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Usefulness of Tirapazamine as a Combined Agent in Chemoradiation and Thermo-chemoradiation Therapy at Mild Temperatures: Reference to the Effect on Intratumor Quiescent Cells

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C3H/He mice bearing SCC VII tumors received 5-bromo-2′-deoxyuridine (BrdU) continuously for 5 days via implanted mini-osmotic pumps to label all proliferating (P) cells. The mice then received one of six different DNA-damaging agents with or without mild temperature hyperthermia (40°C, 30 min, MTH). These agents were adriamycin (ADM), mitomycin C (MMC), cyclophosphamide (CPA), bleomycin (BLM), cisplatin (CDDP), and tirapazamine (TPZ). After the drug treatment, the tumor-bearing mice were irradiated with a series of doses of γ-rays. Immediately after irradiation, the tumors were excised, minced and trypsinized. The tumor cell suspensions thus obtained were incubated with cytochalasin-B (a cytokinesis blocker), and the micronucleus (MN) frequency in cells without BrdU labeling (=quiescent (Q) cells) was determined using immunofluorescence staining for BrdU. The MN frequency in the total (P+Q) tumor cells was determined from the tumors that had not been pretreated with BrdU. MTH significantly increased the MN frequency of total cells in tumors irradiated with γ-rays combined with CPA, BLM, CDDP or TPZ, and that of Q cells in tumors irradiated with γ-rays combined with BLM or TPZ. The sensitivity difference in the MN frequency between total and Q tumor cells was significantly decreased by the combination with TPZ. TPZ combined with radiotherapy and TPZ combined with thermo-radiotherapy at mild temperatures appear to be promising modalities for sensitizing tumor cells *in vivo*, including Q tumor cells.

Key words: Quiescent cell — Mild temperature hyperthermia — γ-Ray — DNA-damaging agent — Tirapazamine

Heat treatment has been demonstrated to be effective as an adjuvant modality to radiotherapy.[1)] Laboratory experiments showed that heating for 30 to 60 min at relatively high temperatures, i.e. >43 to 44°C, damages intratumor blood vessels and kills tumor cells.[2)] In addition, hyperthermia causes direct cellular radiosensitization.[3)] However, currently available hyperthermia devices have been ineffective in raising the temperature of human tumors sufficiently to cause vascular damage, kill tumor cells, and directly radiosensitize the tumor cells. Thus, Oleson suggested that hyperthermia might improve tumor oxygenation and so indirectly radiosensitize tumors through an increase in tumor blood flow in a clinical study in which heat treatment was shown to improve the effectiveness of radiotherapy.[4)] Actually, mild temperature hyperthermia (MTH) was reported to increase the tumor response to radiation[5)] and some chemotherapeutic agents.[6)]

Recent studies have suggested that tumor hypoxia could be exploited by the use of bioreductive agents that preferentially kill hypoxic cells.[7,8)] Tirapazamine (TPZ, SR-4233, WIN 59075, 3-amino-1,2,4-benzotriazine-1,4-dioxide), one of the most well-known bioreductive agents, was reported to have highly selective cytotoxicity toward hypoxic cells in both *in vivo* and *in vitro* tumor systems.[7–9)] Severe hypoxia was not required and the level of hypoxia found in many human tumors was sufficient for the toxicity.[8)]

In general, far more patients are diagnosed with solid tumors than with hematologic malignancies. These tumors present major treatment challenges for clinicians and research is focused on expanding the options for effective management. Clinicians and researchers are recognizing the potential value of combining chemotherapy and radiation to treat many of these tumors. Hence, in this study,

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the significance of MTH combined with chemoradiation therapy using γ-rays and one of six different drugs that have different relative toxicities to tumor cells within murine solid tumors (SCC VII squamous cell carcinoma) was investigated. These drugs were adriamycin (ADM), mitomycin C (MMC), cyclophosphamide (CPA), bleomycin (BLM), *cis*-diamminedichloroplatinum [II] (cisplatin, CDDP) and TPZ.

