Effects of Bioreductive Agents, Tirapazamine and Mitomycin C, on Quiescent Cell Populations in Solid Tumors, Evaluated by Micronucleus Assay

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Mice bearing transplantable solid tumors received 10 intraperitoneal administrations of 5-bromo-2′-deoxyuridine (BrdU) to label the proliferating (P) tumor cells, and were then irradiated with ^{60}Co gamma-rays or injected with cis-diamminedichloroplatinum (II) (cisplatin). The tumor cells were isolated and incubated with cytochalasin-B (a cytokinesis blocker). The micronucleus (MN) frequency in the cells without BrdU labeling, which were regarded as quiescent (Q) cells in the tumor, was determined using immunofluorescence staining for BrdU. The MN frequency in the total (P+Q) tumor cell population was determined from tumors that were not pretreated with BrdU. Pretreatment with tirapazamine, a bioreductive agent, could enhance the sensitivity of tumor cells, including Q cells, to radiation more markedly than mitomycin C pretreatment as judged from an in vivo assay immediately after irradiation. Post-irradiation administration of tirapazamine produced a large post-irradiation radiosensitizing effect on both the total and Q tumor cell populations in vivo. Cisplatin treatment combined with tirapazamine demonstrated that tirapazamine also has a chemosensitizing potential for both the total and Q tumor cell populations. We confirmed that the sensitivity of Q cell populations to radiation and chemotherapy using cisplatin can be enhanced by combined treatment with tirapazamine.

Key words: Quiescent cell — Tirapazamine — Mitomycin C — Hypoxic cell

Recent studies have suggested that tumor hypoxia could be exploited by the use of bioreductive agents which preferentially kill hypoxic cells.[1, 2] Brown and colleagues reported that tirapazamine (SR 4233, WIN 59075) showed highly selective toxicity toward hypoxic cells in both in vitro and in vivo murine tumor systems.[3, 4] Severe hypoxia was not required and the level of hypoxia found in many human tumors (< 10–20 mmHg) was sufficient for toxicity.[5] Tirapazamine has been shown to interact with radiation[1] or various anticancer agents including cisplatin[6–8] to enhance tumor cell killing. Tirapazamine is currently undergoing clinical trials.[1, 9, 10]

It is known that many tumor cells in solid tumors are non-proliferating (quiescent), and it has been made clear that plateau-phase cultures in vitro contain large numbers of quiescent (Q) cells.[11, 12] Although the nature of Q cells has been extensively studied over the last 25 years, many aspects of these cells are still unknown.[13] Q cells are more likely to be hypoxic,[14] and therefore more sensitive to tirapazamine and mitomycin C, and Q cells should be less accessible to drugs since they are thought to be more distant from blood supply.[15] To improve the treatment of cancer, it is necessary to determine the response of Q

cells in solid tumors to various anticancer therapeutic drugs, since many tumor cells are quiescent in situ, but are still clonogenic.[16] However, no studies on the effect of bioreductive agents on Q cells in vivo has been reported.

In this study, we evaluated the combination effects of bioreductive agents, tirapazamine and mitomycin C, with gamma-ray irradiation or cisplatin administration in the treatment of transplantable murine solid tumors, especially from the viewpoint of their killing effect on Q cells in vivo, using our method for selectively detecting the response of Q cells in solid tumors.[17]

MATERIALS AND METHODS

Tumors, mice and labeling with BrdU SCC VII squamous cell carcinomas, derived from C3H/He mice, were maintained in vitro in Eagle's minimum essential medium containing 12.5% fetal bovine serum. Cells were collected from monolayer cultures, and approximately 1.0×10^5 cells were inoculated subcutaneously into the left hind legs of 8-week-old female C3H/He mice. Fourteen days after inoculation, the tumor had reached approximately 1 cm in diameter. Nine days after inoculation, 5-bromo-2′-deoxyuridine (BrdU) dissolved in physiological saline (100 mg/kg) was administered intraperitone-

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ally 10 times, at 12-h intervals to label all proliferating (P) cells in the tumors. Administration of BrdU did not change the tumor growth rates. The tumor was 1 cm in diameter upon treatment. The labeling index after 10 doses of BrdU was $55.3 \pm 4.5\%$ (mean$\pm$SD), and had reached a plateau level at this stage. Therefore, in this study, we regarded tumor cells not incorporating BrdU after 10 doses as Q cells for all practical purposes.

