Usefulness of combined treatment with mild temperature hyperthermia and/or tirapazamine in the treatment of solid tumors: its independence of p53 status

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Human head and neck squamous cell carcinoma cells transfected with mutant *TP53* (SAS/mp53) or with neo vector as a control (SAS/neo) were inoculated subcutaneously into both hind legs of Balb/cA nude mice. Mice bearing the tumors received 5-bromo-2′-deoxyuridine (BrdU) continuously to label all proliferating (P) cells in the tumors. The mice then received tirapazamine (TPZ) with or without mild temperature hyperthermia (40°C, 60 min) (MTH), γ-ray irradiation with or without MTH and/or TPZ, cisplatin (CDDP) with or without MTH and/or TPZ, or paclitaxel (TXL) with or without MTH and/or TPZ. After each treatment, the tumors were excised, minced and trypsinized. The tumor cell suspensions thus obtained were incubated with a cytokinesis blocker (cytochalasin-B), and the micronucleus (MN) frequency in cells without BrdU labeling (i.e., quiescent (Q) cells) was determined by using immunofluorescence staining for BrdU. Meanwhile, 6 h after γ-ray irradiation or 24 h after other cytotoxic treatments, tumor cell suspensions obtained in the same manner were used for determining the frequency of apoptosis in Q cells. The MN frequency and apoptosis frequency in total (P+Q) tumor cells were determined from the tumors that were not pretreated with BrdU. On the whole, γ-ray irradiation and CDDP injection induced a higher frequency of apoptosis and lower frequency of MN in SAS/neo cells than SAS/mp53 cells. There were no apparent differences in the induced frequency of apoptosis and MN between SAS/neo and SAS/mp53 cells after TPZ or TXL treatment. MTH sensitized cells to TPZ-inducing cytotoxicity more markedly in SAS/mp53 and Q cells than in SAS/neo cells and total cells, respectively. In γ-ray irradiation and CDDP treatment, the enhancement in combination with MTH and/or TPZ was more remarkable in SAS/mp53 cells and Q cells than in SAS/neo and total tumor cells, respectively. Also in the case of TXL treatment, the combination with MTH and/or TPZ induced a slightly greater enhancement effect in SAS/mp53 cells and Q cells. In view of the difficulty in controlling mutated p53 status tumors and intratumor Q cells, combination treatment with MTH and/or TPZ as a cooperative modality in cancer therapy is considered to have potential for controlling solid tumors as a whole. (Cancer Sci 2003; 94: 125–133)

It has been shown that the *p53* tumor suppressor gene serves a critical role in maintaining genomic stability during a cell cycle checkpoint in G1 and the G2/M transition,[1)] and as an effector of DNA repair[2)] and apoptosis.[3)] Wild-type p53 is needed to activate apoptosis in sensitive cells in response to DNA damage.[4)] These actions of p53 are potentially critical in determining the effectiveness of ionizing radiation and/or chemotherapeutic agents. Actually, mutations in the *p53* tumor suppressor gene have been shown to have an impact on the clinical course of several human cancers: patients with cancers harboring p53 mutations often have a worse prognosis than those with tumors harboring wild-type p53.[5)] Thus, the genetic and functional status of the *p53* gene is thought to be an important factor in guiding therapeutic strategies for cancer patients.

Meanwhile, mild temperature hyperthermia (MTH) was reported to increase the tumor response to radiation by improving tumor oxygenation through an increase in tumor blood flow,[6)] and tirapazamine (TPZ, SR-4233, WIN 59075, 3-amino-1,2,4-benzotriazine-1,4-dioxide), a well-known bioreductive agent, has highly selective cytotoxicity toward hypoxic cells in *in vivo* tumor systems.[7)] In addition, MTH was also shown to enhance the tumor response, especially of the intratumor quiescent (Q) cell population, to chemotherapy.[8)] Based on these reports, we have already demonstrated that combined treatment with MTH and/or TPZ is a promising modality in radiotherapy or chemotherapy from the viewpoint of the killing effect on intratumor Q tumor cells in comparison with the total (proliferating (P)+Q) tumor cell population, using several tumor systems with wild-type p53 status.[9)]

Accordingly, to investigate the usefulness of MTH and/or TPZ in the treatment of solid tumors consisting of cells with a mutated p53 status, we analyzed the killing effects of several DNA-damaging cytocidal treatments combined with or without MTH and/or TPZ on intratumor Q and total cells, employing two different tumor cell lines identical in genetic background except for p53 status[10)] and our method for detecting the intratumor Q cell response in terms of the micronucleus (MN) frequency and apoptosis frequency.[9)] As DNA-damaging cytocidal treatments, γ-ray irradiation, TPZ administration, cisplatin (CDDP) injection and paclitaxel (TXL) treatment were employed.

Materials and Methods

Cells, tumors and mice. The human head and neck squamous cell carcinoma cell line SAS (provided by JCRB, Tokyo) was cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 20 m*M* 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 12.5% fetal bovine serum in a conventional humified 5% CO_2 incubator. SAS cells show the phenotype of wild-type p53 in radiation- and heat-induced signal transduction.[11)] Plasmids pC53-248, which contains an *mp53* gene (codon 248, from Arg to Trp) producing a dominant negative mp53 protein, and pCMV-Neo-Bam, which contains a neo-resistance marker, were provided by B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). These plasmids

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were linearized with *Hin*dIII. Confluent SAS cells, approximately 2×10^6 cells in a 75-cm^2 flask, were trypsinized, and the resulting cell suspension in phosphate-buffered saline (PBS) (1 ml) was transferred into an electroporation chamber. Cells were supplemented with linearized DNA (10 μg/10 μl of pC53-248 or pCMV-Neo-Bam), and electroporated three times at 600 V. After standing for 30 min at room temperature, cells were plated onto dishes 10 cm in diameter in DMEM and incubated at 37°C. Forty-eight hours later, cells were treated with G418 (geneticin, 200 μg/ml, Sigma Chemical Co., St. Louis, MO), an agent for selection of transfected clones, and then incubated at 37°C for 14 days to allow colony formation. Colonies resistant to G418 were isolated with cloning cylinders. Through these manipulations, two stable transfectants SAS/mp53 and SAS/neo were established. SAS/neo cells have a functionally wild-type p53 protein, and SAS/mp53 cells express a dominant negative p53 protein. The procedure used for transfection is described in detail elsewhere.[12)]

Cells were collected from exponentially growing cultures, and 5.0×10^5 cells were inoculated subcutaneously into the left hind legs of 6- to 7-week-old syngeneic female Balb/cA nude mice. Three weeks after inoculation, a tumor with a diameter of approximately 7 mm could be observed at each implanted site, whichever stable transfectant was used.

