Title Page

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

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Running head:

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Key words: acute hypoxia, quiescent cell, antiangiogenic agent, mild temperature hyperthermia

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Abstract

Purpose: To evaluate the influence of bevacizumab on intratumor oxygenation status and lung metastasis following radiotherapy, specifically with reference to the response of quiescent (Q) cell populations within irradiated tumors.

Materials and Methods: B16-BL6 melanoma tumor-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 hyperthermia at mild temperatures (MTH) with or without the administration of bevacizumab under aerobic conditions or totally hypoxic conditions achieved by clamping the proximal end of the tumors. Immediately after the irradiation, cells from some tumors 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 other tumor-bearing mice, 17 days after irradiation, macroscopic lung metastases were enumerated.

Results: Three days after bevacizumab administration, acute hypoxia-rich total cell population in the tumor showed the remarkably enhanced radio-sensitivity to γ-rays, and the hypoxic fraction (HF) was reduced, even after MTH treatment. However, the HF 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 the perfusion-limited acute hypoxia and some potential to cause the decrease in the number of lung metastases as well as nicotinamide.

Introduction

It was believed that antiangiogenic therapy prevents the tumor vascular growth and proliferation, thus depriving the tumor of oxygen and nutrients necessary for survival [1]. Subsequent study, however, suggested that antiangiogenic therapy may also "normalize" the tumor vasculature for a short period of time, thereby providing a window of opportunity for improved drug delivery and enhanced sensitivity to radiation [1,2]. Tumor hypoxia results from either limited oxygen diffusion (chronic hypoxia) or limited perfusion (acute hypoxia) [3]. Further, it was reported that acute and cyclic, but not chronic, hypoxia significantly increased the number of spontaneous lung metastases, and that this effect was due to the influence of acute hypoxia treatment on the primary tumor [4,5]

Here, we tried to analyze on hypoxia in solid tumors 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 tumor response and lung metastasis compared with irradiation combined with mild temperature hyperthermia (MTH), already shown to have the potential to release tumor cells from diffusion-limited chronic hypoxia [6,7]. In addition, concerning the local tumor response, the effect not only on the total (= proliferating (P) + quiescent (Q)) tumor cell population but also on the Q cell population was evaluated using our original method for detecting the response of Q cells in solid tumors [8].

Materials and Methods

Mice and tumors

B16-BL6 murine melanoma cells (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. Tumor cells (1.25 x 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). Eighteen days later, the tumors, approximately 7 mm in diameter, were employed for the cytotoxic treatment, and the body weight of the tumor-bearing mice was 20.1 ± 2.1 (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 at our university. The p53 of B16-BL6 tumor cells is the wild type [9].

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

Twelve days after the inoculation, mini-osmotic pumps (Durect Corporation, Cupertino, CA) containing BrdU dissolved in physiological saline (250 mg/ml) were implanted subcutaneously into the animals' backs for 6 days to label all P cells. The percentage of labeled cells after the continuous treatment with BrdU reached a plateau at this stage. Therefore, tumor cells not incorporating BrdU after continuous labeling were regarded as Q cells.

Treatment

Fifteen days after the tumor cell inoculation, bevacizumab (a humanized monoclonal antibody against VEGF, Roche) dissolved in physiological saline was intravenously administered at a dose of 10 mg/kg through a tail vein in a single injection. Bevacizumab was already

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shown to induce the period of vascular normalization in B16-F10 murine melanoma tumors originating from B16-F1 murine melanoma cells [10]. Thus, in B16-BL6 tumors also originating from B16-F1 murine melanoma cells, bevacizumab was also thought to work the same as in B16-F10 tumors. Three days later (=18 days after inoculation, on day 18), the percentages of labeled cells after the continuous administration of BrdU for 6 days were 60.1 ± 6.8 % and 54.3 ± 6.0 % treated with bevacizumab and those not, respectively. Solid tumors grown in the left hind legs of mice were irradiated with a cobalt-60 γ-ray irradiator at 2.5 Gy/min 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 under no anesthetic.