Treatment After labeling with BrdU, the tumor-bearing C3H/He mice were assigned to 3 groups. For groups 1 and 2, whole-body irradiation was carried out 1 h after the last dose of BrdU had been given. Mice received irradiation from a cobalt-60 gamma-ray irradiator at a dose rate of 5.97 Gy/min. In group 1, tirapazamine (50 mg/kg) or mitomycin C (5 mg/kg), dissolved in phosphate-buffered saline (PBS), was intraperitoneally administered 30 min before irradiation. Tumors were excised immediately after irradiation. In group 2, tumors were excised 24 h after irradiation alone, or 24 h after the intraperitoneal injection of tirapazamine (50 mg/kg) or mitomycin C (5 mg/kg) immediately following irradiation. In group 3, cisplatin was intraperitoneally administered at a dose of 1/20, 1/10, 1/4 and 1/2 LD_{50} (mean lethal dose, 17.7 mg/kg) 120 min after the administration of the last dose of BrdU. Intraperitoneal cisplatin administration was also performed 120 min after the intraperitoneal injection of tirapazamine or 30 min after the intraperitoneal injection of mitomycin C. Cisplatin has been shown to have a sufficient anti-tumor effect in an *in vivo-in vitro* assay by extirpating tumors more than 30 min after the intraperitoneal injection.[18] Therefore, we excised tumors from mice 1 h after the administration of cisplatin.

On the other hand, each group also included mice not pretreated with BrdU.

The concentrations and time course employed in this study have been shown to be appropriate for these bioreductive drugs to function completely.[7, 19, 20] Hori synthesized tirapazamine (SR 4233, WIN 59075, 3-amino-1,2,4-benzotriazine-1,4-dioxide), and mitomycin C was supplied by Kyowa Hakko Kogyo Co., Ltd. (Tokyo).

Immunofluorescence staining of BrdU-labeled cells and observation of micronucleus formation These procedures have been described in detail elsewhere.[17] After the above-mentioned treatments, excised tumors were minced and trypsinized at 37°C for 15 min, using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). Tumor cell suspensions were inoculated in 60-mm tissue culture dishes containing 5 ml of complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while allowing nuclear division. The proportion of binuclear cells reached a maximum 48 h and 72 h after the initiation of the cultures for total and Q cell populations, respectively. The cultures were trypsinized and

single cell suspensions were fixed with 70% ethanol. After centrifugation, the cell pellet was resuspended in 0.4 ml of cold Carnoy's fixative (3 volumes of ethanol and 1 volume of acetic acid). The suspension (30 μl) was then placed on a glass microscope slide using a dropper and the sample was dried at room temperature. The slides were treated with 2 N hydrochloric acid for 30 min at room temperature to dissociate the histones and partially denature the DNA. The slides were then immersed in a borax-borate buffer (pH 8.5) to neutralize the acid. BrdU-labeled cells were detected by indirect immunofluorescence staining using monoclonal anti-BrdU antibody and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody. To observe the double-staining of tumor cells with FITC and propidium iodide (PI), cells on the slides were treated with 30 μl of PI (1–5 μg/ml in PBS) while under the fluorescence microscope. When the intensity of the red fluorescence produced by PI became similar to the intensity of the green fluorescence in nuclei prestained with FITC, the treatment was stopped by rinsing the slide with water. The micronucleus (MN) frequency in BrdU-unlabeled Q cells 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.[21]

In tumors from mice not pretreated with BrdU, the ratio obtained indicated the MN frequency of all phases of the total tumor (P+Q) cell population in the tumors.

The MN frequency of BrdU-labeled cells, which could be regarded as P cells upon treatment, was modified because the radiosensitization effect of the incorporated

Table I. Micronucleus Frequencies without Irradiation or Cisplatin[a] Administration

Assay	Total tumor cells	Quiescent cells
Immediately after treatment (group 1)		
No drug	0.040 ± 0.019[b]	0.069 ± 0.021
With MMC[c]	0.077 ± 0.032	0.085 ± 0.034
With TPZ[d]	0.083 ± 0.035	0.109 ± 0.046
24 h after treatment (group 2)		
No drug	0.065 ± 0.024	0.115 ± 0.048
With MMC	0.085 ± 0.036	0.138 ± 0.052
With TPZ	0.120 ± 0.049	0.139 ± 0.051
Immediately after treatment (group 3)		
No drug	0.070 ± 0.030	0.081 ± 0.031
With MMC	0.082 ± 0.033	0.092 ± 0.041
With TPZ	0.104 ± 0.043	0.111 ± 0.048

a) *cis*-diamminedichloroplatinum (II).
b) Mean$\pm$1 SD.
c) Mitomycin C.
d) Tirapazamine.

