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Wortmannin efficiently suppresses the recovery from radiation-induced damage in pimonidazole-unlabeled quiescent tumor cell population

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Labeling of proliferating (P) cells in mice bearing EL4 tumors was achieved by continuous administration of 5-bromo-2′-deoxyuridine (BrdU). Tumors were irradiated with γ-rays at 1 h after pimonidazole administration followed by caffeine or wortmannin treatment. Twenty-four hours later, assessment of the responses of quiescent (Q) and total (= P + Q) cell populations were based on the frequencies of micronucleation and apoptosis using immunofluorescence staining for BrdU. The response of the pimonidazole-unlabeled tumor cell fractions was assessed by means of apoptosis frequency using immunofluorescence staining for pimonidazole. The pimonidazole-unlabeled cell fraction showed significantly enhanced radio-sensitivity compared with the whole cell fraction more remarkably in Q cells than total cells. However, a significantly greater decrease in radio-sensitivity in the pimonidazole-unlabeled than the whole cell fraction, evaluated using an assay performed 24 hours after irradiation, was more clearly observed in Q cells than total cells. In both the pimonidazole-unlabeled and the whole cell fractions, wortmannin efficiently suppressed the reduction in sensitivity due to delayed assay. Wortmannin combined with γ-ray irradiation is useful for suppressing the recovery from radiation-induced damage especially in the pimonidazole-unlabeled cell fraction within the total and Q tumor cell populations.

Keywords: Quiescent cell; recovery from radiation-induced damage; pimonidazole; wortmannin; caffeine

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 tumor cell population has never been shown to be fully hypoxic [2]. Actually, the sizes of the HF of Q and total (= proliferating (P) + 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, were 55.1 ± 6.2% (mean ± SE) and 11.1 ± 4.0 %, respectively [3]. However, the value for Q cell population 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 substituted 2-nitroimidazole, and a mouse IgG1 monoclonal antibody (MAb1) to stable covalent adducts formed through reductive activation of pimonidazole in hypoxic cells [4]. Here, we tried to selectively detect the response of the pimonidazole-unlabeled cell fraction of the Q cell population. To achieve this we combined our method for selectively detecting the response of Q cells in solid tumors with the method for detecting cell and tissue hypoxia using pimonidazole and MAb1 to pimonidazole.

Two major pathways for the repair of potentially lethal DNA double-stranded breaks (DSBs) exist in mammalian cells. The non-homologous end-joining (NHEJ) pathway is imprecise, error-prone and mutagenic, and mutant cell lines lacking key components of this pathway all exhibit impaired kinetics of DNA DSB repair and exquisite radiosensitivity [5, 6]. Homologous recombination (HR) is a more precise (error-free) repair mechanism and is more important for the repair of DSBs in late-S and G2 when a sister chromatid is available for the recombination reaction. Cell lines with defects in HR also exhibit increased radio-sensitivity and decreased fidelity of repair [5, 7]. Wortmannin was thought to have the potential to hinder NHEJ repair by inhibiting a catalytic subunit of DNA-dependent protein kinase [8]. Caffeine was thought to have the potential to inhibit HR by targeting ataxia telangiectasia-mutated protein kinase (ATM) and ATM- and Rad3-related protein kinase (ATR) [9].

In the present study, the radio-sensitivity of the pimonidazole-unlabeled cell fraction of the Q cell population after cobalt-60 γ-ray irradiation followed with or without post-irradiation administration of caffeine or wortmannin, was determined, to examine the effect of caffeine and wortmannin on the repair of radiation-induced damage in a pimonidazole-unlabeled Q tumor cell population.

MATERIALS AND METHODS

Mice and tumors

EL4 lymphoma cells (Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan) 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 [10]. 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, USA) 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. The percentage of labeled cells after continuous labeling with BrdU was 66.1 ± 3.8% and plateaued 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 γ-ray irradiation. γ-ray irradiation was performed with a cobalt-60 γ-ray irradiator at a dose rate of 2.75 Gy/min with tumor-bearing mice held in a specially constructed device with the tail firmly fixed with an adhesive tape. Lead blocks were used to avoid irradiating other body parts than the tumor-bearing left hind leg. In addition, wortmannin (6 mg/kg) or caffeine (68.5 mg/kg = 1/2 LD50 (mean lethal dose)) dissolved in physiological saline [8, 9] was also administered intraperitoneally immediately after irradiation.