Determining the timing for apoptosis detection. The tumor-bearing mice were assigned to four groups for treatment with four different DNA-damaging treatments. Mice in group 1 received γ-ray irradiation from a cobalt-60 γ-ray irradiator at a dose rate of approximately 5.0 Gy/min. For group 2, TPZ (40 mg/kg) dissolved in physiological saline was intraperitoneally administered. For groups 3 and 4, CDDP and TXL were injected intraperitoneally at a dose of 8.85 mg/kg and 40 mg/kg, respectively, which are near the maximum tolerated doses for mice.[13–15)] TXL was supplied by Bristol Myers Squibb (Tokyo).

At various time points after treatment, mice were sacrificed and tumors were excised. Excised tumors were minced, and trypsinized at 37°C for 15 min, using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). Single cell suspensions were fixed with 70% ethanol overnight at 4°C. After centrifugation, the cell pellet was resuspended in 0.4 ml of cold modified Carnoy's fixative (three volumes of ethanol and one volume of acetic acid). The suspension was then placed on glass microscope slides using a dropper and the sample was dried at room temperature. Cells on the slides were treated with 30 μl of propidium iodide (PI, 1–5 μg/ml in PBS) and monitored under a fluorescence microscope. Tumor cells and bodies with apoptotic morphological characteristics were counted manually among at least 600 tumor cells. Standard criteria for the morphological characteristics of apoptosis were chromatin condensation, nucleolar disintegration and the formation of crescent caps of condensed chromatin at the nuclear periphery.[16, 17)] Single, relatively large (≥4 μm in diameter) and roundish nuclear residues existing in extra- or intratumoral cells with intensive staining were identified as apoptotic bodies.

Main DNA-damaging treatment experiment.

Labeling with 5-bromo-2′-deoxyuridine (BrdU): Two weeks after tumor cell inoculation, mini-osmotic pumps (Alzet model 2001, DURECT Corp., Cupertino, CA) containing BrdU dissolved in physiological saline (250 mg/ml) were implanted subcutaneously to label all proliferating (P) cells for 7 days. Administration of BrdU did not change the tumor growth rate. The tumors were approximately 7 mm in diameter on treatment. The labeling index (LI) after continuous labeling with BrdU was 48.4 (41.7–55.1) % (mean (95% confidence limit)) and 43.2 (37.0–49.4) % for SAS/neo and SAS/mp53 tumor cells, respectively, and reached a plateau level at these stages. Therefore, we regarded tumor cells not incorporating BrdU after continuous labeling as Q cells.

DNA-damaging treatment: After labeling with BrdU, the following four different cytotoxic treatments were given.

In the first mouse group, TPZ dissolved in physiological saline was administered intraperitoneally at doses of 10, 20 and 40 mg/kg immediately after MTH or without MTH. Nagasawa, Uto and Hori synthesized TPZ according to the established method,[18)] which Brown and colleagues also employed,[19)] and analyzed it 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 to the TPZ used world-wide. The tumors grown in the left hind legs of mice were heated at 40°C for 60 min by immersing the animal's foot in a water bath. We employed the same heating method as reported previously.[20)] 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 tumors were excised 1 h after TPZ administration.

The second group of mice received MTH at first, then TPZ, and finally γ-ray irradiation. A γ-ray dose of 4 Gy through 24 Gy was delivered from a cobalt-60 γ-ray irradiator, 30 min after the intraperitoneal administration of TPZ at a dose of 40 mg/kg combined with or without MTH.

The third group of mice received MTH at first, then TPZ, and finally CDDP. CDDP at a dose of 8.85 mg/kg was administered intraperitoneally, 2 h after the intraperitoneal administration of TPZ at a dose of 40 mg/kg combined with or without MTH.

The last group of mice received MTH at first, then TPZ, and finally TXL. TXL at a dose of 40 mg/kg was administered intraperitoneally, 24 h after the intraperitoneal administration of TPZ at a dose of 40 mg/kg combined with or without MTH.

The concentrations and time course employed here have been shown to be appropriate for TPZ, CDDP and TXL to function completely.[13–15, 21, 22)] Each treatment group also included mice that were not pretreated with BrdU.

Immunofluorescence staining of BrdU-labeled cells and observation of apoptosis and micronucleus formation: Based on the result of the experiment for determining the timing of apoptosis detection, 6 h and 24 h after γ-ray irradiation and other DNA-damaging treatments, respectively, for the apoptosis assay, and right, 1 h and 24 h after γ-ray irradiation, TPZ or CDDP administration and TXL treatment, respectively, for the MN assay, tumors were excised from mice given BrdU, minced, and trypsinized. For the apoptosis assay, the single tumor cell suspensions obtained were fixed with 70% ethanol overnight at 4°C. For the MN assay, tumor cell suspensions were incubated for 48–72 h in tissue culture dishes containing complete medium and 1.0 μg/ml of cytochalasin-B to inhibit cytokinesis while allowing nuclear division, and 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. 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 (Becton Dickinson, San Jose, CA) and fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody (Sigma). To observe double staining of tumor cells with green-emitting FITC and red-emitting PI, cells on the slides were treated with PI and monitored under a fluorescence microscope.