BrdU[22] has the potential to influence the frequency of MN and binuclear cell appearance in BrdU-labeled cells. Therefore, the correct MN frequency of P cells without the BrdU effect could not be obtained.

Three mice were used to assess each set of conditions and each experiment was repeated 3 times. To examine the differences between pairs of values, Student's t test was used when the variances of the two groups could be assumed to be equal; otherwise, the Welch t test was used.

RESULTS

Table I shows the MN frequency without gamma-ray irradiation or cisplatin administration in the total (P+Q) and the Q tumor cell population. Under each condition, Q cells showed higher MN frequency than the total cells, although the difference was not significant. In each assay, the use of a bioreductive drug, especially TPZ, increased the MN frequency for each cell population, although again the difference was not significant. When a bioreductive drug was administered before tumor exci-

sion, even if no radiation or cisplatin was given, the MN frequency was higher than when no bioreductive drug was administered, because of the genotoxicity of the drug. Therefore, we used the normalized MN frequency to exclude the effects of the genotoxicity of the drug on the MN frequency. The normalized MN frequency is the MN frequency in the irradiated tumors or cisplatin-administered tumors minus the MN frequency in the unirradiated tumors or in tumors not treated with cisplatin.

Fig. 1 shows the dose-response curves for the normalized MN frequency in total (Fig. 1a) and Q (Fig. 1b) tumor cell populations when the tumors were excised immediately after irradiation (i.e., results from group 1). In general, the normalized MN frequency for the Q cells was lower than that for the total tumor cell population under each set of treatment conditions. We calculated the dose-modifying factors (DMF) for Q cells in tumors not treated with bioreductive drugs; these factors were used to compare the radiation or cisplatin doses necessary to obtain various normalized MN frequencies in Q cells with the doses required in the total tumor cell population.
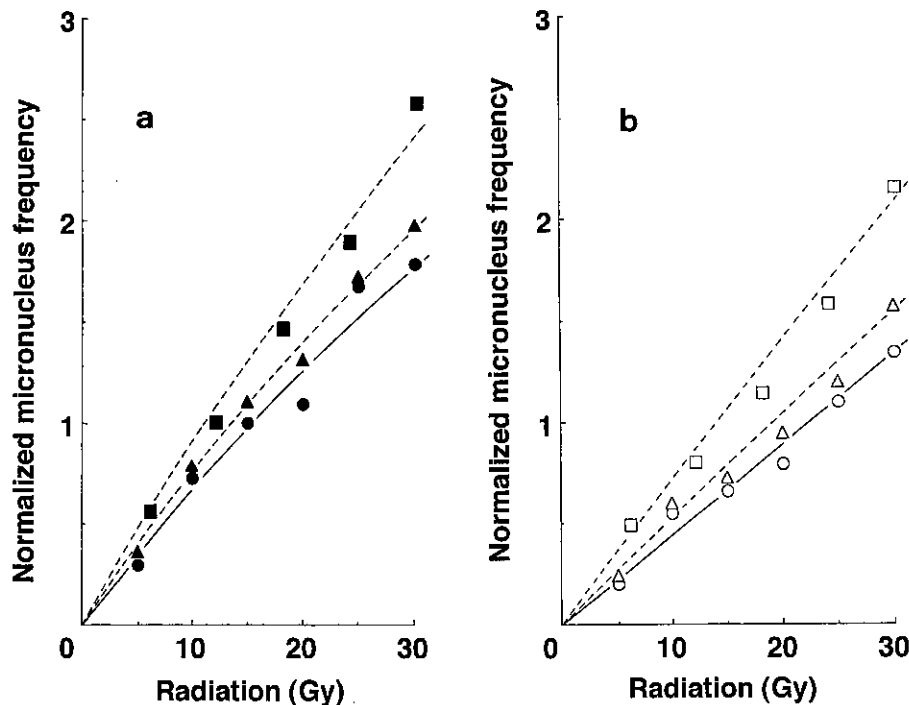

