

Radio-sensitivity of pimonidazole-unlabeled intratumor quiescent cell population to γ -rays, accelerated carbon ion beams and boron neutron capture reaction

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1 **Radio-sensitivity of pimonidazole-unlabeled intratumor quiescent**
2 **cell population to γ -rays, accelerated carbon ion beams and boron**
3 **neutron capture reaction**
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12 **Abstract**

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14 Objectives: Detecting the radio-sensitivity of intratumor
15 quiescent (Q) cells unlabeled with pimonidazole to accelerated
16 carbon ion beams and the boron neutron capture reaction (BNCR).
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20 Methods: EL4 tumor-bearing C57BL/J mice received
21 5-bromo-2'-deoxyuridine (BrdU) continuously to label all
22 intratumor proliferating (P) cells. After the administration of
23 pimonidazole, tumors were irradiated with γ -rays, accelerated
24 carbon-ion beams, or reactor neutron beams with the prior
25 administration of a ^{10}B -carrier. Responses of intratumor Q and total
26 (= P + Q) cell populations were assessed based on frequencies of
27 micronucleation and apoptosis using immunofluorescence staining
28 for BrdU. The response of pimonidazole-unlabeled tumor cells was
29 assessed by means of apoptosis frequency using immunofluorescence
30 staining for pimonidazole.
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44 Results: Following γ -ray irradiation, the pimonidazole-unlabeled
45 tumor cell fraction showed significantly enhanced
46 radio-sensitivity compared with the whole tumor cell fraction more
47 remarkably in the Q than total cell populations. However, a
48 significantly greater decrease in radio-sensitivity in the
49 pimonidazole-unlabeled cell fraction, evaluated using a delayed
50 assay or a decrease in radiation dose rate, was more clearly observed
51 among the Q than total cells. These changes in radio-sensitivity
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1 were suppressed following carbon-ion beam and neutron beam only
2 irradiation. In BNCR, the use of a ^{10}B -carrier, especially
3 *L-para-boronophenylalanine- ^{10}B* , enhanced the sensitivity of the
4 pimonidazole-unlabeled cells more clearly in the Q than total cells.
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9 Conclusions: The radio-sensitivity of the pimonidazole-unlabeled
10 cell fraction depends on the quality of radiation delivered and
11 characteristics of the ^{10}B -carrier used in BNCR.
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15 Advances in knowledge: The pimonidazole-unlabeled sub-fraction of
16 Q tumor cells may be a critical target in tumor control.
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23 **Keywords :**

24 quiescent cell; hypoxia; α -rays; carbon ion beam; boron neutron
25 capture reaction; pimonidazole
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34 **Authors have no conflict of interest concerning this manuscript.**
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Introduction

Human solid tumors are thought to contain moderately large fractions of quiescent (Q) tumor cells, which are not involved in the cell cycle and have stopped dividing, but which are as viable as established experimental animal tumor lines [1]. The presence of Q cells is probably due, at least in part, to hypoxia and the depletion of nutrition in the tumor core as a consequence of poor vascular supply [1]. As a result, with the exception of non-viable Q cells at the very edge of the necrotic rim where there is diffusion-limited hypoxia, Q cells are viable and clonogenic but have ceased dividing.

Using our method for selectively detecting the response of Q cells in solid tumors to treatment that damages DNA, the Q cell population in solid tumors has been shown to exhibit more resistance to conventional radio- and chemotherapy [2]. The Q cell population has also been demonstrated to have greater capacity to recover from radiation- and chemotherapeutic agent-induced damage and to have a significantly larger hypoxic fraction (HF) irrespective of the *p53* status of tumor cells [2]. However, the Q cell population in solid tumors has never been shown to be fully hypoxic [2]. Actually, the size of the HF of Q cell populations in SCC VII squamous cell carcinomas, implanted in the hind legs of C3H/He mice and with a diameter of 1 cm, was 55.1 ± 6.2 (mean \pm SE) % [3]. Thus, this value was significantly less than 100 %, indicating that the Q cell population undoubtedly includes oxygenated tumor cells.

A few years ago, the universal detection of hypoxic cells in both tissues and cell cultures became possible using pimonidazole, a

1 substituted 2-nitroimidazole, and a mouse IgG1 monoclonal antibody
2 (MAb1) to stable covalent adducts formed through reductive
3 activation of pimonidazole in hypoxic cells [4]. Here, we tried
4 to selectively detect the response of the pimonidazole-unlabeled
5 and probably oxygenated cell fraction of the Q cell population.
6 To achieve this we combined our method for selectively detecting
7 the response of Q cells in solid tumors with the method for detecting
8 cell and tissue hypoxia using pimonidazole and MAb1 to pimonidazole.
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10 High-linear energy transfer (LET) radiation including neutrons
11 is more effective [2] than low-LET X- or γ -radiation at inducing
12 biological damage. High-LET radiation shows a higher relative
13 biological effectiveness (RBE) value for cell killing, a reduced
14 oxygen effect, and a reduced dependence on the cell-cycle [2,5],
15 making it potentially superior to low-LET radiation in the treatment
16 of malignant tumors. Reactor thermal and epithermal neutron beams
17 available at our institute had been also shown to have a
18 significantly higher RBE value than γ -rays in irradiated tumor cells
19 *in vivo* [2]. Due to a selective physical dose distribution and
20 enhanced biological damage in target tumors, particle radiation
21 therapy with protons or heavy ions has gained increasing interest
22 worldwide, and many clinical centers are considering introducing
23 radiation therapy with charged particles. However, almost all
24 reports on the biological advantages of charged particle beams are
25 based on effects only on total tumor cell populations as a whole
26 using *in vitro* cell cultures or *in vivo* solid tumors [1,5].
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57 Intensity modulated radiotherapy and stereotactic irradiation
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1 have become common as new radiotherapy modalities for the treatment
2 of malignancies. These techniques often require precise positioning
3 of patients and longer exposure times in a single treatment session
4 [6, 7]. Prolongation of irradiation time may induce adverse
5 radiation effects and evokes major concern related to the dose-rate
6 effect. Thus, there is a need to clarify the effect of a reduction
7 in dose-rate on the radio-sensitivity of tumors *in vivo*.
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10 In the present study, the radio-sensitivity of the
11 pimonidazole-unlabeled cell fraction of the Q cell population,
12 following cobalt-60 γ -ray or 290 MeV/u accelerated carbon ion beam
13 irradiation at both a high dose rate (HDR) and a reduced dose rate
14 (RDR), was determined, compared with irradiation using reactor
15 thermal neutron beams following the administration of a ^{10}B -carrier
16 at our institute. This is the first attempt to evaluate the
17 sensitivity of oxygenated fractions of Q tumor cells *in vivo* in
18 response to particle radiation.
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Methods

Mice and Tumors

EL4 lymphoma cells (Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University) derived from C57BL/6J mice were maintained *in vitro* in RPMI 1640 medium supplemented with 12.5 % fetal bovine serum. The p53 status of the EL4 tumor cells was the wild type [8]. Cells were collected from exponentially growing cultures and approximately 1.0×10^5 tumor cells were inoculated subcutaneously into the left hind legs of 9-week-old syngeneic female C57BL/6J mice (Japan Animal Co., Ltd., Osaka, Japan). Fourteen days after the inoculation, the tumors, approximately 1 cm in diameter, were employed for irradiation in this study, and the body weight of the tumor-bearing mice was 22.1 ± 2.3 g. Mice were handled according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety Handling Regulations for Laboratory Animal Experiments.