The frequency of apoptosis in BrdU-unlabeled cells (=Q cells at treatment) could be determined by counting apoptotic cells among tumor cells that showed only red fluorescence, according to the above-mentioned criteria. The frequency was defined as the ratio of the number of apoptotic cells to the total number of observed tumor cells. The MN frequency in BrdU-unlabeled 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.[17)]

The ratios obtained in tumors not pretreated with BrdU indicated the apoptosis frequency and the MN frequency at all phases 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 MN frequency, respectively.

Cell survival assay: The cell survival assay was also performed in mice given no BrdU using an *in vivo-in vitro* assay method at the same time points after treatments as for the MN assay. 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 1.5 $(1.2–1.8)\times10^7$/g and 3.4 $(2.6–4.2)\times10^6$/g for SAS/neo and SAS/mp53 tumors, respectively.

To confirm the stability of transfectants SAS/neo and SAS/mp53, part of the tumor cell suspensions obtained after cytotoxic treatment and tumor cells from part of the colonies grown through the *in vivo-in vitro* assay method were subjected to western blotting analysis for p53 and Bax proteins as described by Ota *et al*.[23)] Not only the level, but also the function of p53 protein can be detected because the *bax* gene is a target of the *p53* gene. As a result, it was confirmed that the p53 status of each transfectant was not changed by these experimental procedures. 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

Fig. 1 shows the time course of the change in the values of the apoptosis frequency for each tumor cell line following γ-ray ir-

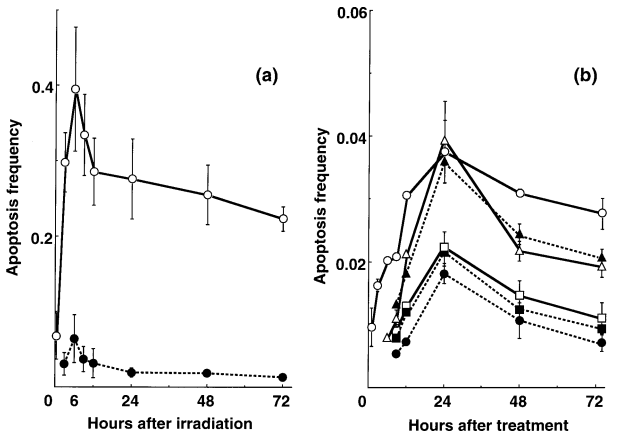

Fig. 1. Effect of time after γ-ray irradiation (20 Gy) (a) and intraperitoneal administration of tirapazamine (TPZ, 40 mg/kg, squares), cisplatin (CDDP, 8.85 mg/kg, circles) or paclitaxel (TXL, 40 mg/kg, triangles) (b) on apoptosis frequency. The time course of change in the values of the apoptosis frequency for SAS/neo tumor cells (open symbols and solid lines) and SAS/mp53 tumor cells (solid symbols and dotted lines). Bars represent 95% confidence limits.

Table 1. Plating efficiencies, apoptosis frequencies and micronucleus frequencies at 0 Gy

	Total cells	Quiescent cells
Plating efficiency (%)		
Without tirapazamine or mild temperature hyperthermia		
SAS/neo	45.4 (36.3–54.5)[1)]	—
SAS/mp53	23.5 (17.4–29.6)	—
With mild temperature hyperthermia		
SAS/neo	44.0 (34.5–53.5)	—
SAS/mp53	23.1 (17.1–29.1)	—
With tirapazamine		
SAS/neo	15.1 (12.5–17.7)	—
SAS/mp53	5.0 (4.3–5.7)	—
With tirapazamine and mild temperature hyperthermia		
SAS/neo	14.8 (12.4–17.2)	—
SAS/mp53	4.8 (4.2–5.4)	—
Apoptosis frequency		
Without tirapazamine or mild temperature hyperthermia		
SAS/neo	0.083 (0.076–0.090)	0.106 (0.091–0.121)
SAS/mp53	0.009 (0.007–0.011)	0.033 (0.029–0.037)
With mild temperature hyperthermia		
SAS/neo	0.088 (0.080–0.096)	0.116 (0.102–0.130)
SAS/mp53	0.017 (0.015–0.019)	0.045 (0.040–0.050)
With tirapazamine		
SAS/neo	0.103 (0.096–0.110)	0.138 (0.118–0.158)
SAS/mp53	0.030 (0.021–0.039)	0.068 (0.060–0.074)
With tirapazamine and mild temperature hyperthermia		
SAS/neo	0.108 (0.088–0.128)	0.148 (0.125–0.171)
SAS/mp53	0.038 (0.034–0.042)	0.080 (0.071–0.089)
Micronucleus frequency		
Without tirapazamine or mild temperature hyperthermia		
SAS/neo	0.038 (0.032–0.044)	0.056 (0.049–0.063)
SAS/mp53	0.072 (0.064–0.080)	0.111 (0.101–0.121)
With mild temperature hyperthermia		
SAS/neo	0.088 (0.080–0.096)	0.156 (0.135–0.177)
SAS/mp53	0.152 (0.136–0.168)	0.211 (0.191–0.231)
With tirapazamine		
SAS/neo	0.318 (0.258–0.378)	0.366 (0.305–0.427)
SAS/mp53	0.382 (0.322–0.442)	0.541 (0.491–0.591)
With tirapazamine and mild temperature hyperthermia		
SAS/neo	0.368 (0.308–0.428)	0.466 (0.406–0.526)
SAS/mp53	0.462 (0.402–0.522)	0.641 (0.580–0.702)

1) 95% confidence limit.

radiation (Fig. 1(a)) or DNA-damaging drug treatment with TPZ, CDDP or TXL (Fig. 1(b)). In each cell line, the apoptosis frequency showed a maximum value 6 h after irradiation and 24 h after the drug treatment. After γ-ray irradiation or CDDP injection, the values for SAS/neo tumor cells were significantly higher than those for SAS/mp53 cells ($P<0.05$) throughout all these experiments. Following TPZ or TXL treatment, the values were almost the same and there were no significant differences between SAS/neo and SAS/mp53 cells.

