Improvement of the Tumor-Suppressive Effect of Boron Neutron Capture Therapy for Amelanotic Melanoma by Intratumoral Injection of the Tyrosinase Gene

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Abstract
Boron neutron capture therapy (BNCT) is successful when there is a sufficient 10B concentration in tumor cells. In melanoma, 10B-para-boronophenylalanine (BPA) accumulation is proportional to melanin-producing activity. This study was done to confirm enhancement of the tumor-suppressive effect of BNCT on amelanotic melanoma by intratumoral injection of the tyrosinase gene. D178 or FF amelanotic melanomas were implanted s.c. in Syrian hamsters. One group of D178- or FF-bearing hamsters (TD178 or TFF group) received intratumoral injections of pcDNA-Tyrs constructed as a tyrosinase expression plasmid. The other hamsters (pD178 and pFF groups) were injected with pUC119, and control hamsters (D178 and FF groups) only with transfection reagents. All the groups underwent immunofluorescence analysis of tyrosinase expression and BPA biodistribution studies. BNCT experiments were done at the Kyoto University Research Reactor. Tyrosinase expression increased in the tumors of the TD178 and TFF groups but remained the same in the pD178 and pFF groups. Tumor boron concentrations in the TD178 and TFF groups increased significantly (TD178: 49.7 ± 12.6 versus D178: 27.2 ± 4.9 μg/g, P < 0.0001; TFF: 30.7 ± 6.6 versus FF: 13.0 ± 4.7 μg/g, P < 0.0001). The BNCT tumor-suppressive effect was marked in the TD178 and TFF groups. In vivo transfection with the tyrosinase gene increased BPA accumulation in the tumors, the BNCT tumor-suppressive effect on amelanotic melanoma being significantly enhanced. These findings suggest a potential new clinical strategy for the treatment of amelanotic melanoma with BNCT. (Cancer Res 2006; 66(7): 3747-53)

Introduction
Boron neutron capture therapy (BNCT) is a radiotherapeutic modality that theoretically has a selective killing effect on tumors. Thermal neutrons are captured by the 10B atom, resulting in the emission of linear recoiling α-particles and 7Li nuclei, with respective traveling ranges of ~9 and ~5 μm. These particles have high linear energy transfer radiation and lethally damage DNA when they penetrate a cell nucleus (1-3). The number of 10B atoms incorporated into tumor cells is a critical factor that determines the specific, powerful killing effect of BNCT. It is reported that 15 to 30 μg 10B/g must be present in tumors and low levels in normal tissue, and that a sufficient number of thermal neutrons must reach a tumor for BNCT to be successful (4, 5).

BNCT for malignant melanoma that uses 10B-para-boronophenylalanine (BPA) as the capture agent and is used clinically was developed by Mishima et al. (6). To date, successful BNCT treatment of 18 melanoma patients has been reported (7, 8). In one study, histologic subtypes of melanomas treated with BNCT were nodular, superficial spreading, acral lentiginous, and lentigo maligna melanomas, but no amelanotic variant was included (9).

Amelanotic melanoma is a variant that may be devoid of clinically apparent pigmentation. Amelanotic tumors, therefore, often become advanced because of delay in diagnosis (10). In general, the prognosis for amelanotic tumors is worse than for their pigmented counterparts. In one study, patients with pigmented tumors had a 10-year survival rate of 73%, whereas the rate was only 54% for those with nonpigmented lesions (11).

Coderre et al. (12) reported BPA accumulation in nonpigmented melanomas to be about half that in melanotic counterparts, but enough selectively was present in the nonpigmented tumors compared with normal tissues for effective BNCT. When, however, the clinical safety limit of skin damage (13, 14) is considered, there is the strong possibility that the therapeutic dose given such tumors may be insufficient. Therefore, it is necessary to improve the selective accumulation of BPA in amelanotic melanoma cells relative to contiguous normal tissues. A recent study of human amelanotic melanoma cells supports the view that melanogenic gene transfection improves BPA uptake and the effects of BNCT (15). Tsuboi et al. (16) studied a melanoma-bearing mouse model and reported that induction of the formation of melanin monomers and polymers by human tyrosinase cDNA transfer effectively improved BPA uptake by amelanotic melanoma cells and the therapeutic outcome of BNCT. Their gene transfection experiment, however, was done in vitro before transplanting the melanoma cells to the mice. Furthermore, they reported neither the extent of damage nor the dose absorbed to tumor-surrounding normal skin in their animal model after BNCT.

