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Impact of the p53 Status of the Tumor Cells on the Effect of Reactor Neutron Beam Irradiation, with Emphasis on the Response of Intratumor Quiescent Cells

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Human head and neck squamous cell carcinoma cells transfected with mutant p53 (SAS/mp53) or with neo vector as a control (SAS/neo) were inoculated subcutaneously into both the hind legs of Balb/cA nude mice. Tumor-bearing mice received 5-bromo-2′-deoxyuridine (BrdU) continuously to label all proliferating (P) cells in the tumors. After administration of sodium borocaptate-^{10}B (BSH) or *p*-boronophenylalanine-^{10}B (BPA), the tumors were irradiated with neutron beams. The tumors not treated with ^{10}B-compound were irradiated with neutron beams or γ-rays. The tumors were then excised, minced and trypsinized. The tumor cell suspensions thus obtained were incubated with a cytokinesis blocker, and the micronucleus (MN) frequency in cells without BrdU labeling (=quiescent (Q) cells) was determined using immunofluorescence staining for BrdU. Meanwhile, 6 h after irradiation, tumor cell suspensions obtained in the same manner were used for determining the frequency of apoptosis in Q cells. The MN and apoptosis frequencies in total (P+Q) tumor cells were determined from the tumors that were not pretreated with BrdU. Without ^{10}B-carriers, in both tumors, the relative biological effectiveness of neutrons was greater in Q cells than in total cells, and larger for low than high cadmium ratio neutrons. With ^{10}B-carriers, the sensitivity was increased for each cell population, especially for total cells. BPA increased both frequencies for total cells more than BSH. Nevertheless, the sensitivity of Q cells treated with BPA was lower than that of BSH-treated Q cells. These sensitization patterns in combination with ^{10}B-carriers were clearer in SAS/neo than in SAS/mp53 tumors. The p53 status of the tumor cells had the potential to affect the response to reactor neutron beam irradiation following ^{10}B-carrier administration.

Key words: p53 — Apoptosis — Micronucleus — Neutron capture therapy — Quiescent cell

Recently, it has been shown that the *p53* tumor suppressor gene serves a critical role in maintaining genomic stability during the cell cycle checkpoint in G1 and G2/M transition,[1, 2] and as an effector of DNA repair[3, 4] and apoptosis.[5, 6] Wild-type p53 is needed to activate apoptosis in sensitive cells in response to DNA damage.[7] 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 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.[8] Thus, the genetic and functional status of the *p53* gene is an important factor in guiding therapeutic strategies for cancer patients.

The neutron capture reaction in boron (^{10}B(n,α)^{7}Li) is, in principle, very effective in destroying tumors, provided that a sufficient amount of ^{10}B can be accumulated in the target tissue and a sufficient number of very low energy thermal neutrons can be delivered there. The two particles generated in this reaction show high linear energy transfer (LET) and have a range of approximately 14 μm or roughly the diameter of one or two cells. Thus, most of the ionizing energy imparted to tissue is localized to ^{10}B-loaded cells, resulting in induction of a high relative biological effectiveness (RBE) when ^{10}B can be distributed homogeneously in the target tissue.[9] However, at present, it is difficult to deliver a sufficient amount of ^{10}B nearly homogeneously throughout the target tumor tissue.[10]

Many of the cells in solid tumors are non-proliferating (quiescent, Q). To improve cancer treatment, the response of Q cells in solid tumors to various anticancer treatments should be determined, since many tumor cells are quiescent *in situ* but are still clonogenic.[11, 12] In analyzing the response of Q cells in solid tumors, we have developed a method utilizing both the micronucleus (MN) assay and the identification of proliferating (P) cells with 5-bromo-2′-deoxyuridine (BrdU) and anti-BrdU monoclonal antibody.[13] Micronuclei appear in dividing cells with chromosomal aberrations after irradiation. The frequency of their

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appearance correlates with the dose of radiation and extent of cell killing.[14, 15] However, some cells die through apoptosis after irradiation and this is related to the tumor sensitivity to irradiation.[16] Very recently, our method for detecting the Q cell response to irradiation using P cell labeling with BrdU and the MN frequency assay was also shown to be applicable to apoptosis detection assay.[17, 18]

Accordingly, to investigate the relationship between the radiobiological responses of tumor cells, that is, total (P+Q) cells and Q cells in solid tumors, to reactor neutron beams following ^{10}B-carrier administration and the p53 status of the cells constituting the solid tumors, we analyzed the killing effects of neutron beams with two different cadmium (Cd) ratios on intratumor total and Q cells, using two different tumor cell lines with identical genetic backgrounds except for p53 status. The neutron capture reaction was performed using two different ^{10}B-carriers, $Na_2B_{12}H_{11}SH$ (sodium borocaptate-^{10}B, BSH) and *p*-boronophenylalanine-^{10}B (BPA), both of which have been clinically employed.[9]

MATERIALS AND METHODS

Cells, tumors and mice The human head and neck squamous cell carcinoma cell line SAS (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 humidified 5% CO_2 incubator. SAS cells show the phenotype of wild-type p53 in radiation- and heat-induced signal transduction.[19, 20] Plasmid pC53-248, which contains an *mp53* gene (codon 248, from Arg to Trp) producing a dominant negative mp53 protein, and plasmid pCMV-Neo-Bam, which contains a neo-resistance marker, were provided by B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). These plasmids 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.[21]

Cells were collected from exponentially growing cultures, and approximately 5.0×10^5 cells were inoculated subcutaneously into both 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.