Many intratumor cells do not proliferate (quiescent) *in situ*, but are still clonogenic.[7] In analyzing the response of quiescent (Q) cells in solid tumors, we have developed a combined method involving micronucleus (MN) assay and identification of proliferating (P) cells with 5-bromo-2′-deoxyuridine (BrdU) and anti-BrdU monoclonal antibody.[10] Using this method, the response of Q tumor cells as well as total tumor (P+Q) cells within murine solid tumors to chemoradiation therapy combined with and without MTH was also evaluated.

MATERIALS AND METHODS

Tumors, mice, and labeling with BrdU SCC VII squamous cell carcinoma derived from C3H/He mice (Department of Radiology, Faculty of Medicine, Kyoto University) was maintained *in vitro* in Eagle's minimum essential medium. Cells were collected from monolayer cultures, and approximately 1.0×10^5 cells were inoculated subcutaneously into the left hind legs of 8- to 11-week-old syngeneic female C3H/He mice. Nine days after inoculation, mini-osmotic pumps (Alzet model 2001, Palo Alto, CA) containing BrdU dissolved in physiological saline (250 mg/ml) were implanted subcutaneously to label all P cells for 5 days. Administration of BrdU did not change the tumor growth rate. The tumors were 1 cm in diameter on treatment. The labeling index after continuous labeling with BrdU was 55.3 (50.8–59.8) % (mean (95% confidence limit)), and reached a plateau level at this stage. Therefore, in this study, we regarded tumor cells not incorporating BrdU after continuous labeling as Q cells.[10]

Treatment After labeling with BrdU, the tumor-bearing mice received intraperitoneal administration of one of six different drugs dissolved in physiological saline, or not. These drugs were ADM, MMC, CPA, BLM, CDDP and TPZ, administered at doses of 8, 5, 150, 20, 10 and 40 mg/kg, respectively, which are near the maximum tolerated doses for C3H/He mice. TPZ was synthesized according to the established method,[11] which Zeman and colleagues also employed,[9] and analyzed by chemical ionization mass spectrometry [m/z, 179(MH+)], electron ionization mass spectrometry [m/z, 178(M+)] and infrared spectrometry [KBr]. This synthesized TPZ was regarded as identical with commonly used TPZ.

Immediately after drug administration, the tumors grown in the left hind legs of mice were heated at 40°C for 30 min by immersing the animal's foot in a water bath, or not. We employed the same heating method as reported previously.[12] Temperatures at the tumor center equilibrated within 3 to 4 min after immersion in the water bath and remained 0.2–0.3°C below the water bath temperature. The temperature difference between the tumor center and the periphery was within 0.1°C. The water bath temperature was maintained at 0.3°C above the desired tumor temperature.

The tumor-bearing mice then received a series of radiation doses of 4 to 25 Gy from a cobalt-60 γ-ray irradiator at a dose rate of approximately 5.0 Gy/min, right after the heat treatment. The tumors were then excised immediately after the γ-ray irradiation. Each treatment group included mice pretreated with and without BrdU.

Immunofluorescence staining of BrdU-labeled cells and observation of micronucleus formation The procedures we used have been described in detail elsewhere.[10] Excised tumors from mice given BrdU were minced and trypsinized. Tumor cell suspensions were incubated for 48 h in tissue culture dishes containing complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis, while allowing nuclear division. The cultures were trypsinized and single cell suspensions were fixed with 70% ethanol. After centrifugation, the cell pellet was resuspended with cold Carnoy's fixative. The suspension was then placed on a glass microscope slide and the sample was dried at room temperature. The slides were treated with 2 *N* hydrochloric acid for 45 min at room temperature to dissociate the histones and partially denature the DNA. The slides were then immersed in borax-borate buffer (pH 8.5) to neutralize the acid. BrdU-labeled tumor cells were detected by indirect immunofluorescence staining using monoclonal anti-BrdU antibody and fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody. To observe double staining of tumor cells with FITC and propidium iodide (PI), cells on the slides were treated with PI (1–5 μg/ml in phosphate-buffered saline) and monitored under a fluorescence microscope. The MN frequency in BrdU-unlabeled cells (=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.[13]

The ratio obtained in tumors not pretreated with BrdU indicated the MN frequency of all phases of the total (P+Q) tumor cell populations. More than 300 binuclear cells were counted to determine the MN frequency.