Our purpose was to clarify whether the increase in BPA uptake by such tumors and enhancement of the BNCT effect can be produced by intratumor injection of the tyrosinase gene. We report here that two hamster amelanotic melanoma cell lines, D178 (17) and FF (18), had statistically significant increases in BPA uptake by the tumor tissues, and the tumor-suppressive effect in animals that had received an intratumor injection of the tyrosinase gene showed improved.

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Materials and Methods

Cell lines. D178 hamster amelanotic melanoma, obtained from Dr. H.S. Greene (17), was maintained in MEM with 10% fetal bovine serum (FBS). Hamster-derived FF amelanotic melanoma purchased from Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan (18) was maintained in RPMI 1640 with 10% FBS.

Western blot analysis. For the detection of tyrosinase, tyrosinase-related protein 1 (TRP-1), and TRP-2 protein expression, cell lysates were prepared in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). Total proteins (10 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (TEFCO Co., Ltd., Tokyo, Japan) by a semidry transfer system NEB-3000W (Nichiryo Co., Ltd., Tokyo, Japan). Blots were blocked in 5% milk/0.05% Tween 20-PBS then incubated in the same solution containing goat polyclonal antityrosinase antibody, goat polyclonal anti-TRP-1 antibody, and goat polyclonal anti-TRP-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), after which they were incubated with horseradish peroxidase–conjugated bovine anti-goat secondary antibody. Immunoreactive sites were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, United Kingdom).

Plasmid constructs. We constructed a plasmid vector, pcDNA-Tyrs, that expressed a fusion protein consisting of a V5 epitope tag and full-length mouse tyrosinase to distinguish between native and extrinsic tyrosinase after gene transfer. The V5 epitope is present in P and V proteins of the paramyxovirus SV5. The anti-V5 antibody recognizes the 14 amino acid sequence Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr, which permits detection of recombinant proteins containing the V5 epitope. Mouse tyrosinase cDNA was amplified by a reverse transcription-PCR of the total RNA extracted from B16 mouse melanoma with two primers (sense primer, 5′-CACATGTTCCTGGGATGTTTGGTGACCGGTGACACGAGTTCGTT-3′; antisense primer, 5′-TGACTGCTCGTATCAGCAACAGCAGTTCG-3′) which are separated in a 1.2% agarose gel, excised, purified, and ligated to the pcDNA 3.2/V5/GW/D-C2 cocktail (Invitrogen, Carlsbad, CA). The pcDNA insert was sequenced with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). plC119 was the control plasmid against pUC119 (pD178 group, the control plasmid transfectant group). The remaining hamsters, the no gene transfection controls (D178 group), were injected with 200 μL of ExGen500. Melanoma-bearing hamsters received gene injections of FF melanomas, in the same manner as the D178 melanoma animals (TF, pFF, and FF groups). Three days after injection, these hamsters were used in the BNCT experiment or were anesthetized and killed to analyze protein expression and BPA uptake by the tumors.

H&E stains and immunofluorescence microscopy. After transfection, paraffin-embedded tumors were sectioned (5-μm thick) then stained with H&E for observation of melanin granules. The other sections were stained with goat polyclonal antityrosinase antibody 1:100 (Santa Cruz Biotechnology) and mouse monoclonal anti-V5 antibody 1:200 (Invitrogen) to analyze native tyrosinase and V5-tagged tyrosinase protein expression. The respective secondary antibodies were used with Texas red–conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology) and FITC-conjugated bovine anti-mouse IgG (Santa Cruz Biotechnology). Immunofluorescence microscopy was done with a Nikon ECLIPSE E800 fluorescence microscope (Nikon Instech Co., Ltd., Kanagawa, Japan). Images were captured by a DXM1200 camera (Nikon Instech) equipped with ACT-1 control software (Nikon Instech).

BPA biodistribution studies. BPA (1-enantiomer; >95% 10B enriched) was obtained from STELLA CHEMIFA Corp, Osaka, Japan. Because BPA solubility in water is slight at a physiologic pH, Yoshino et al. (19) developed a BPA-fructose complex to increase it.