Taking the potential of future enlargement of disease fields for neutron capture therapy (NCT) into consideration, we used the head and neck tumor model that is not glioma or melanoma, the only two diseases on which clinical NCT is actually performed at present.[9] Since head and neck tumors are often not so deep-seated as brain tumors and are comparatively well-defined, these tumors may represent a suitable site for NCT.

Irradiation Tumor-bearing mice then received γ-ray irradiation from a cobalt-60 γ-ray irradiator at a dose rate of approximately 5.0 Gy/min. Other tumor-bearing mice were irradiated with a neutron beam generated at the Kyoto University Reactor (KUR) with or without ^{10}B-carrier administration. A polyethylene holder on which 16 mice can be fixed radially was made and tumor-bearing mice were fixed in place with adhesives. Neutron beams with two different Cd ratios, 150 and 1.0, were used. The one with a Cd ratio of 150 includes the largest component of thermal neutrons available at our facility. The other with a Cd ratio of 1.0 contains the largest component of epithermal neutrons. In other words, a high Cd ratio means that thermal neutrons predominate and a low Cd ratio means that epithermal neutrons predominate. Only thermal neutrons can produce high LET and RBE particles through neutron capture reaction at the ^{10}B nucleus.[9] The neutron fluence was measured by radioactivation of gold foil (3 mm diameter, 0.05 mm thickness) both at the front and back of tumors. Since the tumors were small and located just beneath the surface, intratumor neutron fluence was assumed to decrease linearly from the front to the back of the tumors. Thus, we used the averaged neutron fluence from the measured values on both sides of tumors. γ-Ray doses, including secondary γ-rays, were measured with thermoluminescent dosimeter powder at the back of the tumors.[22] For the estimation of neutron energy spectra, eight kinds of activation foils and fourteen kinds of nuclear reactions were used. Neutron absorbed dose was calculated using flux-to-dose conversion factors.[23] The weight percentage of the tumors was assumed to be H (10.7%), C (12.1%), N (2%), O (71.4%) and others (3.8%).[24, 25] The average neutron fluence and Kerma rate, and the measured γ-ray dose rate for each neutron beam are shown in Table I. The Kerma rate for boron dose per F $n\cdot cm^{-2}\cdot s^{-1}$ of thermal neutron flux for 1 $\mu g\cdot g^{-1}$ of ^{10}B was 2.67×10^{-8} F $cGy\cdot h^{-1}$.

Determining the timing for apoptosis detection Without ^{10}B-carrier administration, γ-rays at a dose of 20 Gy or neutron beams were applied to the two different tumors. Neutron beams with a Cd ratio of 150 and 1.0 were applied for 165 min and 123 min, being equivalent to 6.4 Gy and 6.0 Gy as a physical absorbed dose, respectively. Thirty minutes after the intraperitoneal injection of BSH dissolved in physiological saline at a dose of 150 mg·kg^{-1} or 3 h after oral administration of BPA also dissolved in physiological saline at a dose of 750 mg·kg^{-1}, both tumors were irradiated with the neutron beam at a Cd ratio of 150 for 45 min. This irradiation was equivalent to 2.1 Gy as a physical absorbed dose (Fig. 1).

At various time points after irradiation, 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^{-1} 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, and at least 5 slides were counted per cell suspension. Standard criteria for the morphological characteristics of apoptosis were chromatin condensation, nucleolar disintegration and formation of crescent caps of condensed chromatin at the nuclear periphery.[26, 27] 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 irradiation experiment

Labeling with BrdU: Two weeks after tumor cell inoculation, mini-osmotic pumps (Durect Corporation, Cupertino, CA) containing BrdU dissolved in physiological saline (250 mg·ml^{-1}) were implanted subcutaneously to label all 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, respec-

Table I. Neutron Flux (n·cm^{-2}·s^{-1}) and Kerma Rate (cGy·h^{-1}) for Each Neutron Beam

Cadmium ratio	Thermal neutrons (≤0.6 eV)	Epithermal neutrons (0.6–10 keV)	Fast neutrons (≥10 keV)	γ-Rays
150				
Neutron flux	2.0×10^{9}	2.5×10^{7}	6.6×10^{6}	
Kerma rate	98.1	0.384	22.9	100
1.0				
Neutron flux	3×10^{7}	7.3×10^{8}	4.7×10^{7}	
Kerma rate	0.24	22	160	70

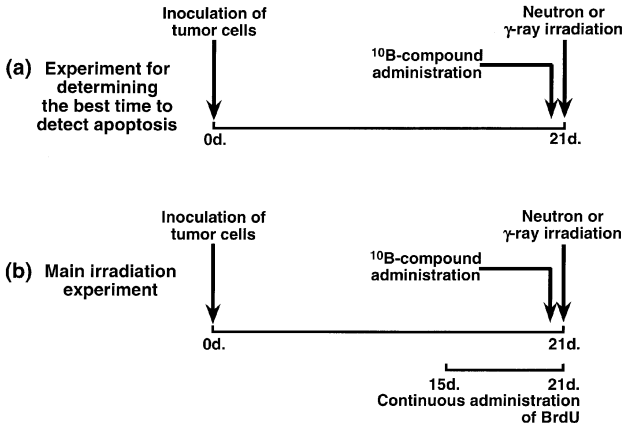

Fig. 1. Flow diagram summarizing the experiment for determining the best time to detect apoptosis (a) and the main irradiation experiment (b).