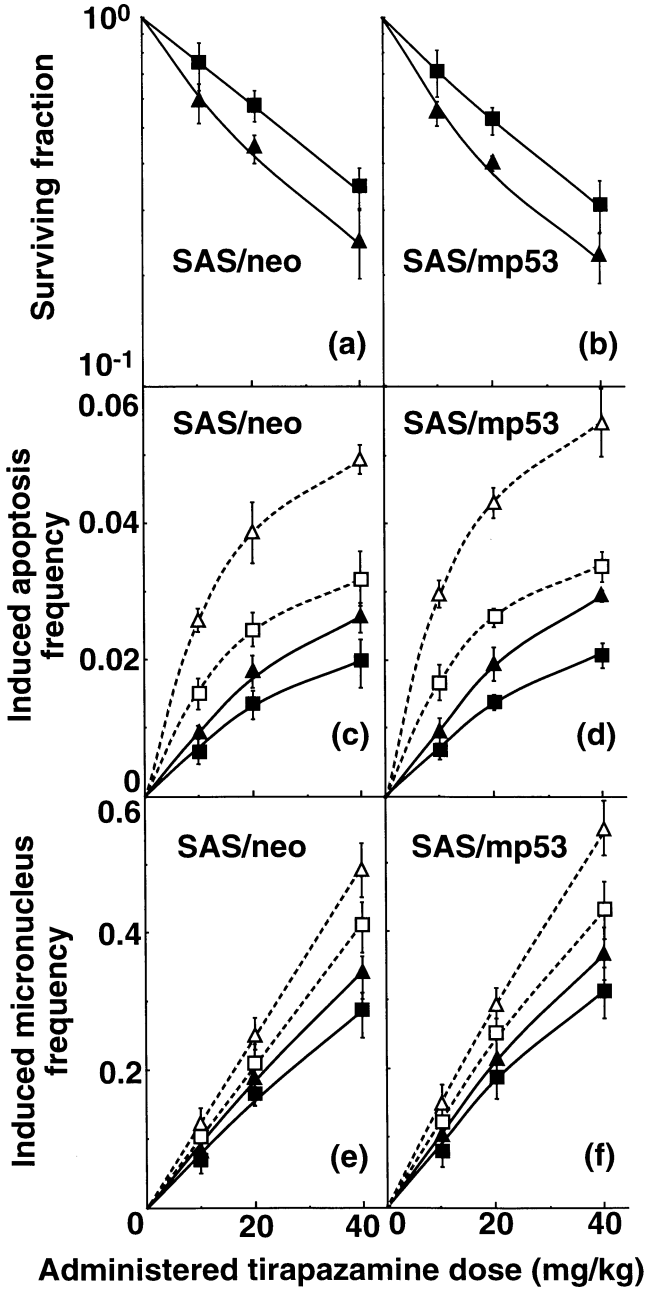

Fig. 2. Cell-survival curves for SAS/neo (a) and SAS/mp53 (b) cells, tirapazamine dose-response curves for induced apoptosis frequency in SAS/neo (c) and SAS/mp53 cells (d), and tirapazamine dose-response curves for induced micronucleus frequency in SAS/neo (e) and SAS/mp53 cells (f) as a function of the intraperitoneally administered dose of tirapazamine (10, 20 and 40 mg/kg). In both SAS/neo and SAS/mp53 tumors, combined treatment with mild temperature hyperthermia (MTH, 40°C, 60 min, triangles) decreased surviving fractions, compared with tirapazamine alone (squares). In both total (solid symbols) and quiescent (open symbols) cells, combined treatment with MTH (triangles) increased induced apoptosis and micronucleus frequencies, compared with tirapazamine alone (squares). Bars represent 95% confidence limits.

Table 1 shows the plating efficiencies for total tumor cells and the apoptosis and MN frequencies without γ-ray radiation for total and Q tumor cells in each tumor. Without radiation, Q tumor cells showed higher MN and apoptosis frequencies than total tumor cells under each set of conditions in each tumor, although the differences were not significant.

Fig. 2, (a) and (b) show the cell survival curves for SAS/neo and SAS/mp53 tumor cells, respectively, as a function of the administered TPZ dose in the total tumor cell population. Fig. 2, (c) through (f) show the induced apoptosis and MN frequencies, for combinations with or without MTH as a function of the administered TPZ dose in total and Q tumor cells within SAS/neo and SAS/mp53 tumors. The induced apoptosis and MN frequencies were the apoptosis and MN frequency in treated tumors minus the apoptosis and MN frequency in no-treatment control tumors, respectively. There was no apparent difference in the sensitivity to TPZ between SAS/neo and SAS/mp53 in each tumor cell population. In both cell lines, the combination with MTH decreased the surviving fraction (SF) of total tumor cells and increased the apoptosis and MN frequencies in total and Q tumor cells. To evaluate the effects of MTH on SF in total cells and on apoptosis and MN frequencies in total and Q tumor cells, we calculated the enhancement ratio (ER) at various endpoints, using the values from the fitted curves shown in Fig. 2 (Table 2). ER was defined as the ratio of the TPZ dose needed to obtain equivalent endpoints without and with MTH. The ERs for Q cells were higher than those for total tumor cell, and the values for SAS/mp53 were slightly larger than those for SAS/neo.