Fig. 1. Dose-response curves for normalized micronucleus (MN) frequency (MN frequency — C, where C is the MN frequency in cells from tumors in animals not exposed to gamma-ray radiation) in total cell populations (a) and quiescent (Q) cells (b). Tumors were excised immediately after irradiation alone (● ○), immediately after irradiation combined with mitomycin C (▲ △), and immediately after irradiation combined with tirapazamine (■ □). Each of the bioreductive drugs was intraperitoneally injected into the tumor-bearing mice 30 min before irradiation. Only mean values are shown, to avoid confusion. In the total and Q tumor cell populations, there were significant differences between those that received TPZ and those that received no drugs ($P < 0.05$).

For this calculation, we used the values from tumors excised immediately after irradiation alone, as shown in Fig. 1 (Table II). The values of DMF for Q cells were

Table II. Increased Resistance[a] of Quiescent Cells Relative to the Total Tumor Cell Population

Normalized micronucleus frequency	Assay performed immediately after irradiation alone	Assay using cisplatin[b] alone
0.1	1.48	3.19
0.2	1.71	2.80
0.3	1.60	2.75
0.5	1.55	—
0.75	1.46	—
1.0	1.45	—
1.25	1.39	—

a) The dose of radiation or cisplatin needed to obtain each normalized micronucleus frequency in quiescent cells/the dose of radiation or cisplatin needed to obtain each normalized micronucleus frequency in the total tumor cell population.
b) *cis*-diamminedichloroplatinum (II).

Table III. Enhancement Ratios[a]

Assay	Normalized micronucleus frequency	Total tumor cells		Quiescent cells	
		MMC[b]	TPZ[c]	MMC	TPZ
Immediately after irradiation (Fig. 1)					
	0.25	1.13	1.36	1.18	1.60
	0.5	1.13	1.39	1.20	1.59
	0.75	1.11	1.34	1.19	1.54
	1.0	1.11	1.36	1.17	1.55
	1.25	1.12	1.38	1.15	1.53
	1.5	1.13	1.39	—	—
	1.75	1.14	1.41	—	—
24 h after irradiation (Fig. 2)					
	0.25	1.20	1.92	1.22	1.93
	0.5	1.15	1.55	1.23	1.94
	0.75	1.13	1.45	1.29	2.06
	1.0	1.11	1.41	—	—
	1.25	1.10	1.40	—	—
	1.5	1.09	1.40	—	—
With cisplatin administration (Fig. 3)					
	0.1	1.43	2.40	1.44	3.38
	0.2	1.37	2.10	1.42	2.69
	0.3	1.33	1.90	1.45	2.58
	0.5	1.25	1.66	—	—
	0.75	1.22	1.51	—	—
	1.0	1.19	1.43	—	—

a) The enhancement ratio is the dose of radiation or cisplatin needed to obtain each normalized micronucleus frequency in tumor cells that received no drugs/the dose of radiation or cisplatin needed to obtain each normalized micronucleus frequency in tumor cells treated with MMC or TPZ.
b) Mitomycin C.
c) Tirapazamine.

significantly higher than 1.00 ($P < 0.05$). In both the total and Q cell populations, tirapazamine or mitomycin C administration before irradiation increased the normalized MN frequencies. To evaluate the increase in the normalized MN frequency induced by each bioreductive agent, we calculated the enhancement ratio (ER), this being the ratio between the radiation or cisplatin doses needed to obtain an equivalent normalized MN frequency without and with bioreductive drugs, using the mean values of the data given in Fig. 1 (Table III). Preirradiation treatment with tirapazamine resulted in significantly large ER values ($P < 0.05$) in both tumor cell populations, especially in the Q cells. Preirradiation injection of mitomycin C could not bring about as large an ER value as tirapazamine, but the ER values for Q cells were a little larger than those for the total cell population.