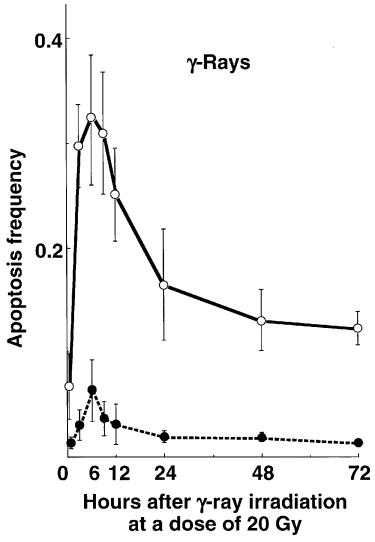

Fig. 2. Effect of time after γ-ray irradiation at a dose of 20 Gy on apoptosis frequency. The time course of change in the values of the apoptosis frequency for SAS/neo tumor cells (open circles and solid lines) and SAS/mp53 tumor cells (solid circles and dotted lines). Bars represent 95% confidence limits.

tively, and reached a plateau level at these stages. Therefore, in this study, we regarded tumor cells not incorporating BrdU after continuous labeling as Q cells.

Irradiation treatment: After labeling with BrdU, the tumor-bearing mice that were not given ^{10}B-carrier received irradiation from the cobalt-60 γ-ray irradiator at doses of 4, 11 and 18 Gy or from the reactor neutron beam with a Cd ratio of 150 at physical absorbed doses of 2.4, 4.7 and 6.4 Gy or with a Cd ratio of 1.0 at physical absorbed doses of 1.8, 3.9 and 6.0 Gy. Other tumor-bearing mice that were given ^{10}B-carrier received the neutron beam with a Cd ratio of 150 at physical absorbed doses of 0.7, 1.4 and 2.1 Gy.

Each treatment group also included mice that were not pretreated with BrdU (Fig. 1(b)).

Some of the tumors that were not irradiated were used to determine the ^{10}B concentration in the tumors. The ^{10}B concentration in the tumors was measured by prompt γ-ray spectrometry using a thermal neutron guide tube at KUR.[18)]

Immunofluorescence staining of BrdU-labeled cells and observation of apoptosis and MN formation Based on the result of the experiment for determining the timing of apoptosis detection, 6 h after irradiation for apoptosis assay, and right after irradiation for 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 h in tissue culture dishes containing complete medium and 1.0 $\mu g \cdot ml^{-1}$ 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 immunoglobulin G (whole molecule) antibody (Sigma, St. Louis, MO). 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 irradiation) 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, 27)]

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.

Needless to say, the induction of a MN requires division of the cell nucleus.[13)] The duration of incubation with cytochalasin-B allowed Q cells to be recruited into the cell cycle. Thus, the optimal incubation period was determined so that the maximum rate of binuclear tumor cells could be observed. The frequencies of apoptosis and MN for BrdU-labeled cells were modified because the radiosensitization effect of the incorporated BrdU could potentially influence the frequencies in BrdU-labeled cells. Thus, the correct frequencies of BrdU-labeled cells without the BrdU effect are not able to be determined. During continuous labeling with BrdU, the shift of cells from P to Q population could result in labeled Q cells. These cells were excluded when we scored micronuclei in binuclear cells and apoptotic cells in tumor cells showing only red fluo-

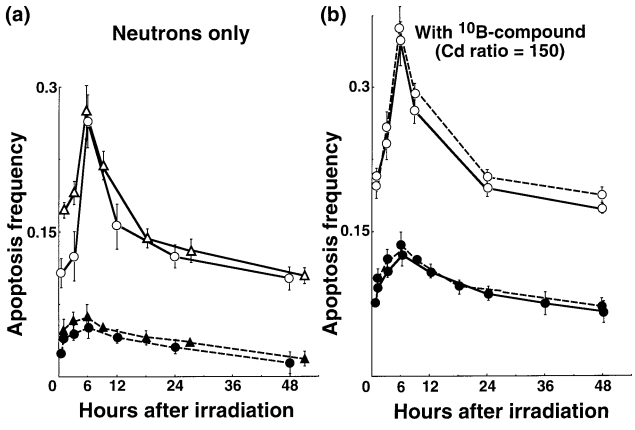

Fig. 3. Effect of time after neutron beam irradiation with a cadmium (Cd) ratio of 150 (circles) or 1.0 (triangles) on apoptosis frequency. The time course of change in the values of the apoptosis frequency for SAS/neo tumor cells (open symbols) and SAS/mp53 tumor cells (solid symbols) following irradiation alone (a) and irradiation after BSH (solid lines) or BPA (dotted lines) administration (b). BSH, sodium borocaptate-^{10}B; BPA, *p*-boronophenylalanine-^{10}B. Bars represent 95% confidence limits.

Table II. Plating Efficiencies, Micronucleus Frequencies and Apoptosis Frequencies at 0 Gy

Tumor cell fraction	No ^{10}B-carrier	BSH[a)]	BPA[b)]
Plating efficiency for total cells (%)			
SAS/neo	57.0 (50.0–64.0)[c)]	39.5 (33.7–45.8)	34.7 (24.7–44.7)
SAS/mp53	46.5 (40.4–52.6)	37.1 (33.5–40.7)	28.5 (21.3–35.7)
Apoptosis frequency			
SAS/neo			
Total cells	0.030 (0.020–0.040)	0.051 (0.042–0.060)	0.059 (0.047–0.071)
Quiescent cells	0.043 (0.034–0.052)	0.058 (0.045–0.071)	0.082 (0.067–0.097)
SAS/mp53			
Total cells	0.009 (0.007–0.011)	0.011 (0.008–0.014)	0.019 (0.014–0.024)
Quiescent cells	0.012 (0.008–0.016)	0.016 (0.012–0.020)	0.029 (0.020–0.038)
Micronucleus frequency			
SAS/neo			
Total cells	0.036 (0.030–0.042)	0.060 (0.048–0.072)	0.095 (0.077–0.113)
Quiescent cells	0.050 (0.037–0.063)	0.090 (0.071–0.109)	0.11 (0.095–0.125)
SAS/mp53			
Total cells	0.067 (0.055–0.079)	0.090 (0.070–0.110)	0.11 (0.090–0.130)
Quiescent cells	0.074 (0.061–0.087)	0.094 (0.071–0.117)	0.13 (0.105–0.155)

a) Sodium borocaptate-^{10}B.
b) *p*-Boronophenylalanine-^{10}B.
c) Numbers in parentheses are 95% confidence limits.