Each irradiation group also included mice that were not pretreated with BrdU.

Immunofluorescence staining of BrdU-labeled and/or pimonidazole-labeled cells and the observation of apoptosis and micronucleation

Based on our previous report related to the determination of the timing of apoptosis [11], as an immediate assay, an apoptosis assay was undertaken at 6 h after irradiation and a micronucleus assay was carried out immediately after irradiation. Tumors were excised from mice given BrdU, weighed, minced and trypsinized (0.05% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA) in phosphate-buffered saline (PBS) at 37°C for 20 min). Furthermore, as a delayed assay, tumors were also excised from mice given BrdU, weighed, minced and trypsinized at 30 h after irradiation for the apoptosis assay, and at 24 h after irradiation for the micronucleus assay. For the apoptosis assay, single cell suspensions were fixed without further treatment. For the micronucleus assay, tumor cell suspensions were incubated for 72 h in tissue culture dishes containing complete culture medium and 1.0 μg/ml of cytochalasin-B, to inhibit cytokinesis while allowing nuclear division. The cultures were then trypsinized and cell suspensions were fixed. For both assays, after the centrifugation of fixed cell suspensions, the cell pellet was resuspended with cold Carnoy's fixative (ethanol: acetic acid = 3:1 in volume). The suspension was placed on a glass microscope slide and the sample was dried at room temperature. Slides were treated with 2 M hydrochloric acid for 60 min at room temperature to dissociate the histones and partially denature the DNA. They were then immersed

in borax-borate buffer (pH 8.5) to neutralize the acid. BrdU-labeled tumor cells were detected using indirect immunofluorescence staining with a rat monoclonal anti-BrdU antibody (Abcam plc, Cambridge, UK) and a goat Alexa Fluor 488-conjugated anti-rat IgG antibody (Invitrogen Corp., Carlsbad, CA, USA). Pimonidazole-labeled tumor cells were detected using indirect immunofluorescence staining with a mouse monoclonal anti-pimonidazole antibody (Hypoxyprobe Inc., Burlington, MA, USA) and a rabbit Alexa Fluor 594-conjugated anti-mouse IgG antibody (Invitrogen Corp.). To enable the observation of the triple staining of tumor cells with green-emitting Alexa Fluor 488 and red-emitting Alexa Fluor 594, cells on the slides were treated with blue-emitting 4′6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml in PBS) and imaged using a fluorescence microscope.

The frequency of apoptosis in cells not labeled with BrdU (= Q cells at irradiation) and tumor cells not labeled with pimonidazole was determined by counting apoptotic cells in tumor cells that did not show green fluorescence from Alexa Fluor 488 and red fluorescence from Alexa Fluor 594, respectively [12]. The apoptosis frequency was defined as the ratio of the number of apoptotic cells to the total number of observed tumor cells [11]. The micronucleus frequency in BrdU-unlabeled cells was examined by counting the micronuclei in the binuclear cells that did not show green fluorescence emitted by Alexa Fluor 488. The micronucleus frequency was defined as the ratio of the number of micronuclei in the binuclear cells to the total number of binuclear cells observed [2, 13]. The representative pictures of the micronuclei and apoptotic tumor cells have already been shown in our previously published reports [12, 13].

The ratios obtained in tumors not pretreated with BrdU indicated the apoptosis frequency and the micronucleus frequency in the total (P + Q) tumor cell populations. More than 300 tumor cells and binuclear cells were counted to determine the apoptosis frequency and the micronucleus frequency, respectively.