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

Nine days after the tumor inoculation, mini-osmotic pumps (Durect Corporation, Cupertino, CA) containing BrdU dissolved in physiological saline (250 mg/ml) were implanted subcutaneously to enable the labeling of all P cells over a 5-day period [9]. The percentage of labeled cells after continuous labeling with BrdU was 66.1 ± 3.8 % and plateau at this stage. Therefore, tumor cells not incorporating BrdU after continuous exposure were regarded as Q cells.

Treatment

After the labeling with BrdU, tumor-bearing mice received an intraperitoneal administration of pimonidazole hydrochloride

1 (Hypoxyprobe Inc., Burlington, MA, USA) dissolved in physiological
2 saline at a dose of 60 mg/kg. Ninety minutes later, mice received
3 -ray or accelerated carbon-ion beam irradiation, or reactor
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5 neutron beam irradiation following administration of the
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9 ^{10}B -carrier with no anesthetic.

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11 -Rays were delivered using a cobalt-60 -ray irradiator at dose
12 rates of 2.5 and 0.039 Gy/min representing HDR and RDR irradiation,
13 respectively. Carbon-12 ions were accelerated up to 290 MeV/u by
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15 the synchrotron of the Heavy Ion Medical Accelerator installed at
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17 the National Institute of Radiological Sciences in Chiba, Japan.
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19 The dose rate was regulated through a beam attenuation system, and
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21 irradiation was conducted using horizontal carbon beams with a dose
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23 rate of 1.0 or 0.035 Gy/min. The LET of a carbon ion beam with a
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25 6-cm spread-out Bragg peak (SOBP) ranges from 14 keV/ μm to greater
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27 than 200 keV/ μm , depending on depth. The desired LET beam was
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29 obtained by selecting the depth along the beam path using a Lucite
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31 range shifter. An LET of 50 keV/ μm at the middle of the SOBP was
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33 employed here.

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35 Sodium mercaptoundecahydrododecaborate- ^{10}B (sodium
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37 borocaptate- ^{10}B , BSH, $\text{Na}_2^{10}\text{B}_{12}\text{H}_{11}\text{SH}$) ($125 \text{ mg}\cdot\text{kg}^{-1}$) and
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39 boronophenylalanine- ^{10}B (BPA, $\text{C}_9\text{H}_{12}^{10}\text{BNO}_4$) ($250 \text{ mg}\cdot\text{kg}^{-1}$) were
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41 purchased from KatChem Ltd. (Czech Republic) and prepared freshly
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43 by dissolving in physiological saline and injected
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45 intraperitoneally in a volume of $0.02 \text{ mL}\cdot\text{g}^{-1}$ mouse body weight. In
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47 accordance with our previous study [10], at a dose of less than
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49 $500 \text{ mg}\cdot\text{kg}^{-1}$ for BSH and less than $1,500 \text{ mg}\cdot\text{kg}^{-1}$ for BPA, no apparent
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51 toxicity was observed. Based on the certificate of analysis and
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53 Material Safety Data Sheet provided, BSH was not contaminated with
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55 the borocaptate dimer (BSSB, $[\text{B}_{24}\text{H}_{22}\text{S}_2]^{4-}$). Since the intratumor
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¹⁰B concentration during neutron irradiation is a crucial determinant of the cell-kill effect in BNCR, to obtain similar intratumor ¹⁰B concentrations during exposure to the neutron beam, irradiation was started at selected time points after the intraperitoneal injection of the ¹⁰B-carriers at a selected dose of ¹⁰B. Based on a preliminary study of the biodistribution of ¹⁰B, irradiation was started from 45 min after the intraperitoneal injection of 125 and 250 mg·kg⁻¹ (71.0 and 12.0 mg ¹⁰B·kg⁻¹) of BSH and BPA, respectively. ¹⁰B concentrations were determined with a thermal neutron guide tube installed at the Kyoto University Research Reactor (KUR) [11].

The tumor-bearing mice were irradiated with a reactor neutron beam at a power of 1 MW at KUR. A LiF thermoplastic shield was employed to avoid irradiating other body parts except implanted solid tumors. Neutron irradiation was performed using a reactor neutron beam with a cadmium ratio of 9.4. The cadmium ratio is the ratio of the response of an uncovered neutron detector to that of the same detector under identical conditions when it is covered with cadmium of a specified thickness. The neutron fluence was measured from the radioactivation of gold foil at both the front and back of the tumors. Since the tumors were small and located just beneath the surface, the neutron fluence was assumed to decrease linearly from the front to back of the tumors. Thus, we used the average neutron fluence determined from the values measured at the front and back.

Contaminating γ -ray doses including secondary γ -rays were measured with a thermoluminescence dosimeter (TLD) powder at the back of the tumors. The TLD used was Beryllium Oxide (BeO) enclosed in a quartz glass capsule. BeO itself has some sensitivity to thermal neutrons. The thermal neutron fluence of $8 \times 10^{12} \text{ cm}^{-2}$ is equal to

1 an approximately 1 cGy γ -ray dose. We usually use the TLD together
2 with gold activation foil for the neutron-sensitivity correction.
3 The details have been described previously [12]. For the estimation
4 of neutron energy spectra, eight kinds of activation foil and
5 fourteen kinds of nuclear reaction were used [12]. The absorbed
6 dose was calculated using the flux-to-dose conversion factor [13].
7 The tumors contained H (10.7 % in terms of weight), C (12.1 %),
8 N (2 %), O (71.4 %), and others (3.8 %) [14]. The average neutron
9 flux and Kerma rate of the employed beam were $1.0 \times 10^9 \text{ n}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$
10 and $48.0 \text{ cGy}\cdot\text{h}^{-1}$ for the thermal neutron range (less than 0.6 eV),
11 $1.6 \times 10^8 \text{ n}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ and $4.6 \text{ cGy}\cdot\text{h}^{-1}$ for the epithermal neutron range
12 (0.6 through 10 keV), and $9.4 \times 10^6 \text{ n}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ and $32.0 \text{ cGy}\cdot\text{h}^{-1}$ for the
13 fast neutron range (more than 10 keV), respectively. The Kerma rate
14 for boron dose per $\Phi \text{ n}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$ of thermal neutron flux for $1 \mu\text{g}\cdot$
15 g^{-1} of ^{10}B was $2.67 \times 10^{-8} \Phi \text{ cGy}\cdot\text{h}^{-1}$. The contaminating γ -ray dose
16 rate was $66.0 \text{ cGy}\cdot\text{h}^{-1}$.