Some tumor-bearing mice received an intraperitoneal administration of nicotinamide (1000 mg/kg) dissolved in physiological saline 1 hour before the irradiation. Others were subjected to local mild temperature hyperthermia (MTH) at 40°C for 60 min by immersing the implanted tumor in a water bath immediately before the irradiation [11]. Temperatures at the tumor center equilibrated within 3 to 4 min after immersion in the water bath and remained 0.2-0.3°C below the bath’s temperature. The water bath’s temperature was maintained at 0.3°C above the desired tumor temperature [11]. Some tumors implanted in other tumor-bearing mice were made totally hypoxic by clamping the proximal end of the tumors 5 min before the irradiation, as reported previously [12]. The tumors 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 pretreated with BrdU.

Immunofluorescence staining of BrdU-labeled cells and micronucleus (MN) assay

Immediately after irradiation, some 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 hours in tissue culture dishes containing complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while allowing nuclear division. The cultures were trypsinized, and cell suspensions were fixed and resuspended with cold Carnoy's fixative (ethanol:acetic acid = 3:1 in volume). 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 neutralize the acid. BrdU-labeled tumor 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 the tumor cells between stained with and without green-emitting FITC and observe them separately, cells on the slides were treated with red-emitting propidium iodide (PI, 2 μg/ml in PBS) as a background staining 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 [8].

The ratios obtained in tumors not pretreated with BrdU indicated the MN frequency at all phases in the total tumor 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 tumors in mice given no BrdU using an *in vivo-in vitro* assay method immediately after irradiation. Tumors 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 ± 0.4 x 10^7/g tumor weight. Appropriate numbers of viable tumor cells from the single cell suspension were plated on 60 or 100 mm tissue culture dishes, and, 12 days later, colonies were fixed with ethanol, stained with Giemsa, and counted. For the tumors that received no irradiation, the plating efficiencies for the total tumor 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 tumors 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.

As stated above, the MN frequencies for Q cells were obtained from unlabeled tumor cells after continuous BrdU labeling. The MN frequencies and surviving fractions (SFs) for total cell populations were obtained from cells in tumors 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 (HF) in B16-BL6 tumors

The MN frequency in tumor cells not labeled with BrdU (= Q cells) were translated to the surviving fraction (SF), using the regression line for the relationship between the normalized MN frequency and the SF determined for the total cell populations in tumors from mice that were not pretreated with BrdU [6,8].

To determine the HF of the tumors, 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 HFs were determined from the vertical displacement of the parallel lines [6,7,8].

Growth of B16-BL6 tumors

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 tumors implanted in the left hind legs of some tumor-bearing mice was checked 2-3 times a week for about 20 days. Tumor 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 tumor measured with calipers. There was no significant difference in tumor growth between the non-irradiated tumors treated with the intravenous administration of bevacizumab on day 15 and those not.

Metastasis assessment

Seventeen days after irradiation (= 35 days after the inoculation of B16-BL6 melanoma cells), the tumor-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]. Eighteen 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.

Data analysis and statistics

Three mice with a tumor in the left hind leg were used to assess each set of conditions and each experiment was repeated three times. Namely, nine 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. The data of cell survival and MN frequencies were fitted to the linear quadratic dose relationship [15].

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Results

Table 1 shows the plating efficiencies for the total tumor 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 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 without bevacizumab, nicotinamide, or MTH. **Figures 2** shows normalized MN frequencies as a function of irradiated dose with or without bevacizumab, nicotinamide, or MTH in the total (left panels) and Q (right panels) tumor cell populations. The normalized MN frequency was the MN frequency in tumors that received γ-ray irradiation minus that in tumors that did not. Overall, the normalized MN frequencies were significantly smaller in Q cells than 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 **Figs. 1** and **2** were used (**Table 2**). In both cell populations, each combined treatment significantly enhanced the radio-sensitivity compared with irradiation only ($p < 0.05$). Nicotinamide only, bevacizumab only, and bevacizumab + nicotinamide significantly enhanced the radio-sensitivity 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 radio-sensitivity between the total and Q cell populations, dose-modifying factors, were calculated using the data in **Figs 1** and **2** (**Table 3**). The difference in radio-sensitivity, especially without bevacizumab, was widened with nicotinamide and reduced with MTH. However, the difference under irradiation after MTH was significantly enlarged with bevacizumab ($p < 0.05$). In contrast, the difference under irradiation only and irradiation after nicotinamide administration was not changed very much with bevacizumab.