Clonogenic cell survival assay

The clonogenic cell survival assay was also performed in mice given no BrdU or pimonidazole using an *in vivo–in vitro* assay method. Tumors were disaggregated by stirring for 20 min at 37°C in PBS containing 0.05% trypsin and 0.02% EDTA. The cell yield was $(1.1 \pm 0.3) \times 10^8$/g tumor weight. A colony formation assay using the *in vivo–in vitro* assay method was performed with the culture medium mixed with methylcellulose (15.0 g/l) (Aldrich, Milwaukee, WI, USA).

The apoptosis and micronucleus frequencies and surviving fractions for the total cell population were obtained from cells in tumors that were not pretreated with BrdU or pimonidazole. The apoptosis and micronucleus frequencies for Q cells were obtained from unlabeled tumor cells after continuous BrdU labeling without pimonidazole loading [13]. The apoptosis frequencies for the total tumor cell populations that were not labeled with pimonidazole were obtained from tumor cells that were not labeled with pimonidazole after pimonidazole loading without BrdU pretreatment. The apoptosis frequencies for Q cells that were not labeled with pimonidazole were obtained from tumor cells that were not labeled with BrdU or pimonidazole after both continuous BrdU labeling and pimonidazole loading [12]. Thus, there was no effect of interaction between BrdU and irradiation or between pimonidazole and irradiation on the values for the apoptosis and micronucleus frequencies and surviving fractions. Incidentally, since the rate of pimonidazole-labeled tumor cells could change during culturing with cytochalasin-B over 3 days, following the production of single tumor cell suspensions by excising and mincing the tumors from mice that underwent pimonidazole loading, the micronucleus frequency for the cell fraction that was not labeled with pimonidazole after pimonidazole loading was not determined. As a consequence, the radiosensitivity of the pimonidazole-unlabeled cell fractions was only determined in relation to apoptosis induction. This was the reason for using the EL4 leukemia cell line with its much greater capacity for the induction of apoptosis rather than other solid tumor-originating cell lines [11].

Data analysis and statistics

Three tumor-bearing mice were used to assess each set of conditions and each experiment was repeated at least twice. This means the number of tumor-bearing mice employed in each treatment group is nine. To examine the differences between pairs of values, Student's *t*-test was used when variances of the two groups were assumed to be equal with the Shapiro–Wilk normality test; otherwise the Welch *t*-test was used.

RESULTS

Table 1 shows the surviving fractions (SFs) without γ-ray radiation for the total tumor cell population and the MN and apoptosis frequencies without γ-ray radiation for the total and Q cell populations. Q cells showed significantly higher MN and apoptosis frequencies at 0 Gy than the total cell population under each set of conditions ($P < 0.05$). Wortmannin and caffeine induced significantly lower SFs and significantly higher MN and apoptosis frequencies at 0 Gy in both the total and Q cell populations than absolutely no treatment ($P < 0.05$). Furthermore, the values of the SFs and the MN and apoptosis frequencies at 0 Gy for both drugs were almost the same. Thus, it follows that the dose of each drug employed here had an almost iso-effect in the absence of γ-ray irradiation.

Cell survival curves for the total tumor cell population as a function of radiation dose are shown in Fig. 1. The SFs

Table 1. Surviving fraction, micronucleus frequency and apoptosis frequency at 0 Gy

	Total tumor cells	Quiescent cells
<Surviving fraction at 0 Gy (%)>		
Without drug	25.5 ± 6.8[a]	–
With wortmannin	15.5 ± 3.1	–
With caffeine	15.2 ± 3.0	–
<Micronucleus frequency at 0 Gy>		
Without drug	0.053 ± 0.006	0.073 ± 0.008
With wortmannin	0.101 ± 0.013	0.138 ± 0.015
With caffeine	0.106 ± 0.013	0.133 ± 0.014
<Apoptosis frequency at 0 Gy>		
Without drug	0.040 ± 0.005	0.067 ± 0.007
With wortmannin	0.080 ± 0.009	0.107 ± 0.011
With caffeine	0.083 ± 0.010	0.113 ± 0.012
Especially in pimonidazole-unlabeled cell fraction		
Without drug	0.017 ± 0.002	0.028 ± 0.003
With wortmannin	0.035 ± 0.004	0.048 ± 0.005
With caffeine	0.037 ± 0.004	0.049 ± 0.006

[a]Mean ± standard error ($n = 9$).

showed an increasing tendency in Fig. 1 in the following order: immediately after irradiation alone < 24 h after irradiation with wortmannin < 24 h after irradiation with caffeine < 24 h after irradiation alone.