Fig. 2 shows the dose-response curves for the normalized MN frequency in total (Fig. 2a) and Q (Fig. 2b) tumor cell populations, when the tumors were excised 24 h after irradiation alone, or 24 h after the intraperitoneal injection of tirapazamine or mitomycin C right after irradiation (i.e., results from group 2). Following the intraperitoneal injection of bioreductive agents just after irradiation, the decrease in the normalized MN frequency, i.e., potentially lethal damage repair (PLDR),[17] shown by assays performed 24 h after irradiation alone, was suppressed in each cell population. The DMFs due to PLDR at various normalized MN frequencies were calculated using the data shown in Fig. 2 (Table IV). The Q cells had a higher DMF than the tumor cell population as a whole at each normalized MN frequency. At a normalized MN frequency of 0.75, the DMF for Q cells was significantly higher than that for the total tumor cell population ($P < 0.05$). In each cell population, PLDR after the injection of the bioreductive drugs was less than that seen without any drugs. To evaluate the inhibition of PLDR by each bioreductive agent, we calculated the ER, using the mean values for the data given in Fig. 2 (Table III). Mitomycin C inhibited PLDR to some extent when administered immediately after irradiation. This inhibition was a little more marked in Q cells than in the total cell population. When tirapazamine was used, larger ER values than the DMF values due to PLDR for both the total and Q tumor cells were obtained. Especially in Q cells, the ER values were significantly larger than the DMF values due to PLDR ($P < 0.05$). In other words, tirapazamine not only inhibited PLDR, but also had a post-irradiation radiosensitization effect on both tumor cell populations, especially on Q cells.

Fig. 3 shows the dose-response curves for the normalized MN frequency in total and Q tumor cells after the administration of cisplatin (i.e., results from group 3). The normalized MN frequency in Q cells was lower than
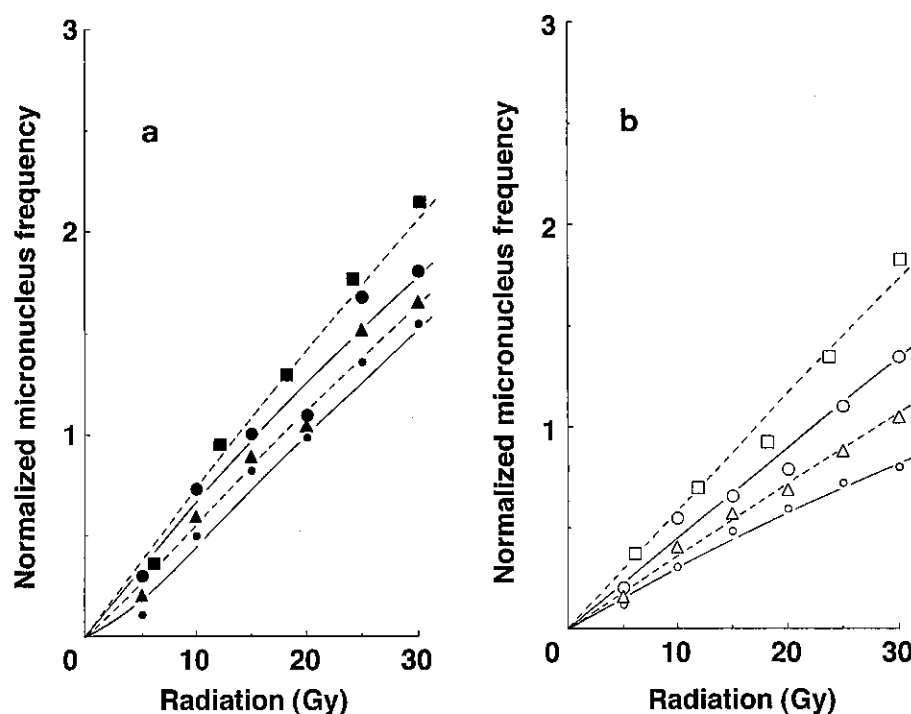
Fig. 2. Dose-response curves of the normalized micronucleus (MN) frequency (MN frequency — C, where C is the MN frequency in cells from tumors not exposed to radiation) for the total cell population (a) and quiescent (Q) cells (b). Tumors were excised immediately after irradiation alone (● ○), 24 h after irradiation alone (• ○), 24 h after the intraperitoneal administration of mitomycin C immediately following irradiation (▲ △), and 24 h after the intraperitoneal administration of tirapazamine (TPZ) immediately following irradiation (■ □). To avoid confusion, only the mean values are shown. In the total and Q tumor cell populations, there were significant differences between those that received TPZ and those that were obtained from 24 h after irradiation alone ($P < 0.05$).