Fig. 3, (a) and (b) show the γ-ray irradiation dose-response curves for both tumor cells. To compare the cell survival curves between these two tumor cells, we calculated the dose modifying factors (DMFs) for SAS/mp53 tumor cells relative to SAS/neo tumor cells. The factors were calculated by comparing the radiation doses to obtain each endpoint in SAS/mp53 tumor

Table 2. Enhancement ratios[1] by mild temperature hyperthermia

Tumor	Endpoint	Total cells	Quiescent cells
Surviving fraction			
SAS/neo	0.6	1.65 (1.5–1.8)[2]	—
	0.4	1.5 (1.3–1.7)	—
SAS/mp53	0.6	1.7 (1.55–1.85)	—
	0.4	1.7 (1.5–1.9)	—
Induced apoptosis frequency[3]			
SAS/neo	0.02	1.6 (1.45–1.75)	2.0 (1.8–2.2)
	0.01	1.3 (1.2–1.4)	1.65 (1.5–1.8)
SAS/mp53	0.02	1.7 (1.55–1.85)	2.1 (1.9–2.3)
	0.01	1.3 (1.2–1.4)	1.75 (1.6–1.9)
Induced micronucleus frequency[3]			
SAS/neo	0.2	1.15 (1.05–1.25)	1.2 (1.1–1.3)
	0.1	1.15 (1.05–1.25)	1.2 (1.1–1.3)
SAS/mp53	0.2	1.2 (1.1–1.3)	1.25 (1.1–1.4)
	0.1	1.2 (1.1–1.3)	1.25 (1.1–1.4)

1) Ratio of radiation dose required to obtain each endpoint without combined treatment in relation to the radiation dose required to obtain each endpoint with mild temperature hyperthermia.
2) 95% confidence limit.
3) Induced frequency was the frequency minus the frequency in absolutely untreated control tumors.

cells with the doses required in SAS/neo tumor cells (Table 3). The values of the DMFs decreased in the following order; without MTH or TPZ>with MTH alone>with TPZ alone>with both MTH and TPZ. Especially, combined treatment with both MTH and TPZ not only reduced the difference in the radiosensitivity between SAS/mp53 and SAS/neo, but also made more radioresistant SAS/mp53 cells slightly more radiosensitive than SAS/neo cells.

Fig. 3, (c) through (f) show the normalized apoptosis and MN frequencies, for the combination with or without MTH and/or TPZ as a function of the radiation dose in total and Q tumor cells within SAS/neo and SAS/mp53 tumors. When the combined treatment with MTH and/or TPZ was performed before γ-ray irradiation, even if no radiation was given, these frequencies were higher than when no combined treatment was achieved, because of the effect of the combined treatment itself (Table 1). Therefore, for background correction, we used the normalized frequency to exclude the effects of the combined treatment on the frequency. The normalized frequency is the frequency in the irradiated tumors minus the frequency in the non-irradiated tumors.

On the whole, SAS/neo showed significantly higher normalized apoptosis frequencies ($P<0.05$) and, although not significantly, lower normalized MN frequencies than SAS/mp53. To evaluate the effect of combined treatment with MTH and/or TPZ on each endpoint in total and Q cell populations, the ER was calculated at various endpoints using the data given in Fig. 3 (Table 4). The ER values for each cell population in each tumor increased in the following order; with MTH alone<with TPZ alone<with both MTH and TPZ. In addition, the ER values for Q cells were higher than those for total tumor cells, and the values for SAS/mp53 were slightly larger than those for SAS/neo. Thus, the DMFs for Q cells relative to total tumor cells were decreased in the following order; with MTH alone>with TPZ alone>with both MTH and TPZ (Table 5). These factors were obtained by comparing the radiation doses necessary to obtain various endpoints in Q cells with the doses required in the total tumor cells, using the values shown in Fig. 3, (c) through (f).

Fig. 4 shows the changes in SF for total tumor cells and in the induced apoptosis and MN frequencies for total and Q tumor cells in SAS/neo and SAS/mp53 tumors administered intraperitoneally (i.p.) with CDDP at a dose of 8.85 mg/kg, according to combined treatment with MTH and/or TPZ (40 mg/kg, i.p.). Without MTH or TPZ, total tumor cells in the SAS/neo tumor showed a significantly lower SF, significantly higher induced apoptosis frequency and slightly lower induced MN frequency than the SAS/mp53 tumors. Q tumor cells of the SAS/neo showed higher induced apoptosis and slightly lower induced MN frequencies than SAS/mp53. With combined treatment, the SFs were decreased and the induced apoptosis and MN frequencies were increased in the following order; with MTH alone<with TPZ alone<with both MTH and

Table 3. Dose modifying factors[1] for SAS/mp53 tumor cells relative to SAS/neo tumor cells in cell survival curves

Treatment	Surviving fraction	
	0.03	0.1
γ-Rays alone		
	1.2 (1.1–1.3)[2]	1.15 (1.05–1.25)
With mild temperature hyperthermia		
	1.05 (0.9–1.2)	1.0 (0.9–1.1)
With tirapazamine		
	0.95 (0.85–1.05)	0.95 (0.85–1.05)
With tirapazamine and mild temperature hyperthermia		
	0.9 (0.8–1.0)	0.9 (0.8–1.0)

1) Radiation dose required to obtain each endpoint in SAS/mp53 tumor cells in relation to the radiation dose required to obtain each endpoint in SAS/neo tumor cells.
2) 95% confidence limit.