For baseline correction, we used the net micronucleus frequency to exclude the micronucleus frequency in non-irradiated tumors. The net micronucleus frequency was defined as the micronucleus frequency in the irradiated tumors minus the micronucleus frequency in the non-irradiated tumors. Dose–response curves for the net micronucleus frequency in total and Q tumor cell populations as a function of radiation dose are shown in Fig. 2. Overall, the net micronucleus frequencies were significantly lower in the Q cells than the total cell population ($P < 0.05$). In both the total and Q cell populations, the net micronucleus frequencies showed a decreasing tendency in Fig. 2 in the following order: immediately after irradiation alone > 24 h after irradiation with wortmannin > 24 h after irradiation with caffeine > 24 h after irradiation alone.

For another baseline correction, we used the net apoptosis frequency to exclude the apoptosis frequency in

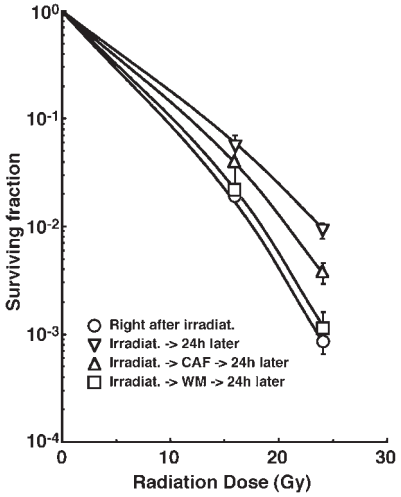

Fig. 1. Cell survival curves for the whole tumor cell fraction in the total tumor cell population of EL4 tumors as a function of γ-ray radiation dose. Circles, reverse triangles, triangles and squares represent the surviving fractions immediately after γ-ray irradiation only, at 24 h after γ-ray irradiation only, at 24 h after γ-ray irradiation followed by caffeine administration and at 24 h after γ-ray irradiation followed by wortmannin administration, respectively. Bars represent standard errors ($n = 9$).

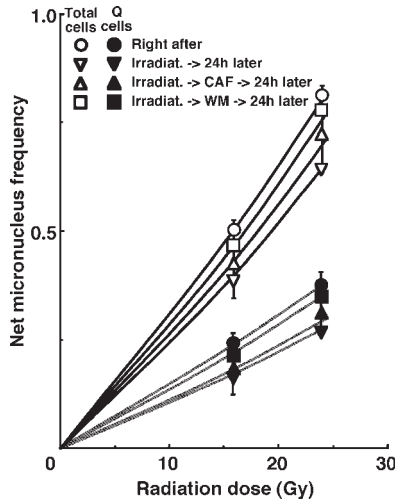

Fig. 2. Dose–response curves of the net micronucleus frequency for the whole tumor cell fraction in the total and quiescent (Q) tumor cell populations of EL4 tumors as a function of γ-ray radiation dose. Open and solid symbols represent the net micronucleus frequencies for total and quiescent tumor cell populations, respectively. Circles, reverse triangles, triangles and squares represent the net micronucleus frequencies immediately after γ-ray irradiation only, at 24 h after γ-ray irradiation only, at 24 h after γ-ray irradiation followed by caffeine administration and at 24 h after γ-ray irradiation followed by wortmannin administration, respectively. Bars represent standard errors ($n = 9$).

non-irradiated tumors. The net apoptosis frequency was the apoptosis frequency in the irradiated tumors minus that in the non-irradiated tumors. Dose–response curves for the net apoptosis frequency in the total and Q tumor cell populations as a function of radiation dose are shown in Fig. 3. Overall, the net apoptosis frequencies were significantly lower in the Q than total cell population ($P < 0.05$). Moreover, the net apoptosis frequency was significantly higher for the cell fraction that was not labeled with pimonidazole than for the whole tumor cell fraction in both the Q and total cell populations under each set of conditions ($P < 0.05$). For both the pimonidazole-unlabeled and the whole cell fractions, in the Q as well as total tumor cell population, the net apoptosis frequencies showed a decreasing tendency in Fig. 3 in the following order: immediately after irradiation alone > 24 h after irradiation with wortmannin > 24 h after irradiation with caffeine > 24 h after irradiation alone.