The BPA biodistribution studies were done 13 days after melanoma implantation. The melanoma-bearing hamsters received BPA in a BPA-fructose complex (400 mg/kg body weight) via i.p. injection, and their melanomas were resected 2 hours later under sodium pentobarbital anesthesia (40 mg/kg body weight). Each sample was dissolved in a mixture of HClO4 (60%, 0.9 mL) and H2O2 (30%, 1.8 mL) then passed through a 0.45 μm filter. The tumor boron concentration was assayed by inductively coupled plasma-atomic emission spectrometry.

BNCT experiment. Thirteen days after melanoma implantation, 7 hamsters from each group were administered BPA as described above, and two hours later anesthetized and kept in a specially designed acrylic cage. Except for the melanoma-bearing region of the buttocks, the body of each hamster was shielded from thermal neutrons by LiF tiles (13). Hamsters were placed 10 cm from the bismuth surface of the reactor. Each D178 melanoma lesion was treated with thermal neutrons at 5 MW for 10 minutes at the Kyoto University Research Reactor. FF melanoma lesions were irradiated for 40 minutes. Thermal neutron fluxes were obtained by gold foil activation analysis, and γ-rays of the tumor surfaces with a thermoluminescence dosimeter.

Treatment results were monitored by measuring tumor size as a function of time postirradiation. Vernier calipers were used to measure the largest (a) and smallest (b) tumor diameters. Tumor volume was calculated by the formula: volume (mm3) = a × b2 / 2 (20).

The animal protocols used in these experiments were approved by the Animal Care and Use Committee of Kawasaki Medical School (nos. 03-083, 2003, and 03-106, 2004).

Statistical analysis. Student’s t test was used to compare boron concentrations. Differences in experimental results on tumor growth were assessed by two-way repeated measures ANOVAs with SigmaStat 3.1 statistical software (Systat Software Inc., Richmond, CA).

Results

Tyrosinase expression and melanoma cell radiosensitivity. Expressions of tyrosinase, TRP-1, and TRP-2 in D178 and FF melanoma cells are shown in Fig. 1A. The expression tyrosinase protein is weak in the D178 cells and very weak in the FF cells. Both the TRP-1 and TRP-2 expressions are very weak in the D178 and FF cells. Figure 1B shows the survival curves of the D178 and FF cells derived from the clonogenic assay. Respective D50 values of
these cells were 0.78 ± 0.02 and 1.29 ± 0.20 Gy. FF cells were more resistant than D178 cells in the X-ray survival tests.

**Tyrosinase expression after in vivo gene transfection.** Tyrosinase protein expression results for the TD178, pD178, and D178 melanomas are presented in Fig. 2A. Three days after transfection, total tyrosinase had increased in the TD178 group tumors, whereas tyrosinase expression remained the same in pD178 ones. To confirm that the increase in tyrosinase was due to pcDNA-Tyrs transfection, tumors were first incubated with mouse anti-V5 antibody then with FITC-conjugated anti-mouse IgG. TD178 group tumor expression of the V5 antigen corresponded to the region with elevated total tyrosinase, whereas pD178 group tumor expression did not. No melanin granules were present in the H&E-stained slides of TD178, pD178, and D178 group tumors under light microscopy. In contrast, dark brown melanin granules were seen in the H&E slide of D179, a melanotic variant of D178 (Fig. 2B).

Tyrosinase protein expression results for TFF, pFF, and FF melanomas are shown in Fig. 3A. Total tyrosinase in the TFF melanomas was elevated somewhat by pcDNA-Tyrs, whereas it remained the same in the pFF group tumors. No melanotic variant was compared directly with FF, but none of the dark brown melanin granules in D179 were present in the FF series H&E slides (Fig. 3B).

**Tumor growth curves after gene transfection.** Neither pcDNA-Tyrs nor pUC119 gene transfection had an observable suppressive effect on tumor growth (Fig. 2C). Tumor volumes in the D178 group increased slowly up to day 11 after transplantation and then increased rapidly. Tumor growth rates of hamsters that underwent gene transfection did not differ significantly from those of nontransfected animals (TFF versus FF group, \( P = 0.42 \); pFF versus FF group, \( P = 0.92 \)). In addition, the TFF group tumor growth rate was equivalent to that of the pFF group (\( P = 0.40 \)).