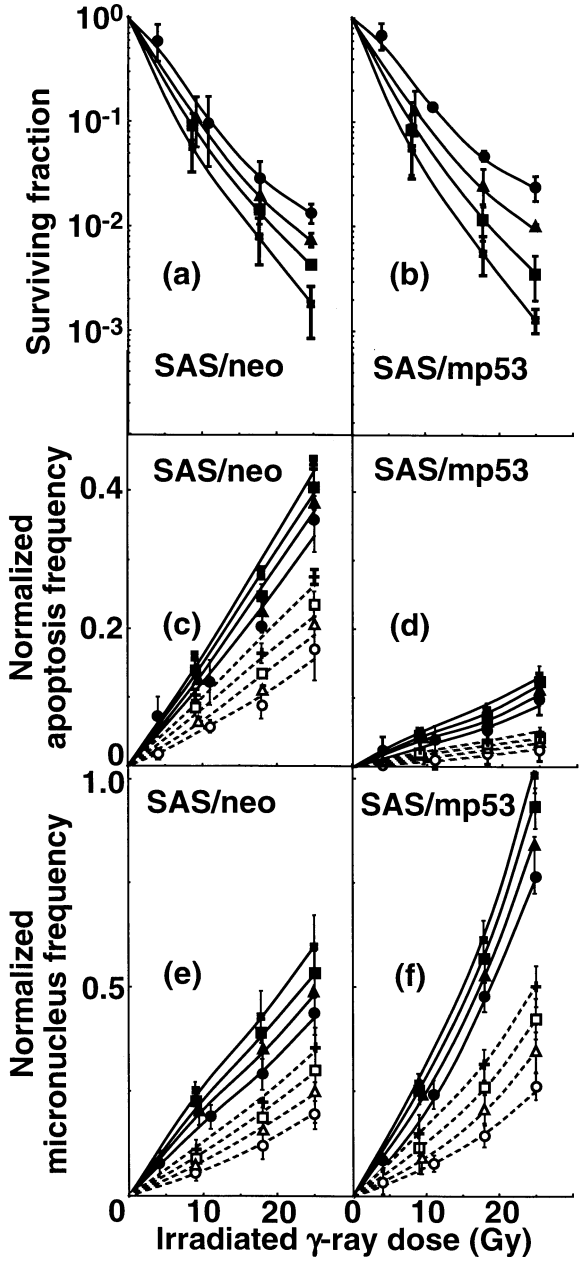

Fig. 3. Cell-survival curves for SAS/neo (a) and SAS/mp53 (b) cells, γ-ray dose-response curves for normalized apoptosis frequency in SAS/neo (c) and SAS/mp53 cells (d), and γ-ray dose-response curves for normalized micronucleus frequency in SAS/neo (e) and SAS/mp53 cells (f) as a function of irradiated dose of γ-rays. In both SAS/neo and SAS/mp53 tumors, cell survival decreased in the following order; without mild temperature hyperthermia (MTH, 40°C, 60 min) or tirapazamine (TPZ, 40 mg/kg, i.p.) (circles)>with MTH alone (triangles)>with TPZ alone (squares)>with both MTH and TPZ (crosses). In both tumors, normalized apoptosis and micronucleus frequencies for both total (solid symbols) and quiescent (open symbols) cells increased in the following order; without MTH or TPZ (circles)<with MTH alone (triangles)<with TPZ alone (squares)<with both MTH and TPZ (crosses). Bars represent 95% confidence limits.

Table 4. Enhancement ratios[1] by combined treatments

Tumor	Endpoint	Combined treatments		
		MTH[2]	TPZ[3]	MTH+TPZ
Surviving fraction (in total cells only)				
SAS/neo	0.1	1.15 (1.1–1.2)[4]	1.35 (1.2–1.5)	1.7 (1.5–1.9)
	0.03	1.15 (1.1–1.2)	1.3 (1.2–1.4)	1.6 (1.45–1.75)
SAS/mp53	0.1	1.3 (1.2–1.4)	1.6 (1.4–1.8)	2.1 (1.9–2.3)
	0.03	1.35 (1.2–1.5)	1.65 (1.5–1.8)	2.15 (1.9–2.4)
Normalized apoptosis frequency[5]				
SAS/neo				
Total cells	0.3	1.1 (1.0–1.2)	1.2 (1.1–1.3)	1.35 (1.15–1.55)
	0.15	1.1 (1.0–1.2)	1.2 (1.1–1.3)	1.3 (1.2–1.4)
Q cells	0.15	1.2 (1.1–1.3)	1.4 (1.3–1.5)	1.7 (1.5–1.9)
SAS/mp53				
Total cells	0.08	1.15 (1.05–1.25)	1.3 (1.2–1.4)	1.45 (1.25–1.65)
	0.02	1.2 (1.1–1.3)	1.35 (1.2–1.5)	1.5 (1.3–1.7)
Q cells	0.02	1.4 (1.2–1.6)	1.55 (1.4–1.7)	1.8 (1.55–2.05)
Normalized micronucleus frequency[5]				
SAS/neo				
Total cells	0.4	1.1 (1.0–1.2)	1.2 (1.1–1.3)	1.3 (1.2–1.4)
	0.2	1.2 (1.1–1.3)	1.35 (1.2–1.5)	1.5 (1.4–1.6)
Q cells	0.2	1.2 (1.1–1.3)	1.4 (1.3–1.5)	1.6 (1.5–1.7)
SAS/mp53				
Total cells	0.4	1.1 (1.0–1.2)	1.25 (1.1–1.4)	1.4 (1.25–1.55)
	0.2	1.2 (1.1–1.3)	1.4 (1.25–1.55)	1.55 (1.4–1.7)
Q cells	0.2	1.2 (1.1–1.3)	1.45 (1.3–1.6)	1.85 (1.7–2.0)

1) Ratio of radiation dose required to obtain each endpoint without combined treatment in relation to the radiation dose required to obtain each endpoint with combined treatment.
2) Mild temperature hyperthermia.
3) Tirapazamine.
4) 95% confidence limit.
5) Normalized frequency was the frequency minus the frequency in non-irradiated tumors.

Table 5. Dose modifying factors[1] for quiescent tumor cells relative to the total tumor cell populations

Tumor	Combined treatment			
Endpoint	γ-Rays alone	MTH[2]	TPZ[3]	MTH+TPZ
Normalized apoptosis frequency[4]				
SAS/neo				
0.15	2.05 (1.9–2.2)[5]	1.8 (1.6–2.0)	1.7 (1.5–1.9)	1.55 (1.4–1.7)
SAS/mp53				
0.02	2.3 (2.1–2.5)	1.9 (1.7–2.1)	1.8 (1.6–2.0)	1.75 (1.6–1.9)
Normalized micronucleus frequency[4]				
SAS/neo				
0.2	2.2 (1.9–2.5)	2.15 (1.95–2.35)	2.1 (2.0–2.2)	2.05 (1.9–2.2)
SAS/mp53				
0.2	2.2 (2.0–2.4)	2.15 (1.95–2.35)	2.0 (1.9–2.1)	1.8 (1.6–2.0)

1) Radiation dose required to obtain each endpoint in quiescent tumor cells in relation to the radiation dose required to obtain each endpoint in total tumor cells.
2) Mild temperature hyperthermia.
3) Tirapazamine.
4) Normalized frequency was the frequency minus the frequency in non-irradiated tumors.
5) 95% confidence limit.