To evaluate the radio-sensitivity of the cell fraction that was not labeled with pimonidazole, as compared with the whole cell fraction in both the total and Q cell populations, dose-modifying factors (DMFs) were calculated using the data shown in Fig. 3 (Table 2). Overall, DMF values tended to be higher for the Q cell than for the total cell population, and in particular immediately after irradiation there was a much larger difference, although not significantly. In both the total and Q cell population, the DMF values had a tendency to decrease in the following order: immediately after irradiation alone > 24 h after irradiation with wortmannin > 24 h after irradiation with caffeine > 24 h after irradiation alone, without any significant differences.

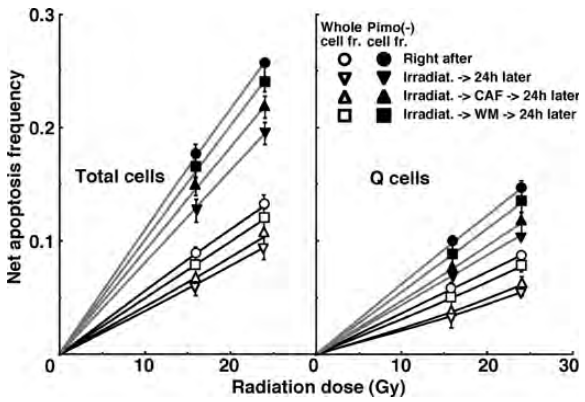

Fig. 3. Dose–response curves for the net apoptosis frequency of the total (left panel) and quiescent (Q) (right panel) tumor cell populations of EL4 tumors as a function of γ-ray radiation dose. Open and solid symbols represent the net apoptosis frequencies for the whole tumor cell fraction and the cell fraction not labeled with pimonidazole (Pimo (–)) in both the total and Q tumor cell populations, respectively. Circles, reverse triangles, triangles and squares represent the net apoptosis frequencies immediately after γ-ray irradiation only, at 24 h after γ-ray irradiation only, at 24 h after γ-ray irradiation followed by caffeine administration and at 24 h after γ-ray irradiation followed by wortmannin administration, respectively. Bars represent standard errors ($n = 9$).

Table 2. Dose-modifying factors for the pimonidazole unlabeled cell fraction compared with the whole cell fraction in the total or quiescent cell population[a]

	Total cells	Quiescent cells
Immediately after irradiation		
	1.95 ± 0.15[b]	2.15 ± 0.15
24 h after irradiation		
Without drug	1.7 ± 0.1	1.9 ± 0.15
With wortmannin	1.9 ± 0.15	2.0 ± 0.2
With caffeine	1.75 ± 0.15	1.95 ± 0.15

[a]The ratio of the dose of radiation necessary to obtain the net apoptosis frequency of 0.05 in a whole cell fraction to that needed to obtain the frequency in the pimonidazole-unlabeled cell fraction.
[b]Mean ± standard error ($n = 9$).

To investigate the reduction in radio-sensitivity caused by a delayed assay, DMFs were calculated using the data given in Figs 1–3 (Table 3). On the whole, in the fraction unlabeled with pimonidazole or the whole cell fraction, the values decreased in the following order: after irradiation alone > 24 h after irradiation with caffeine > 24 h after irradiation with wortmannin in both the total and Q cell populations, particularly in the latter population. Concerning the significant differences observed in this decreasing tendency, the details are shown in the notes for Table 3. The DMF values were higher in the Q cell than total cell population in both the pimonidazole-unlabeled and whole cell fractions. In both the Q and total cell populations, the values were higher for pimonidazole-unlabeled cell fractions than whole cell fractions, particularly in the case of the Q cells.