For each set of irradiation conditions, the regression lines for the relationship between the normalized MN frequency and the clonogenic SF determined for the total tumor 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 ($p < 0.001$) (**Fig. 3**). The normalized MN frequency of Q cells was translated to the clonogenic SF of Q cells, using the regression line determined for the total tumor cell population.

Based on directly determined clonogenic SFs of the total tumor cell populations, the HF of total cells was determined for all conditioned tumors except totally hypoxic tumors (**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 tumors (**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 tumor growth curves after irradiation with or without treatment with bevacizumab, nicotinamide, or MTH 18 days after the tumor cell inoculation. To evaluate tumor growth, the period required for each tumor to become three times as large as on day 18 was obtained using the data shown in **Fig. 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 ($p < 0.05$). 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 seemed 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 radio-sensitizing or -protecting effect was observed in terms of the numbers of lung metastases. However, at the doses of 8 and 16 Gy, bevacizumab combination seemed to decrease the numbers a little.

The numbers of lung metastases from the local tumors 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 **Fig. 5** (**Table 6**). Irrespective of bevacizumab combination, irradiation combined with nicotinamide resulted in smaller numbers than any other combination. Irrespective of nicotinamide or MTH combination, the addition of bevacizumab administration seemed to reduce the numbers of metastases.

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Discussion

Perfusion-related (acute) hypoxia is caused by inadequate blood flow in tissues. Tumor microvasculatures frequently have severe structural and functional abnormalities, such as a disorganized 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 O_2 delivery leads to ischemic hypoxia, which is often transient. Thus, acute hypoxic areas are distributed throughout the tumor depending on these causative factors [3,5,13]. Nicotinamide, a vitamin B_3 analogue, prevents these transient fluctuations in tumor blood flow that lead to the development of acute hypoxia [17]. Diffusion-related (chronic) hypoxia is caused by an increase in diffusion distances with tumor expansion. This results in an inadequate O_2 supply for cells distant (>70 μm) from the nutritive blood vessels. Diffusion-related hypoxia may also be caused by deterioration of diffusion "geometry," for example, concurrent versus countercurrent blood flow within the tumor microvessel network [5,13]. MTH before irradiation decreased the HF, even combined with nicotinamide administration. In contrast, MTH did not decrease the HF when tumor-bearing mice were placed in a circulating carbogen (95% O2 / 5% CO2) chamber during irradiation [6]. Thus, MTH was shown to increase the tumor response to radiation by improving tumor oxygenation through an increase in tumor blood flow [18], thereby preferentially overcoming chronic hypoxia rather than acute hypoxia.

As shown in **Fig. 3**, the normalized MN frequency can fully reflect radiosensitivity as precisely as clonogenic cell survival because of

a statistically significant positive correlation with SF. Actually, similar finding was already shown in the previous report [19]. In B16-BL6 tumors, the radio-sensitization was a little higher with nicotinamide than MTH in the total cell population, and a little larger with MTH than nicotinamide in Q cells (**Figs. 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 supported that the HFs in the total and Q cell populations of B16-BL6 tumors, like SCC VII tumors, are predominantly composed of acute and chronic HFs, respectively [6].

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 total than in Q cell population (**Table 4**). These results indicated that bevacizumab preferentially oxygenated the acutely HF rather than the chronically HF in this tumor. In other words, the HF in the tumor treated with bevacizumab may be preferentially composed of the chronic HF. It is true that bevacizumab was also shown to decrease the size of the chronically HF to some degree [10], but the reduction of acute hypoxia was thought to be much more remarkable than that of chronic hypoxia, based on our findings observed here. Meanwhile, the changes in tumor growth as a whole (**Fig. 4**, **Table 5**) were reasonably consistent with and well supported the changes in the radio-sensitivity of total tumor cell populations in cell survival curves (**Fig. 1**) and dose-response curves of the normalized MN frequency (**Fig. 2**).