17 Individual animals were secured in a specially designed device
18 made of acrylic resin with the tail firmly fixed in position with
19 adhesive tape. Each treatment group also included mice that had
20 not been pretreated with BrdU.

21 ***Immunofluorescence Staining of BrdU-Labeled and/or***

22 ***Pimonidazole-Labeled Cells and the Observation of Apoptosis and*** 23 ***Micronucleation***

24 Based on our previous report related to the determination of the
25 timing of apoptosis [10], as an immediate assay, an apoptosis assay
26 was undertaken at 6 h after irradiation and a micronucleus assay
27 was carried out immediately after irradiation. Tumors were excised
28 from mice given BrdU, weighed, minced and trypsinized (0.05% trypsin
29 and 0.02% ethylenediamine-tetraacetic acid (EDTA) in
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1 phosphate-buffered saline (PBS) at 37 °C for 20 min). Furthermore,
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3 as a delayed assay, tumors were also excised from mice given BrdU,
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5 weighed, minced and trypsinized at 30 h after irradiation for the
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7 apoptosis assay, and at 24 h after irradiation for the micronucleus
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9 assay. For the apoptosis assay, single cell suspensions were fixed
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11 without further treatment. For the micronucleus assay, tumor cell
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13 suspensions were incubated for 72 h in tissue culture dishes
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15 containing complete culture medium and 1.0 µg/ml of cytochalasin-B,
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17 to inhibit cytokinesis while allowing nuclear division. The
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19 cultures were then trypsinized and cell suspensions were fixed.
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21 For both assays, after the centrifugation of fixed cell suspensions,
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23 the cell pellet was resuspended with cold Carnoy's fixative
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25 (ethanol:acetic acid = 3:1 in volume). The suspension was placed
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27 on a glass microscope slide and the sample was dried at room
28
29 temperature. Slides were treated with 2 M hydrochloric acid for
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31 60 min at room temperature to dissociate the histones and partially
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33 denature the DNA. They were then immersed in borax-borate buffer
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35 (pH 8.5) to neutralize the acid. BrdU-labeled tumor cells were
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37 detected using indirect immunofluorescence staining with a rat
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39 monoclonal anti-BrdU antibody (Abcam plc, Cambridge, UK) and a goat
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41 Alexa Fluor 488-conjugated anti-rat IgG antibody (Invitrogen Corp.,
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43 Carlsbad, CA, USA). Pimonidazole-labeled tumor cells were detected
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45 using indirect immunofluorescence staining with a mouse monoclonal
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47 anti-pimonidazole antibody (Hypoxyprobe Inc., Burlington, MA, USA)
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49 and a rabbit Alexa Fluor 594-conjugated anti-mouse IgG antibody
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51 (Invitrogen Corp., Carlsbad, CA, USA). To enable the observation
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53 of the triple staining of tumor cells with green-emitting Alexa
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55 Fluor 488 and red-emitting Alexa Fluor 594, cells on the slides
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57 were treated with blue-emitting 4'6-diamidino-2-phenylindole
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1 (DAPI) (0.5 µg/ml in PBS) and imaged using a fluorescence
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3 microscope.

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5 The frequency of apoptosis in cells not labeled with BrdU (= Q
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7 cells at irradiation) and tumor cells not labeled with pimonidazole
8
9 was determined by counting apoptotic cells in tumor cells that did
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11 not show green fluorescence from Alexa Fluor 488 and red
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13 fluorescence from Alexa Fluor 594, respectively. The apoptosis
14
15 frequency was defined as the ratio of the number of apoptotic cells
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17 to the total number of observed tumor cells [10]. The micronucleus
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19 frequency in BrdU-unlabeled cells was examined by counting the
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21 micronuclei in the binuclear cells that did not show green
22
23 fluorescence emitted by Alexa Fluor 488. The micronucleus frequency
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25 was defined as the ratio of the number of micronuclei in the
26
27 binuclear cells to the total number of binuclear cells observed
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29 [2].
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32 The ratios obtained in tumors not pretreated with BrdU indicated
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34 the apoptosis frequency and the micronucleus frequency in the total
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36 (P + Q) tumor cell populations. More than 300 tumor cells and
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38 binuclear cells were counted to determine the apoptosis frequency
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40 and the micronucleus frequency, respectively.
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43 **Clonogenic Cell Survival Assay**

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45 The clonogenic cell survival assay was also performed in mice
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47 given no BrdU or pimonidazole using an *in vivo-in vitro* assay method.
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49 Tumors were disaggregated by stirring for 20 min at 37 °C in PBS
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51 containing 0.05 % trypsin and 0.02% EDTA. The cell yield was (1.1
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53 ± 0.3) × 10⁸/g tumor weight. A colony formation assay using the *in*
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55 *vivo-in vitro* assay method was performed with the culture medium
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57 mixed with methylcellulose (15.0 g/L) (Aldrich, Milwaukee, WI,
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59 USA).
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1 The apoptosis and micronucleus frequencies and surviving
2 fractions for the total cell population were obtained from cells
3 in tumors that were not pretreated with BrdU or pimonidazole. The
4 apoptosis and micronucleus frequencies for Q cells were obtained
5 from unlabeled tumor cells after continuous BrdU labeling without
6 pimonidazole loading. The apoptosis frequencies for the total tumor
7 cell populations that were not labeled with pimonidazole were
8 obtained from tumor cells that were not labeled with pimonidazole
9 after pimonidazole loading without BrdU pretreatment. The apoptosis
10 frequencies for Q cells that were not labeled with pimonidazole
11 were obtained from tumor cells that were not labeled with BrdU or
12 pimonidazole after both continuous BrdU labeling and pimonidazole
13 loading. Thus, there was no effect of interaction between BrdU and
14 irradiation or between pimonidazole and irradiation on the values
15 for the apoptosis and micronucleus frequencies and surviving
16 fractions. Incidentally, since the rate of pimonidazole-labeled
17 tumor cells could change during culturing with cytochalasin-B over
18 3 days, following the production of single tumor cell suspensions
19 by excising and mincing the tumors from mice that underwent
20 pimonidazole loading, the micronucleus frequency for the cell
21 fraction that was not labeled with pimonidazole after pimonidazole
22 loading was not determined. As a consequence, the radiosensitivity
23 of the pimonidazole-unlabeled cell fractions was only determined
24 in relation to apoptosis induction. This was the reason for using
25 the EL4 leukemia cell line with its much greater capacity for the
26 induction of apoptosis than other solid tumor-originating cell
27 lines [10].