To examine the difference in radio-sensitivity between the total and Q cell populations, DMFs that allow us to compare the dose of radiation necessary to obtain each endpoint in the two cell populations were calculated using the data in Figs 2 and 3 (Table 4). All DMF values were significantly higher than 1.0 ($P < 0.05$). The DMF values increased in the following order: immediately after irradiation alone < 24 h after irradiation with wortmannin < 24 h after irradiation with caffeine < 24 h after irradiation alone, although not significantly. The values were lower for the sub-population that was not labeled with pimonidazole as compared with the whole cell population without any significant differences.

DISCUSSION

The fraction of cells that were not labeled with pimonidazole showed significantly higher radio-sensitivity than the

Table 3. Dose-modifying factors due to a delayed assay*

	Without drug	With wortmannin	With caffeine
<Surviving fraction = 0.01>			
Total cells	1.35 ± 0.1**,a,b	1.05 ± 0.05[a]	1.15 ± 0.05[b]
<Net micronucleus frequency = 0.25>			
Total cells	1.25 ± 0.1[c]	1.05 ± 0.05[c]	1.15 ± 0.05
Quiescent cells	1.4 ± 0.1[d]	1.1 ± 0.05[d]	1.25 ± 0.1
<Net apoptosis frequency = 0.05>			
Total cells	1.25 ± 0.1[e]	1.1 ± 0.05[e]	1.15 ± 0.1
Especially in pimonidazole-unlabeled cell fraction			
	1.45 ± 0.1[f]	1.15 ± 0.05[f,g]	1.35 ± 0.1[g]
Quiescent cells	1.4 ± 0.1[h]	1.1 ± 0.05[h,i]	1.3 ± 0.1[i]
Especially in pimonidazole-unlabeled cell fraction			
	1.65 ± 0.15[j]	1.15 ± 0.05[j,k]	1.5 ± 0.15[k]

*The ratio of the dose of radiation necessary to obtain each end-point with a delayed assay to that needed to obtain each end-point with an assay immediately after irradiation alone.
** Mean ± standard error ($n = 9$)
Letters [a–k] represent the significant differences between two values ($P < 0.05$).

Table 4. Dose-modifying factors for quiescent cells relative to total tumor cells[a]

Immediately after irradiation	24 h after irradiation		
	Without drug	With wortmannin	With caffeine
<Net micronucleus frequency = 0.25>			
2.05 ± 0.2[b]	2.25 ± 0.2	2.05 ± 0.2	2.2 ± 0.2
<Net apoptosis frequency = 0.05>			
1.8 ± 0.2	1.95 ± 0.2	1.85 ± 0.2	1.9 ± 0.2
Especially in pimonidazole-unlabeled cell fraction			
1.6 ± 0.15	1.8 ± 0.2	1.6 ± 0.15	1.75 ± 0.15

[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 ($n = 9$).

whole cell fraction in both the Q and total cell populations, and amongst the Q cells in particular (Table 2). This was probably because the pimonidazole-unlabeled cells were more oxygenated than the whole cell fraction, which comprised oxygenated and hypoxic tumor cells, in both the Q and total tumor cell populations [4]. Additionally the Q cell population as a whole included a larger hypoxic fraction than the total tumor cell population [2, 13]. As shown in Table 3, the pimonidazole-unlabeled cell fraction had a greater recovery capacity than the whole cell fraction, especially in the case of the Q cells. When combined with wortmannin or caffeine, especially with wortmannin, the recovery capacity in both the pimonidazole-unlabeled and the whole cell fractions was efficiently suppressed, especially in the Q cell population (Table 3). Thus, the decreases in the DMFs for the pimonidazole-unlabeled relative to the whole cell fractions were repressed with wortmannin or caffeine, especially with wortmannin (Table 2).