The recombinant humanized 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, tumor hypoxia decreased 2 days after antiangiogenic treatment such as vascular endothelial growth factor (VEGF)-blocking therapy, was almost abolished by day 5, and increased again by day 8 [2,10]. In addition to reducing hypoxia, antiangiogenic treatment is thought to be associated with the recruitment of pericytes – cells that help shore up vessel walls – to the tumor blood vessels, which stabilize the leaky and dilated vasculature, common characteristics of tumor vessels [21]. The pericyte-covered vessels were also reported to decrease in number by day 8 [20]. Vascular normalization including the recruitment of pericytes is thought to occur 2 to 5 days after the blocking of VEGF [1,3]. During this window some 2 to 5 days after the blocking of VEGF, pericyte coverage of tumor vessels [1,2] and a decrease in tumor vessel permeability and interstitial fluid pressure [22] occur, resulting in normalization of tumor 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 tumor cells (**Table 1**). Q cells were shown to have significantly less radiosensitivity than the total cell population ($p < 0.05$) (**Fig. 2, Table 3**). 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 radiotherapy for controlling local tumors. 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 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 population 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 recently reported the significance of the injection of an acute hypoxia-releasing agent, nicotinamide, into tumor-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 (**Fig. 5, Table 4**). During the window of vascular normalization, acute hypoxia may be preferentially released and this release seems to be more important in suppressing metastasis from the primary tumor 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 tumor cells in the primary tumor (**Fig. 5**). Metastasis-repressing effect achieved through a reduction in the number of clonogenic tumor cells by irradiation is much greater than that achieved by reducing tumor 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 tumor can have the potential to give an impact to control local tumors and lung metastases, respectively.

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The authors report no declarations of interest.

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Table 1.

Plating efficiency and micronucleus frequency at 0 Gy

	Total cell population	Quiescent cells
<Plating efficiency (%)>		
Absolutely control	84.4 ± 8.2[a]	----
Totally hypoxic	73.9 ± 7.3	----
Nicotinamide only	81.4 ± 7.3	----
MTH[b] only	83.5 ± 8.7	----
BV[c] 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

a; Mean ± standard error (n = 9)
b; Mild temperature hyperthermia.
c; Bevacizumab

Table 2.

Enhancement ratios[a] due to combined treatment with bevacizumab, nicotinamide, or mild temperature hyperthermia

	Total cell population	Quiescent cells
<Surviving fraction = 0.05>		
+ Nicotinamide	1.3 ± 0.1[b]	----
+ MTH[c]	1.1 ± 0.05[1]	----
+ BV[d] only	1.2 ± 0.1	----
+ BV + Nicotinamide	1.25 ± 0.1	----
+ BV + MTH	1.35 ± 0.1[1]	----
<Normalized micronucleus frequency = 0.3>		
+ Nicotinamide	1.2 ± 0.1[2]	1.05 ± 0.05[2]
+ MTH	1.05 ± 0.05[3,4]	1.25 ± 0.05[3]
+ BV only	1.15 ± 0.05[5]	1.05 ± 0.05[5,6]
+ BV + Nicotinamide	1.15 ± 0.05[7]	1.05 ± 0.05[7]
+ BV + MTH	1.25 ± 0.1[4]	1.3 ± 0.1[6]

[a]; The 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]; Mean ± standard error (n = 9)

[c]; Mild temperature hyperthermia.

[d]; Bevacizumab

[1,2,3,4,5,6,7]; The differences between two values were significant ($p < 0.05$).

Table 3.

Dose-modifying factors for quiescent cells relative to the total cell population[a]

	Without Bevacizumab	With Bevacizumab
<Normalized micronucleus frequency = 0.3>		
Radiation only	1.7 ± 0.1[b,1,2]	1.75 ± 0.1
+ Nicotinamide	1.9 ± 0.1[1]	1.85 ± 0.1
+ MTH[c]	1.5 ± 0.1[2,3]	1.7 ± 0.1[3]

[a]; The 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 tumor cell population.

[b]; Mean ± standard error (n = 9)

[c]; Mild temperature hyperthermia

[1,2,3]; The differences between two values were significant ($p < 0.05$).

