinamide	7.5 ± 1.6 ^{b,d}	8.1 ± 1.7
+MTH	11.5 ± 1.6 ^{c,d,e}	6.3 ± 1.5 ^e
Quiescent tumour cell population		
Without nicotinamide or MTH	51.3 ± 5.5 ^{f,g}	44.6 ± 5.3 ^h
+Nicotinamide	38.5 ± 6.4 ^{f,i}	41.2 ± 6.5 ^j
+MTH	25.5 ± 3.4 ^{g,i,k}	17.8 ± 3.6 ^{h,j,k}

Values are presented as mean ± standard error (*n*=9).

MTH, mild temperature hyperthermia.

Letters ^{a-k} represent the significant differences between two values (*p*<0.05). Overall, the values were significantly larger in Q cells than the total cell population under each set of conditions (*p*<0.05).

^fBased on the paired survival curve method using the dose-survival curves under both aerobic and hypoxic conditions for more than 16 Gy.

Figure 4. Tumour growth curves for B16-BL6 tumours after γ -ray irradiation with or without treatment with bevacizumab (BV), nicotinamide or mild temperature hyperthermia (MTH) on day 18 after tumour cell inoculation. Tumour growth was determined by comparing tumour volume with that on γ -ray irradiation. $\circ$ and $\bullet$, irradiation under aerobic conditions; $\square$ and $\blacksquare$, irradiation under aerobic conditions following nicotinamide treatment; $\triangle$ and $\blacktriangle$, irradiation under aerobic conditions following MTH; $\circ$, $\square$ and $\triangle$, without irradiation; $\bullet$, $\blacksquare$ and $\blacktriangle$, with irradiation at a dose of 16 Gy; broken line, irradiation without BV; solid line, irradiation with BV. Bars represent standard errors ($n=9$).

($p<0.05$). For irradiation only and irradiation after MTH, the period was also significantly prolonged in combination with bevacizumab ($p<0.05$).

Figure 5 shows the numbers of lung metastases on day 35 after inoculation as a function of the dose of γ -rays with or without bevacizumab, nicotinamide or MTH. Without irradiation, irrespective of bevacizumab combination, nicotinamide and MTH appeared to decrease and increase the numbers of macroscopic metastases, respectively. With irradiation, as the delivered dose of γ -rays increased, the numbers decreased. Essentially, since there was an almost parallel shift in all the curves, no apparent radiosensitising or protecting effect was observed in terms of the numbers of lung metastases. However, at the doses of 8 and 16 Gy, bevacizumab combination appeared to decrease the numbers a little.

The number of lung metastases from local tumours that received γ -rays under each irradiation condition, which produced an identical SF of 0.05 as an initial effect, were estimated using the data shown in Figure 5 (Table 6). Irrespective of bevacizumab combination, irradiation combined with nicotinamide resulted in smaller numbers

Table 5. The period in days required for each tumour to become 3 times as large as on day 18 after tumour cell inoculation

	Without bevacizumab	With bevacizumab
No irradiation		
No treatment	3.4 ± 0.6	3.1 ± 0.5
+Nicotinamide	3.4 ± 0.6	3.3 ± 0.6
+MTH	4.3 ± 0.9	3.6 ± 0.7
Irradiated dose=16 Gy		
Radiation only	$13.5 \pm 2.0^{a,b,c}$	$22.4 \pm 1.4^{a,d}$
+Nicotinamide	24.7 ± 1.5^b	23.7 ± 1.5
+MTH	$20.7 \pm 2.0^{c,e}$	$27.3 \pm 2.1^{c,d,e}$

Values are presented as mean $\pm$ standard error ($n=9$).

MTH, mild temperature hyperthermia.

Letters^{a-e} represent the significant differences between two values ($p<0.05$). Whether for irradiation only, irradiation plus nicotinamide, or irradiation plus MTH with or without bevacizumab, the differences among the values for 0 Gy and 16 Gy were significant ($p<0.05$).

of metastases than any other combination. Irrespective of nicotinamide or MTH combination, the addition of bevacizumab appeared to reduce the number of metastases.