On the other hand, the Q cell population as a whole showed a greater recovery capacity than the total tumor cell population, and the combination with wortmannin or caffeine, especially with wortmannin showed a greater potential to suppress a recovery capacity in Q than total tumor cell population (Table 3). Therefore, the increases in the DMFs for the Q relative to total tumor cell populations were also repressed with wortmannin or caffeine, especially with wortmannin.

In recent years the concept of cancer stem cells (CSCs), or tumor-initiating cells (tumor clonogens), has attracted a great deal of interest because of the potential clinical significance [14]. In part, these cells are thought to exist in a pathophysiological microenvironment where hypoxia, low pH and nutrient deprivation occur. Under these microenvironmental conditions, dividing tumor cells have also been thought to become quiescent. Actually, a subset of CSCs or tumor clonogens consists of non-dividing quiescent cells [15]. One mechanism of CSC or tumor clonogen resistance to cytotoxic treatment is based on an enhanced DNA repair capacity [16]. Here, the pimonidazole-unlabeled Q cell fraction showed a much greater recovery capacity than the Q cell population as a whole, even if the recovery capacity was significantly greater in the entire Q cell population than in the total tumor cell population as a whole. In other words, from the viewpoint of not only quiescent status but also enhanced DNA repair capacity, some of the characteristics of the pimonidazole-unlabeled cell fraction in the Q cell population were found to be similar to those of CSCs or tumor clonogens.

Potentially lethal damage (PLD) is the component of radiation damage that can be modified by post-irradiation conditions [17]. Under ordinary circumstances, PLD causes cell death. Changing cellular growth conditions or the microenvironment of cells influences the expression of PLD or its recovery (PLDR), and thereby influences sensitivity to radiation. PLDR is favored by conditions that maintain cells without encouraging or allowing them to divide. Conditions found in solid tumors, regions of which may be far from blood vessels and low in glucose and oxygen, have a low extracellular pH, and show high concentrations of cellular waste products, may prevent cells from proliferating and thereby promote the recovery of PLD. Extensive studies on PLDR suggest that DNA DSBs are potentially lethal lesions that can be converted into lethal damage [17].

Wortmannin was originally described as a potent inhibitor of phosphoinositide 3-kinases (PI-3Ks), which are lipid kinases involved in insulin and other mitogenic signaling pathways. The kinase domain of PI-3K shares homology with ATM protein, which is a protein kinase that functions in DNA damage-responsive signaling pathways by phosphorylating target proteins. ATM is related to several other protein kinases involved in the regulation of eukaryotic cell cycle progression and DNA damage-triggered responses [7, 8]. These proteins all contain a carboxy-terminal kinase domain that shares significant sequence homology with the kinase domains of mammalian and yeast PI-3Ks. In this family of PI-3K-related kinases, there is a catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) that functions in the NHEJ DNA repair pathway. Thus, wortmannin is thought to show the potential to inhibit the catalytic activities of DNA-PKcs, thereby hampering the NHEJ repair pathway [8]. However, wortmannin is also well known to inhibit the PI3K-Akt-mTOR signaling pathway, which facilitates proliferation and survival of cells [7]. Thus, in order to analyze the involvement of the PI3K signaling pathway, DNA-PK and/or ATM knockdown studies are needed in the future.

ATM and ATR are NHEJ-independent checkpoint regulators that facilitate repair by HR and are inhibited by caffeine [18]. Irradiation-induced DNA DSBs activate NHEJ and HR repair pathways and ATM- and ATR-dependent pathways that regulate checkpoint responses. Caffeine inhibits both the ATM- and ATR-regulated checkpoint responses, which mainly affect HR [18]. Thus, caffeine is thought to preferentially inhibit HR repair.

