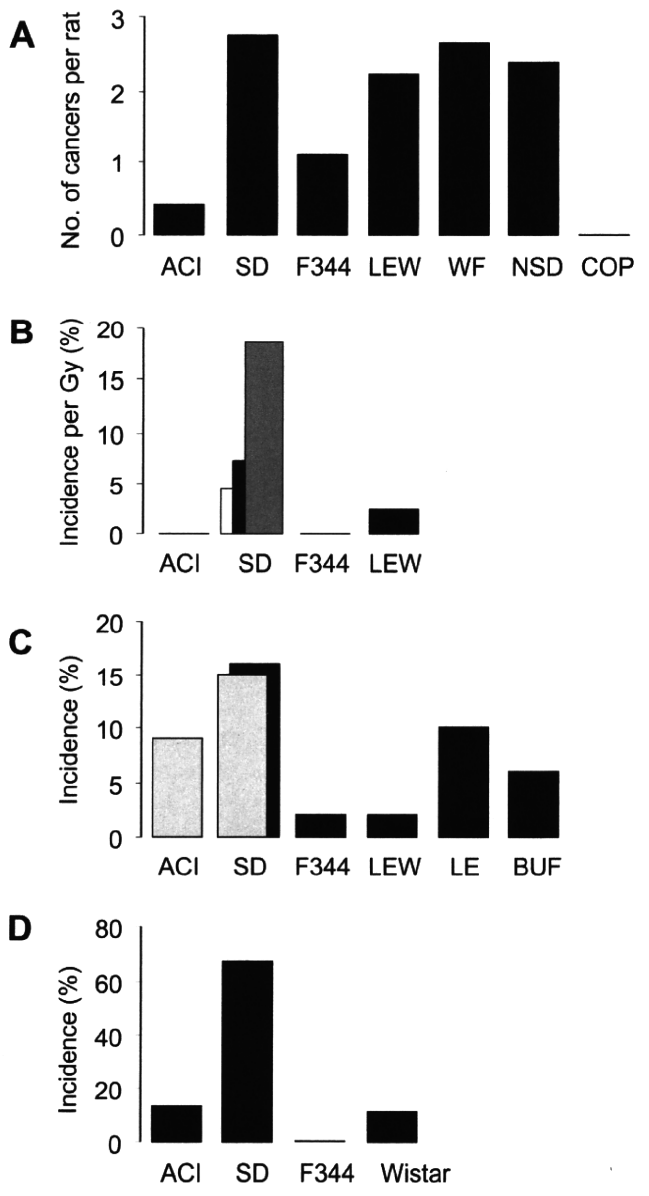


**Fig. 8.** Rat strain difference in susceptibility to mammary carcinogens. Panel *a*, The average number of cancers per rat after exposure to the chemical carcinogen 7,12-dimethylbenz(a)anthracene.[144] Panel *b*, Slope of linear equations fitted to dose-effect relationship for X-ray–induced mammary cancer incidence. Sprague-Dawley (data from three literatures are shown by *white*,[145] *black*[146] and *gray*[135] *columns*), F344[148] and Lewis[146] rats were observed for 300–350 days, and ACI rats for 190 days.[147] Panel *c*, Incidence of mammary cancer after exposure to 0.05 Gy of fission neutrons[150] (*black column*) and 0.004 Gy of 430 keV fast neutrons[149] (*gray column*). Panel *d*, Incidence of mammary cancer after exposure to 1 Gy of 290 MeV/u carbon ions with a spread-out Bragg peak.[137] All graphs were constructed from data in indicated literature. SD, Sprague-Dawley; LEW, Lewis; WF, Wistar-Furth; LE, Long-Evans; BUF, Buffalo.

susceptible in contrast to F344 and Lewis strains (Fig. 8*c*).[149,150] In addition, the Sprague-Dawley rat also has higher susceptibility to heavy ion–induced carcinogenesis than F344 and ACI strains (Fig. 8*d*).[137] The high susceptibility of the Sprague-Dawley strain thus seems to be independent of the carcinogen species. Given that variations in cancer susceptibility also exist for human populations, secondary cancer risk after heavy-ion therapy might well differ among individuals.

### *Biological characteristics of induced tumors*

Although the biological characteristics of heavy ion–induced cancers are poorly understood, some insights are available from animal experiments. Data on neon ion–irradiated rats have indicated that the incidence of fibroadenoma (a benign tumor) of the mammary gland was higher than that of carcinoma (or cancer).[135] The same tendency has been observed in carbon ion–, X-ray– and γ-ray–irradiated rats.[135,137] Iron ion–induced mammary cancers have also been reported to contain fibroadenoma and carcinoma.[136] Such high prevalence of fibroadenoma is characteristic of radiation induction models, making a stark contrast to chemical induction, where carcinoma predominantly develops.[144] In addition, carbon ion–induced mammary cancer is reported to be highly metastatic to lung compared to γ-ray–induced one.[137] As metastasis has been observed after irradiation with a dose as low as 0.05 Gy,[137] it is unlikely that heavy ion–induced tissue reactions of the lung have promoted the metastatic process. The mechanism underlying the strong metastatic potential is currently unclear.

The ovarian hormone estrogen plays important roles in the development of most mammary cancers, and estrogen receptor expression is indicative of the hormone dependence of tumors. Because the majority (> 70%) of both carbon ion– and γ-ray–induced mammary cancers express estrogen receptor,[137] estrogen may play crucial roles in both models. The H-*ras* protooncogene is an estrogen-regulated gene, and its mutation, together with its induction by estrogen, plays a major role in chemically-induced rat mammary carcinogenesis.[151] Carbon ion–induced rat mammary cancer is, however, devoid of H-*ras* mutations, as is γ-ray–induced cancer, nor does it have *p53* mutation.[137] Thus, causative gene mutation for heavy-ion induction remains unidentified. Because comprehensive genetic analysis using microarray has revealed altered genes in γ-ray–induced rat mammary cancer,[152] such analysis on heavy ion–induced cancers is indeed warranted.

### *Implication for secondary cancer risk from medical exposure*

Evidence suggests that heavy ions with LET of 33 and 40–90 keV/μm have high RBE for inducing mammary cancer in the Sprague-Dawley rat. Normal tissue in the close vicinity of cancer may be exposed to the high-LET compo-

nent of the SOBP beam during therapy and thus be at high risk of developing secondary cancer. In contrast, low-LET carbon ions (~13 keV/μm) have both low *in vitro* transformation efficiency[153] and weak tumorigenicity in vivo,[139] suggesting a relatively low risk from exposure of normal tissue to therapeutic heavy-ion beams. The variable sensitivity among rat strains implies that individual cancer susceptibility needs to be taken into account, such as genetic polymorphisms related to breast cancer risk (e.g., *BRCA* genes). More information from experimental carcinogenesis study is awaited for a more concrete estimation of secondary cancer risk from heavy-ion therapy.

## 8. PERSPECTIVES

In spite of a series of studies, how heavy ions inactivate cells more effectively than photons continues to remain a fascinating question. The question concerning the LET dependence of induction of DNA DSBs and clustered DNA damage is still fully open.[11,12] Growing evidence now suggests that heavy ions kill cells via various modes of cell death (e.g., autophagy and premature senescence in addition to apoptosis) and overcome radioresistance, although the current evidence is still only phenomenological. A deeper understanding of the mechanism of action of heavy ions and the molecular underpinnings of heavy ion–induced cell death would be necessary, which may in turn lead to (*i*) mechanism-based designing of biological approaches that enhance tumor control without aggravating, or even with assuaging, normal tissue complications, (*ii*) prediction of both the heavy-ion responsiveness of tumors and normal tissue complications (including acute reactions and secondary cancers) prior to treatment and (*iii*) tailor-made therapy for individual patients. In this regard, recent studies have been conducted to molecularly profile the heavy-ion response of tumor cells irradiated in vivo,[154,155] and this encourages further investigation to clarify the genes responsible for susceptibility to heavy ions.