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 BrdU could potentially influence the frequency of micronuclei and binuclear cells in BrdU-labeled cells.[14] The correct MN frequency of P cells without the BrdU effect is

not able to be determined. During the continuous labeling with BrdU over 5 days, a change from P to Q populations resulted in labeled Q cells. Therefore, micronuclei in binuclear cells showing only red fluorescence by PI were scored and cells stained with FITC were excluded.

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. *P* values were from two-sided tests.

RESULTS

Table I shows the MN frequencies at 0 Gy for total cells and for Q cells in tumors treated with drugs and/or MTH and without drug or MTH. When any drug, except MMC and TPZ, was combined with or without MTH, Q cells showed significantly lower MN frequencies than total cells ($P<0.05$). In contrast, when TPZ was combined, Q cells showed a significantly higher MN frequency than total cells ($P<0.05$). In total tumor cells, when CPA, BLM, CDDP or TPZ was used, and in Q cells, when BLM or TPZ was used, MTH produced a significantly higher MN frequency ($P<0.05$).

Figs. 1 and 2 show γ-ray dose-response curves for corrected MN frequency in total tumor cells and Q cells from tumors in animals not given any drugs and given one of the six different drugs, respectively. For baseline correction, we used the corrected MN frequency to exclude the effects of MTH or administered drugs on the MN frequency. The corrected MN frequency is the MN frequency in the γ-ray-irradiated tumors minus the MN frequency in the non-irradiated tumors. Whether drug and MTH were combined or not, there were significant differences between total tumor cells and Q cells ($P<0.05$). In total cells, MTH produced significant increases in the corrected MN frequency when combined with CPA, BLM, CDDP, or TPZ ($P<0.05$). In Q cells, MTH produced a significant increase in the corrected MN frequency when combined with BLM or TPZ ($P<0.05$).

To evaluate the effects of MTH on the corrected MN frequency in total and Q tumor cells, we calculated the enhancement ratio (ER) at various corrected MN frequencies, using the values from the fitted curves shown in Figs. 1 and 2 (Table II). ER was defined as the ratio of the γ-ray radiation dose needed to obtain equivalent corrected MN frequencies without and with MTH. All values of the ER were larger than 1.00. There was a tendency for the ERs for total cells to be higher than or nearly equal to those for Q cells except when no drug was administered. No drug resulted in higher ERs for Q cells than for total cells. The ERs for total cells treated with CPA, BLM, CDDP or TPZ and those for Q cells treated with BLM or TPZ were significantly larger than 1.00 ($P<0.05$).

The dose-modifying factor (DMF), which is the ratio of the γ-ray radiation doses needed to obtain various corrected MN frequencies in Q cells and those in total cells, was calculated using the values from the fitted curves in Figs. 1 and 2 (Table III). All values shown in Table III