**Boron concentrations and the tumor/normal tissue boron concentration ratio.** Boron concentrations in the tumors and surrounding normal skin, and the tumor/normal tissue (T/N) boron concentration ratio 2 hours after i.p. BPA administration

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**Figure 1.** A, Western blot analysis for detection of tyrosinase expression (lanes 1), TRP-1 (lanes 2), and TRP-2 (lanes 3) in D178 and FF cells. B, survival curves for D178 and FF melanoma cells after 4 MV X-ray irradiation. Data were fitted to a single-hit multitarget model of X-ray dose survival. The \( D_0 \) value for D178 is 0.78 ± 0.02 Gy, for FF 1.29 ± 0.20 Gy. Results are from triplicate independent experiments. Points, survival fraction; bars, ± SD.

**Figure 2.** A, tyrosinase expression in TD178 (tyrosinase transfectant), pD178 (control plasmid transfectant), and D178 (carrier solvent alone control) tumors under a fluorescence microscope. B, light microscopy images of sections of TD178, pD178, D178, and D179 tumors stained with H&E. No melanin granules are present in the TD178, pD178, and D178 group tumors in contrast to the many melanin granules (arrows) in the D179 melanotic variant. Bars, 50 \( \mu \)m. C, effect of intratumor gene transfer on D178 tumor growth. Tumor volume was monitored over time after tumor cell inoculation. ●, intratumor injection of pcDNA-Tyrs (TD178); ○, intratumor injection of pUC119 plasmid (pD178); ×, carrier solvent alone (D178). Arrow, time at which transfection was done. Points, means for five hamsters per group; bars, SD. There were no significant differences among the three groups (TD178 versus D178 group, \( P = 0.11 \); pD178 versus D178 group, \( P = 0.42 \); TD178 versus pD178 group, \( P = 0.88 \)).
significantly from that for the D178 group (P < 0.0001). In contrast, the value for the pD178 group does not differ. Averages for the three groups are similar (8.7-10.0 g/g). Tumor volumewas monitored over time after tumor cell inoculation.

**Figure 3.**

A, tyrosinase expression in TFF (tyrosinase transfectant), pFF (control plasmid transfectant), and FF (carrier solvent alone control) tumors observed under a fluorescence microscope. Photographs of tumor sections in the same field stained for tyrosinase or V5-tagged with tyrosinase are presented in parallel. The FF tumor section was stained only for tyrosinase. Bars, 100 μm. B, light microscopy images of sections of TFF, pFF, and FF tumors stained with H&E. Bars, 50 μm. C, effect of intratumor gene transfer on FF tumor growth. Tumor volume was monitored over time after tumor cell inoculation. ●, intratumor injection of pcDNA-Tyrs (TFF); □, intratumor injection of pUC119 plasmid (pFF); ▼, carrier solvent alone (FF). Arrow, time at which transfection was done. Points, means for five hamsters per group; bars, SD. There were no significant differences among the three groups (TFF versus FF group, P = 0.42; pFF versus FF group, P = 0.92; TFF versus pFF group, P = 0.40).

data are given in Table 1. In D178-bearing hamsters, the respective tumor boron concentrations for the TD178, pD178, and D178 groups are 49.7 ± 12.6, 27.8 ± 4.3, and 27.2 ± 4.9 μg/g. The value for the TD178 group is ~1.8 times that for the D178 group (P < 0.0001). In contrast, the value for the pD178 group does not differ significantly from that for the D178 group (P = 0.75). Skin values for the three groups are similar (8.7-10.0 μg/g). T/N boron concentration ratios for the TD178, pD178, and D178 groups, respectively, are 5.1, 3.3, and 3.4. The ratio for the TD178 group is significantly higher than that for the pD178 group (P = 0.0037).

Respective tumor boron concentrations of the TFF, pFF, and FF groups are 30.7 ± 6.6, 12.8 ± 3.1, and 13.0 ± 4.7 μg/g. The value for the TFF group is ~2.4 times that for the FF group (P < 0.0001), whereas the value for the pFF group does not differ significantly from that for the FF group (P = 0.91). The skin's boron concentration was similar in all three groups (8.4-8.9 μg/g). T/N boron concentration ratios for the TFF, pFF, and FF groups, respectively, are 3.6, 1.5, and 1.5. The ratio for the TFF group is significantly higher than that for the pFF group (P < 0.0001).