## ACKNOWLEDGMENTS

N. H. and Y. K. are indebted to Drs. Takamitsu Hara, Sakura Hamada, Keiko Kataoka (Gunma Univ., Japan), Tetsuya Sakashita, Tomoo Funayama and Yuichiro Yokota (JAEA, Japan) for help with irradiation experiments; the work was supported in part by a Grant-in-Aid for the 21st Century Center-of-Excellence Program for Biomedical Research Using Accelerator Technology to Gunma University Graduate School of Medicine from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and by the budget of Nuclear Research of MEXT based on screening and counseling by the Atomic Energy Commission of Japan. T. I. and Y. S. acknowledge Drs. Mayumi Nishimura, Shizuko Kakinuma, Takashi Takabatake, Kazuhiko Daino (NIRS, Japan) and Daisuke Iizuka (Hiroshima Univ., Japan) for kind collaboration and critical reading of the manuscript; the work was supported in part by a research grant from the Takeda Science Foundation, and Grants-in-Aid for Young Scientists (B) (No. 20710049) from MEXT and for Scientific Research (A and C) (No. 19201011 and 21610029) from the Japan Society for the Promotion of Science (JSPS), and Health and Labor Sciences Research Grants (No. 19141201 and 19S-1) from the Ministry of Health, Labor and Welfare of Japan. S. M. and K. O. acknowledge Drs. Ryoichi Hirayama and Akiko Uzawa (NIRS, Japan) for dedicated support for animal experiments; the work was supported in part by Grants-in-Aid for Scientific Research (C) (No. 18591380 and 20591493) from JSPS. T. Ogata and T. T. acknowledge Drs. Nariaki Matsuura, Yutaka Takahashi (Osaka Univ., Japan) and Yoshiya Furusawa (NIRS, Japan). R. O. and T. K. greatly appreciate Drs. Akira Fujimori, Miho Noguchi, Mr. Kentaro Seo and Mr. Yoshihiro Fujii (NIRS, Japan) for invaluable help in preparing the manuscript; the work was supported by a Grant-in-Aid for Scientific Research (A) (No. 16209036) from JSPS. A. T. and T. Ohnishi acknowledge Drs. Tadaaki Kirita and Nobuhiro Yamakawa (Nara Medical Univ., Japan); the work was supported by Grants-in-Aid for Scientific Research from MEXT, a grant from the Central Research Institute of Electric Power Industry, and a grant for Exploratory Research for Space Utilization from the Japan Space Forum. Some works herein were conducted as part of the Research Project with Heavy Ions at NIRS-HIMAC. The cost for the English editing of the manuscript was supported by Space Radiation Research Unit, International Open Laboratory, NIRS, Japan, and the authors are grateful to Dr. Yukio Uchihori (NIRS, Japan).

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*Received on November 20, 2009*
*Accepted on December 14, 2009*

J. Radiat. Res., 51, 27–35 (2010)

Regular Paper

# Usefulness of Hexamethylenetetramine as an Adjuvant to Radiation and Cisplatin in the Treatment of Solid Tumors: its Independency of p53 Status

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**Hexamethylenetetramine/p53 status/Quiescent cell/Hypoxia.**

The usefulness of hexamethylenetetramine as an adjuvant to radiation and cisplatin in the treatment of solid tumors and its dependency on the p53 status of tumor cells were examined. 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 the hind legs of Balb/cA nude mice. The tumor-bearing mice received 5-bromo-2'-deoxyuridine (BrdU) continuously to label all proliferating (P) cells in the tumors. Then, they received hexamethylenetetramine (HMTA), intraperitoneally or continuously, combined with or without γ-ray irradiation or cisplatin treatment. Immediately after treatment following HMTA, the response of quiescent (Q) cells was assessed in terms of the micronucleus frequency using immunofluorescence staining for BrdU. The response of the total (= P + Q) tumor cells was determined from the BrdU non-treated tumors. A higher toxicity of HMTA to Q cells than total cells, especially in SAS/neo, was made less clear by continuous administration. There was no apparent difference in the radio- and cisplatin-sensitivity enhancing effects by HMTA combination between SAS/neo and SAS/mp53 tumors, with a slightly greater effect in SAS/mp53. In both SAS/neo and SAS/mp53 tumors, continuous HMTA administration produced higher radio- and cisplatin-sensitivity enhancing effects than intraperitoneal single administration. Therefore, the use of HMTA as an adjuvant to radiation or cisplatin might be promising in curing solid tumors with large fraction of hypoxic cells and also with frequent loss-of-function in p53.

## INTRODUCTION

One main theme of radiobiology has been the importance of hypoxia in radiotherapy. In the early 1950s, Gray and colleagues stressed the importance of hypoxia in reducing the effectiveness of radiation treatments.[1)] In the early 1970s, Sartorelli's group proposed that the presence of hypoxia could be used to an advantage if a prodrug could be designed that was metabolized to a cytotoxic compound only in hypoxic cells;[2)] this would provide a method of killing these treatment-resistant cells while having a little systemic toxicity.

The development of bioreductive agents that are particularly toxic to hypoxic cells is considered a promising approach to solving the problem of radio-resistant tumor hypoxia in cancer radiotherapy.[4)] Tirapazamine (TPZ, 1,2,4-benzotriazine 1,4-di-N-oxide, SR4233), a lead compound in the development of a bioreductive hypoxic cytotoxin, in combination with radiation or standard cytotoxic chemotherapy agents has been shown to be very useful for controlling solid tumors as a whole, especially for controlling hypoxia-rich intratumor quiescent (Q) cell populations irrespective of p53 status of tumor cells.[5)] Tumor hypoxia results from either limited oxygen diffusion (chronic hypoxia) or limited perfusion (acute hypoxia, transient hypoxia or ischemic hypoxia).[6)] Chronically hypoxic tumor cells existing at the rim of the oxygen diffusion distance can be killed by just a

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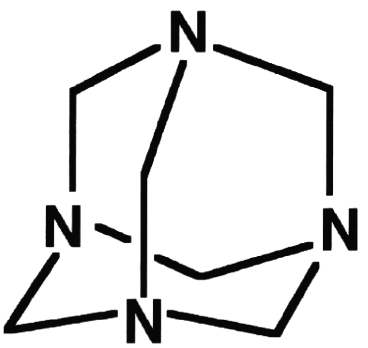
doi:10.1269/jrr.09072

single administration of TPZ. Acutely hypoxic tumor cells occurring sporadically throughout solid tumors can be killed by TPZ during long-term continuous administration. In other words, the long-term continuous administration of TPZ can kill both chronically and acutely hypoxic tumor cells.[7] Although TPZ has enhanced some standard clinical regimens, the results are variable and in some combinations toxicity was enhanced.[8] As a result, TPZ has had so far no clear beneficial effects compared to conventional drugs in clinical trials.

It has been shown that the p53 tumor suppressor gene serves a critical role in maintaining genomic stability during a cell cycle checkpoint in not only G1 but also the G2/M transition,[9] as an effector of DNA repair and apoptosis. Wild-type p53 status tumor cells are liable to activate apoptosis in response to DNA damage. p53 is mutated in a majority of human solid tumors and play a central role in the cellular response to DNA damaging treatments like ionizing radiation, chemotherapy or hypoxic stress.[10] Hypoxic stress also induces p53 protein accumulation and p53-dependent apoptosis, but does not induce p53-dependent cell cycle arrest.[10] Loss of p53 function may result in resistance to DNA-damaging agents, including ionizing radiation and hypoxic stress.[11,12] Thus, the genetic and functional status of the p53 gene is thought to be an important factor in guiding therapeutic strategies for cancer patients.

Formaldehyde preserves or fixes tissue or cells by irreversibly cross-linking primary amine groups in proteins with other nearby nitrogen atoms in protein or DNA through a -CH2- linkage.[13] An acid-dependent formaldehyde donor, hexamethylenetetramine (HMTA) (Fig. 1), has been used as an antiseptic for urinary tract infections and it has been supposed to be characterized as a non-carcinogen in animals.[13] In the hypoxic condition in a solid tumor, pyruvate generated by glycolysis induces a low pH environment[14] that produces formaldehyde through dissociation of HMTA. So far, some studies using HMTA were reported, especially referring to its combined effect with adriamycin.[14] Except for surgical resection, chemotherapy using cis-diamminedichloroplatinum (cisplatin) and radiotherapy are the most frequently employed anticancer therapeutic modalities in real clinics. Based on these clinical backgrounds, using a p53 wild-type SCC VII tumor, the combined effect of HMTA with γ-ray irradiation or cisplatin treatment was examined, resulting in producing almost similar radio- and cisplatin sensitivity enhancing effects to TPZ.[15] Accordingly, in this study, using two different solid tumors identical in genetic background except for p53 status and our method for selectively detecting the response of the quiescent (Q) cell populations within solid tumors,[5] we evaluated the usefulness of combined use of HMTA in solid tumor treatment based on its dependency on p53 status. In addition, the usefulness of continuous administration of HMTA was also evaluated.