Table I. Micronucleus Frequencies at 0 Gy

Cell fraction Drugs used	Mild heating (–)	Mild heating (+)
Total tumor cells		
Drug (–)	0.044 (0.039–0.049)[a)]	0.049 (0.043–0.055)
Adriamycin	0.40 (0.35–0.45)	0.42 (0.36–0.48)
Mitomycin C	0.27 (0.24–0.30)	0.30 (0.26–0.34)
Cyclophosphamide	0.22 (0.19–0.25)	0.44 (0.38–0.50)
Bleomycin	0.22 (0.18–0.26)	0.31 (0.26–0.36)
Cisplatin	0.47 (0.41–0.53)	0.71 (0.63–0.79)
Tirapazamine	0.28 (0.26–0.30)	0.33 (0.30–0.36)
Quiescent cells		
Drug (–)	0.055 (0.049–0.061)	0.057 (0.051–0.063)
Adriamycin	0.21 (0.19–0.23)	0.24 (0.22–0.26)
Mitomycin C	0.22 (0.18–0.26)	0.28 (0.25–0.31)
Cyclophosphamide	0.13 (0.10–0.16)	0.16 (0.12–0.20)
Bleomycin	0.13 (0.11–0.15)	0.18 (0.15–0.21)
Cisplatin	0.25 (0.21–0.29)	0.33 (0.28–0.38)
Tirapazamine	0.39 (0.33–0.45)	0.56 (0.49–0.63)

a) Numbers in parentheses are 95% confidence limits, determined using mean values, standard deviations and the numbers of observations on which the means and the standard deviations were based.

were significantly larger than 1.00 ($P<0.05$). This means that Q cells were significantly more resistant than total cells ($P<0.05$). Whether combined with MTH or not, the values for TPZ were significantly lower than those for any other drug or no drug at each corrected MN frequency ($P<0.05$). Namely, TPZ significantly reduced the sensitivity difference between total and Q cells. The sensitivity difference was slightly increased by combination treatment with MTH when a drug was administered. In contrast, combination treatment with MTH slightly reduced the sensitivity difference only when no drug was administered.

DISCUSSION

The effect of cytochalasin-B on chromosome damage in cells has not been completely clarified. However, a close relationship has been reported between cell survival and the MN frequency obtained with cytochalasin-B treatment after X-ray irradiation.[13, 15] As in the case of X-ray irradiation, DNA is considered to be the primary target for cell killing by the chemotherapeutic agents employed here and TPZ.[8, 16] Furthermore, in the total cell populations of the SCC VII tumors, after intraperitoneal injection of CDDP,

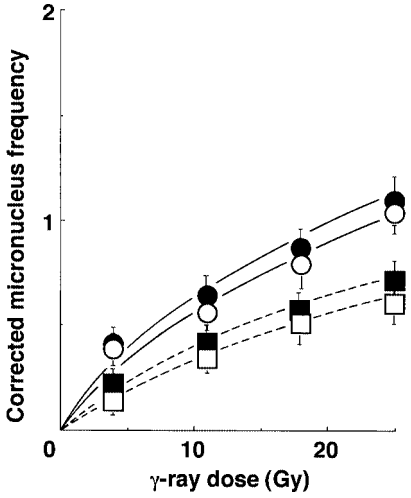

Fig. 1. γ-Ray dose-response curves for corrected micronucleus (MN) frequency (MN frequency−C, where C is the MN frequency in cells from tumors in animals not given γ-rays) in total cells and quiescent (Q) cells from tumors in animals not given any drugs. Total cells: ○, mild heating (−); ●, mild heating (+). Q cells: □, mild heating (−); ■, mild heating (+).