TPZ. Further, the changes caused by combined treatment in these induced frequencies were more marked in Q cells than in total cells, and the changes in the SFs and these induced frequencies were slightly greater in SAS/mp53 than in SAS/neo. As a result, combined treatment with both TPZ and MTH could greatly reduce the difference in the sensitivity to CDDP between SAS/neo and SAS/mp53 and finally make the sensitivity identical in terms of the SF, which is thought to reflect total cell death directly.

Fig. 5 shows the changes in SF for total tumor cells and in the induced apoptosis and MN frequencies for total and Q tumor cells in SAS/neo and SAS/mp53 tumors administered intraperitoneally with TXL at a dose of 40 mg/kg, according to combined treatment with MTH and/or TPZ (40 mg/kg). With-

out MTH or TPZ, total tumor cells in the SAS/neo tumor showed a slightly lower SF, slightly higher induced apoptosis frequency and slightly lower induced MN frequency than the SAS/mp53 tumors. Q tumor cells of the SAS/neo showed higher induced apoptosis and slightly lower induced MN frequencies than SAS/mp53. With combined treatment, the SFs were decreased and the induced apoptosis and MN frequencies were increased in the same order as described above for Fig. 4. Further, the changes caused by combined treatment in these induced frequencies were slightly more marked in Q cells than in total cells, and the changes in the SFs and these induced frequencies were slightly greater in SAS/mp53 than in SAS/neo. Thus, the originally not so marked difference in sensitivity to TXL between SAS/neo and SAS/mp53 was reduced by these combined treatments, until finally the sensitivity was identical in terms of the SF.

Discussion

As shown in our previous report,[10] as far as these two tumor cells are concerned, the fluorescence staining method was thought to reflect apoptotic events as well as the conventional TUNEL (deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling) method. However, the contribution of apoptosis to determining the response of tumors depends on the cell type[24] as well as the induction of MN after DNA-damaging treatment.[25] The time course change in the value of the apoptosis frequency (Fig. 1) showed that death by apoptosis was well related to a response to γ-rays or CDDP in SAS/neo tumors, compared with SAS/mp53 tumors. That is to say, the ratio of apoptosis to total cell death is relatively high in the SAS/neo tumor cells following these DNA-damaging treatments. Therefore, the normalized and induced MN frequencies in SAS/neo tumor cells conversely showed smaller values as a whole (Figs. 3 and 4), compared with SAS/mp53 tumor cells. In contrast, there were no apparent differences in the values of the apoptosis frequency between SAS/neo and SAS/mp53 at any time point after TPZ or TXL administration. Namely, apoptosis induced by TPZ or TXL was independent of p53 status. Thus, both tumor cells might show almost the same values of MN frequency (Figs. 2 and 5).

In our study[26] on the use of an administration of nicotinamide before irradiation to release intratumor acute hypoxia or the placement of mice in a circulating carbogen (95% O_2/5% CO_2) chamber for 30 min before and during irradiation to release intratumor chronic hypoxia, it was demonstrated that the hypoxic fraction (HF) of Q cells is much larger than that of total tumor cells and largely comprised the chronically HF with a smaller acutely HF. In addition, the combination with nicotinamide administration and/or MTH and with the placement in a circulating carbon chamber and/or MTH indicated that MTH preferentially oxygenated the chronically HF in the solid tumor probably through an increase in the tumor blood flow.[27] Thus, as also shown in another of our reports,[28] MTH could predominantly decrease the HF of chronic hypoxia-rich intratumor Q cell fractions. Moreover, in our subsequent study,[13] the cytotoxicity of TPZ was also found to be raised in combination with MTH. Based on these findings, the use of hypoxia-selective cytotoxin TPZ and/or chronic hypoxia-releasing MTH combined with radiation and/or chemotherapy was thought to be promising for enhancing the response of intratumor Q cells. Actually, this combination was very effective in terms of enhancing the Q-cell response in radiotherapy or chemotherapy.[29, 30] Recently, it was also reported that TPZ is still needed to enhance the Q-cell response on chemoradiation using the mitotic spindle poison paclitaxel, because no radiosensitization effect in combination with paclitaxel was induced in Q tumor cells.[31] However, all these studies on combined treatment with MTH and/or TPZ were performed using mouse tumor cell lines with wild-type p53 status.

Now, p53 is supposed to be mutated in a majority of human solid tumors and to play a central role in the cellular response to ionizing radiation or chemotherapy-induced DNA damage.[32, 33] After DNA damage, p53 promotes cell cycle arrest, apoptosis, and transcription of DNA repair complexes. DNA damage may lead to cycle arrest at the G1 check-point after p53-dependent transcription of p21,[34] association with DNA replication/repair complexes such as proliferating cell nuclear antigen (PCNA) and growth arrest DNA damege protein (GADD45),[35, 36] and induction of apoptosis.[3, 37] Loss of p53 function may result in resistance to DNA-damaging agents, including ionizing radiation.[5, 38] Actually, since mutations in p53 are closely associated with a worse prognosis for solid tumors,[5] it is pertinent to ask whether cells with a mutated p53 are more resistant to combined treatment with MTH and/or TPZ or not, as has been suggested for many anticancer agents.[5]

In agreement with previous reports,[39–41] the cell-killing effect on both total and Q cell populations by a high dose of TPZ or

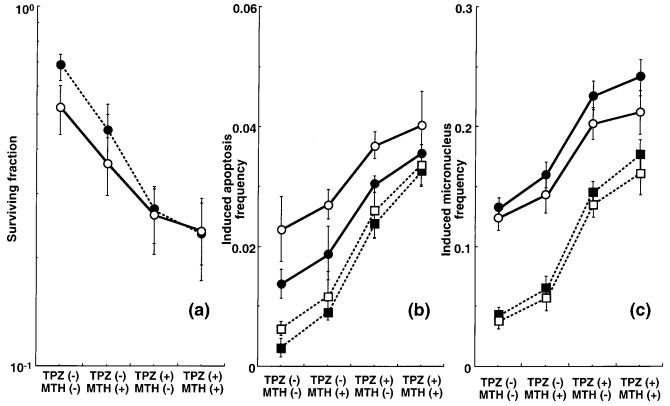

Fig. 4. Changes in surviving fraction (a) for total tumor cells, induced apoptosis frequency (b) and induced micronucleus frequency (c) for total (circles) and quiescent (squares) tumor cells in SAS/neo (open symbols) and SAS/mp53 (solid symbols) tumors treated with cisplatin (8.85 mg/kg, i.p.) combined with mild temperature hyperthermia (MTH, 40°C, 60 min) and/or tirapazamine (TPZ, 40 mg/kg, i.p.). Bars represent 95% confidence limits.