**Hexamethylenetetramine**
**(C6H12N4, M.W. = 140.19)**

**Fig. 1.** Chemical structure of hexamethylenetetramine (HMTA).

## 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 Dulbeco's Modified Eagle's medium (DMEM) containing 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) and 12.5% fetal bovine serum in a conventional humified 5% CO2 incubator. Although SAS cells have a homozygous point mutation (GAG to TAG) at codon 336 of exon 10 in the oligomerization domain of an mp53 gene, they show the phenotype of wild-type p53 in radiation- and heat-induced signal transduction.[16] SAS/mp53 and SAS/neo cells were obtained by the transfection of dominant-negative p53 (Arg248Trp) expressing plasmid, pC53-248, and control empty plasmid pCMV-Neo-Bam (provided by B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD). As a result, SAS/neo cells have a functional p53 protein, and SAS/mp53 cells express a dominant negative p53 protein. The procedure is described in detail elsewhere.[17]

Cells were collected from exponentially growing cultures, and $5.0 \times 10^5$ cells were injected subcutaneously into the left hind legs of 6–7 weeks 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. At this size, no necrotic component was observed within the both SAS/neo and SAS/mp53 tumors. Mice were handled according to the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety Handling Regulations for Laboratory Animal Experiments, Kyoto University.

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

Two weeks after tumor cell inoculation, mini-osmotic

pumps (Alzet model 2001, DURECT Corporation, 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 percentage of labeled cells after continuous labeling with BrdU was 48.4 ± 6.7% [mean ± SD] and 43.2 ± 6.2% 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.

### *Treatment*

*Treatment with HMTA alone*

After the labeling with BrdU, HMTA in physiological saline were administered at doses of 6.56–65.6 millimole/kg. This was carried out either by administration of a single intraperitoneal injection or continuously for 24 hours by subcutaneously implanting mini-osmotic pumps (Alzet model 2001D, Durect Corporation, Cupertino, CA) containing HMTA dissolved in physiological saline. One hour after the intraperitoneal injection or after the 24-hour continuous subcutaneous infusion, implanted tumors were excised. Employed doses were based on the previous report[15] and the toxicity data shown at the Material Safety Data Sheet for HMTA (intravenous, mouse, LD50 = 9200 mg/kg).

*Combination with γ-ray irradiation*

After the labeling with BrdU, HMTA dissolved in physiological saline was administered at doses of 65.6 millimole/kg, singly by intraperitoneal injection or continuously for 24 hours by subcutaneously implanting mini-osmotic pumps. One hour after the intraperitoneal injection or after the 24-hour continuous subcutaneous infusion of HMTA, γ-rays were delivered with a cobalt-60 γ-ray irradiator at a dose rate of approximately 2.5 Gy/min for detecting the radiosensitizing effect of HMTA. Based on the reported results concerning TPZ-induced cytotoxicity,[18] the employed doses of HMTA induced almost the same cytotoxicity to total tumor cell population in terms of cell survival and induced micronucleus (MN) frequency as the dose of 40 mg/kg (= 0.224 millimole/kg) of TPZ when administered intraperitoneally to SAS/neo tumors. Immediately after irradiation, implanted tumors were excised.

*Combination with cisplatin treatment*

After the labeling with BrdU, HMTA dissolved in physiological saline was administered at doses of 65.6 millimole/kg singly by intraperitoneal injection or continuously for 24 hours by subcutaneously implanting mini-osmotic pumps. One hour after the intraperitoneal injection or after the 24-hour continuous subcutaneous infusion of HMTA, cisplatin was intraperitoneally administered at a dose of 0.1 through 0.5 $LD_{50}$ (the dose required to kill 50%, $LD_{50}$ = 17.7 mg/kg) for detecting the cisplatin-sensitizing effect of HMTA. One hour after the administration of cisplatin, implanted tumors were excised. All above-mentioned sequences and timing of each treatment were appropriate enough to function completely, based on our previous work.[7,15]

### *Immunofluorescence staining of BrdU-labeled cells and micronucleus (MN) assay*

Tumors excised from the mice given BrdU were minced and trypsinized [0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in PBS, 37°C, 15 min]. Tumor cell suspensions thus obtained 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. After the centrifugation of fixed cell suspensions, the cell pellet was resuspended with cold Carnoy's fixative (ethanol:acetic acid = 3:1 in volume). The suspension was then placed on a glass microscope slide and the sample was dried at room temperature. To enable detection of BrdU in P cells, the slides were treated with 2 M hydrochloric acid for 60 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, St. Louis, MO). To observe the double staining of tumor cells with green-emitting FITC and red-emitting propidium iodide (PI), cells on the slides were treated with PI (2 μg/ml in PBS) and monitored under a fluorescence microscope.

The MN frequency in cells not labeled with BrdU 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.[5]

The ratios obtained in tumors not pretreated with BrdU indicated the MN frequency at all phases in the total (P + Q) tumor cell population. More than 300 (334 ± 33) binuclear cells were counted to determine the MN frequency. The respective MN frequencies under the absolutely no treatment control condition were 0.033 ± 0.003 and 0.040 ± 0.005 in the SAS/neo tumor, and 0.058 ± 0.005 and 0.062 ± 0.007 in SAS/mp53 tumor for total tumor and Q cell populations.

### *Clonogenic cell survival assay*

The clonogenic cell survival assay was also performed in the mice given no BrdU using an *in vivo-in vitro* assay method. Tumors were excised from the mice, minced and disaggregated by stirring for 20 min at 37°C in PBS containing 0.05% trypsin and 0.02% EDTA. Through these procedures, single tumor cell suspensions were obtained from the

whole tumor. The cell yield was (1.5 ± 0.3) × $10^7$ /g and (3.4 ± 0.8) × $10^6$/g for SAS/neo and SAS/mp53 tumors, respectively. Appropriate numbers of viable tumor cells from the single cell suspension, that were identified under blood cell counting chamber, were plated on 60 or 100-mm tissue culture dishes, and, 16 days later, colonies were fixed with ethanol, stained with Giemsa, and counted. The plating efficiency under no treatment control condition was 47.1 ± 8.5% for SAS/neo and 23.5 ± 5.9% for SAS/mp53 tumors.

The sensitivity of Q cells was assessed in terms of the MN frequency using immunofluorescence staining for BrdU. That of the total (= P + Q) tumor cells was determined from the BrdU non-treated tumors in terms of the MN frequency and clonogenic cell survival.

Five mice were used to assess each set of conditions and each experiment was repeated twice. To examine the differences between pairs of values, Student's *t*-test was used when variances of the two groups could be assumed to be equal; otherwise the Welch *t*-test was used. *p*-Values are from two-sided tests.

**Table 1.** Dose-modifying factors[a] of continuous administration compared with single intraperitoneal administration

| | SAS/neo | SAS/mp53 |
|---|---|---|
| <Surviving fraction = 0.5> | | |
| Total tumor cells | 1.7 (1.6–1.8)[b] | 1.3 (1.2–1.4) |
| <Normalized micronucleus frequency = 0.2> | | |
| Total tumor cells | 1.85 (1.7–2.0) | 1.55 (1.45–1.65) |
| Quiescent cells | 1.35 (1.25–1.45) | 1.3 (1.2–1.4) |

[a]; The ratio of the drug dose required to obtain each endpoint with single intraperitoneal administration to the drug dose required to obtain each endpoint with continuous administration.

[b]; Numbers in parentheses are 95% confidence limits, determined using standard errors. When the ranges of 95% confidence limits have no overlap between any two values, the difference between the two values is considered significant ($p < 0.05$).

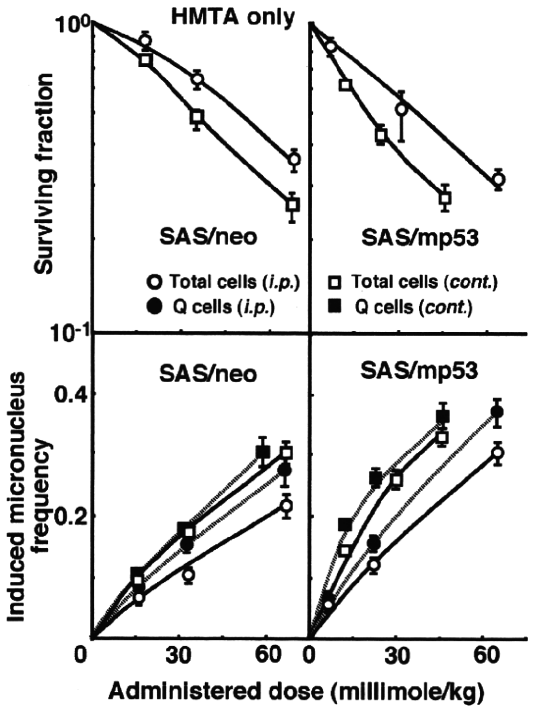



**Fig. 2.** The clonogenic cell survival curves for total tumor cell populations (upper panels) and the induced micronucleus frequencies for total and quiescent (Q) cell populations (lower panels) following intraperitoneal (*i.p.*) single or subcutaneous 24 hour continuous (*cont.*) administration of hexamethylenetetramine (HMTA) into SAS/neo (left panel) or SAS/mp53 (right panel) tumors. Assays were performed 1 hour after *i.p.* single administration or after the 24 hour *cont.* administration. Bars represent standard deviations.