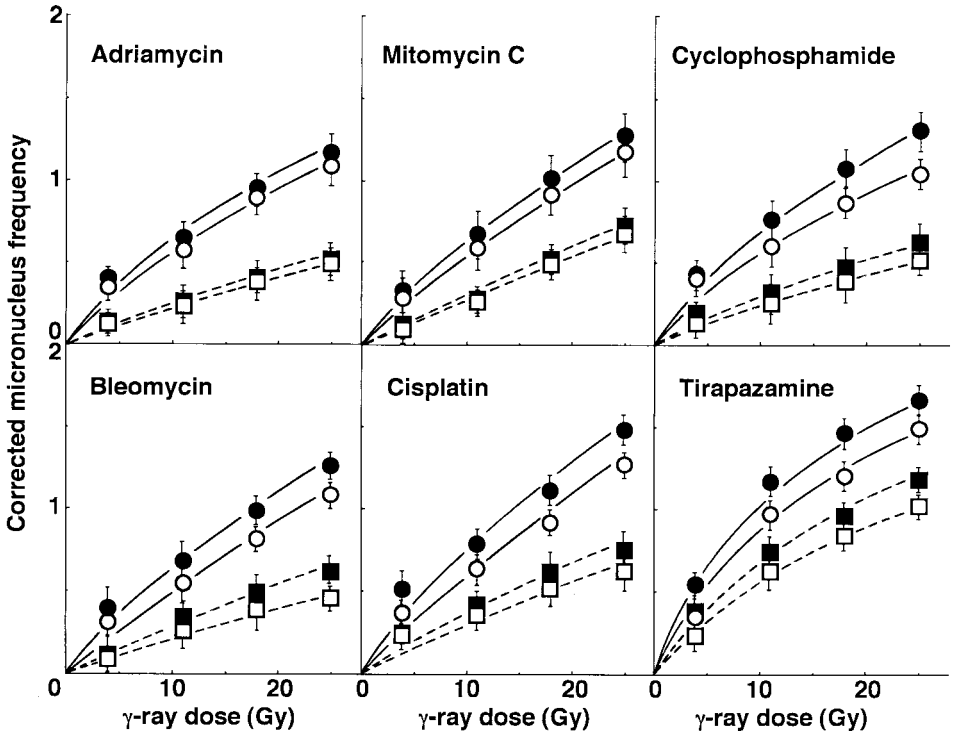

Fig. 2. γ-Ray dose-response curves for corrected micronucleus (MN) frequency (MN frequency−C, where C is the MN frequency in cells from tumors in animals not given γ-rays) in total cells and quiescent (Q) cells from tumors in animals given adriamycin, mitomycin C, cyclophosphamide, bleomycin, cisplatin, or tirapazamine. Total cells: ○, mild heating (−); ●, mild heating (+). Q cells: □, mild heating (−); ■, mild heating (+).

there was a close relationship between the MN frequency obtained with cytochalasin-B treatment and the surviving fraction determined by the *in vivo-in vitro* assay method.[17] Therefore, the MN frequency would reflect sensitivity to DNA damaging treatment.

The MN frequencies of cells from animals not treated with γ-rays or drugs (Table I) showed that the MTH employed here could not induce direct thermal cytotoxicity. It has been reported that this level of MTH cannot delay tumor growth or cause direct thermal radiosensitization.[3, 12] In contrast, when combined with drugs, MTH increased the MN frequency in both cell populations. In total cells, MTH could significantly sensitize the tumor cells *in vivo* in combination with CPA, BLM, CDDP or TPZ ($P<0.05$). This is compatible with previous reports that the thermal enhancement ratio for solid tumor response to chemotherapy using CPA, CDDP, BLM or TPZ was significantly larger than 1.0, but that for ADM or

Table II. Enhancement Ratios Due to Mild Heating

Drugs	Corrected micronucleus frequency	Enhancement ratio[a]	
		Total tumor cells	Quiescent tumor cells
Drug (–)	0.2	1.11	1.25
	0.4	1.15	1.25
	0.5	1.15	1.27
	0.8	1.14	—
	1.0	1.14	—
Adriamycin	0.2	1.22	1.06
	0.4	1.11	1.07
	0.5	1.11	1.08
	0.8	1.10	—
	1.0	1.09	—
Mitomycin C	0.2	1.12	1.12
	0.4	1.12	1.09
	0.5	1.13	1.09
	0.8	1.12	—
	1.0	1.12	—
Cyclophosphamide	0.2	1.31	1.11
	0.4	1.32	1.14
	0.5	1.32	1.17
	0.8	1.33	—
	1.0	1.31	—
Bleomycin	0.2	1.40	1.27
	0.4	1.36	1.34
	0.5	1.34	1.34
	0.8	1.26	—
	1.0	1.24	—
Cisplatin	0.2	1.52	1.22
	0.4	1.41	1.24
	0.5	1.36	1.25
	0.8	1.28	—
	1.0	1.25	—
Tirapazamine	0.2	1.48	1.27
	0.4	1.48	1.27
	0.5	1.47	1.27
	0.8	1.42	1.27
	1.0	1.39	1.29
	1.3	1.35	—