The imprecise NHEJ pathway is the predominant repair process for cells in G0, G1 or early S-phase, and the precise HR pathway is more important for the repair of DSBs in late-S and G2 [5]. It has been reported that wortmannin sensitized plateau-phase cultured cells, not P cells, to radiation *in vitro* [19]. Actually, in our previous reports and here, Q cells and the pimonidazole-unlabeled tumor cells showed greater recovery capacity than the total cell population and the whole tumor cell fraction, respectively, in solid tumors, and the recovery in both total and Q cell populations and in pimonidazole-unlabeled cell fraction was more efficiently inhibited in combination with wortmannin than caffeine, in spite of an almost isoeffect induced by the drugs in the absence of γ-ray irradiation (Figs 2 and 3, Tables 1 and 3). Meanwhile, it has also been reported that the expression of RAD51, a critical mediator of HR, is repressed by hypoxia in numerous cell lines, irrespective of p53 status [5, 20]. Taking into account that the intratumor Q cell population includes a much larger hypoxic fraction than the total cell population, as shown in our previous report [21], the recovery in Q cells in p53-expressing solid tumors is thought to be predominantly due to the DNA-PK-mediated NHEJ pathway. Furthermore, also in the pimonidazole-unlabeled tumor cell fraction, the recovery was more efficiently repressed with wortmannin than caffeine, which is also thought to be mainly due to the DNA-PK-mediated NHEJ pathway. Wortmannin may be a promising candidate as a recovery inhibitor in radiotherapy for solid tumors with wild-type p53, if its toxicity is within the tolerance level for patients. In the future, we would like to evaluate the effectiveness of wortmannin as a recovery inhibitor in the pimonidazole-unlabeled tumor cell fraction within solid tumors with mutated-type p53.

The microenvironmental conditions under which dividing tumor cells become quiescent might promote the formation of micronuclei and apoptosis at 0 Gy in the whole Q tumor cell fractions, partly due to hypoxic stress (Table 1) [1]. In this study, the Q cells were shown to be significantly

less radio-sensitive ($P < 0.05$) and to have a greater recovery capacity than the total cell population (Figs 2 and 3, Table 4). This finding indicated that more Q cells survive radiation therapy than P cells. In particular, in the cell fraction that was not labeled with pimonidazole, the difference in radio-sensitivity between the Q and total cell populations was markedly increased when evaluated using the delayed assay. This was due to the greater recovery capacity of the unlabeled Q cell fraction as compared with the unlabeled cell fraction in the total tumor cell population (Table 3). Therefore, whether in the pimonidazole-unlabeled or the whole Q cell population, it follows that control of the Q cells has a great impact on the outcome of radiation therapy and that the Q cell population can be a critical target in the control of solid tumors.

In this study, the pimonidazole-unlabeled Q cell fraction showed a greater recovery capacity than the Q cell population as a whole. Thus, there was similarity between the pimonidazole-unlabeled Q cell fraction and CSCs or tumor clonogens in terms of quiescent status and enhanced recovery capacity. However, CSCs or tumor clonogens are thought to exist under rather hypoxic conditions [14–16]. Meanwhile, hypoxia that develops in solid tumors stabilizes the hypoxia inducible factor-1α (HIF-1α) subunit of the HIF-1 transcription factor, leading to up-regulation of dozens of hypoxia-regulated genes that increase glycolysis and oxygen delivery. HIF-1α is an indicator of the current oxygen status because it is rapidly stabilized under hypoxia and rapidly degraded when oxygen is available, and expressed below ~2% O_2 [22]. In contrast, chemical hypoxia marker pimonidazole is metabolically reduced under hypoxia to produce adducts that can be detected using antibodies, and the pimonidazole adducts are formed only below 1% O_2 [22]. Furthermore, HIF-1 is also known to induce cell cycle arrest at G1 phase [23]. Therefore, it follows that some pimonidazole-unlabeled Q tumor cells are expressing HIF-1 under hypoxia between 1 and 2% O_2. In terms of the radiobiological characteristics, there may be a great similarity between the HIF-1 expressing pimonidazole-unlabeled Q tumor cells and CSCs or tumor clonogens. In the future, the characteristics of the intratumor Q cell population in connection with those of CSCs or tumor clonogens using human tumor cell lines and the spatial relationship between the Q cells (BrdU-unlabeled cells), hypoxic cells (pimonidazole-positive cells) and blood vessels with immunohistochemical analysis will need to be elucidated.

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