rescence by PI for DNA staining, because these cells were stained with FITC.

Cell survival assay The cell survival assay was also performed in mice given no BrdU using an *in vivo-in vitro* assay method. Tumors were disaggregated by stirring for 20 min at 37°C in PBS containing 0.05% trypsin and 0.02% EDTA. The cell yield was 1.5 $(1.2–1.8)\times10^7$ g^{-1} and 3.4 $(2.6–4.2)\times10^6$ g^{-1} 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 irradiation 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.*[19)] Not only the level, but also the function of p53 protein could 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 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

The ^{10}B concentrations for the BSH and BPA administration groups were 14.6 ± 1.4 μg/g and 16.0 ± 2.8 μg/g in SAS/neo tumors, and 13.4 ± 1.2 μg/g and 15.5 ± 2.8 μg/g in SAS/mp53 tumors, respectively. There were no significant differences.

Figs. 2 and 3 show the time course of the change in the values of the apoptosis frequency for each tumor cell line following γ-ray irradiation (Fig. 2) or neutron beam irradiation with a Cd ratio of 150 or 1.0 (Fig. 3). Whichever irradiation was employed, the apoptosis frequency showed

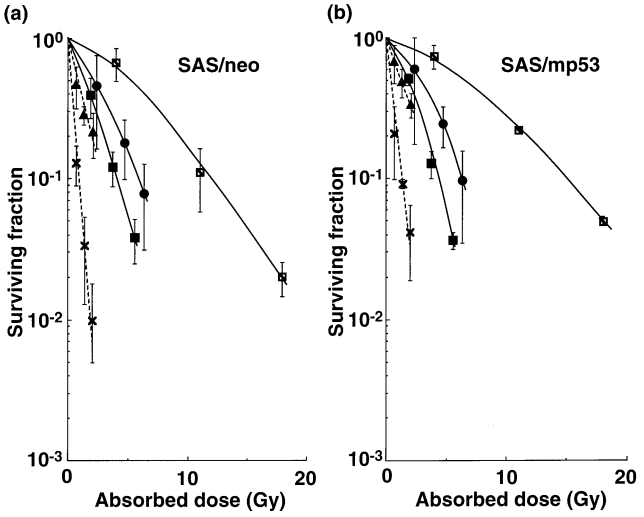

Fig. 4. Cell survival curves for SAS/neo tumor cells (a) and SAS/mp53 tumor cells (b). Tumors were irradiated with γ-rays (▨), reactor neutron beams alone with a cadmium (Cd) ratio of 150 (●) or 1.0 (■), or reactor neutron beams with a Cd ratio of 150 following BSH (▲) or BPA (✖) administration. BSH, sodium borocaptate-^{10}B; BPA, *p*-boronophenylalanine-^{10}B. Bars represent 95% confidence limits.

Table III. Dose Modifying Factors[a)] for SAS/mp53 Tumor Cells Relative to SAS/neo Tumor Cells in Cell Survival Curves

Treatment	Surviving fraction	
	0.05	0.3
γ-Rays	1.2 (1.1–1.3)[b)]	1.25 (1.15–1.35)
Neutron beam (Cd=1.0)	1.0 (1.0–1.1)	1.1 (1.0–1.2)
Neutron beam (Cd=150)	—	1.1 (1.0–1.2)
BSH[c)] on Cd:150 neutron beam	—	1.3 (1.2–1.4)
BPA[d)] on Cd:150 neutron beam	1.3 (1.2–1.4)	1.35 (1.2–1.5)

a) 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.
b) 95% confidence limits.
c) Sodium borocaptate-^{10}B.
d) *p*-Boronophenylalanine-^{10}B.

a maximum value 6 h after irradiation in each cell line when no ^{10}B-carrier was administered. Even when ^{10}B-carrier was administered before the neutron beam irradiation with a Cd ratio of 150, the maximum value of the apoptosis frequency was also observed 6 h after irradiation in both tumor cells. On the whole, the values for SAS/neo tumor cells were significantly higher than those for SAS/mp53 cells ($P<0.05$) throughout all these experiments.

Table II shows the plating efficiencies for total tumor cells and the apoptosis and MN frequencies without 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, though the differences were not significant.

Fig. 4 shows the cell survival curves for SAS/neo tumor cells (a) and SAS/mp53 tumor cells (b) as a function of the physical absorbed radiation dose. 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 cells with the doses required in SAS/neo tumor cells (Table III). The values of the DMF were significantly larger than 1.0 for the treatment with γ-ray irradiation alone or neutron beam irradiation following ^{10}B-carrier administration.