a) The ratio of radiation dose needed to obtain each corrected micronucleus frequency without, and with, mild hyperthermia.

Table III. Dose Modifying Factors for Quiescent Cells Relative to Total Tumor Cells

Drugs	Corrected micronucleus frequency	Dose modifying factor[a]	
		Radiation alone	Radiation+ mild hyperthermia
Drug (–)	0.2	2.01	1.92
	0.3	2.03	1.94
	0.4	2.10	1.90
	0.5	2.15	1.86
Adriamycin	0.2	2.65	3.05
	0.3	2.81	2.93
	0.4	2.88	2.98
	0.5	2.98	3.07
Mitomycin C	0.2	2.07	2.19
	0.3	2.44	2.58
	0.4	2.36	2.43
	0.5	2.21	2.31
Cyclophosphamide	0.2	3.12	3.69
	0.3	3.06	3.54
	0.4	2.98	3.44
	0.5	3.00	3.37
Bleomycin	0.2	2.51	2.80
	0.3	2.58	2.71
	0.4	2.72	2.72
	0.5	2.78	2.78
Cisplatin	0.2	2.11	2.63
	0.3	2.14	2.53
	0.4	2.21	2.51
	0.5	2.28	2.48
Tirapazamine	0.2	1.66	1.75
	0.3	1.68	1.76
	0.4	1.67	1.73
	0.5	1.67	1.75
	0.8	1.72	1.75
	1.0	1.83	1.77

a) The dose modifying factor is the radiation dose needed to obtain each corrected micronucleus frequency in quiescent tumor cells/the radiation dose needed to obtain each corrected micronucleus frequency in total tumor cells.

MMC was almost equal to 1.0.[6, 18] The response of Q tumor cells to BLM and TPZ was significantly enhanced by MTH ($P<0.05$).

Based on our previous report, P cells of this tumor include a large acutely (perfusion-limited) hypoxic fraction and Q cells consist of a large chronically (diffusion-limited) hypoxic fraction.[19] Further, it was shown that MTH mainly oxygenated the chronically hypoxic fraction, probably through an increase in tumor blood flow induced by MTH.[20] Thus, in both total and Q cell populations, especially in Q cells, higher doses of each drug could be distributed through an increase in blood flow, and oxygen tension could be increased through its oxygenation effect mainly in the chronically hypoxic fraction in combination with MTH. Therefore, MTH could significantly increase the sensitivity to the preferentially oxic cell cytotoxin BLM in both cell populations. On the other hand, it was also shown that moderately oxygenated cells are important for tumor response to hypoxic cell cytotoxins and such cells could constitute a significant proportion of solid tumors.[21] Thus, MTH might change some hypoxic fraction to a level of oxygenation intermediate between fully oxygenated and hypoxic through an increase in tumor blood flow, and at the same time distribute higher doses of TPZ and kill more tumor cells at these intermediate oxygen tensions.[8] This may be why MTH could sensitize both total and Q cells significantly in combination with TPZ. It is worth noting that P cells are thought to have greater potential to take up drugs than Q cells owing to their aerobic and well-nourished condition mainly due to the rich and homogeneous vascular distribution within solid tumors,[7] and CPA must be activated in the liver before entering the target tissue.[6] Accordingly, significantly higher doses of CDDP or CPA-derived active metabolite might be taken up into P cells, but not into Q cells, through MTH. This may be why MTH could significantly sensitize only total cells in combination with CPA or CDDP. With ADM and MMC, this level of thermal dose (40°C, 30 min) might be too small *in vivo* to sensitize each tumor cell significantly.[6]