**Tumor and skin response after BNCT.** Growth curves of the TD178 and pD178 group tumors after BNCT were plotted against time (Fig. 4A). Unlike the therapeutic response of the pD178 group after BNCT, tumor suppression was significant in the TD178 group (P < 0.0001). Two of seven TD178 tumors were suppressed immediately and had disappeared completely 15 to 17 days after BNCT. In contrast, none of seven hamsters with pD178 tumors achieved complete remission after BNCT. Erythema was present in both the TD178 and pD178 groups several days after BNCT and continued during the observation period, but there was no desquamation. Thermal neutron fluence was 1.67 × 10¹³ n/cm², and γ-rays 0.36 Gy in the BNCT experiment with the D178 melanoma-bearing hamsters. Assuming that the RBE of the high linear energy transfer irradiation was 2.5 (13, 21), the respective calculated biologically weighted absorbed doses by the tumors and normal skin are 16.8 and 4.5 RBE-Gy in the TD178 group, and 10.0 and 4.2 RBE-Gy in the pD178 group (Table 2).

Average tumor volumes of the TFF and pFF groups were monitored over time after BNCT (Fig. 4B). As compared with tumor growth in the pFF group, that in the TFF group was significantly suppressed after BNCT (P < 0.0001). One of seven TFF tumors was in complete remission, and the remainder were in >70% remission. In contrast, none of the pFF tumors showed >50% remission; at days 8 to 10 after BNCT, they showed regrowth. In both groups, erythema was observed immediately after BNCT, advancing to dry desquamation and hair loss. Thermal neutron fluence was 6.21 × 10¹² n/cm² and γ-rays 1.52 Gy in the BNCT experiment with the FF melanoma-bearing hamsters. The respective calculated biologically weighted absorbed doses in the tumors and normal skin were 40.6 and 15.2 RBE-Gy for the TFF groups and 20.0 and 14.9 RBE-Gy for the pFF groups (Table 2). Direct intratumor injection of the tyrosinase gene increased the T/N ratio from 1.5 to 3.6 in the FF melanoma-bearing hamsters. Assuming that the RBE of the high linear energy transfer irradiation was 2.5 (13, 21), the respective calculated biologically weighted absorbed doses by the tumors and normal skin are 16.8 and 4.5 RBE-Gy in the TD178 group, and 10.0 and 4.2 RBE-Gy in the pD178 group (Table 2).

**Figure 4.**

15.2 RBE-Gy for the TFF groups and 20.0 and 14.9 RBE-Gy for the pD178 group (Table 2).

**Table 1. Tumor and normal tissue boron concentrations 2 hours after BPA administration**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Tumor concentration (μg/g)</th>
<th>Ratio (T/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tumor</td>
<td>Normal tissue (skin)</td>
</tr>
<tr>
<td>TD178</td>
<td>10</td>
<td>27.2 ± 4.9</td>
<td>8.5 ± 2.7</td>
</tr>
<tr>
<td>pD178</td>
<td>10</td>
<td>27.8 ± 4.3</td>
<td>9.0 ± 2.2</td>
</tr>
<tr>
<td>FF</td>
<td>10</td>
<td>49.7 ± 12.6</td>
<td>10.0 ± 2.0</td>
</tr>
<tr>
<td>pFF</td>
<td>10</td>
<td>13.0 ± 4.7</td>
<td>8.5 ± 1.9</td>
</tr>
<tr>
<td>TFF</td>
<td>10</td>
<td>30.7 ± 6.6</td>
<td>8.7 ± 2.1</td>
</tr>
</tbody>
</table>

*Number of hamsters per group.

†Measured by inductively coupled plasma-atomic emission spectrometry; each value is a mean ± SD.

‡Calculated for individual hamsters in an experimental group. Values are means ± SD.
produces melanin pigment in amelanotic melanoma cells and in line with previous reports that tumors even when tyrosinase expression was increased. This is not distinct black melanin granules in the TD178 and TFF group hamsters per group; bars, SD. Tumor growth in the TD178 group was significantly suppressed by BNCT compared with that in the pD178 group (P < 0.0001). B, tumor suppression effect of BNCT on the TFF and pFF groups. ●, TFF tumors (pDNA-Tyrs tyrosinase transfectant); ○, pD178 tumors (pUC119 control plasmid transfectant). Points, means for seven hamsters per group; bars, SD. After BNCT, tumor volume in the TFF group was significantly decreased compared with that in the pFF group (P < 0.0001).