Table 4.

The sizes of the hypoxic fractions (%)
in total and quiescent tumor cell populations[a]

	Without Bevacizumab	With Bevacizumab
<Total tumor cell population>		
Without nicotinamide or MTH	16.1 ± 2.8[b,1,2,3]	8.4 ± 1.7[1]
With Nicotinamide	7.5 ± 1.6[2,4]	8.1 ± 1.7
With MTH[c]	11.5 ± 1.6[3,4,5]	6.3 ± 1.5[5]
<Quiescent tumor cell population>		
Without nicotinamide or MTH	51.3 ± 5.5[6,7]	44.6 ± 5.3[8]
With Nicotinamide	38.5 ± 6.4[6,9]	41.2 ± 6.5[10]
With MTH	25.5 ± 3.4[7,9,11]	17.8 ± 3.6[8,10,11]

[a]; Based on the paired survival curve method using the dose-survival curves under both aerobic and hypoxic conditions for more than 16 Gy.

[b]; Mean ± standard error (n = 9)

c; Mild temperature hyperthermia.

[1,2,3,4,5,6,7,8,9,10,11]; The differences between two values were significant ($p < 0.05$). Overall, the values were significantly larger in Q cells than the total cell population under each set of conditions ($p < 0.05$).

Table 5.

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

	Without Bevacizumab	With Bevacizumab
<No irradiation>		
No treatment	3.4 ± 0.6[a]	3.1 ± 0.5
+ Nicotinamide	3.4 ± 0.6	3.3 ± 0.6
+ MTH[b]	4.3 ± 0.9	3.6 ± 0.7
<Irradiated dose = 16 Gy>		
Radiation only	13.5 ± 2.0[1,2,3]	22.4 ± 1.4[1,4]
+ Nicotinamide	24.7 ± 1.5[2]	23.7 ± 1.5
+ MTH	20.7 ± 2.0[3,5]	27.3 ± 2.1[3,4,5]

[a]; Mean ± standard error (n = 9)

[b]; Mild temperature hyperthermia.

[1,2,3,4,5]; The differences between two values were significant ($p < 0.05$). 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 ($p < 0.05$).

Table 6.

The numbers of metastases from the irradiated tumors 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[b]	14.8	13.1

[a]; Using the data shown in Figure 5, the numbers of lung metastases were estimated from local tumors 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.

[b]; Mild temperature hyperthermia.

Figure legends

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

Fig. 2 Dose response curves of the normalized micronucleus frequency for total (left panels) and quiescent (right panels) cell populations from B16-BL6 tumors irradiated with or without bevacizumab (BV) in combination with nicotinamide (upper panels) or mild temperature hyperthermia (MTH) (lower panels) on day 18 after inoculation. Symbols are as in Figure 1. Bars represent standard errors (n = 9).

Fig. 3 The correlation between the normalized micronucleus frequency and the surviving fraction of the total cell population for each tumor under each set of treatment conditions. The regression line had a significant positive correlation: ln Y = - 5.11 X (r - -0.96, $p < 0.001$). Symbols are as in Figure 1. Bars represent standard errors (n = 9).

Fig. 4 Tumor growth curves for B16-BL6 tumors after γ-ray irradiation

with or without treatment with bevacizumab (BV), nicotinamide, or mild temperature hyperthermia (MTH) on day 18 after tumor cell inoculation. Tumor growth was determined by comparing tumor volume with that on γ-ray irradiation. ○, ● irradiation only under aerobic conditions; □, ■ irradiation under aerobic conditions following nicotinamide treatment; △, ▲ irradiation under aerobic conditions following MTH; ○, □, △ without irradiation; ●, ■, ▲ with irradiation at a dose of 16 Gy; ·········· irradiation without bevacizumab; ———— irradiation with bevacizumab . Bars represent standard errors (n = 9).

Fig. 5 Counted numbers of macroscopic metastases in the lung on day 35 after tumor 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 tumor cell inoculation. ○, ● irradiation only under aerobic conditions; □, ■ irradiation under aerobic conditions following nicotinamide treatment; △, ▲ irradiation under aerobic conditions following MTH; ○, □, △ irradiation without bevacizumab; ●, ■, ▲ irradiation with bevacizumab. Bars represent standard errors (n = 9).