When combined with a series of γ-ray doses of 4 to 25 Gy, MTH could radiosensitize Q cells more strongly than total cells if no drug was administered (Table II). In contrast, when combined with a drug, MTH could radiosensitize Q cells less strongly than, or at best similarly to, total cells, resulting in higher values of the DMF in the combination treatment with MTH (Table III). The effect that MTH oxygenates mainly the chronically hypoxic fraction was predominantly observed in chronic hypoxia-rich Q cell fractions. Therefore, without drug, the γ-ray sensitivity of Q cells was raised more markedly than that of total cells (Table II), and the radiosensitivity difference between total and Q cells was reduced by MTH (Table III).[20] However, when γ-ray irradiation was combined with a drug, the effect on γ-ray sensitivity induced by MTH was less clear. This is probably because of the difference in the combination effect on tumor cells, which depends on the characteristics of the combined drugs in relation to oxygen tension, tissue pH, heterogeneity in drug distribution within tumors, and other microenvironmental factors.[7]

On the other hand, MTH combined with γ-ray irradiation significantly radiosensitized total cells from tumors treated with CPA, BLM, CDDP or TPZ, and Q cells from tumors treated with BLM or TPZ, compared with γ-ray irradiation alone ($P<0.05$) (Table II). This radiosensitization pattern by combination treatment with MTH was the same as that for no irradiation. In other words, in chemotherapy for solid tumors, the mechanism of sensitization due to combination treatment with MTH was thought to be similar, whether γ-ray irradiation was administered or not. This means interactions between γ-rays and drugs and between γ-rays and MTH do not have to be taken into account when these modalilities are combined with chemotherapy *in vivo*. Namely, the effect of single fraction γ-ray irradiation is almost additive *in vivo* when combined with chemotherapy or thermo-chemotherapy. This was in contrast to the above-mentioned finding that the combination effect with MTH in γ-ray irradiation became ambiguous on drug treatment. Consequently, the characteristics of combination therapy using antitumor chemotherapy with single fraction γ-ray irradiation and/or MTH are thought to depend greatly on those of the employed chemotherapeutic agents.

The presence of Q 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.[7] Therefore, Q cells may have shown higher MN frequencies without γ-ray irradiation or drug administration than total cells (Table I). Solid tumors, especially human tumors, are thought to contain a high proportion of Q cells.[7] It has been reported that Q cells have lower radiosensitivity than P cells in solid tumors.[7] Further, Q cells have greater potentially lethal damage repair capacities and significantly lower radio- and chemosensitivity than the total cell population within solid tumors *in vivo*.[10, 19, 20] Consequently, the control of Q cells is considered to be critical to the outcome of anticancer treatment. In this regard, in the treatment of solid tumors, TPZ was thought to be the most effective of the six agents examined, when combined with radiation or thermo-radiation at mild temperatures because of its significant effect to reduce the radiosensitivity difference between total and Q tumor cells (Table III). In addition, even without irradiation, only TPZ produced a larger MN frequency in Q cells than total cells (Table I) because of its characteristics as a hypoxia-specific cytotoxin and the presence of much larger hypoxic fractions in Q tumor cell populations.[8, 19, 20] Thus, from the viewpoint of Q tumor cell control, TPZ combined with

radiotherapy and TPZ combined with thermo-radiotherapy at mild temperatures are very promising modalities for the treatment of solid tumors, due to the strong cytotoxicity for Q tumor cells which was demonstrated by the MN method combined with immunofluorescence staining for BrdU, a promising method to evaluate Q cell sensitivity. In future, to draw a clinically useful conclusion, we plan to perform TCD_{50} (radiation dose yielding 50% local tumor control) assay and to examine therapeutic efficacy in combination with fractionated radiotherapy.

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