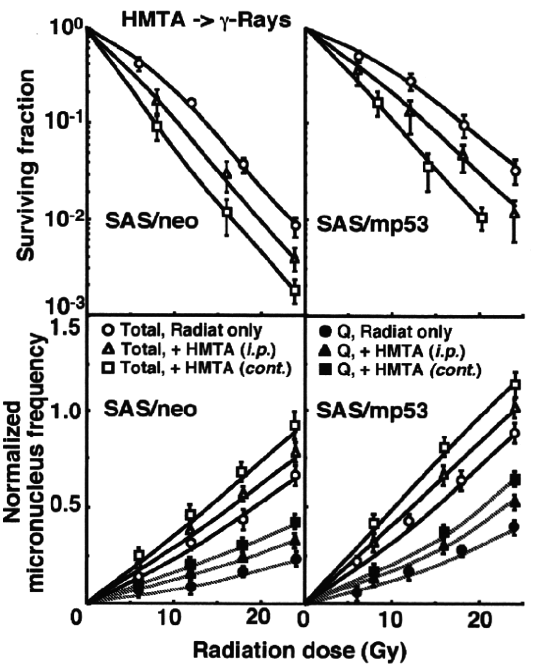



**Fig. 3.** The clonogenic cell survival curves for total tumor cell populations (upper panels) and the normalized micronucleus frequencies for total or quiescent (Q) cell populations (lower panels) immediately after γ-ray irradiation combined with or without intraperitoneal (*i.p.*) single or subcutaneous continuous (*cont.*) administration of hexamethylenetetramine (HMTA, 65.6 millimole/kg) into SAS/neo (left panel) or SAS/mp53 (right panel) tumors. Assays were performed immediately after irradiation 1 hour after *i.p.* single administration or after 24 hour *cont.* administration. Bars represent standard deviations.

## RESULTS

### *Effects of HMTA alone*

Upper panels of Fig. 2 show the clonogenic cell survival curves of total tumor cell populations following HMTA administration. Lower panels of Fig. 2 show the induced MN frequencies, i.e., the MN frequency in the HMTA administered tumors minus that in the no drug administered tumors, of total or Q cell populations. In both SAS/neo and SAS/mp53 tumors, subcutaneous continuous administration induced significantly lower surviving fractions (SFs) in total cells and significantly larger induced MN frequencies in both total and Q cells than intraperitoneal single administration ($P < 0.05$). In both SAS/neo and SAS/mp53 tumors, induced MN frequencies in Q cells were significantly larger than those in total cells ($P < 0.05$). To analyze the effect of continuous administration, the values of dose modification factors for continuous administration compared with an intraperitoneal single administration were calculated using the data shown in Fig. 2 (Table 1). In both tumors, the values for total cells were significantly larger than those for Q cells ($P < 0.05$). In total tumor cell population, the values for SAS/neo were significantly larger than those for SAS/mp53 ($P < 0.05$). In contrast, in Q cells, the values for SAS/neo were only slightly larger than those for SAS/mp53. According to the reported result concerning cytotoxicity of TPZ,[18] the employed doses of 65.6 millimole/kg of HMTA induced almost the same cytotoxicity to total tumor cell population of SAS/neo tumors in terms of cell survival and induced MN frequency as the dose of 40 mg/kg (= 0.224 millimole/kg) of TPZ when administered intraperitoneally to SAS/neo tumors.[19] Therefore, the employed doses of HMTA combined with γ-ray irradiation or cisplatin treatment in the subsequent experiments were determined to be 65.6 millimole/kg.

**Table 2.** Radio-sensitizing effect[a] of pre-radiation use of HMTA[b] in combination with γ-ray irradiation

| | SAS/neo | SAS/mp53 |
|---|---|---|
| <Surviving fraction = 0.05> | | |
| Total tumor cells | | |
| i.p.[c] | 1.25 (1.15–1.35)[d] | 1.35 (1.25–1.45) |
| cont.[e] | 1.55 (1.4–1.7) | 1.6 (1.45–1.75) |
| <Normalized micronucleus frequency = 0.2> | | |
| Total tumor cells | | |
| i.p. | 1.25 (1.15–1.35) | 1.35 (1.25–1.45) |
| cont. | 1.55 (1.45–1.65) | 1.6 (1.5–1.7) |
| Quiescent tumor cells | | |
| i.p. | 1.45 (1.35–1.55) | 1.5 (1.4–1.6) |
| cont. | 1.7 (1.6–1.8) | 1.75 (1.65–1.75) |

[a]; The ratio of the radiation dose necessary to obtain each end-point without any drug and the radiation dose necessary to obtain each end-point with each drug.
[b]; Hexamethylenetetramine.
[c]; Single intraperitoneal administration.
[d]; Numbers in parentheses are 95% confidence limits, determined using standard errors. When the ranges of 95% confidence limits have no overlap between any two values, the difference between the two values is considered significant ($p < 0.05$).
[e]; Continuous subcutaneous administration.

### *Effects of HMTA combined with radiation*

Upper panels of Fig. 3 show the clonogenic cell survival curves of total tumor cell populations following γ-ray irradiation combined with or without HMTA administration before the γ-ray irradiation. Lower panels of Fig. 3 show the normalized MN frequencies, i.e., the MN frequency in the irradiated tumors minus that in the non-irradiated tumors, of total or Q cell populations. To analyze the radio-sensitizing

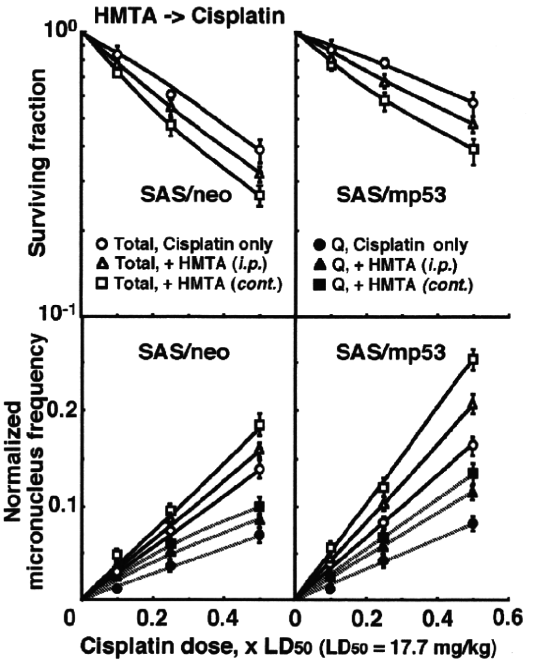



**Fig. 4.** The clonogenic cell survival curves for total tumor cell populations (upper panels) and the normalized micronucleus frequencies for total or quiescent (Q) cell populations (lower panels) 1 hour after cisplatin treatment combined with or without intraperitoneal (*i.p.*) single or subcutaneous continuous (*cont.*) administration of hexamethylenetetramine (HMTA, 65.6 millimole/kg) into SAS/neo (left panel) or SAS/mp53 (right panel) tumors. Assays were performed 1 hour after cisplatin treatment 1 hour after *i.p.* single administration or after 24 hour *cont.* administration. Bars represent standard deviations.

effect of the combined use of HMTA, the values of dose modification factors for the use of HMTA in combination with irradiation compared with irradiation alone were calculated using the data shown in Fig. 3 (Table 2). For both SAS/neo and SAS/mp53 tumors, HMTA combination showed significant radio-sensitization effects on both total and Q cells ($P < 0.05$), with a significantly larger enhancement effect of continuous administration than that of intraperitoneal single administration ($P < 0.05$). Whether intraperitoneally or continuously administered, the radio-sensitizing effects of HMTA were slightly larger in Q cells and in SAS/mp53 tumors than in total cells and in SAS/neo tumors, respectively, without a significant difference. In both SAS/neo and SAS/mp53 tumors, the increase in the radio-sensitizing effect by continuous administration compared with intraperitoneal single injection was slightly more remarkable in total cells than in Q cells, again although not significantly.

*Effects of HMTA combined with cisplatin*

Upper panels of Fig. 4 show the clonogenic cell survival curves of total tumor cell populations following cisplatin treatment combined with or without HMTA administration before the cisplatin tretment. Lower panels of Fig. 4 show the normalized MN frequencies, i.e., the MN frequency in the treated tumors with cisplatin minus that in the non-treated tumors with cisplatin, of total or Q cell populations. To analyze the cisplatin-sensitizing effect of the combined use of HMTA, the values of dose modification factors for the use of each drug in combination with cisplatin treatment compared with cisplatin treatment alone were calculated using the data shown in Fig. 4 (Table 3). HMTA combination showed a significant sensitization effect on both total and Q cells ($P < 0.05$), with a significantly larger enhancement effect of continuous administration than that of intraperitoneal single administration ($P < 0.05$). Whether intraperitoneally or continuously administered, in both SAS/neo and SAS/mp53 tumors, the sensitizing effect was significantly larger in Q cells than in total cells ($P < 0.05$). However, in both total and Q cell populations, the sensitizing effect was only slightly larger on SAS/mp53 than on SAS/neo tumors without a significant difference. In both SAS/neo and SAS/mp53 tumors, the increase in the sensitizing effect by continuous administration compared with intraperitoneal single injection was slightly more marked in total cells than in Q cells, again although not significantly.