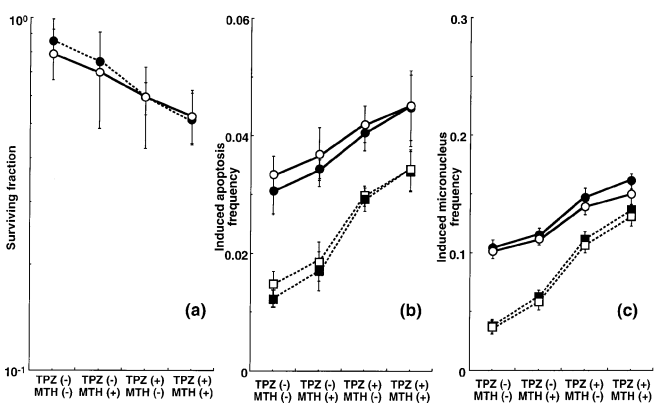

Fig. 5. Changes in surviving fraction (a) for total tumor cells, induced apoptosis frequency (b) and induced micronucleus frequency (c) for total (circles) and quiescent (squares) tumor cells in SAS/neo (open symbols) and SAS/mp53 (solid symbols) tumors treated with paclitaxel (40 mg/kg, i.p.) combined with mild temperature hyperthermia (MTH, 40°C, 60 min) and/or tirapazamine (TPZ, 40 mg/kg, i.p.). Bars represent 95% confidence limits.

TXL itself did not depend on p53 status (Figs. 2, (a) and (b), and 5 (a)), and SAS/neo showed greater sensitivity to γ-rays or CDDP than SAS/mp53 (Figs. 3, (a) and (b), and 4 (a)). Potentiation of the killing effect on both cell fractions of TPZ in combination with MTH did not depend on p53 status (Table 2), but, if anything, SAS/mp53 was slightly more markedly potentiated in both cell fractions. Also when combined with γ-ray irradiation, there were no apparent differences in the potentiation of the cell-killing effect on either cell fraction between SAS/neo and SAS/mp53 by MTH and/or TPZ (Table 4). Similarly, the effects on both cell fractions were slightly more enhanced in SAS/mp53. When MTH and/or TPZ was combined with CDDP or TXL treatment, the pattern of enhancement of the killing effect on both cell fractions was almost the same as when combined with γ-rays (Figs. 4 and 5).

This p53-independency of the potentiation of the cytocidal effect by combined treatment with MTH and/or TPZ is very advantageous and useful for controlling solid tumors consisting of cells with a mutated p53 status, which are often harder to control by DNA-damaging treatments than solid tumors with a wild-type p53 status.[5, 38] In addition, the tendency that the killing effect on mutated tumors could be enhanced more markedly, although only slightly, showed this combined treatment is much more useful for controlling solid tumors as a whole. This might be because SAS/mp53 tumors have significantly higher HFs (14.1 (10.9–17.3) % vs. 7.9 (4.9–10.9) %) and higher Q cell fractions (56.8 (50.6–63.0) % vs. 51.6 (44.9–58.3) %) than SAS/neo tumors as shown in our report.[10] As a result, since Q cells include much larger HF and larger chronically HF than P cells, combination treatment with chronic hypoxia-releasing MTH and/or hypoxia-selective cytotoxin TPZ not only reduced the sensitivity difference to γ-rays, CDDP or TXL between SAS/neo and SAS/mp53, but also shifted, although only slightly, the sensitivity of SAS/mp53 to make it more sensitive than SAS/neo (Table 3, Figs. 3, (a) and (b), 4 and 5). Furthermore, in SAS/mp53 tumors, combined treatment with MTH and/or TPZ reduced the sensitivity difference between total and Q cell populations slightly more than in SAS/neo tumors (Table 5, Figs. 4 and 5). This might be because of higher Q cell fractions and higher HFs of Q cells (54.6 (49.1–60.1) % vs. 43.3 (38.8–46.8) %) in SAS/mp53 tumors than in SAS/neo tumors.[10] Anyway, regardless of the p53 status of tumor cells, combined treatment with MTH and/or TPZ is thought to be very useful in conventional DNA-damaging cancer therapy.

Solid tumors, especially human tumors, are thought to contain a high proportion of Q cells.[7] 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.[7] This might promote MN formation and apoptosis in Q tumor cells, partly due to the effect of hypoxia-induced apoptosis (Table 1).[16] It has been reported that Q cells have lower sensitivity to radiation and chemotherapeutic agents than P cells in solid tumors *in vivo*.[7, 9] This means that more Q cells survive after radiotherapy and chemotherapy than P cells. Consequently, the control of Q cells has a great impact on the outcome of anticancer radiation therapy. However, as shown in Table 5 and Figs. 4 and 5, the difference in sensitivity between total and Q cells did not depend on the p53 status of tumor cells. Thus, from the viewpoint of the tumor cell killing effect including intratumor Q cell control, a treatment modality for enhancing the Q cell response has to be taken into account. Meanwhile, combined treatment with MTH and/or TPZ enhanced the response of intratumor Q cells very effectively, irrespective of p53. Thus, this cooperative modality in combination with conventional anticancer DNA-damaging treatment is very promising for controlling not only wild-type p53 status tumors, but also mutated p53 status tumors, especially in terms of controlling intratumor Q cells.

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