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/c nu/o 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-10B (BSH) or p-boronophenylalanine-10B (BPA), the tumors were irradiated with neutron beams. The tumors not treated with 10B-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 10B-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 10B-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 10B-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 10B-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 repair3, 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, 9 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 (10B(n,α)Li) is, in principle, very effective in destroying tumors, provided that a sufficient amount of 10B 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 10B-loaded cells, resulting in induction of a high relative biological effectiveness (RBE) when 10B can be distributed homogeneously in the target tissue.9 However, at present, it is difficult to deliver a sufficient amount of 10B 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
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 mM 2-(4-(2-hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES) and 12.5% fetal bovine protein, and plasmid pCMV-Neo-Bam, which contains a neo-resistance marker, were provided by B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). These neo-resistance marker, were provided by B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). These plasmids were linearized with a Cd ratio of 0.1 contains 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 10B 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.23 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 cm⁻²s⁻¹ of thermal neutron flux for 1 µg g⁻¹ of 10B was 2.67×10⁻⁸ F cGy h⁻¹.
Determining the timing for apoptosis detection

Without 10B-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, respectively.

### 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           | 2.0×10\(^9\)              | 2.5×10\(^7\)                   | 6.6×10\(^6\)           | 98.1   |
| Neutron flux  | 98.1                      | 0.384                           | 22.9                   | 100    |
| 1.0           | 3×10\(^7\)                | 7.3×10\(^5\)                   | 4.7×10\(^3\)           | 0.24   |
| Neutron flux  | 7.3×10\(^5\)              | 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).](image)
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 $\gamma$-ray irradiator at doses of 4.1, 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 $^{10} \text{B}$-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 $^{10} \text{B}$ concentration in the tumors. The $^{10} \text{B}$ concentration in the tumors was measured by prompt $\gamma$-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 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 in 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.](image-url)
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) × 10⁷ g⁻¹ and 3.4 (2.6–4.2) × 10⁶ 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 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 ¹⁰B 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

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

| Tumor cell fraction | No ¹⁰B-carrier | BSH | BPA |
|---------------------|----------------|-----|-----|
| Plating efficiency for total cells (%) | 57.0 (50.0–64.0) | 39.5 (33.7–45.8) | 34.7 (24.7–44.7) |
| 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 |
| 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) |

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a) Sodium borocaptate-¹⁰B.
b) p-Borophenylalanine-¹⁰B.
c) Numbers in parentheses are 95% confidence limits.
a maximum value 6 h after irradiation in each cell line when no 10B-carrier was administered. Even when 10B-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). Tumors were irradiated with \(\gamma\)-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.

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 \(\gamma\)-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 \(\gamma\)-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 $\gamma$-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 $\gamma$-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 $\gamma$-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, 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 $\gamma$-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.](image1)

![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 $\gamma$-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.](image2)
In this study, the differences in radiosensitivity between total and Q cells are consistently observed. The time course of change in the value of the apoptotic frequency between total and Q cells was markedly reduced by using the neutron beams. However, there was no apparent relationship between the difference in sensitivity to cell cycle and p53 status can be remarkably reduced by using the neutron beams. Therefore, the induced normalized MN frequencies 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 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 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. 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Table V. Relative Biological Effectiveness\(^{a)}\) in Total and Quiescent Tumor Cell Populations

| Tumor cell fraction | End point | Neutron beam C\(_d\) ratio:1.0 | C\(_d\) 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             | 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            | 0.02      | 2.45 (2.3–2.6)                | 2.3 (2.1–2.5)   |
|                     | 0.02      | 4.9 (4.5–5.3)                 | 4.1 (3.8–4.4)   |
| Normalized micro-nucleus frequency\(^{d)}\) |            |                               |                 |
| SAS/neo             | 0.1       | 2.8 (2.6–3.0)                 | 2.2 (2.0–2.4)   |
|                     | 0.1       | 4.6 (4.2–5.0)                 | 3.4 (3.1–3.7)   |
| SAS/mp53            | 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)  |
| a) Radiation dose required to obtain each endpoint with \(\gamma\)-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 \(^{10}\text{B}-\text{Carriers}^{a)}\)

| Tumor cell fraction | End point | \(^{10}\text{B}-\text{Carrier}\) |
|---------------------|-----------|-------------------------------|
|                     | BSH\(^{a)}\ | BPA\(^{a)}\ |
| Surviving fraction  |           |                               |                 |
| (in total cells only) |          |                               |                 |
| SAS/neo             | 0.3       | 2.4 (2.2–2.6)\(^{a)}\)       | 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\(^{c)}\) |            |                               |                 |
| SAS/neo             | 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            | 0.02      | 1.6 (1.3–1.9)                 | 2.5 (2.2–2.8)   |
|                     | 0.02      | 1.5 (1.3–1.7)                 | 1.4 (1.3–1.5)   |
| Normalized micro-nucleus frequency\(^{e)}\) |            |                               |                 |
| SAS/neo             | 0.1       | 2.2 (1.9–2.5)                 | 4.8 (4.2–5.4)   |
|                     | 0.1       | 2.1 (1.9–2.3)                 | 1.9 (1.7–2.1)   |
| SAS/mp53            | 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 \(^{10}\text{B}-\text{carrier}\) in relation to the radiation dose required to obtain each endpoint with \(^{10}\text{B}-\text{carrier}\).

b) Sodium borocaptate-\(^{10}\text{B}\).
c) \(p\)-Boronoophenylalanine-\(^{10}\text{B}\).
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.13,14 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 10B-carrier than SAS/neo (P<0.05) (Table III). Concerning the irradiation with neutron beams alone without the 10B-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.3,14,15)

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 apoptosis20) (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 10B in the tumors employed here, to evaluate the usefulness of 10B-carrier administration before neutron beam irradiation, the DMFs of Q cells following 10B-carrier administration were determined using the data given in Figs. 5 and 6 (Table VII). Whichever 10B-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 10B-carrier, in contrast with the neutron beam irradiation alone. As stated above, this is mainly because within solid tumors, less 10B from the 10B-carrier, and much less 10B 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 10B within solid tumors following 10B-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 Factors13) for Quiescent Tumor Cells Relative to the Total Tumor Cell Populations Following 10B-Carrier Administration

| Tumor | End point | 10B-Carrier | BSH10 | BPA12 |
|-------|-----------|-------------|-------|-------|
| SAS/neo | 0.05 | 1.3 (1.1–1.5)13) | 3.4 (2.9–3.9) | |
| SAS/mp53 | 0.02 | 1.3 (1.15–1.45) | 3.8 (3.2–4.4) | |
| SAS/neo | 0.02 | 1.6 (1.4–1.8) | 2.15 (2.0–2.3) | |
| SAS/mp53 | 0.1 | 1.3 (1.1–1.5) | 2.35 (2.15–2.55) | |
| SAS/neo | 0.2 | 1.9 (1.7–2.1) | 2.5 (2.2–2.8) | |
| SAS/mp53 | 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–10B.
c) p-Boronophenylalanine–10B.
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 10B-carrier administration, irrespective of the p53 status.

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