28 **Data Analysis and Statistics**

29 More than three tumor-bearing mice were used to assess each set
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1 of conditions and each experiment was repeated at least twice. To
2 examine the differences between pairs of values, Student's *t*-test
3 was used when variances of the two groups were assumed to be equal
4 with Shapiro-Wilk normality test; otherwise the Welch *t*-test was
5 used.
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Results

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3 The plating efficiency and the micronucleus and apoptosis
4 frequencies after a radiation dose of 0 Gy are shown in **Table 1**.
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6 The micronucleus and apoptosis frequencies were significantly
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8 higher for the Q cell population than for the total cell population.
9
10 In contrast, the apoptosis frequency was significantly lower for
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12 the cell fraction that was not labeled with pimonidazole than for
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14 the whole tumor cell fraction in both the Q and total tumor cell
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16 populations.
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19 Cell survival curves for the total tumor cell population as a
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21 function of radiation dose are shown in **Figure 1**. In the irradiation
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23 of γ -rays and carbon-ion beams, the surviving fractions (SFs)
24
25 increased in the following order with a more remarkable change for
26
27 γ -rays than carbon-ion beams: immediately after HDR irradiation <
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29 24 h after HDR irradiation < immediately after RDR irradiation.
30
31 In BNCR, the SFs increased in the following order: with BPA < with
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33 BSH < without a ^{10}B -carrier.
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37 For baseline correction, we used the net micronucleus frequency
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39 to exclude the micronucleus frequency in non-irradiated tumors.
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41 The net micronucleus frequency was defined as the micronucleus
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43 frequency in the irradiated tumors minus the micronucleus frequency
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45 in the non-irradiated tumors. Dose response curves for the net
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47 micronucleus frequency in total and Q tumor cell populations as
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49 a function of radiation dose are shown in **Figure 2**. Overall, the
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51 net micronucleus frequencies were significantly lower in the Q cells
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53 than the total cell population. In both the total and Q cell
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55 populations after the irradiation of γ -rays and carbon-ion beams,
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57 the net micronucleus frequencies decreased in the following order
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59 with a more remarkable change for γ -rays than carbon-ion beams:
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1 immediately after HDR irradiation > 24 hours after HDR irradiation
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3 > immediately after RDR irradiation. In BNCR, the net micronucleus
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5 frequencies for the total cell population increased in the following
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7 order: without a ^{10}B -carrier < with BSH < with BPA. However, those
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9 for the Q cell population increased in the following order: without
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11 a ^{10}B -carrier < with BPA < with BSH.
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13 For another baseline correction, we used the net apoptosis
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15 frequency to exclude the apoptosis frequency in non-irradiated
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17 tumors. The net apoptosis frequency was the apoptosis frequency
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19 in the irradiated tumors minus that in the non-irradiated tumors.
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21 Dose response curves for the net apoptosis frequency in the total
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23 and Q tumor cell populations as a function of radiation dose are
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25 shown in **Figure 3**. Overall, the net apoptosis frequencies were
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27 significantly lower in the Q than total cell population, with much
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29 larger differences for γ -rays than carbon-ion beams. Moreover, the
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31 net apoptosis frequency was significantly higher for the cell
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33 fraction that was not labeled with pimonidazole than for the whole
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35 tumor cell fraction in both the Q and total cell populations under
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37 each set of conditions, again with a much larger difference for
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39 γ -rays than carbon-ion beams. For both the pimonidazole-unlabeled
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41 and the whole cell fractions, in the Q as well as total tumor cell
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43 population, the net apoptosis frequencies decreased in the
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45 following order with a more remarkable change for γ -rays than
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47 carbon-ion beams: immediately after HDR irradiation > 24 h after
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49 HDR irradiation > immediately after RDR irradiation. Also in BNCR,
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51 the pimonidazole-unlabeled cells showed higher net apoptosis
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53 frequencies than the whole tumor cell fractions with little
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55 remarkable change in the Q compared to total cell population and
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57 with increased change in the following order: without a ^{10}B -carrier
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1 < with BSH < with BPA.

2
3 To evaluate the radio-sensitivity of the cell fraction that was
4 not labeled with pimonidazole, as compared with the whole cell
5 fraction in both the total and Q cell populations, dose-modifying
6 factors (DMFs) were calculated using the data obtained under γ -ray
7 and carbon-ion beam irradiation conditions (**Fig. 3, Table 2**).

8
9 Overall, DMF values tended to be higher for the Q cell than total
10 cell population, and in particular immediately after HDR
11 irradiation with a much larger difference for γ -rays than carbon-ion
12 beams. In the total cell population, the DMF values were almost
13 constant. However, for Q cells, the DMF values had a tendency to
14 decrease in the following order: immediately after HDR irradiation
15 > 24 h after HDR irradiation > immediately after RDR irradiation.

16
17 Also in BNCR (**Table 3**), DMF values tended to be higher for the Q
18 cell than total cell population, and in both Q and Total cell
19 populations, the DMF values had a tendency to increase in the
20 following order: without a ^{10}B -carrier < with BSH < with BPA.