**Table 3.** Cisplatin-sensitizing effect[a] of pre-administration use of HMTA[b]

| | SAS/neo | SAS/mp53 |
|---|---|---|
| <Surviving fraction = 0.6> | | |
| Total tumor cells | | |
| i.p.[c] | 1.25 (1.15–1.35)[d] | 1.4 (1.3–1.5) |
| cont.[e] | 1.55 (1.45–1.65) | 1.65 (1.55–1.75) |
| <Normalized micronucleus frequency = 0.05> | | |
| Total tumor cells | | |
| i.p. | 1.15 (1.05–1.25) | 1.25 (1.15–1.35) |
| cont. | 1.45 (1.35–1.55) | 1.5 (1.4–1.6) |
| Quiescent tumor cells | | |
| i.p. | 1.45 (1.35–1.55) | 1.5 (1.4–1.6) |
| cont. | 1.65 (1.55–1.75) | 1.7 (1.6–1.8) |

[a]; The ratio of the cisplatin dose necessary to obtain each end-point without any drug and the cisplatin dose necessary to obtain each end-point with each drug.
[b]; Hexamethylenetetramine.
[c]; Single intraperitoneal administration.
[d]; Numbers in parentheses are 95% confidence limits, determined using standard errors. When the ranges of 95% confidence limits have no overlap between any two values, the difference between the two values is considered significant ($p < 0.05$).
[e]; Continuous subcutaneous administration.

## DISCUSSION

Humans are continuously exposed to exogenous formaldehyde. This chemical is a naturally occurring biological compound that is present in all tissues, cells, and bodily fluids.[13] Formaldehyde functions as key intermediates in the "one-carbon pool" used for the biosynthesis of purines, thymidine, and some amino acids. It is usually rapidly metabolized by reduction, oxidation, and reduced glutathione-dependent pathways.[13] However, saturation in formaldehyde metabolism may lead to DNA damage.[20] It has been well documented that cells exposed to formaldehyde exhibit, as a major form of DNA damage, DNA-protein crosslinks.[21] On the other hand, in a limited oxygen environment, pyruvate generated by glycolysis in the cell cytoplasm is preferentially converted into lactic acid by lactate dehydrogenase, which induces a low pH environment (pH 6.4–6.8).[22] Furthermore, through the Warburg effect, many cancer cells vigorously consume glucose and preferentially produce lactic acid even in the presence of adequate oxygen.[22] Thus, due to the extracellular acidic conditions adjacent to solid tumors, it could be expected that an acid-dependent formaldehyde donor, HMTA should dissociate to release formaldehyde into nearby tissues. Based on these backgrounds, the usefulness of HMTA in combination with γ-ray irradiation or cisplatin treatment, both of which are the most frequently employed antitumor therapeutic modalities, was examined especially in terms of the effects on intratumor hypoxia-rich Q cell population.

In cytotoxicity study using HMTA only, HMTA showed significantly larger toxicity to Q cells than total cells as

shown in Table 4 indicating the difference in the sensitivity between total and Q cells under each treatment. This finding was compatible with the previous report showing that Q cells include much higher hypoxic fractions (HFs) than total cells.[5,7] Continuously administered HMTA was also able to induce more cytotoxicity to tumor cells *in vivo* than intraperitoneal single administration through adding the sensitizing effect on acutely hypoxia-rich total cell population to the effect on chronically hypoxia-rich Q cell population. Meanwhile, SAS/mp53 tumors include a larger size of not only the HF but also the chronically HF than SAS/neo tumors, and Q cell populations in both tumors include a larger HF, particularly the chronically HF, than total cell populations, especially concerning SAS/neo tumors.[23] Therefore, the addition of sensitizing effect through continuous administration was significantly larger for total cells than for Q cells, especially in SAS/neo tumors.

HMTA combination showed significantly sensitizing effects on both total and Q cells whether combined with γ-ray irradiation or cisplatin treatment with larger enhancement effects on Q cells and in SAS/mp53 tumors than on total cells and in SAS/neo tumors, respectively. This was probably due to the larger HFs in Q cells and SAS/mp53 tumors than total tumor cells and SAS/neo tumors, respectively. Especially when administered continuously, the enhancement effect on acutely hypoxia-rich total cells was added to that on chronically hypoxia-rich Q cells. However, in combination with cisplatin treatment, on the whole, slightly more remarkable sensitizing effects were found than in combination with γ-ray irradiation. This may be partly because HMTA with a good permeation potential[14] could induce an efficient enhancement effect even under heterogeneous distribution of cisplatin in the solid tumors. The distribution of cisplatin was mainly dependent on blood flow through intratumor vessels, in contrast to almost homogeneously delivered irradiated γ-ray doses throughout the tumors. Meanwhile, concerning the radio- and cisplatin-sensitizing effects, the enhancement was supra-additive effect. As these dose-response curves were normalized with the values for HMTA only treatment conditions, a clearly supra-additive effect could be observed when combined with γ-rays or cisplatin in the treatment of both SAS/neo and SAS/mp53 tumors.

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

| | No combination | i.p.[b] | cont.[c] |
|---|---|---|---|
| Toxicity | | | |
| <Normalized micronucleus frequency = 0.2> | | | |
| SAS/neo | ---- | 0.75 (0.7–0.8)[d] | 0.7 (0.65–0.75) |
| SAS/mp53 | ---- | 0.75 (0.7–0.8) | 0.7 (0.65–0.75) |
| Radio-sensitization | | | |
| <Normalized micronucleus frequency = 0.2> | | | |
| SAS/neo | 2.05 (1.95–2.15) | 1.7 (1.6–1.8) | 1.8 (1.65–1.95) |
| SAS/mp53 | 2.0 (1.9–2.1) | 1.55 (1.45–1.65) | 1.65 (1.55–1.75) |
| Cisplatin-sensitization | | | |
| <Normalized micronucleus frequency = 0.05> | | | |
| SAS/neo | 2.5 (2.3–2.7) | 2.2 (2.1–2.3) | 2.3 (2.2–2.4) |
| SAS/mp53 | 2.35 (2.2–2.5) | 2.0 (1.9–2.1) | 2.1 (2.0–2.1) |

[a]; The ratio of the radiation dose or cisplatin dose necessary to obtain each normalized micronucleus frequency in quiescent cells and the radiation dose or cisplatin dose necessary to obtain each normalized micronucleus frequency in total tumor cells.
[b]; Single intraperitoneal administration.
[c]; Continuous subcutaneous administration.
[d]; Numbers in parentheses are 95% confidence limits, determined using standard errors. When the ranges of 95% confidence limits have no overlap between any two values, the difference between the two values is considered significant ($p < 0.05$).

In SAS/mp53 solid tumors, cells with mutant p53 could escape from the checkpoint mechanism for maintaining genomic stability by wild-type p53, and proliferate without cell cycle arrest or apoptosis.[10] However, as is often the case with advanced malignant solid tumors, in due course, neovascularization could not catch up with the rapid tumor cell proliferation, resulting in a large HF and Q cell fraction in solid tumors. p53 status has the potential to have an effect on microenvironmental conditions, such as the cellularity and the size of the HF in solid tumors.

Solid tumors, especially human tumors, are thought to contain a high proportion of Q cells.[24] 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.[24] This might promote the formation of micronuclei in Q cells within the control solid tumors. Due to the findings that Q cell population has less sensitivity, a greater recovery capacity, and a larger HF than P cell population *in vivo* under γ-ray irradiation or conventional anticancer chemotherapeutic agent treatment,[5] more Q cells can survive after conventional radiation therapy or chemotherapy than P cells. Meanwhile, it should also be noted that the role of Q cells as determinants of treatment success or failure is not fully established, and their impact may vary from tumor to tumor. It is thought that during fractionated radiotherapy or chemotherapy, the Q cell population substantially decreases because of recruitment from Q to P status *in vivo* due to preferential death of P cells.[25] However, even after fractionated radiotherapy or a series of chemotherapy, Q cell population still remain as long as solid

tumors cannot fully regress to an extent where Q status cannot be present. Consequently, the control of Q cells has a great impact on the outcome of radiation therapy and conventional chemotherapy. As shown in Table 4, the use of HMTA reduced the sensitivity difference between total and Q cells in both SAS/neo and SAS/mp53 tumors to some extent in combination with γ-ray irradiation or cisplatin treatment, especially when administered intraperitoneally. This was compatible with the previous finding that not only chronically hypoxia-rich Q cell population but also acutely hypoxia-rich total cell population can be sensitized through subcutaneous continuous administration with a hypoxia-oriented agent in contrast to the fact that chronically hypoxia-rich Q cell population is preferentially sensitized through intraperitoneal single administration.[7] Anyway, the combined use of HMTA, with conventional radiotherapy or chemotherapy using cisplatin was thought to be useful for reducing the sensitivity difference between total and Q cells to some extent, irrespective of p53 status of treated tumors.