Discussion

In the present study, BPA tumor accumulation was significantly improved by direct in vivo tyrosinase gene transfection. Consequently, the tumor-suppressive effect of BNCT was markedly enhanced in amelanotic melanoma-bearing hamsters. In contrast, there was neither increased BPA uptake by tumors nor improvement of the BNCT effect after pUC119 plasmid transfection. These findings agree with the report that tumor boron accumulation was increased and the killing effect of BNCT enhanced by transfection of the tyrosinase gene to mouse amelanotic melanoma cells (16). Moreover, our investigation showed that intratumor injection of the tyrosinase gene is nontoxic to hamsters and has no suppressive effect on tumor growth.

In recent years, many new drugs have been developed and tested as boron delivery agents for neutron capture therapy (22–27), but no new boron-carrier has been found for clinical use. As one of two boron delivery agents (4, 6, 7, 28), BPA has been used in clinical trials on various kinds of tumors, but there are some tumors for which no therapeutic BNCT gain occurs because of the small amount of BPA taken up. It is therefore necessary to devise a method for improving the BPA uptake of tumors. Various methods for increasing BPA-mediated boron delivery to tumor cells have been reported (29–31). In melanoma, BPA accumulation and the melanin-producing activity of the tumor cells are correlated because BPA is a tyrosine analogue, a precursor of melanin (32). The conversion of L-tyrosine to melanin is mediated by the enzymatic activities of the tyrosinase family of proteins, which includes tyrosinase, TRP-1, and TRP-2. Tyrosinase is the rate-limiting enzyme in this pathway (33). Therefore, we used in vivo tyrosinase gene transfection as a means of increasing BPA uptake by melanomas because melanin synthesis is a very important factor in BPA accumulation in melanoma cells. We showed that the up-regulation of tyrosinase in two amelanotic melanoma models significantly enhanced BPA uptake and had a tumor-suppressive effect in BNCT. Light microscopy (Figs. 2B and 3B) showed no distinct black melanin granules in the TD178 and TFF group tumors even when tyrosinase expression was increased. This is not in line with previous reports that tyrosinase gene transfection produces melanin pigment in amelanotic melanoma cells and albino melanocytes (15, 16, 34). TRP-1 and TRP-2 protein expressions were scarcely detectable in Western blots of the D178 and FF group tumors (Fig. 1A), but the mechanism(s) that blocked production of melanin granules in our study has yet to be established. Deletion or mutation of the P gene (35, 36), mutations at the Tyrp locus (37, 38), and less polymerization of 5,6-dihydroxyindole-2-carboxylic acid melanin monomers to melanin offer partial explanations (39, 40). High-performance liquid chromatography showed that tyrosinase gene transfection tended to increase the dihydroxyindole-2-carboxylic acid melanin monomers to melanin (data not shown). Yoshino’s (41) use of 11B nuclear magnetic resonance and his report that melanin monomers and BPA form chemical complexes within malignant melanomas, together with our results, suggest that the effect of BNCT on amelanotic melanoma depends on the amount of melanin monomer present and has nothing to do with the presence of melanin polymer. Further studies are needed to clarify whether in vivo model transfection with other melanogenic genes, such as TRP-1 and TRP-2, increases BPA uptake and induces melanin synthesis.

Table 2. Doses delivered to tumor and normal (skin) tissue during boron neutron capture therapy

<table>
<thead>
<tr>
<th>Group</th>
<th>Region</th>
<th>Biologically weighted dose (RBE-Gy)</th>
<th>10B(n,α)Li&lt;sup&gt;*&lt;/sup&gt;</th>
<th>14N(n, p)14C&lt;sup&gt;*&lt;/sup&gt;</th>
<th>γ-Ray&lt;sup&gt;†&lt;/sup&gt;</th>
<th>Total</th>
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<td>pD178</td>
<td>Tumor</td>
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<td>1.0</td>
<td>0.4</td>
<td>10.0</td>
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<td>1.0</td>
<td>0.4</td>
<td>4.2</td>
<td></td>
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<td>1.0</td>
<td>0.4</td>
<td>16.8</td>
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<tr>
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<td>Skin</td>
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<td>1.0</td>
<td>0.4</td>
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<td>1.5</td>
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</tr>
<tr>
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<td>3.7</td>
<td>1.5</td>
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<sup>*</sup>The RBE of 10B(n,α)Li and 14N(n, p)14C<sup>*</sup> is 2.5.