Figure 1
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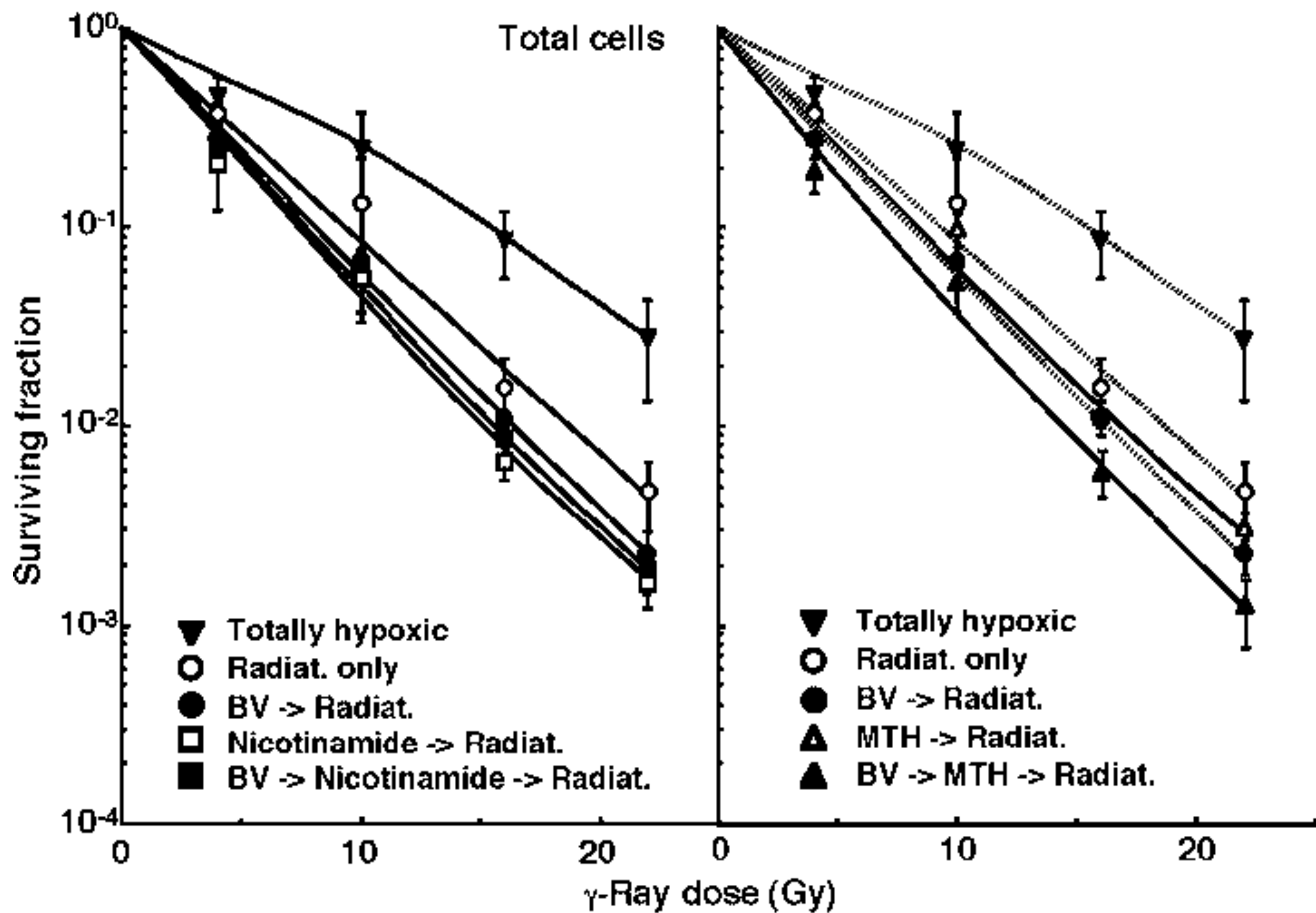

Figure 2

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Total cells

Quiescent cells

▼ Totally hypoxic
○ Radiat. only
● BV -> Radiat.
□ Nicotinamide -> Radiat.
■ BV -> Nicotinamide -> Radiat.