According to our previous study, TPZ also exhibited a similar extent of sensitization toward radiation and cisplatin to HMTA at the dose producing same cytotoxity.[15] However, considering the fact that HMTA has long been used as an antiseptic for urinary tract infection,[14] HMTA might have an advantage as an adjuvant to radiation or cisplatin for clinical use, although further studies on toxicity of HMTA to normal tissues still have to be carried out to guarantee safety. Furthermore, considering the effectiveness on Q cells and its independency of p53 status, radiotherapy and chemotherapy using cisplatin combined with HMTA are thought to be very promising.

## ACKNOWLEDGMENTS

This study was supported, in part, by a Grant-in-aid for Scientific Research (C) (20591493) from the Japan Society for the Promotion of Science.

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*Received on June 19, 2009*
*Revision received on August 11, 2009*
*Accepted on August 25, 2009*
*J-STAGE Advance Publication Date: October 3, 2009*

Mutation Research 695 (2010) 69–74

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

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# Ascorbic acid 2-glucocide reduces micronucleus induction in distant splenic T lymphocytes following head irradiation

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ARTICLE INFO

*Article history:*
Received 13 April 2009
Received in revised form 2 November 2009
Accepted 6 December 2009
Available online 16 December 2009

*Keywords:*
Abscopal radiation effect
Ascorbic acid
T lymphocyte
Micronucleus

ABSTRACT

*Purpose:* Evidence from in vivo studies suggests there are enhanced radiation effects in abscopal regions after local head gamma ray irradiation. Splenocyte apoptosis and T lymphocyte micronuclei were induced at higher rates than what would be estimated given the dose at a shielded, distant position. In addition, we evaluated the radio-protective effects of ascorbic acid, acting as a radical scavenger on enhanced radiation effects in the shielded spleen following local head irradiation.

*Methods and materials:* The heads of C3H mice were exposed to γ-rays (10–20 Gy), while the other parts of the body were shielded with a 5 cm-thick lead block. The effective dose for the spleen was calculated at 1.0–2.0 Gy. Splenocytes were isolated 24 h after cranial irradiation and their apoptosis was measured with an Elisa kit (Roche). The induction of T lymphocyte micronuclei was studied using the cytokinesis-block micronucleus assay. The ascorbic acid glucoside, 2-O-alpha-D-glucopyranosyl-L-ascorbic acid (AA-2G), was orally administered to mice 1 h before whole body irradiation. The radio protective effects of AA-2G were estimated by comparing the induction of splenocyte damage (by apoptosis) and micronucleus induction.

*Results:* The splenocyte damage, as measured by the above two methods, was more excessive than what would be expected given exposure to 1.0–2.0 Gy of radiation. Our results suggest that the effects,were enhanced in a distant, non-irradiated organ after localized irradiation. Plasma ascorbic acid concentrations were increased 8–10× over control. Treatment with ascorbic acid slightly protected mouse splenocytes from the induction of apoptosis by the enhanced effects of radiation in the abscopal region. However, ascorbic acid significantly inhibited micronucleus induction in splenic T lymphocytes following local head irradiation.

*Conclusions:* Our results suggest that ascorbic acid effectively scavenged radiation-induced radicals and protected against the enhanced effects of radiation in an abscopal region after local head gamma ray irradiation.



## 1. Introduction

The abscopal effects of radiation were first reported in 1969 and were defined as significant responses to radiation in tissues that are separate from the area exposed to the radiation [1,2].

The enhanced effects of radiation in shielded organs are thought to be based on the phenomena of so-called bystander effects. Their mechanism is thought to involve radiation signals that are transduced from the radiation-targeted organ to shielded organs [3,4]. Most have been observed in the low-dose range [3,5,6]. In fact, Prise et al. [6] noted that most bystander effects appear to saturate at higher dose levels, and that other factors must switch to hypersensitivity of a non-targeted response.

Abscopal effects from radiation have also been previously reported at therapeutic doses. For example, in studies with partially irradiated lungs, animals and patients were reported to have higher than expected tissue damage in unirradiated parts of the lung [7,8]. The mechanism for this hypersensitivity of non-targeted responses has not been elucidated, but inflammatory responses and reactive oxygen species, such as superoxide radicals, are involved [2,9].

We previously published that bystander effects were observed after boron neutron capture therapy (BNCT), and found that the radical scavenger ascorbate could effectively protect from distant damage [10]. Another group reported that radical scavengers were protective against radiation-induced bystander effects in an in vitro study [11]. Here, we investigated the possibility that 2-O-alpha-D-glucopyranosyl-L-ascorbic acid (AA-2G) had clinically relevant

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doi:10.1016/j.mrgentox.2009.12.002

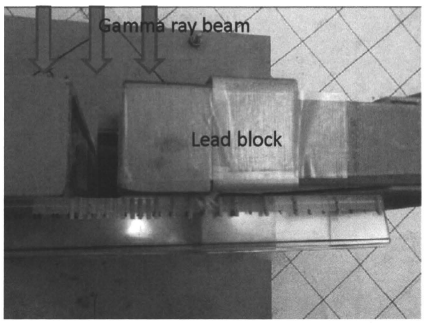


**Fig. 1.** A view from above showing the strategy for local head irradiation. The head of a mouse was irradiated while being held in a restrainer through a 2 cm slit, while the rest of its body was located behind a 5 cm lead block.

radio-protective effects. Its radio-protective effects were evaluated after induction of apoptosis in mouse splenocytes and micronuclei in splenic T cells, at a site that was distant from local head irradiation.

## 2. Materials and methods

### 2.1. Mice and ascorbic acid administration

Six-week-old female C3H/He mice were obtained from Japan Animal Inc. and acclimated to our laboratory for 8–10 weeks prior to use in experiments. AA-2G was purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). C3H female mice (14–16 weeks old) were given AA-2G orally (dissolved in water, 1 mg/g of body weight), 1 h before gamma-ray irradiation. The concentration of AA-2G administration was decided after referencing previous experiments [12–14] and considering the high plasma accumulation and toxicity of ascorbic acid. Note: 1 mg of AA-2G is the equivalent of 0.52 mg of ascorbic acid. The ascorbic acid concentration in mouse plasma after AA-2G administration was measured by HPLC.

### 2.2. Irradiation

Gamma rays were delivered with a $^{60}$Co gamma-ray machine at a rate of 1.0 Gy/min. Mice were restrained in a plastic box on a radiation shelf. For partial head irradiation, heads were placed in a 2.0 cm slit in the front side of the restrainer and the rest of their bodies were shielded behind a 5 cm-thick lead block (Fig. 1). The absorbed doses for the head and the body are shown in Fig. 2. For total body irradiation (to evaluate direct splenic damage following irradiation), the whole body was irradiated (up to 5 Gy).

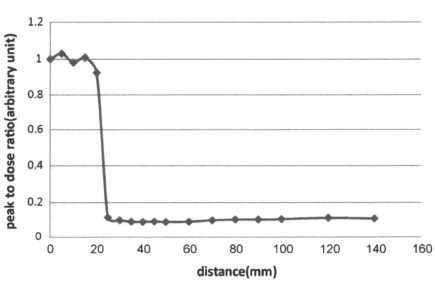


**Fig. 2.** Estimation of the amount of radiation passing through the 2 cm slit (the irradiated head dose) and into the area shielded by the lead block (the spleen dose). Each point corresponds to a red mark on the scale in Fig. 1. The intervals between the points were 5 mm (0–5 cm), 1 cm (5–10 cm) and 2 cm (10–14 cm). The head of the mouse was located in the first 2 cm (in the open region). The spleen was located at 4 cm. The dose given to the spleen was estimated at 1 Gy when 10 Gy of irradiation was given to the head.