Discussion

Perfusion-related (acute) hypoxia is caused by inadequate blood flow in tissues. Tumour microvasculatures frequently have severe structural and functional abnormalities, such as a disorganised vascular network, dilations, an elongated and tortuous shape, an incomplete endothelial lining, a lack of physiological/pharmacological receptors, an absence of flow regulation and intermittent stasis [16]. Perfusion-related oxygen delivery leads to ischaemic hypoxia, which is often transient. Thus, acute hypoxic areas are distributed throughout the tumour depending on the causative factor [3, 5, 13]. Nicotinamide, a vitamin B₃ analogue, prevents these transient fluctuations in tumour blood flow that lead to the development of acute hypoxia [17]. Diffusion-related (chronic) hypoxia is caused by an increase in diffusion distance with tumour expansion; this results in inadequate oxygen supply for cells distant ($>70 \mu\text{m}$) from the nutritive blood vessels. Diffusion-related hypoxia may also be caused by deterioration of diffusion "geometry," for example, concurrent *vs* countercurrent blood flow within the tumour microvessel network [5, 13]. MTH before irradiation decreased the HF, even combined with nicotinamide administration. In contrast, MTH did not decrease the HF when tumour-bearing mice were placed in a circulating carbogen (95% O₂/5% CO₂) chamber during irradiation [6]. Thus, MTH was shown to increase the tumour response to radiation by improving tumour oxygenation through an increase in tumour blood flow [18], thereby preferentially overcoming chronic hypoxia rather than acute hypoxia.

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. A similar finding has already been demonstrated in a previous report [19]. In B16-BL6 tumours, the radiosensitisation was a little

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 with or without treatment with bevacizumab (BV), nicotinamide, or mild temperature hyperthermia (MTH) on day 18 after tumour cell inoculation. ○ and ●, irradiation under aerobic conditions; □ and ■, irradiation under aerobic conditions following nicotinamide treatment; △ and ▲, irradiation under aerobic conditions following MTH; ○, □ and △, irradiation without bevacizumab; ●, ■ and ▲, irradiation with bevacizumab. Bars represent standard errors ($n=9$).

higher with nicotinamide than with MTH in the total cell population, and a little larger with MTH than with nicotinamide in Q cells (Figures 1 and 2, Table 2). Further, nicotinamide and MTH remarkably reduced the size of the HF in total and Q cell populations, respectively (Table 4). These findings support the theory that the HFs in the total and Q cell populations of B16-BL6 tumours, like SCC VII tumours, are predominantly composed of acute and chronic HFs, respectively [6].

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

	Without bevacizumab	With bevacizumab
Surviving fraction = 0.05		
Radiation only	14.9	11.8
+Nicotinamide	14.0	11.3
+MTH	14.8	13.1

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 with or without bevacizumab, nicotinamide or mild temperature hyperthermia, which produced an identical surviving fraction of 0.05 after *in vivo-in vitro* assays.

The decrease in the HF induced by combining bevacizumab with MTH was more remarkable than that achieved by combining bevacizumab with nicotinamide treatment in both total and Q cell populations (Table 4). In both bevacizumab and/or nicotinamide, and bevacizumab and/or MTH, bevacizumab alone decreased the HF more markedly in the total than in the Q cell population (Table 4). These results indicated that bevacizumab preferentially oxygenated the acute HF rather than the chronic HF in this tumour. In other words, the HF in the tumour treated with bevacizumab may be preferentially composed of chronic HF. Bevacizumab has been shown to decrease the size of chronic HF to some degree [10], but the reduction of acute hypoxia is thought to be much more remarkable than that of chronic hypoxia, based on our findings. The changes in tumour growth as a whole (Figure 4 and Table 5) were reasonably consistent with, and supported, the changes in the radiosensitivity of total tumour cell populations in cell survival curves (Figure 1) and dose-response curves of the normalised MN frequency (Figure 2).