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22 To investigate the reduction in radio-sensitivity caused by a
23 delayed assay or a decrease in the radiation dose rate, DMFs were
24 calculated using the data for γ -ray and carbon-ion beam irradiation
25 conditions given in **Figures 1 through 3 (Table 4)**. Overall, carbon
26 beams showed lower DMF values than γ -rays under all sets of
27 conditions. On the whole, in the fraction unlabeled with
28 pimonidazole or the whole cell fraction, the values were higher
29 after RDR irradiation than at 24 h after HDR irradiation in both
30 the total and Q cell populations, particularly in the latter
31 population. The DMF values were significantly higher in the Q cell
32 than total cell population in both the pimonidazole-unlabeled and
33 whole cell fractions. In both the Q and total cell populations,
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1 the values were higher for pimonidazole-unlabeled cell fractions
2 than whole cell fractions, particularly in the case of the Q cells.
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4 To estimate the radio-enhancing effect of ^{10}B -carriers,
5 irradiation with BPA and BSH in the total and Q cell populations
6 was compared with neutron beam irradiation only, using the data
7 shown in **Figures 1** through **3 (Table 5)**. Both BPA and BSH enhanced
8 the sensitivity of the total cell population significantly more
9 than that of the Q cell population. Further, BPA tended to affect
10 the total cell population more than did BSH. In contrast, the
11 sensitivity of Q cells was relatively more enhanced with BSH than
12 BPA. In both the Q and total cell populations but especially in
13 Q cells, the values were higher for pimonidazole-unlabeled cell
14 fractions than whole cell fractions.
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28 To examine the difference in radio-sensitivity between the total
29 and Q cell populations, DMFs that allow us to compare the dose of
30 radiation necessary to obtain each end-point in the two cell
31 populations, were calculated using the data in **Figures 2** and **3**
32 (**Tables 6** and **7**). All DMF values were significantly higher than
33 1.0, and carbon beams showed smaller values than γ -rays under each
34 set of conditions (**Table 6**). The DMF values increased in the
35 following order: immediately after HDR irradiation < 24 h after
36 HDR irradiation < immediately after RDR irradiation. The values
37 were lower for the sub-population that was not labeled with
38 pimonidazole as compared with the whole cell population (**Table 6**).
39 In BNCR, the DMF values increased in the following order: without
40 ^{10}B -carrier < with BSH < with BPA (**Table 7**). Again, the values were
41 lower for the sub-population not labeled with pimonidazole than
42 the whole cell population.
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Discussion

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3 In recent years the concept of cancer stem cells (CSCs), or
4 tumor-initiating cells (tumor clonogens), has attracted a great
5 deal of interest because of the potential clinical significance
6 [15]. In part, these cells are thought to exist in a
7 pathophysiological microenvironment where hypoxia, low pH and
8 nutrient deprivation occur. Under these microenvironmental
9 conditions, dividing tumor cells have also been thought to become
10 quiescent. Actually, a subset of CSCs or tumor clonogens consists
11 of non-dividing quiescent cells [16]. Thus, in the current study
12 we tried to clarify the radiobiological characteristics of the
13 sub-population in the intratumor Q cell population in the context
14 of CSC or tumor clonogen characteristics.

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28 The fraction of cells that were not labeled with pimonidazole
29 showed significantly higher radio-sensitivity than the whole cell
30 fraction in both the Q and total cell populations, and amongst the
31 Q cells in particular (**Table 2**). This was probably because the
32 pimonidazole-unlabeled cells were more oxygenated than the whole
33 cell fraction, which comprised oxygenated and hypoxic tumor cells,
34 in both the Q and total tumor cell populations [4]. Additionally
35 the Q cell population as a whole included a larger HF than the total
36 tumor cell population [2]. As shown in **Table 4**, the
37 pimonidazole-unlabeled cell fraction had a greater recovery
38 capacity than the whole cell fraction, especially in the case of
39 the Q cells. The radio-sensitivity decreased in the following order:
40 immediately after HDR irradiation, at 24 h after HDR irradiation
41 and immediately after RDR irradiation, particularly in the Q cells
42 (**Table 4**). As a consequence, in the case of the Q cells, the
43 difference in radio-sensitivity between the
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1 pimonidazole-unlabeled and whole cell fractions declined in the
2 same order (**Table 2**). One mechanism of CSC or tumor clonogen
3 resistance to cytotoxic treatment is supposed to be based on an
4 enhanced DNA repair capacity [17]. Here, the pimonidazole-unlabeled
5 Q cell fraction showed a much greater recovery capacity than the
6 Q cell population as a whole, even if the recovery capacity was
7 significantly greater in the entire Q cell population than in the
8 total tumor cell population as a whole. In other words, from the
9 viewpoint of not only quiescent status but also enhanced DNA repair
10 capacity, the characteristics of the pimonidazole-unlabeled cell
11 fraction in the Q cell population were found to be similar to those
12 of CSCs or tumor clonogens.
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26 The microenvironmental conditions under which dividing tumor
27 cells become quiescent might promote the formation of micronuclei
28 and apoptosis at 0 Gy in the whole Q tumor cell fractions, partly
29 due to hypoxic stress (**Table 1**) [1]. In this study, the Q cells
30 were shown to be significantly less radiosensitive and to have a
31 greater recovery capacity than the total cell population (**Figs.**
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1 the Q cell population can be a critical target in the control of
2 solid tumors.
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4 At high LET carbon ion irradiation, tumor radio-sensitivity and
5 the capacity to recover from radiation-induced damage are known
6 to be significantly less dependent on intratumor oxygenation status
7 and the irradiation dose rate [18]. This is thought to be partly
8 because the frequency of closely spaced DNA lesions forming a
9 cluster of DNA damage produced by high LET carbon-ion beams is much
10 less dependent on oxygenation status at the time of irradiation
11 than that of DNA damage produced by low LET γ -ray irradiation [5,
12 18]. Thus, the differences in radio-sensitivity not only between
13 total and Q cell populations but also between
14 pimonidazole-unlabeled cells and the whole cell fraction in both
15 the Q and total cell populations were efficiently reduced. Moreover,
16 the capacity to recover from radiation-induced damage in both the
17 Q and total cell populations as a whole and both the
18 pimonidazole-unlabeled and the whole cell fraction of the Q and
19 total cell populations was remarkably reduced (**Table 2** and **4**). These
20 findings including newly elucidated characteristics concerning the
21 response of the Q cell population and pimonidazole-unlabeled cell
22 fraction in the total and Q cell populations potentially reveal
23 some reliable advantage of high LET radiation over low LET radiation
24 in terms of controlling the CSCs or tumor clonogens that are thought
25 to be resistant to cytotoxic treatment for solid tumors.
26