Figs. 5 and 6 show the normalized apoptosis and MN frequencies for each irradiation condition as a function of the physical absorbed radiation dose in total and Q tumor cells within SAS/neo and SAS/mp53 tumors. When the ^{10}B-carrier was administered before neutron beam irradiation, even if no radiation was given, these frequencies were higher than when no ^{10}B-carrier was administered, because of the slight cytotoxicity of the drug (Table II). Therefore, for background correction, we used the normalized frequency to exclude the effects of the cytotoxicity of the ^{10}B-carrier on the frequency. The normalized frequency is the frequency in the irradiated tumors minus the frequency in the nonirradiated tumors. All frequencies of apoptosis and MN were actual values induced at each irradiation condition. Namely, they include the values induced by contaminating fast neutrons and γ-rays in each neutron beam.

On the whole, SAS/neo cells showed significantly higher normalized apoptosis frequencies ($P<0.05$) and, although not significantly, lower normalized MN frequencies than SAS/mp53 cells.

Without the ^{10}B-carrier, the normalized apoptosis and MN frequencies were lower in Q cells than in the total tumor cells, especially in the case of γ-ray irradiation. We calculated the DMFs of Q cells in tumors not treated with ^{10}B-carriers; these factors were used to compare the radiation doses necessary to obtain various normalized frequencies in Q cells with the doses required in the total tumor

cells. For this calculation, we used the values from tumors excised after irradiation alone without ^{10}B-carrier administration, as shown in Figs. 5 and 6 (Table IV). In both tumor cells, the values of DMF for γ-rays were significantly higher than 1.0 ($P<0.05$). In contrast, for neutron irradiation at each Cd ratio, the values were nearer to 1.0 than those for γ-rays. To investigate the RBE of irradiation with neutron beams, DMFs, which compare the radiation doses necessary to obtain various surviving fractions (SFs) or normalized frequencies in tumors irradiated with γ-rays with those in tumors irradiated using neutron beams without ^{10}B-carrier administration at each Cd ratio, were calculated using the data shown in Figs. 5 and 6 (Table V). These values of DMFs are equivalent to those of RBE. On the whole, all were significantly greater than 1.00 ($P<0.05$), and the values for Q cells were larger than those for total tumor cells. Moreover, in each cell population, the values for low Cd ratio neutron beams tended to be larger than those for high Cd ratio neutron beams.

With ^{10}B-carriers, in both tumors, the SFs for total cells were decreased and the normalized apoptosis and MN frequencies for each cell population in both tumors were increased. To assess the effect of ^{10}B-carrier administration on each endpoint in total and Q cell populations, the enhancement ratio (ER) was calculated at various endpoints using the data given in Figs. 5 and 6 (Table VI). The ER values for BPA administration were significantly larger than those for BSH in total tumor cells ($P<0.05$). This tendency was more clearly observed in SAS/neo than in SAS/mp53. In contrast, in both tumor cells, the values for BSH were larger than those for BPA in Q cells, although not significantly so.

In both total and Q cell populations, similar results concerning the DMF values of Q cells and the RBE and ER values were obtained, whether based on the data of the apoptosis frequency or those of the MN frequency.

DISCUSSION

As shown in our previous report,[28] in both tumor cell lines, the apoptosis frequency showed a similar time course of change after irradiation whether a conventional TUNEL (deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling) method or the fluorescence staining method employed here was used. Hence, as far as these two tumor cell lines are concerned, the fluorescence staining method was thought to reflect apoptotic events as well as the conventional TUNEL method. However, the contribution of apoptosis to determining the response of tumors depends on the cell type,[16]

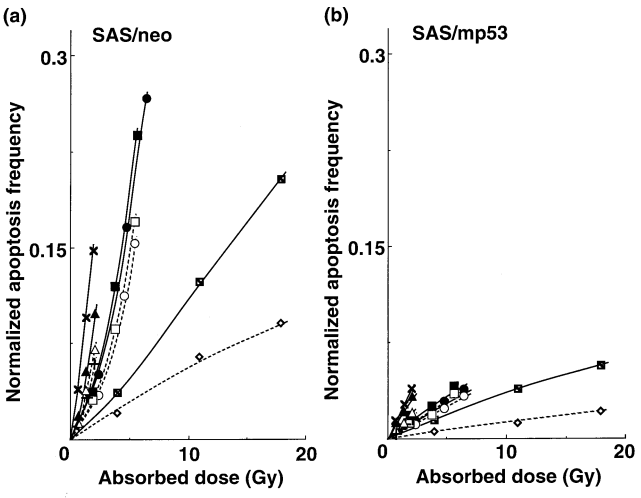

Fig. 5. Dose-response curves of normalized apoptosis frequency as a function of radiation dose for total (▧, ●, ■, ▲, ✖) and quiescent (◇, ○, □, △, ＋) tumor cell populations in SAS/neo (a) and SAS/mp53 (b) tumors. Tumors were irradiated with γ-rays (▧, ◇), reactor neutron beams alone with a cadmium (Cd) ratio of 150 (●, ○) or 1.0 (■, □), or reactor neutron beams with a Cd ratio of 150 following BSH (▲, △) or BPA (✖, ＋) administration. BSH, sodium borocaptate-^{10}B; BPA, *p*-boronophenylalanine-^{10}B. Only mean values are shown, to avoid confusion.