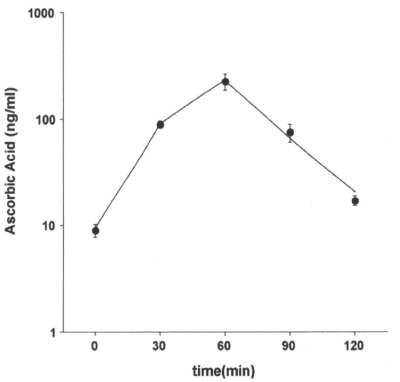


**Fig. 3.** The concentration of ascorbic acid in mouse plasma after AA-2G administration.

### 2.3. Isolation of splenocytes and splenic T lymphocytes

Details of the T lymphocyte isolation have been described elsewhere [15]. Briefly, after gamma irradiation, mice were sacrificed by cervical dislocation, and their spleens were removed, minced and washed twice in Hanks' balanced salt solution. Lymphocytes were separated using Ficoll-Hypaque gradients and were resuspended in RPMI 1640 medium (GIBCO) containing 10% fetal calf serum. The T lymphocytes were cultured at 37 °C in a humidified 5% $CO_2$ incubator. Optimum concentrations of Concanavalin A (Con A, 2 μg/mL) and 2-mercaptoethanol (2-ME, 50 μmol/mL) were used to make lymphocytes transform and divide in culture.

### 2.4. Radiation induced apoptosis and antioxidant enzyme activation

To determine splenocyte apoptosis, mice were sacrificed 24 h after irradiation and their spleens were removed. Single-cell suspensions were eliminated of erythrocytes by incubating at room temperature for 3 min in a solution of Tris-

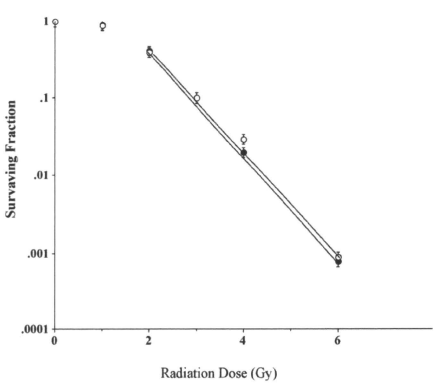


**Fig. 4.** Survival curves for splenic T lymphocytes following gamma irradiation with (open circle) or without (closed circle) ascorbic acid administration. Data represent the means ± SE of three different independent experiments. The curves for doses greater than 2 Gy were fit by linear regression analysis.

**Table 1**
Survival parameters for T lymphocytes after gamma ray irradiation with V.C. (ascorbic acid) treatment.

| Treatment | $D_0$ | $D_{10}$ |
|---|---|---|
| Gamma ray radiation | 0.65 ± 0.2 Gy | 3.0 ± 0.2 Gy |
| Gamma ray radiation with V.C. treatment | 0.85 ± 0.3 Gy | 3.1 ± 0.2 Gy |

Data pooled from three or more experiments; mean ± SE. $D_0$ and $D_{10}$ derived from survival curves following irradiation.

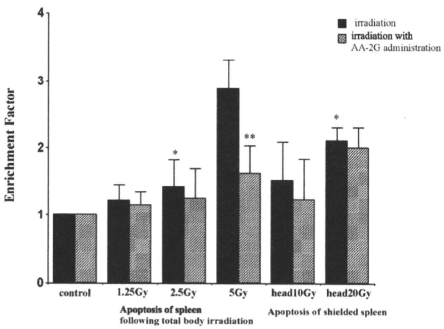


**Fig. 5.** Induction of apoptosis of mouse splenocytes after irradiation (black bars) and the effect of AA-2G administration (gray bars). Histogram bars show the means ± SE for five animals. (*) Significant increase in apoptosis compared to 2.5 Gy total body irradiation. $p < 0.05$. (**) Significant decrease in apoptosis compared to no ascorbic acid administration. $p < 0.05$.

buffered ammonium chloride. After twice washing with PBS, cells were counted and examined for induction of apoptosis. Apoptosis was detected with a sandwich immunoassay system using a cell death detection ELISA kit (Roche Diagnostic Inc.). The assay is based on the quantitative sandwich enzyme immunoassay principle, using mouse monoclonal antibodies directed against DNA and histones, respectively. Apoptosis was measured by following the ELISA protocol. The enrichment factor was the calculated absorbance of each sample divided by the absorbance of corresponding negative control. To measure the activity of catalase (CAT) and superoxide dismutase (SOD), mouse blood was obtained from the main inferior vein 60, 120 and 1200 min after irradiation. The activities of CAT and SOD in mouse plasma were detected using a colorimetric assay (SRL Research Laboratory Inc., Japan).

### 2.5. Determination of T lymphocyte survival and the micronucleus assay

Details of the assays for cell survival and micronucleus frequency have been reported previously [15]. Briefly, the survival data for lymphocytes were obtained by limiting dilution assays. To measure the cloning efficiency of lymphocytes, cells were seeded in culture medium (150 μL) at densities of 10–10,000 cells/well in 96-well tissue culture plates. The cloning efficiency was calculated from the proportion of the wells without clones, using limiting dilution analysis [16]. The cytokinesis-block micronucleus assay for lymphocytes was performed following a method described by Fenech and Morley [17] with slight modifications. Cytochalasin B (Sigma) was added to the cultured T lymphocytes at a final concentration of 5.0 μg/mL, 44 h after Con A stimulation. Eighteen hours later, cells were collected by centrifugation and resuspended in Carnoy's fixative. Next, a drop of the cell suspension was spread on a glass slide and dried, the cells were stained with Hoechst 33258 (50 μg/mL), and the frequency of micronuclei was determined on 10 separate slides by counting the total number of micronuclei per 100 binucleated cells.

**Table 3**
The micronucleus frequency per 100 binucleated T lymphocytes after irradiation and the effect of AA-2G administration.

| Total body irradiation (Gy) | | | Head irradiation 10 Gy |
|---|---|---|---|
| 0 Gy | 1.25 Gy | 2.5 Gy | |
| 2.8 ± 1.5 | 26.8 ± 6.5 | 47.9 ± 13.2 | 62.1 ± 18.5 |
| With AA-2G treatment | | | |
| 2.9 ± 1.5 | 14.9 ± 8.5** | 29.1 ± 11.5** | 24.9 ± 10.5** |

Results show the mean ± SE from at least three independent experiments.
** Significant differences were observed with AA-2G administration (Student's *t*-test; $p < 0.05$).

### 2.6. Statistical analysis

Significance was calculated using Student's tests. Results were considered significant for values of $p < 0.05$.

## 3. Results

### 3.1. The effect of ascorbic acid treatment

The ascorbic acid concentration in mouse plasma increased and was maintained at a high level during the 30–90 min after oral administration of AA-2G. One hour after AA-2G administration (1 mg/g of mouse body weight), the concentration of ascorbic acid in the plasma was increased 4–10× over the control (Fig. 3). The plasma level of ascorbic acid increased sharply, as quickly as 30 min, and was maintained at a high level for 1.5 h after oral administration of AA-2G. The availability of AA-2G as ascorbic acid was compatible with a previous report [13].

Fig. 4 and Table 1 show survival curves and the parameters for T lymphocytes after gamma irradiation, with and without ascorbic acid treatment. These results showed that gamma radiation lethality was not affected by ascorbic acid treatment.

### 3.2. Induction of apoptosis and the activities of anti-oxidative enzymes

Apoptosis was analyzed after 1.25, 2.5 and 5 Gy of whole body irradiation and 10 and 20 Gy of local head irradiation, with and without ascorbic administration. For local head irradiation, the spleen was shielded behind a 5 cm-thick lead block (Fig. 1) and the doses of radiation absorbed by the spleen and head were measured (1.0 Gy for the spleen from 10 Gy of radiation exposure to the head; 2.0 Gy (spleen) from 20 Gy (head); Fig. 2). After 20 Gy head irradiation, the apoptosis that occurred in shielded spleen cells exceeded that which occurred when spleen cells were directly irradiated with 2.5 Gy (Fig. 5). Therefore, the damage to shielded spleen cells was more excessive than what would be expected given a dose of 2.0 Gy.

Fig. 5 also shows the induction of apoptosis in mouse splenocytes after AA-2G treatment. Ascorbic acid protected mouse

**Table 2**
SOD and catalase activity in mouse serum after treatment with AA-2G.

| Time after irradiation (min) | SOD activity (Units/mL) | | Catalase activity (Units/mL) | |
|---|---|---|---|---|
| | 6 Gy irradiation | 6 Gy irradiation with AA-2G | 6 Gy irradiation | 6 Gy irradiation with AA-2G |
| 0 | 7.7 ± 0.8 | 7.1 ± 0.7 | 1.0 ± 0.1 | 1.0 ± 0.1 |
| 60 | 9.2 ± 0.9 | 10.4 ± 1.0 | 3.6 ± 0.4 | 4.2 ± 0.4 |
| 120 | 10.0 ± 0.9 | 11.0 ± 1.0 | 3.6 ± 0.4 | 4.4 ± 0.4 |
| 1200 | 12.5 ± 1.0 | 14.0 ± 1.4 | 2.2 ± 0.2 | 4.4 ± 0.4** |

Results show the mean ± SE from at least three independent experiments.
** Significant increases were observed with AA-2G administration (Student's *t*-test; $p < 0.05$).