Table IV. Increased Resistance[a] due to Potentially Lethal Damage Repair (PLDR)

Normalized micronucleus frequency	Total tumor cell population	Quiescent cells
0.25	1.46	1.48
0.5	1.42	1.52
0.75	1.30	1.61
1.0	1.26	—
1.25	1.22	—
1.5	1.20	—

a) The dose of radiation needed to obtain each normalized micronucleus frequency with PLDR/the dose of radiation needed to obtain each normalized micronucleus frequency without PLDR.

that in total tumor cells. The DMFs of Q cells in the tumors not treated with mitomycin C or tirapazamine were calculated, using the mean values shown in Fig. 3 (Table II). The DMF values of Q cells were significantly greater than 1.00 ($P < 0.05$), and larger than those for the

assay immediately after irradiation alone. To evaluate the effects of the combined administration of bioreductive drugs on MN frequency in total and Q tumor cell populations, we calculated the ER at various normalized MN frequencies, using the mean values for the data given in Fig. 3 (Table III). On the whole, the ERs for Q cells were higher than those for the total cell population with each treatment, and the ER values were relatively larger than those for the assay immediately after irradiation. In this assay, again, tirapazamine gave comparatively larger ERs than mitomycin C, especially in Q cells, and all the ER values when tirapazamine was used were significantly larger than 1.00 ($P < 0.05$).

DISCUSSION

The effect of cytochalasin-B on chromosome damage in cells has not been completely elucidated. However, a close relationship has been reported between cell survival and the MN frequency obtained with cytochalasin-B treatment after X-ray irradiation.[21, 23] Similarly to the
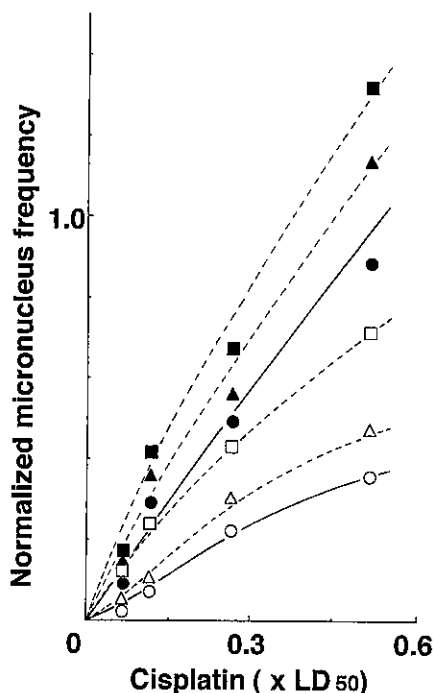
Fig. 3. Dose-response curves for normalized micronucleus (MN) frequency (MN frequency — C, where C is the MN frequency in cells from tumors in animals not given cisplatin) in the total (solid symbols) and quiescent (Q) (open symbols) cell populations. Tumors were excised 1 h after cisplatin administration alone (● ○), 1 h after cisplatin administration combined with mitomycin C (MMC) injection (▲ △), and 1 h after cisplatin administration combined with tirapazamine (TPZ) injection (■ □). TPZ and MMC were intraperitoneally injected into the tumor-bearing mice 120 and 30 min before cisplatin administration, respectively. Only mean values are shown, to avoid confusion. In the total and Q tumor cell populations, there were significant differences between those that received TPZ and those that did not receive MMC or TPZ (P<0.05).

case with X-irradiation, DNA is thought to be the primary target for cell killing by cisplatin.[24] Further, in the total tumor cell populations of SCC VII tumor, after the intraperitoneal injection of cisplatin into tumor-bearing mice, there was a close relationship between the MN frequency obtained with cytochalasin-B treatment and the surviving fraction, as determined by the in vivo-in vitro assay method.[18] Therefore, the sensitivity of these tumor cells to gamma-ray irradiation and cisplatin can be expressed in terms of the frequency of micronuclei instead of the loss of clonogenicity.