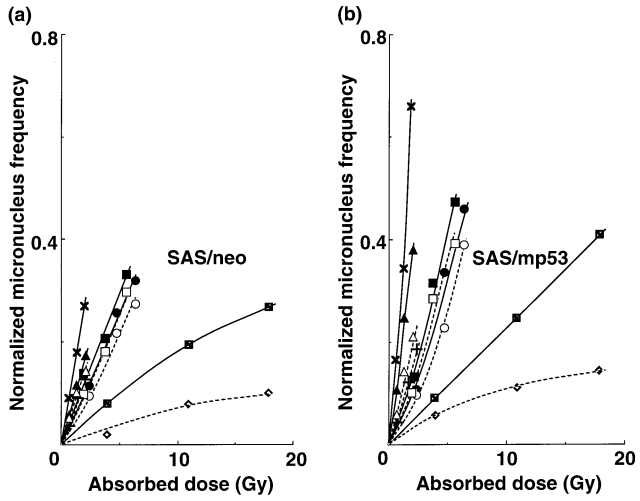

Fig. 6. Dose-response curves of normalized micronucleus frequency as a function of radiation dose for total (▧, ●, ■, ▲, ✖) and quiescent (◇, ○, □, △, ＋) tumor cell populations in SAS/neo (a) and SAS/mp53 (b) tumors. Tumors were irradiated with γ-rays (▧, ◇), reactor neutron beams alone with a cadmium (Cd) ratio of 150 (●, ○) or 1.0 (■, □), or reactor neutron beams with a Cd ratio of 150 following BSH (▲, △) or BPA (✖, ＋) administration. BSH, sodium borocaptate-^{10}B; BPA, *p*-boronophenylalanine-^{10}B. Only mean values are shown, to avoid confusion.

Table IV. Dose Modifying Factors[a)] for Quiescent Tumor Cells Relative to the Total Tumor Cell Populations

Tumor	End point	Neutron beam		γ-Rays
		Cd ratio:1.0	Cd ratio:150	
Normalized apoptosis frequency[b)]				
SAS/neo	0.05	1.25 (1.1–1.4)[c)]	1.3 (1.15–1.45)	1.7 (1.5–1.9)
	0.02	1.2 (1.1–1.3)	1.3 (1.15–1.45)	1.5 (1.3–1.7)
SAS/mp53	0.02	1.15 (1.05–1.25)	1.2 (1.1–1.3)	1.8 (1.55–2.05)
Normalized micronucleus frequency[b)]				
SAS/neo	0.1	1.25 (1.1–1.4)	1.3 (1.15–1.45)	2.0 (1.8–2.2)
SAS/mp53	0.2	1.15 (1.05–1.25)	1.2 (1.1–1.3)	—
	0.1	1.2 (1.1–1.3)	1.3 (1.15–1.45)	2.0 (1.8–2.2)

a) 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.
b) Normalized frequency was the frequency minus the frequency in nonirradiated tumors.
c) 95% confidence limit.

as well as the induction of MN after DNA-damaging treatment.[29)] The time course of change in the value of the apoptosis frequency (Figs. 2 and 3) showed that death by apoptosis was well related to response to γ-ray or reactor neutron beam irradiation 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. Therefore, the induced normalized MN frequencies in SAS/neo tumor cells conversely showed smaller values as a whole (Fig. 6), compared with SAS/mp53 tumor cells.

We showed previously that intratumor Q cells have a larger hypoxic fraction (HF) than the total tumor cells and that, irrespective of p53 status, significant differences in γ-ray sensitivity between total and Q cells are consistently observed.[13, 28)] In this study, the differences in radiosensitivity between total and Q cells were markedly reduced by neutron beam irradiation in both tumors (Table IV). It follows that aerated and hypoxic tumor cells in these solid tumors have almost the same radiosensitivity to reactor neutron beams and that the difference in sensitivity relative to cell cycle and p53 status can be remarkably reduced by using the neutron beams. However, there was no apparent relationship between the difference in sensitivity between total and Q cells and the value of the neutron Cd ratio. Thus, high LET neutrons, irrespective of the neutron Cd ratio and p53 status, should be applied to reduce the difference in sensitivity between total and Q cells.

Meanwhile, in both SAS/neo and SAS/mp53 tumors, the values of RBE for Q cells were larger than those for total cells (Table V). This was mainly because the difference in γ-ray sensitivity between total and Q cells was much greater than that in sensitivity to neutron beams in both tumors (Table IV). Further, in both cell populations, the RBE values for low Cd ratio neutron beams tended to be larger than those for high Cd ratio neutron beams in both p53 status tumors. This might be partially because the low Cd ratio neutrons employed here included a higher percentage of fast neutrons, which include a higher proportion of 24 keV neutrons which induce chromosome aberrations at high efficiency.[22, 30, 31)] In addition, this might be partially because the ratio of high LET neutron components to total radiation components is higher in low Cd ratio neutron beams than in high Cd ratio neutron beams (Table I). The values of RBE for total cells themselves were similar to those in other reports.[9, 32)]

In our previous report concerning reactor neutron beams, we concluded that the use of a ^{10}B-carrier is effective when combined with high Cd ratio neutron beam irradiation.[18)] Therefore, tumor-bearing mice received the neutron beam with a Cd ratio of 150 following ^{10}B-carrier administration. In both tumors, the use of the ^{10}B-carrier increased the sensitivity of both total and Q cell populations, especially that of total cells (Table VI), resulting in a widening of the difference in sensitivity between total and Q cells. This was probably because Q cells cannot take up ^{10}B as efficiently as P cells. In addition, in both p53 status tumors, the sensitivity of Q cells treated with BPA was lower than that of Q cells treated with BSH, although the average ^{10}B concentration for BPA administration was almost the same as that for BSH in the total tumor cells. In other words, when BPA was used, ^{10}B could be distributed to total tumor cells as a whole as well as when BSH was used. However, less ^{10}B could be localized into Q cells than when BSH was administered. That is to say, more ^{10}B was distributed into P cells than when BSH was used. Since it has been thought that Q cells cannot take up ^{10}B as efficiently as P cells,[9)] this indicates that the distribution of ^{10}B, from BPA, in tumor cells is more dependent on the

^{10}B uptake ability of the tumor cells than that from BSH. This might be supported by the report that the cellularity of tumor cells correlated with the ^{10}B concentration following BPA administration.[33] In contrast, when BSH was used, the ER values for total cells were almost the same as those for Q cells. This means that the distribution of ^{10}B from BSH is more dependent on the diffusion of the drug than that from BPA.