27 In boron neutron capture therapy (BNCT), the cellular
28 distribution of ^{10}B from BSH is thought mostly dependent on the
29 diffusion of the drug, whereas that from BPA is more dependent on
30 the ability of the cells to take up ^{10}B [19]. Further, Q cell
31 populations have been shown to have a much larger HF than total
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1 cell populations [2], and hypoxic cells are thought to exhibit less
2 uptake ability than aerobic cells [1]. Therefore, it follows that
3 Q cells have a lower uptake capacity than the total cell population,
4 and that the distribution of ^{10}B from ^{10}B -carriers into Q cells is
5 more dependent on the diffusion of the drugs than on the uptake
6 ability of the cells. **Tables 3** and **5** show that the distribution
7 of ^{10}B in the tumor from BSH relies mostly on passive diffusion,
8 whereas that from BPA relies on uptake capacity in tumor via active
9 transport, the former resulting from a greater effect on Q cells,
10 and the latter, that on the pimonidazole-unlabeled cell fraction
11 and the total tumor cell population. In BNCR, when a ^{10}B -carrier,
12 especially BPA, is employed, the difference in radio-sensitivity
13 not only between total and Q cell populations as a whole but also
14 between pimonidazole-unlabeled cells and the whole cell fraction
15 of the Q and total cell populations is rather extended compared
16 with the case of disuse of ^{10}B -carrier. Consequently, without a
17 reliable method of delivering enough amount of ^{10}B into target tumor
18 cells efficiently irrespective of intratumor microenvironmental
19 conditions including oxygenation status, it is hard to conclude
20 that BNCT in combination with a ^{10}B -carrier can overcome the
21 resistance to cytotoxic treatment of CSCs or tumor clonogens. Also
22 in BNCT, compared with reactor neutron beam irradiation only, Q
23 cells have been shown to have significantly less radiosensitivity
24 than the total cell population when a ^{10}B -carrier, especially BPA,
25 is employed (**Table 7**) [1,2,20]. Thus, more Q cells can survive BNCT
26 than P cells (**Figs. 2** and **3, Table 7**).

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In the present study, the pimonidazole-unlabeled, and probably oxygenated, cell fraction showed a greater recovery capacity than the Q cell population as a whole. However, although there is

1 similarity between the pimonidazole-unlabeled Q cell fraction and
2 CSCs or tumor clonogens in terms of quiescent status and enhanced
3 recovery capacity, CSCs or tumor clonogens are thought to exist
4 under rather hypoxic conditions [15,16,17]. In the future, using
5 human tumor cell lines, the characteristics of the intratumor Q
6 cell population in connection with those of CSCs or tumor clonogens
7 also have to be analyzed.
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Figure legends

Figure 1. Cell survival curves for the whole tumor cell fraction in the total tumor cell population of EL4 tumors as a function of γ -ray (a), accelerated carbon ion beam (b) or reactor neutron beam (c) radiation dose. Circles, triangles and squares represent the surviving fractions immediately after (HDR) and at 24 h after (Delayed) high dose-rate and reduced dose-rate (RDR) γ -ray or accelerated carbon ion beam irradiation, respectively. For reactor neutron beam irradiation, circles, triangles and squares represent the surviving fractions for without a ^{10}B -carrier (Boron (-)), with boronophenylalanine- ^{10}B (BPA), and with sodium mercaptoundecahydrododecaborate- ^{10}B (BSH), respectively. Bars represent standard errors (n = 9).

Figure 2. Dose response curves of the net micronucleus frequency for the whole tumor cell fraction in the total and quiescent (Q) tumor cell populations of EL4 tumors as a function of γ -ray (a), accelerated carbon ion beam (b) or reactor neutron beam (c) radiation dose. Open and solid symbols represent the net micronucleus frequencies for total and quiescent tumor cell populations, respectively. Circles, triangles and squares represent the net micronucleus frequencies immediately after (HDR) and at 24 h after (Delayed) high dose-rate and reduced dose-rate (RDR) γ -ray or accelerated carbon ion beam irradiation, respectively. For reactor neutron beam irradiation, circles, triangles and squares represent the net micronucleus frequencies for without a ^{10}B -carrier (Boron

(-)), with boronophenylalanine- ^{10}B (BPA), and with sodium mercaptoundecahydrododecaborate- ^{10}B (BSH), respectively. Bars represent standard errors ($n = 9$).

Figure 3. Dose response curves for the net apoptosis frequency of the total (left panel) and quiescent (Q) (right panel) tumor cell populations of EL4 tumors as a function of γ -ray (a), accelerated carbon ion beam (b) or reactor neutron beam (c) radiation dose. Open and solid symbols represent the net apoptosis frequencies for the whole tumor cell fraction and the cell fraction not labeled with pimonidazole (Pimo (-)) in both the total and Q tumor cell populations, respectively. Circles, triangles and squares represent the net apoptosis frequencies immediately after (HDR) and at 24 h after (Delayed) high dose-rate and reduced dose-rate (RDR) γ -ray or accelerated carbon ion beam irradiation, respectively. For reactor neutron beam irradiation, circles, triangles and squares represent the net apoptosis frequencies for without a ^{10}B -carrier (Boron (-)), with boronophenylalanine- ^{10}B (BPA), and with sodium mercaptoundecahydrododecaborate- ^{10}B (BSH), respectively. Bars represent standard errors ($n = 9$).

Table 1.**Plating efficiency and micronucleus frequency at 0 Gy.**

	Total tumor cells	Quiescent cells
<Plating efficiency (%)>		
	25.5 ± 6.8^a	----
<Micronucleus frequency>		
	0.053 ± 0.003	0.073 ± 0.006
<Apoptosis frequency>		
<u>In whole cell fraction</u>		
	0.040 ± 0.001	0.067 ± 0.004
<u>In pimonidazole unlabeled cell fraction</u>		
	0.017 ± 0.001	0.028 ± 0.003

^a; Mean ± standard error (n = 9)

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Table 2.

Dose-modifying factors for the pimonidazole unlabeled cell fraction as compared with the whole cell fraction in the total or quiescent cell population^a.

	High dose-rate Immediately after	High dose-rate 24 hours after	Reduced dose-rate
<Net apoptosis frequency = 0.06>			
-Rays			
<u>Total cell population</u>			
	1.55 (1.45-1.65) ^b	1.5 (1.4-1.6)	1.5 (1.4-1.6)
<u>Quiescent cell population</u>			
	1.7 (1.5-1.9)	1.65 (1.55-1.75)	1.6 (1.5-1.7)
Carbon beams			
<u>Total cell population</u>			
	1.25 (1.1-1.4)	1.2 (1.1-1.3)	1.2 (1.1-1.3)
<u>Quiescent cell population</u>			
	1.6 (1.5-1.7)	1.5 (1.4-1.6)	1.4 (1.3-1.5)

^a; The ratio of the dose of radiation necessary to obtain each end-point in a whole cell fraction to that needed to obtain each end-point in the pimonidazole unlabeled cell fraction.

^b; Values in parentheses are 95% confidence limits, determined using standard errors. When the ranges of 95 % confidence limits showed no overlap between two values, the difference between the two values were significant ($p < 0.05$) based on a Chi-squared test.

Table 3.