Total cells

Quiescent cells

▼ Totally hypoxic
○ Radiat. only
● BV -> Radiat.
△ MTH -> Radiat.
▲ BV -> MTH -> Radiat.

Normalized micronucleus frequency

1.5
1.0
0.5
0

0 10 20

γ-Ray dose (Gy)

Figure 3

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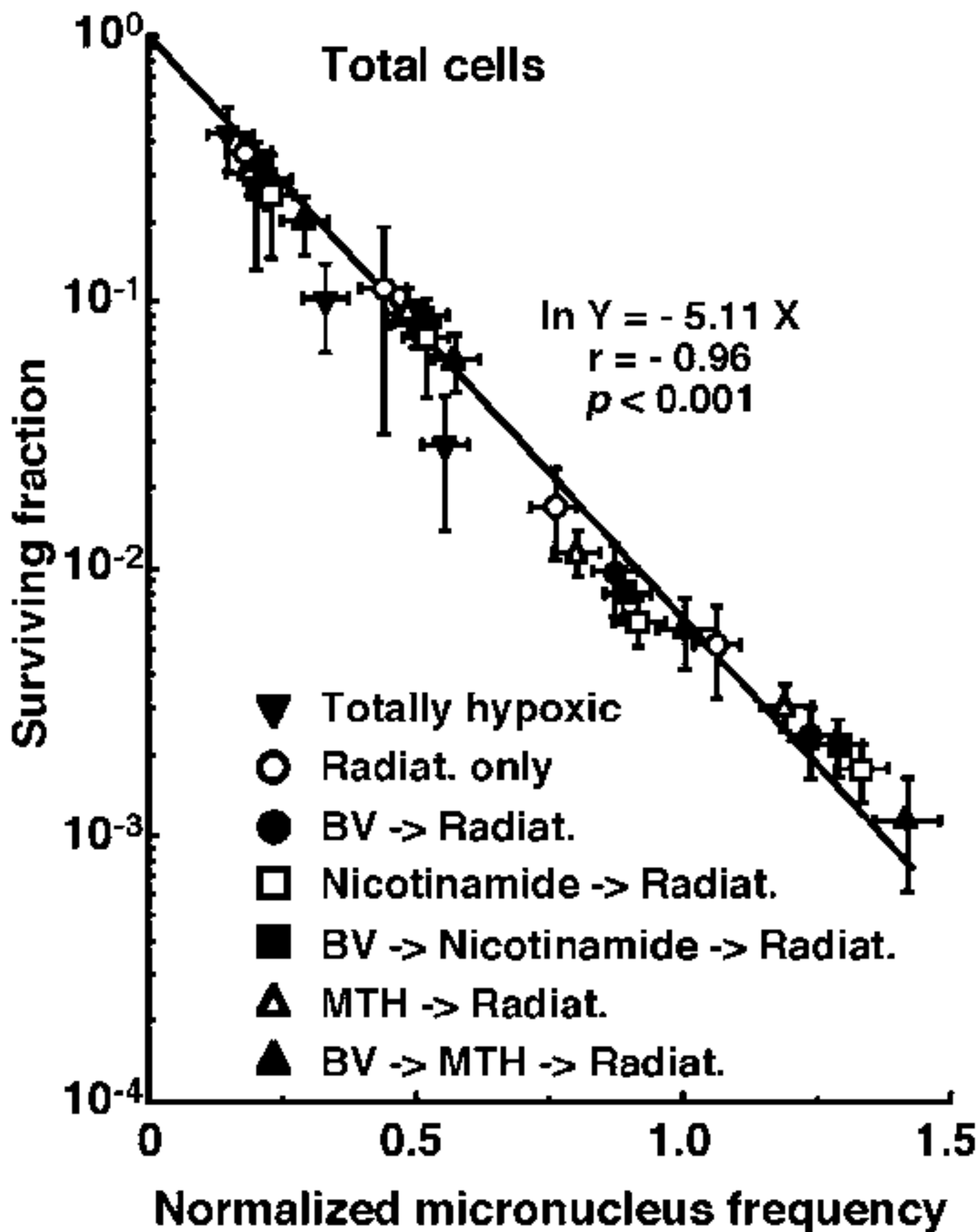