<sup>†</sup>Primary and captured γ-rays. The RBE of γ-ray is 1.0.
The tumor boron concentration and T/N ratio are important factors that markedly affect BNCT results because normal tissues also take up BPA in proportion to metabolic demand (29, 42), and BNCT radiation injury to tumor-surrounding normal tissue is not negligible. In a previous study of D178 melanoma-bearing hamsters, we reported a maximum T/N ratio of ~ 3.1 and a 25 μg/g tumor boron concentration 2 to 3 hours after i.p. administration of BPA (400 mg/kg) and that good tumor control by BNCT was obtained with no serious skin damage (43). In the present study, the T/N ratio was 5.1 and tumor boron concentration 49.7 μg/g in TD178 tumors, and 3.6 and 30.7 μg/g in TFF tumors at 2 hours after a single i.p. injection of 400 mg/kg BPA. Several T/N ratios have been obtained for animal models. Coderre et al. (44) reported that for Harding-Passey melanoma-bearing BALB/c mice, the T/N ratio was 3.5 and tumor boron concentration 12.6 μg/g 6 hours after a single i.p. injection of 300 mg/kg BPA (L-enantiomer). For MRA27 melanoma-bearing rats, Barth et al. (45) reported a T/N ratio of 2.9 and tumor boron concentration of 19.5 μg/g 2.5 hours after a single i.v. injection of 500 mg/kg BPA. Iwakura et al. (46) reported a 3.8 T/N ratio and 2.6 μg/g tumor boron concentration 1.5 hours after a single i.p. injection of 250 mg/kg BPA (d,l-racemic mixture) to A1059 amelanotic melanoma-bearing mice. The same report documented a 6.8 T/N ratio and 6.6 μg/g tumor boron concentration for B16B1b melanotic melanoma-bearing mice. Their data cannot be compared directly with ours because of differences in tumor types, but the values we obtained for the dose of BPA, route of administration, and interval between injection and measurement encouraged us to apply this modality to BNCT for amelanotic melanoma.

As to the tumor control dose, 29.6 B6-GeY were required for Greene’s melanoma, a D178 variant (21). In a study of TA1059 mouse melanoma, constructed by transfecting human tyrosinase c-DNA to parental A1059 amelanotic melanoma, the tumor control dose was 23.3 B6-GeY (16). These tumor doses differ from those reported here for TD178 and TFF. This may indicate that a difference in the inherent radiosensitivities of the tumor types influences the tumor dose of BNCT, in addition to the preferential uptake of BPA. Further studies are needed to clarify the mechanisms that produce the marked difference in tumor dose response between TD178 and TFF.

Skin reactions of FF group animals reached dry desquamation, generally considered the tolerance limit, at a skin dose of 15 B6-GeY. Hiratsuka et al. (13) reported similar estimated respective skin doses that produced dry and moist desquamation, respectively, 14 to 20 and >24 B6-GeY in hamster skin. A study of rat skin that used a thermal neutron beam in conjunction with the DL form of BPA suggested a skin dose of 40 B6-GeY for moist desquamation (47). The reason for these differences in skin response may be related to differences in the skin structures of the hamster and rat.

Because a major limitation of cancer gene therapy is low transfection efficiency to tumor cells, animal study results may not be directly applicable to humans. As, however, highly efficient delivery of the tyrosinase gene to melanoma cells is achievable by vector-targeting strategies (48–50), our method suggests a new clinical approach by which to improve the effect of amelanotic melanoma BNCT.

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References

15. Ando A, Mishima Y, Haneda S, Suzumoto Y, Atobe J, Kurimoto M. Analyses of mixed melanogenesis in tyrosinase cDNA-transfected human amelanotic melano- 

17. Greene HS. A spontaneous melanoma in the hamster with a propensity for amelanotic alteration and sarcomatous transformation during transplanta- 


23. Masunaga S, Nagasawa H, Hiraoka M, et al. Applicability of the 2-nitrimidazole-sodium borocap- 

Improvement of the Tumor-Suppressive Effect of Boron Neutron Capture Therapy for Amelanotic Melanoma by Intratumoral Injection of the Tyrosinase Gene

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