Dose-modifying factors for the pimonidazole unlabeled cell fraction as compared with the whole cell fraction in the total or quiescent cell population^a.

	Neutrons only	With BPA	With BSH
<Net apoptosis frequency = 0.06>			
Neutron beams			
<u>Total cell population</u>			
	1.1 (1.0-1.2)^b	1.3 (1.2-1.4)	1.25 (1.15-1.35)
<u>Quiescent cell population</u>			
	1.15 (1.05-1.25)	1.45 (1.3-1.6)	1.35 (1.25-1.45)

^a; The ratio of the dose of radiation necessary to obtain each end-point in a whole cell fraction to that needed to obtain each end-point in the pimonidazole unlabeled cell fraction.

^b; As in Table 2.

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Table 4.

Dose-modifying factors obtained using a delayed assay or a reduced radiation dose-rate^a.

	High dose-rate 24 hours after	Reduced dose-rate
<Surviving fraction = 0.08>		
Total cells		
-Rays	1.15 (1.1-1.2) ^b	1.4 (1.3-1.5)
Carbon beams	1.1 (1.0-1.2)	1.2 (1.1-1.3)
<Net micronucleus frequency = 0.2>		
Total cells		
-Rays	1.2 (1.1-1.3)	1.35 (1.25-1.45)
Carbon beams	1.05 (1.0-1.1)	1.2 (1.1-1.3)
Quiescent cells		
-Rays	1.4 (1.3-1.5)	1.6 (1.45-1.75)
Carbon beams	1.1 (1.0-1.2)	1.25 (1.15-1.35)
<Net apoptosis frequency = 0.06>		
Total cells		
<u>In whole cell fraction</u>		
-Rays	1.15 (1.1-1.2)	1.3 (1.2-1.4)
Carbon beams	1.05 (1.0-1.2)	1.2 (1.1-1.3)
<u>In pimonidazole unlabeled cell fraction</u>		
-Rays	1.2 (1.1-1.3)	1.3 (1.2-1.4)
Carbon beams	1.05 (1.0-1.2)	1.2 (1.1-1.3)
Quiescent cells		
<u>In whole cell fraction</u>		
-Rays	1.35 (1.25-1.45)	1.55 (1.45-1.65)
Carbon beams	1.15 (1.1-1.2)	1.25 (1.15-1.35)
<u>In pimonidazole unlabeled cell fraction</u>		
-Rays	1.45 (1.35-1.55)	1.65 (1.5-1.8)
Carbon beams	1.15 (1.1-1.2)	1.35 (1.25-1.45)

^a; The 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; As in Table 2.

Table 5.

Enhancement ratios^a due to combination with a ¹⁰B-carrier

¹⁰ B-carrier	Total cell population	Quiescent cells
<Surviving fraction = 0.08>		
BPA ^c	1.95 (1.75-2.15) ^b	----
BSH ^d	1.3 (1.2-1.4)	----
<Net micronucleus frequency = 0.2>		
BPA	2.0 (1.8-2.2)	1.7 (1.6-1.8)
BSH	1.3 (1.2-1.4)	1.8 (1.65-1.95)
<Net apoptosis frequency = 0.06>		
<u>In whole cell fraction</u>		
BPA	1.5 (1.4-1.6)	1.25 (1.15-1.35)
BSH	1.4 (1.3-1.5)	1.35 (1.2-1.5)
<u>In pimonidazole unlabeled cell fraction</u>		
BPA	1.65 (1.5-1.8)	1.65 (1.5-1.8)
BSH	1.5 (1.4-1.6)	1.7 (1.6-1.8)

^a; The ratio of the dose of radiation necessary to obtain each end-point without a ¹⁰B-carrier to that needed to obtain each end-point with a ¹⁰B-carrier.

^b; As in Table 2.

^c; *L-para*-boronophenylalanine-¹⁰B

^d; Sodium mercaptoundecahydrododecaborate-¹⁰B

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Table 6.

Dose-modifying factors for quiescent cells relative to total tumor cells^a.

	High dose-rate immediately	High dose-rate after 24 hours	Reduced dose-rate after
<Net micronucleus frequency = 0.2>			
-Rays			
	1.65 (1.5-1.8) ^b	1.8 (1.65-1.95)	1.9 (1.75-2.05)
Carbon beams			
	1.35 (1.25-1.45)	1.4 (1.25-1.55)	1.45 (1.3-1.6)
<Net apoptosis frequency = 0.06>			
<u>In whole cell fraction</u>			
-Rays			
	1.3 (1.2-1.4)	1.45 (1.35-1.55)	1.55 (1.4-1.7)
Carbon beams			
	1.15 (1.05-1.25)	1.25 (1.15-1.35)	1.3 (1.2-1.4)
<u>In pimonidazole unlabeled cell fraction</u>			
-Rays			
	1.2 (1.1-1.3)	1.4 (1.3-1.5)	1.55 (1.4-1.7)
Carbon beams			
	1.1 (1.0-1.2)	1.2 (1.1-1.3)	1.25 (1.15-1.35)

^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; As in Table 2.

Table 7.

Dose-modifying factors for quiescent cells relative to total tumor cells^a.

	Neutrons only	With BPA	With BSH
<Net micronucleus frequency = 0.2>			
	1.2 (1.1-1.3) ^b	2.15 (1.9-2.4)	1.25 (1.15-1.35)
<Net apoptosis frequency = 0.06>			
<u>In whole cell fraction</u>			
	1.3 (1.2-1.4)	1.6 (1.45-1.75)	1.25 (1.15-1.35)
<u>In pimonidazole unlabeled cell fraction</u>			
	1.25 (1.15-1.35)	1.35 (1.2-1.5)	1.15 (1.05-1.25)

^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; As in Table 2.