The recombinant humanised monoclonal antibody bevacizumab is composed of the human IgG1 framework regions and the antigen-binding regions from the murine IgG1 anti-human VEGF monoclonal antibody [20]. The antibody can be cross-reactive with other species, as shown here. In previous animal experiments, tumour hypoxia decreased 2 days after anti-angiogenic treatment such as VEGF-blocking therapy, had almost disappeared by day 5, and then increased again by day 8 [2, 10]. In addition to reducing hypoxia, anti-angiogenic treatment is thought to be associated with the recruitment of pericytes — cells that help shore up vessel walls — to the tumour blood vessels, which stabilise the leaky and dilated vasculature, common characteristics of tumour vessels [21]. The pericyte-covered vessels were also reported to decrease in number by day 8 [20]. Vascular normalisation including the recruitment of pericytes is thought to occur 2 to 5 days after the blocking of VEGF [1, 3]. During this window, pericyte coverage of tumour vessels [1, 2] and a decrease in tumour vessel permeability and interstitial fluid pressure [22] occur, resulting in normalisation of tumour vessels leading to the release from acute hypoxia.

The presence of Q cells is probably due, at least in part, to hypoxia and the depletion of nutrition, a consequence of poor vascular supply [1, 13, 22]. As a result, Q cells are viable and clonogenic, but have ceased dividing. 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 ($p<0.05$) (Figure 2 and Table 3). This means that more Q cells survive radiation therapy than P cells. Thus, the control of Q cells has a greater impact on the outcome of radiotherapy for controlling local tumours. The difference in radiosensitivity between the total and Q cell populations was increased by adding bevacizumab (Table 3) because of the greater enhancement in radiosensitivity in the total than in the Q cell population through the release from acute hypoxia, except when combined with MTH (Table 2). Nicotinamide and MTH enhanced the radiosensitivity of the total and Q cell populations at irradiation, leading to an increase and a decrease in the difference in radiosensitivity, respectively [6, 7]. MTH

was thought to be more useful than nicotinamide or bevacizumab because of the MTH-induced reduction of the difference in sensitivity between radiosensitive total and radioresistant Q cell populations.

Hypoxia is suggested to enhance metastasis by increasing genetic instability [5]. Acute but not chronic hypoxia increased the number of macroscopic metastases in mouse lungs [4, 5]. We have recently reported the significance of the injection of an acute hypoxia-releasing agent, nicotinamide, into tumour-bearing mice as a combined treatment with γ -ray irradiation in terms of repressing lung metastasis [7]. With or without irradiation, nicotinamide and the VEGF blocking agent bevacizumab seemed to reduce the number of macroscopic lung metastases (Figure 5 and Table 6). During the window of vascular normalisation, acute hypoxia may be preferentially released and this release seems to be more important in suppressing metastasis from the primary tumour than is the release of cells from chronic hypoxia. Without irradiation, MTH seemed to increase the number of metastases, implying that the release from chronic hypoxia is not as important in repressing metastasis as the release from acute hypoxia. However, hyperthermia is not thought to induce metastasis in the clinical setting [23]. Meanwhile, as the dose of γ -rays increased with irradiation, the number of macroscopic lung metastases decreased, reflecting the decrease in the number of clonogenically viable tumour cells in the primary tumour (Figure 5). Metastasis-repressing effect achieved through a reduction in the number of clonogenic tumour cells by irradiation is much greater than that achieved by reducing tumour cells from acute hypoxia. An acute hypoxia-releasing treatment such as the administration of nicotinamide and/or bevacizumab may be promising for reducing numbers of lung metastases. It was shown that control of the chronic hypoxia-rich Q cell population and the acute hypoxia-rich total cell population in the primary tumour can have the potential to give an impact to control local tumours and lung metastases, respectively.

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