In accordance with the results of our previous study using X-ray irradiation to treat solid tumors,[17] we confirmed that the radiosensitivity, using gamma-ray irradi-

ation, and chemosensitivity, using cisplatin, of Q cells is lower than that of the total tumor cell population, and we also showed that Q cell populations have greater PLDR capacities than total tumor cell populations (Tables II and IV). However, when cisplatin was used, the DMF for Q cells was higher than the values we obtained using gamma-ray irradiation (Table II). This difference can probably be attributed to the uneven distribution of cisplatin in the Q cell population, due to the heterogeneity of tumor vasculature. A decline in the average blood flow with tumor growth can lead to randomly distributed regions with altered microenvironments.[25] One physiological consequence of poor tumor perfusion is the occurrence of regions of hypoxia within the tumor mass. In addition, it is now known that this poor tumor perfusion may also limit the clinical efficacy of chemotherapeutic agents, as a result of reduced cell proliferation in areas receiving less nutrients, inadequate drug uptake, and non-optimal distribution of the drug in the tumor tissue.[15] Our findings in Q cells support this theory, since our previous report showed that the Q cell population has a larger hypoxic fraction than the total cell population in solid tumors.[17]

In this study, mitomycin C could not significantly enhance the sensitivity of the total tumor cells or Q cells in solid tumors, even if it was used in combination with gamma-rays or cisplatin. Mitomycin C exhibits toxicity under hypoxic conditions, but its selective toxicity towards hypoxic cells is extremely modest, and extremely low levels of oxygen are required to obtain maximum cytotoxicity.[1] This will minimize the number of cells in the tumor for which it will have preferential cytotoxicity, and it will not kill all of the cells resistant to ionizing radiation or chemotherapeutic agents. In contrast, the use of tirapazamine significantly enhanced the sensitivity of not only the total tumor cells, but also Q cells, if it was combined with radiation therapy or chemotherapy. Additionally, the post-irradiation radiosensitization effect of tirapazamine showed that this compound does not act as an electron-affinic oxygen-mimetic sensitizer like nitroimidazole radiosensitizers. The profile of the cytotoxicity of tirapazamine as a function of the oxygen concentration is different from those of other bioreductive drugs, such as quinone antibiotics (e.g., mitomycin C) and nitroimidazole drugs (e.g., RSU 1069 (1[2-nitro-1-imidazolyl]-3-aziridinyl-2-propanol)).[1] Tirapazamine maintains its "hypoxic cytotoxicity" at oxygen concentrations approximately 10-fold higher than nitroimidazole bioreductive drugs do.[5] This results in an additive killing effect in combination with ionizing radiation or chemotherapeutic agents which is more uniform over the range of oxygen concentrations encountered in vivo than that that of irradiation or chemotherapy combined with other bioreductive drugs.[5] Therefore, tirapazamine has a

greater sensitivity-enhancing effect on Q cells, which have a larger hypoxic cell fraction than the total cell population, when combined with gamma-ray irradiation or cisplatin administration. Meanwhile, in contrast to radiation, which is uniformly distributed, cisplatin is unevenly distributed in solid tumors due to the heterogeneity of tumor vessels. Moreover, bioreductive drugs have the potential to overcome the major cause of solid tumor resistance to conventional anticancer therapies, which results from inadequate oxygenation and drug delivery to tumor cells via the blood vessels. Accordingly, the ER values obtained for tirapazamine combined with cisplatin administration were comparatively larger than those that were obtained from the assays immediately after gamma-ray irradiation.

Solid tumors, especially human tumors, are thought to contain a high proportion of Q cells.[14] The presence of these cells is probably due, in part, to hypoxia and the depletion of nutrition in the tumor core, and this is another consequence of poor vascular supply.[13] It has been reported that Q cells have lower radiosensitivity than P cells in solid tumors *in vivo*.[12-14] As was also shown in this study, Q cells have greater PLDR capacities and significantly lower radio- and chemo-sensitivity than the total cell population within solid tumors *in vivo*. Consequently, the control of Q cells is thought to influ-

ence greatly the outcome of anticancer radiotherapy or chemotherapy. From this viewpoint, the use of tirapazamine, an N-oxide bioreductive drug, combined with radiation therapy or chemotherapy is very effective and advantageous in the treatment of solid tumors, especially Q tumor cell control.

The characterization of Q cells in solid tumors and their sensitivity to various treatments had been greatly hampered by the lack of adequate systems in which Q cells can be identified and obtained in large homogeneous populations. Accordingly, the Q cell assay method used in this study appears to be useful for determining the sensitivity of Q cells populations in solid tumors to ionizing radiation or chemotherapeutic agents. Using this method, we plan to investigate the responses of Q cells to treatments with radiation and chemotherapeutic agents and/or hypoxic cell sensitizers as well as their responses to fractionated irradiation.

ACKNOWLEDGMENTS

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