Although not significantly, the growth fraction, that is the LI after continuous labeling with BrdU, in SAS/mp53 was lower than that in SAS/neo tumors. Further, according to our previous report using the same tumor cells as here,[28] the HF in total cells was significantly higher for SAS/mp53 than for SAS/neo tumors ($P<0.05$). Therefore, it followed that the ER values for BPA in total cells of SAS/mp53 tumors were significantly smaller than those of SAS/neo tumors ($P<0.05$) (Table VI). However, concerning Q cell fractions, our previous study showed that there was no significant difference in the size of the HF although SAS/mp53 had a slightly higher HF than SAS/neo tumors.[28] Thus, there were no significant differences in the values of the ER for Q cells, although SAS/mp53 showed slightly lower values than SAS/neo tumors (Table VI). Although the average ^{10}B concentration in total cells was almost the same for both tumors, the values of the ER for SAS/mp53 were, on the whole, smaller than those for

Table V. Relative Biological Effectiveness[a] in Total and Quiescent Tumor Cell Populations

Tumor cell fraction	End point	Neutron beam	
		Cd ratio:1.0	Cd ratio:150
Surviving fraction (in total cells only)			
SAS/neo	0.3	3.3 (3.0–3.6)[b]	2.2 (2.0–2.4)
	0.1	2.9 (2.7–3.1)	2.1 (2.0–2.2)
SAS/mp53	0.3	3.35 (3.1–3.6)	2.4 (2.2–2.6)
	0.1	3.35 (3.1–3.6)	2.3 (2.1–2.5)
Normalized apoptosis frequency[c]			
SAS/neo			
Total cells	0.05	2.25 (2.05–2.45)	2.05 (1.9–2.2)
	0.02	1.85 (1.7–2.0)	1.5 (1.35–1.65)
Quiescent cells	0.05	3.25 (3.0–3.5)	3.0 (2.7–3.3)
	0.02	2.65 (2.45–2.85)	2.5 (2.3–2.7)
SAS/mp53			
Total cells	0.02	2.45 (2.3–2.6)	2.3 (2.1–2.5)
Quiescent cells	0.02	4.9 (4.5–5.3)	4.1 (3.8–4.4)
Normalized micronucleus frequency[c]			
SAS/neo			
Total cells	0.1	2.8 (2.6–3.0)	2.2 (2.0–2.4)
Quiescent cells	0.1	4.6 (4.2–5.0)	3.4 (3.1–3.7)
SAS/mp53			
Total cells	0.2	3.1 (2.7–3.5)	2.5 (2.2–2.8)
	0.1	3.05 (2.8–3.3)	2.45 (2.3–2.6)
Quiescent cells	0.1	5.4 (4.7–6.1)	4.8 (4.3–5.3)

a) Radiation dose required to obtain each endpoint with γ-rays/radiation dose required to obtain each endpoint with neutron beam alone.
b) 95% confidence limit.
c) Normalized frequency was the frequency minus the frequency in nonirradiated tumors.

Table VI. Enhancement Ratios of ^{10}B-Carriers[a]

Tumor cell fraction	End point	^{10}B-Carrier	
		BSH[b]	BPA[c]
Surviving fraction (in total cells only)			
SAS/neo	0.3	2.4 (2.2–2.6)[d]	7.1 (6.4–7.8)
	0.1	—	6.3 (5.7–6.9)
SAS/mp53	0.3	1.9 (1.7–2.1)	5.2 (4.7–5.7)
	0.1	—	4.5 (4.0–5.0)
Normalized apoptosis frequency[e]			
SAS/neo			
Total cells	0.05	2.4 (2.2–2.6)	4.2 (3.8–4.6)
	0.02	2.2 (1.9–2.5)	4.6 (3.8–5.4)
Quiescent cells	0.05	2.3 (1.9–2.7)	1.6 (1.4–1.8)
	0.02	2.1 (1.8–2.4)	1.65 (1.45–1.85)
SAS/mp53			
Total cells	0.02	1.6 (1.3–1.9)	2.5 (2.2–2.8)
Quiescent cells	0.02	1.5 (1.3–1.7)	1.4 (1.3–1.5)
Normalized micronucleus frequency[e]			
SAS/neo			
Total cells	0.1	2.2 (1.9–2.5)	4.8 (4.2–5.4)
Quiescent cells	0.1	2.1 (1.9–2.3)	1.9 (1.7–2.1)
SAS/mp53			
Total cells	0.2	1.7 (1.5–1.9)	2.8 (2.5–3.1)
	0.1	1.7 (1.4–2.0)	3.3 (2.9–3.7)
Quiescent cells	0.2	1.5 (1.2–1.8)	1.4 (1.2–1.6)
	0.1	1.6 (1.3–1.9)	1.5 (1.2–1.8)

a) Ratio of radiation dose required to obtain each endpoint without ^{10}B-carrier in relation to the radiation dose required to obtain each endpoint with ^{10}B-carrier.
b) Sodium borocaptate-^{10}B.
c) *p*-Boronophenylalanine-^{10}B.
d) 95% confidence limit.
e) Normalized frequency was the frequency minus the frequency in nonirradiated tumors.