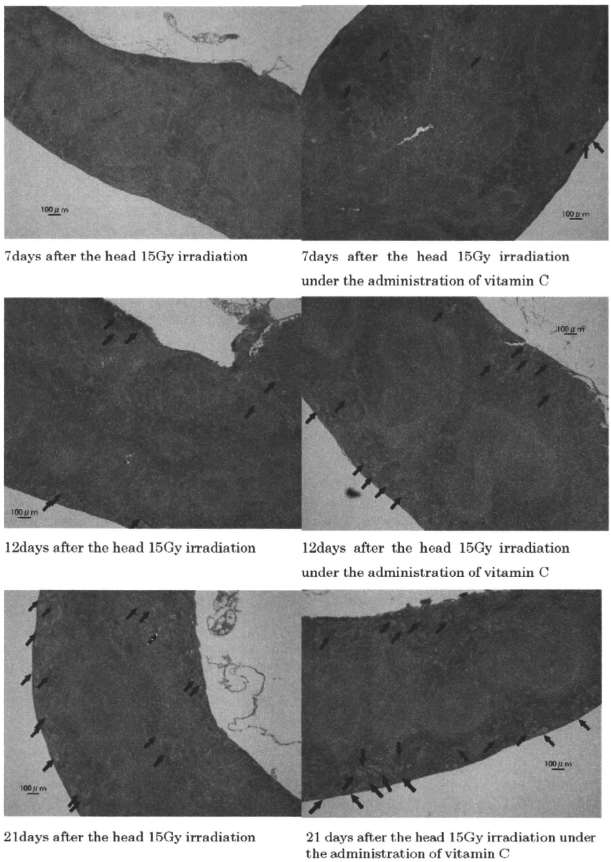


**Fig. 6.** Spleens 7–21 days after 15 Gy of head irradiation. Formalin-fixed paraffin-embedded tissue sections were HE stained. The arrows show macrophage proliferation in the spleen after the head irradiation (magnification 30×. Scale bars 100 μm).

splenocytes from apoptosis after 5 Gy whole-body irradiation. To understand how anti-oxidative enzymes participate in initial DNA damage, we investigated how treating with AA-2G affected the activation of the CAT and SOD in the plasma following a dose of 6 Gy (1 Gy in addition to the 5 Gy irradiation). AA-2G administration increased the activation of CAT and SOD slightly. A significant increase in CAT activity was observed with AA-2G treatment 20 h after irradiation (Table 2).

### 3.3. Micronucleus induction

The induction of micronuclei in mouse T lymphocytes after various treatments is shown in Table 3. Micronuclei were counted after 1.25 and 2.5 Gy of whole body irradiation and 10 Gy of local head irradiation, with and without ascorbic administration. As mentioned above, a 10 Gy dose of radiation exposure to the head corresponded to a dose of 1.0 Gy for the shielded spleen. The frequency of micronuclei induced in shielded splenic T cells with 10 Gy of head irradiation was about 2 times higher than that induced after 2.5 Gy of whole body irradiation (Table 3). Our results confirm that shielded, distant splenic T lymphocytes had more damage than what would have been anticipated, given a dose of 1.0 Gy. This again suggests that the radiation effects in a distant, shielded organ were enhanced after local irradiation. However, while treatment with ascorbic acid protected, although not significantly, against the induction of apoptosis in shielded splenocytes, ascorbic acid significantly inhibited shielded splenic T lymphocytes from forming micronuclei after local head irradiation (Fig. 5 and Table 3). Therefore, unlike its effects on induction of mouse splenocyte apoptosis, AA-2G had radioprotective

effects on the induction of T lymphocyte micronuclei after irradiation.

## 4. Discussion

In high LET therapy, as occurs in boron neutron capture therapy (BNCT) and heavy-ion radiotherapy, hypo-fractionation is acceptable and therapeutic radiation doses are larger than conventional radiotherapies. The normal tissue radiation dose for vascular endothelial cells in BNCT is estimated to be around 10–15 Gy [18]. Enhanced radiation effects in abscopal regions following large local doses of radiation have not previously been investigated. Here, we studied whether shielded splenocytes in abscopal regions suffered enhanced radiation effects after 10–20 Gy of local head gamma ray-irradiation. Splenocyte apoptosis induction and T lymphocyte micronuclei were higher than what would be expected with the estimated dose of radiation in the distant, shielded spleen.

In vivo radiation-induced bystander effects are defined as phenomena that occur when irradiation signals are transduced from an irradiated lesion to a shielded organ and induce a radiation effect in that non-irradiated, shielded organ. A previous group reported that the spleen is a target organ of local-irradiation induced bystander effects in vivo. Koturbash et al. described how cranial X-ray irradiation (1 Gy) induced DNA damage, apoptosis, and increased p53 levels in a shielded spleen [5]. They also suggested the possibility that the induction of indirect DNA damage in the shielded splenocytes was mediated by reactive oxygen species. Mechanistically, the radiation-induced bystander effect in vivo is thought to be mediated by the inflammatory response after exposure to ionizing radiation. Lorimore et al. reported that macrophage activation following a 4 Gy irradiation provided a mechanism for producing damage via bystander effects [19]. Another previous report showed that tumor cell killing by macrophages was activated with more than 10 Gy [20]. These experiments show that high dose radiation might induce bystander signaling by mediating macrophage activation. We confirmed that large dose local head irradiation (10–20 Gy) induced apoptosis and micronuclei in the distant, shielded spleen. These enhanced radiation effects in an abscopal region were induced by a large dose irradiation and may have been mediated by macrophage activation. Shown in the left column of Fig. 6, histological sections revealed macrophage proliferation in the spleen appearing on the 12th day post-irradiation and becoming severe by day 21. Macrophage proliferation in the spleen was found to be more severe after ascorbic acid administration, and to occur earlier (on the 7th day post-irradiation; shown in the right column of Fig. 6).

We evaluated the protective effects of a radical scavenger on enhanced radiation effects in the distant spleen after large doses of local head irradiation. Free radicals are one of the most important bio-chemicals that are triggered by the activation of macrophages following irradiation [21]. This suggests the possibility that radical scavengers might protect abscopal regions from enhanced radiation effects. Our study of apoptosis induction suggests that AA-2G treatment suppressed the induction of apoptosis following total body radiation. In our anti-oxidative enzyme study, AA-2G administration increased catalase activity after 20 h, which was the length of the apoptosis assay, but AA-2G did not significantly affect SOD activity. The C3H/He mouse strain is more radiation-resistant than other Balb/c mouse strains [22,23]. The different sensitivities of mouse strains to irradiation were determined by micronucleus formation in T lymphocytes and fibroblasts, and an intestinal cell survival assay [15]. A previous report demonstrated that hepatic CAT and SOD enzyme activities increased 30 min after whole body ionizing irradiation of C3H mice, suggesting that CAT and SOD may be related to the mechanism of their radiation resistance [24]. We confirmed that these antioxidant enzymes had elevated activities after irradiation and that AA-2G enhanced CAT activity. This result suggests that ascorbic acid may protect from radiation damage by inducing CAT.

We previously reported that radical scavengers are protective against neutron-induced mutations [25,26]. Furthermore, we compared the effects of DMSO (a source of short-lived radical scavengers) and ascorbic acid (a source of long-lived radical scavengers) on the induction of mutations in bystander cells. DMSO treatment slightly reduced the frequency of mutations that were induced by the bystander effect, but post-radiation ascorbic-acid treatment reduced the mutation frequency more than DMSO [10]. Recently, Harada et al. reported that ascorbic acid was an effective radical scavenger for suppressing the bystander response in vitro. They examined three types of radical scavengers, including a nitric oxide scavenger, and found that ascorbic acid was the most effective suppressor of micronucleus induction in non-irradiated bystander cells [27].

We showed that ascorbic acid significantly inhibited shielded splenic T lymphocytes from forming micronuclei following local head irradiation (Fig. 5 and Table 3). However, AA-2G did not protect shielded splenocytes against apoptosis. These results show that AA-2G treatment had radio-protective effects on T-lymphocytes in abscopal regions (in the spleen), but this did not apply to all splenic cells.

Clinically, chromosomal instability [28] and epigenetic dysregulation of DNA [29] were analyzed in non-irradiated or distant organs after irradiation. Enhanced radiation effects in abscopal regions are thought to increase the incidence of secondary, post-radiation therapy cancers. Therefore, effective radioprotection from enhanced radiation effects in abscopal regions is needed. Ascorbic acid is a well-known, important vitamin and a non-toxic radical scavenger that can be effective for protecting against enhanced radiation effects in abscopal regions during radiation therapy.

### Conflict of interest

Authors declare that there are no conflicts of interest.

### Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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