Figure 1
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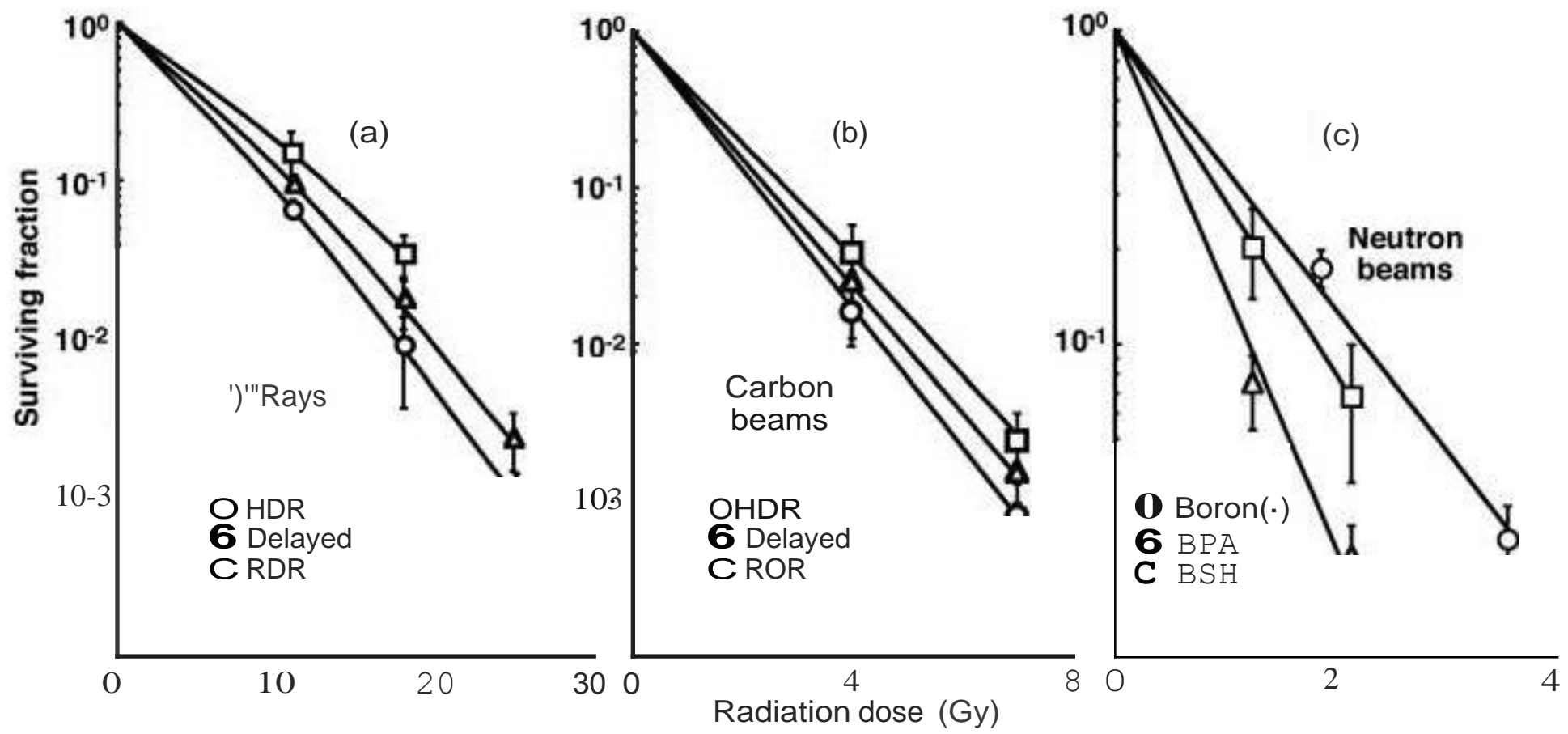


Figure 2
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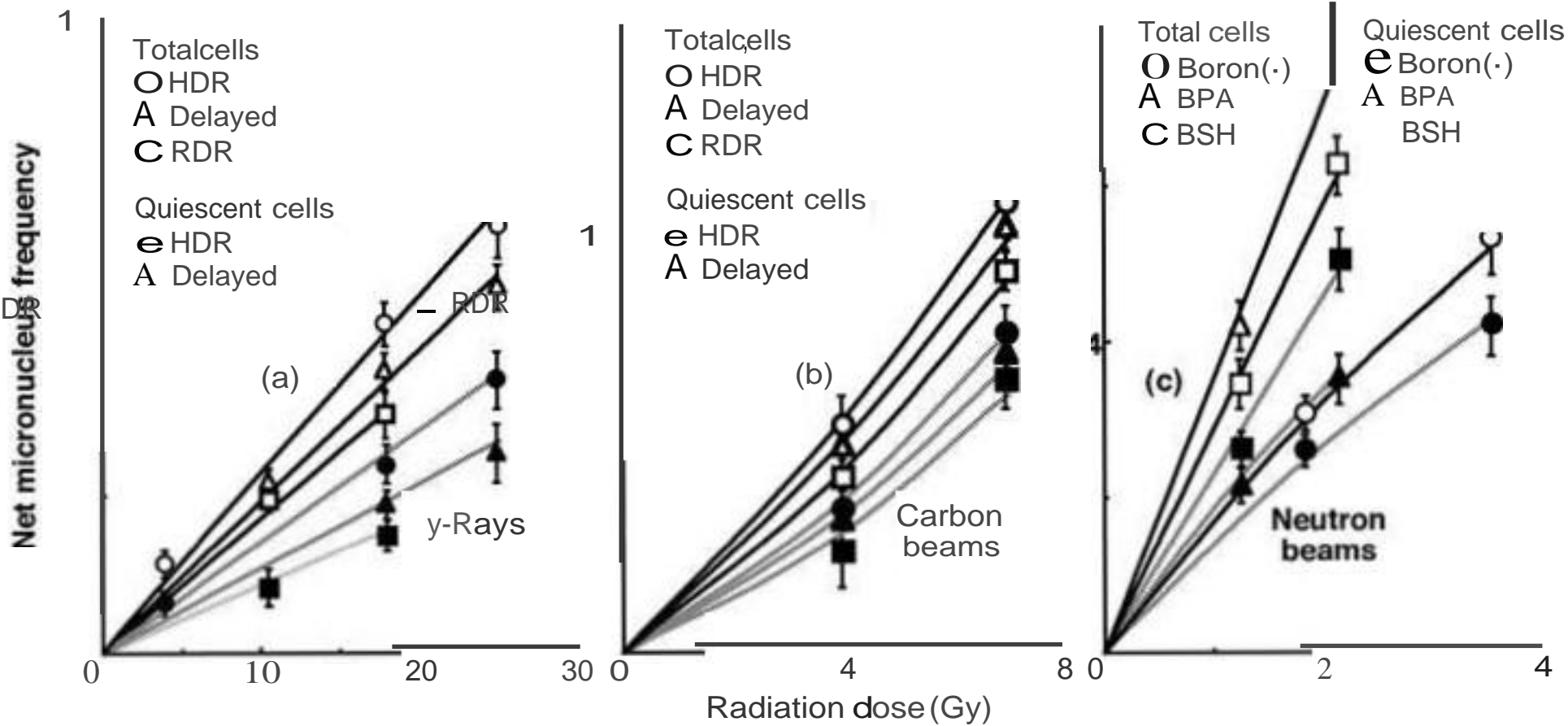


Figure 3
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