SAS/neo tumors in both cell fractions. Smaller cellularity of SAS/mp53 tumor cells also may account for this fact.[33] Actually, SAS/mp53 tumors showed significantly lower values of cell yield than SAS/neo tumors by the *in vivo-in vitro* assay ($P<0.05$).

At present, NCT is indicated only for patients with glioblastoma or malignant melanoma, which is thought to be very resistant and refractory to conventional cancer therapy, resulting in a poor prognosis.[9] Further, the tumors that are highly resistant to conventional cancer therapy are thought to be highly heterogeneous in their radiobiological character.[11, 12] As a result, these tumors have the potential to contain a high percentage of cells with mutated p53. The fact that p53 status influenced the response to radiation dose delivered by neutron capture reaction within solid tumors is very important in determining the efficacy of NCT. The p53 status of tumor cells may also be one of the critical factors in the indication of clinical NCT.

In terms of cell survival curves, SAS/mp53 was significantly more resistant to neutron beams with the ^{10}B-carrier than SAS/neo ($P<0.05$) (Table III). Concerning the irradiation with neutron beams alone without the ^{10}B-carrier, high LET neutron beams, irrespective of the values of the Cd ratio, could markedly reduce the significant sensitivity difference between SAS/neo and SAS/mp53 tumors toward the γ-ray irradiation. This is compatible with reports that showed no significant difference in cellular sensitivity between cells with functional wild-type p53 and those with mutated p53 to high LET radiation, although the radiosensitivity of the former to low LET radiation was higher than that of the latter.[34, 35]

On the whole, in both total and Q cells of both tumors, similar tendencies concerning the values of the DMF of Q cells, RBE and ER were obtained, based on the data of the apoptosis frequency or MN frequency (Tables IV through VI). This implies that the apoptosis frequency, as well as the MN frequency, is an index that reflects the sensitivity to γ-ray and reactor neutron beam irradiation irrespective of p53 status. Additionally, the apoptosis frequency, as well as the MN frequency, could be applied to our method for measuring the Q cell response to γ-ray and reactor neutron beam irradiation within solid tumors embracing p53 mutations or not.

Solid tumors, especially human tumors, are thought to contain a high proportion of Q cells.[3] 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.[3] This might promote apoptosis and MN formation in Q tumor cells, partly due to the effect of hypoxia-induced apoptosis[26] (Table II). It has been reported that Q cells *in vivo* have lower radiosensitivity than P cells in solid tumors.[3, 5] As was also shown in this study, Q cells have significantly lower radiosensitivity to γ-rays than the total cell population within both solid tumors *in vivo*, irrespective of p53 status (Table IV). This means that more Q cells than P cells can survive after anticancer treatment. Consequently, the control of Q cells has a great impact on the outcome of anticancer therapy. In the use of neutron beams in conjunction with the concentration of ^{10}B in the tumors employed here, to evaluate the usefulness of ^{10}B-carrier administration before neutron beam irradiation, the DMFs of Q cells following ^{10}B-carrier administration were determined using the data given in Figs. 5 and 6 (Table VII). Whichever ^{10}B-carrier was used, in both SAS/neo and SAS/mp53 tumors, the DMFs of Q cells relative to total cells were larger, significantly so with the use of BPA ($P<0.05$), than with neutron irradiation alone (Table VII). Namely, the absolute radiation dose required to achieve the same endpoint for Q cells is much higher than that for total cells when combined with the ^{10}B-carrier, in contrast with the neutron beam irradiation alone. As stated above, this is mainly because within solid tumors, less ^{10}B from the ^{10}B-carrier, and much less ^{10}B from BPA, can be localized in Q cells than in P cells in both tumors.

In conclusion, the p53 status of the tumor cells had the potential to influence the distribution of ^{10}B within solid tumors following ^{10}B-carrier administration *in vivo*. The apoptosis frequency, as well as the MN frequency, can be applied to our method for measuring the Q cell response to reactor neutron beam irradiation within solid tumors har-

Table VII. Dose Modifying Factors[a] for Quiescent Tumor Cells Relative to the Total Tumor Cell Populations Following ^{10}B-Carrier Administration

Tumor	End point	^{10}B-Carrier	
		BSH[b]	BPA[c]
Normalized apoptosis frequency[d]			
SAS/neo	0.05	1.3 (1.1–1.5)[e]	3.4 (2.9–3.9)
	0.02	1.3 (1.15–1.45)	3.8 (3.2–4.4)
SAS/mp53	0.02	1.6 (1.4–1.8)	2.15 (2.0–2.3)
Normalized micronucleus frequency[d]			
SAS/neo	0.1	1.3 (1.1–1.5)	2.35 (2.15–2.55)
SAS/mp53	0.2	1.9 (1.7–2.1)	2.5 (2.2–2.8)
	0.1	1.7 (1.55–1.85)	2.7 (2.3–3.1)

a) 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.
b) Sodium borocaptate-^{10}B.
c) *p*-Boronophenylalanine-^{10}B.
d) Normalized frequency was the frequency minus the frequency in nonirradiated tumors.
e) 95% confidence limit.

boring or not harboring p53 mutations. In contrast with reactor neutron beam irradiation alone, which reduces the difference in sensitivity to γ-rays between total and Q cells, greater radiation doses to Q cells are needed to achieve the equivalent endpoint in total cells when combined with ^{10}B-carrier administration, irrespective of the p53 status.

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