Figure 4
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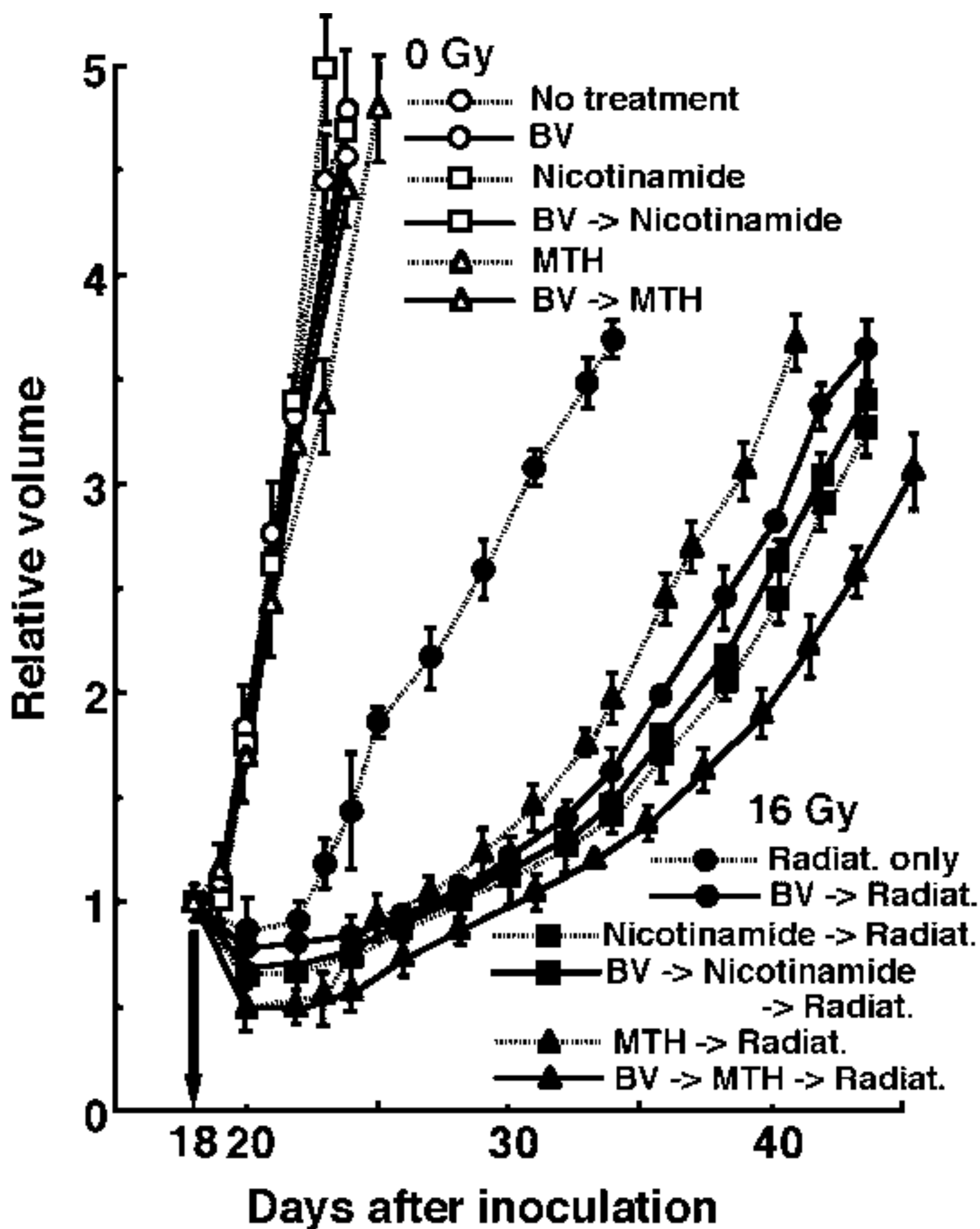

Figure 5

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