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Boron Carbide Particle as a Boron Compound for Boron Neutron Capture Therapy

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Abstract

Background: In boron neutron capture therapy (BNCT), the intravenous administration of boron-containing compounds such as boronophenylalanine (BPA) and borocaptate sodium (BSH) is followed by irradiation with thermal neutrons. In the present study, we determined whether a boron-rich boron carbide (B_4C) nanoparticle could be used for BNCT.

Methods: B_4C nanoparticles were obtained by the laser fragmentation of boron particles in ethyl acetate. Neutron irradiation was delivered via a neutron beam at the Kyoto University Research Reactor (KUR).

Results: Cell viability was lower in oral squamous cell carcinoma (SCC) cells exposed to neutrons in the presence of B_4C particles than in those exposed to neutrons only. The combination of B_4C particles and neutron irradiation also decreased the formation of colonies. Injecting B_4C particles into the oral SCC xenografts of nude mice increased the concentration of ^{10}B in the tumors, but not in the kidney, liver, or spleen. The body weights of animals were not affected by neutron irradiation to B_4C-injected tumors. Tumor volumes were lower in the B_4C-mediated BNCT group than in the neutron group.

Conclusion: These results indicate that B_4C particles can be used locally as a boron compound in BNCT for oral SCC.

Keywords: Boron neutron capture therapy; Boron carbide; Oral squamous cell carcinoma

Introduction

Boron neutron capture therapy (BNCT) is a binary modality, in which the intravenous administration of boron-containing compounds is followed by irradiation with thermal neutrons. ^{10}B in tumors absorbs a thermal neutron and releases two high linear energy transfer (LET) particles: an $\alpha(^4He)$ particle and lithium (^7Li) nucleus. These high LET particles have path lengths in water of 5-10 μm. Thus, BNCT can selectively destroy tumours, if the concentration of ^{10}B in tumors can be increased beyond that in normal tissues [1,2].

Boronophenylalanine (BPA) and borocaptate sodium (BSH) have been the only clinically used boron-containing compounds. BPA, a derivative of the amino acid phenylalanine, can be incorporated into tumor cells through specific uptake mechanisms involving the L family of heterodimeric, sodium-independent, amino acid transporters located at the plasma membrane, particularly L-amino acid transported -1 (LAT-1) [3,4], while BSH can permeate the blood-brain barrier for tumor cell in the brain. Difficulties have been associated with labeling of quiescent cells at the G0 phase with BPA [5,6]. A difficult expression of LAT-1 and arrest of the cell cycle may be responsible for the failed incorporation of BPA into tumor cells. Moreover, these compounds contain a small amount of boron within their molecules.

The use of nanoscale materials in medicine represents an important research area. Numerous nanoscale materials with various components, morphologies, and sizes have been used in drug delivery, bioimaging, chemical sensing, cancer cell diagnosis, and therapeutics [7-10]. A radiofrequency (RF) field-generating system was combined with single- walled carbon nanotubes, which efficiently acted to convert RF irradiation into heat [7]. Gold nanoparticles are a unique photosensitizer in that they exhibit strong localized surface plasma resonance. These nanoparticles have been directly injected into tumors [8,10]. Thereafter, animals received RF treatment or irradiation with near-infrared light and tumors were destroyed by the high temperatures generated. Gadolinium (Gd)-loaded chitosan nanoparticles were used intratumorally in the field of neutron capture therapy (NCT), [11,12].

We previously developed a nanoparticle composed of spherical boron carbide (B_4C). Each B_4C particle obtained was encapsulated in a graphitic layer and the initial grain size was approximately 200 nm [13,14]. Since a concentrated amount of boron is required in a tumor cell for high effective BNCT, the application of B_4C particles with numerous boron atoms may improve the efficiency of this therapy. The intratumoral application of such boron-rich compounds may eradicate BNCT-resistant tumor cells that do not take up BPA. Previous studies reported that BPA-mediated BNCT was effective for the treatment of patients with advanced head and neck cancer [15,16]. Head and neck cancer may be a suitable target for B_4C-mediated BNCT because boron compounds can be directly injected into these tumors. In the present study, we determined whether locally administered B_4C nanoparticles could act as a boron compound for BNCT.

Materials and Methods

Cell culture

The human oral SCC cell line SAS was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). SAS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were then grown in an incubator at $37^\circ C$ in a humidified atmosphere with 5% CO_2. The cells that grew on plastic dishes were dissociated with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). They were suspended in a microtube with a volume of 1.5 ml and incubated while rotating at $37^\circ C$.

Boron compound and BNCT for cultured cells

B_4C particles containing natural boron were prepared as described previously [13,14]. The mean diameter of the particles was 200 nm. Each B_4C particle was encapsulated in a graphitic layer. They were dissolved in phosphate-buffered saline (PBS) and a stock solution was made of B_4C at a concentration of 1,000 ppm.

Regarding *in vitro* BNCT, SAS cells were dissociated by a treatment with EDTA-trypsin and suspended in 200 μl medium containing 50 ppm B_4C particles in a microtube. These tubes were exposed to thermal neutrons 10 min later at the Kyoto University Research Reactor (KUR). Neutron fluence was measured based on the radioactivation of gold foil on the front and back of the tubes, as described previously [17,18]. The average fluence of the thermal neutrons was $3.10×10^{12}$ n/cm^2 at 1 MW. Thermoluminescent dosimeters were used for gamma-ray dosimetry, and the total gamma ray dose was 0.55 Gy after irradiation for 40 min. Thermal neutron fluence was converted to a physical dose using the equation, "$D=\Phi(6.93×10^{-14} ×B+6.78×10^{-14} ×N)+G$".

where D=physical dose, Φi=neutron fluence (n/cm^2), B=boron concentration (ppm), N=nitrogen concentration (2%), and G=gamma-ray dose (Gy).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and colony formation

After BNCT, cells were centrifuged at 500×g for 5 min, suspended in fresh medium, inoculated in 96-well plates, and incubated for 48 h. Ten microliters of a 5 mg/ml MTT (Sigma, St.Louis, MO) solution was added to each well with 100 μl of medium, and cells were incubated at $37^\circ C$ for 4 h. After the addition of 100 μl of 0.04N HCl in isopropanol, the plates were mixed thoroughly to dissolve the dark blue crystals and stood at room temperature overnight. The plates were read on a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA. USA) with a reference wavelength of 630 nm and test wavelength of 570 nm. Background absorbance at 630 nm was subtracted from the 570 nm reading. The values were divided by those of the control and the rate was calculated.

Treated cells were plated onto 60-mm dishes at a cell density that yielded approximately 100 colonies per dish. The cells were incubated for 14 days, fixed in methanol, and stained with 1% crystal violet. Colonies composed of more than 50 cells were counted and the surviving cell fraction was determined by dividing the colony number of the irradiated culture by that of the unirradiated control culture.

^{10}B concentration in tumors and organs

To generate tumors in nude mice, $1×10^6$ cells were inoculated subcutaneously into the back of the leg of 5-week-old female Balb/c nude mice (Clea Japan Inc., Japan). When the tumors reached a suitable size, i.e., 5-10 mm in diameter, the animals were used in the BNCT experiments. B_4C particles were diluted to 250 ppm, and 50 μl of B_4C solution containing 12.5 μg B_4C particles was infiltrated into tumors to cover the whole tumor volume. The tumor, liver, kidney, and spleen were removed 10 min later and placed in teflon tubes. ^{10}B concentrations in these tissues were measured by prompt gamma-ray spectrometry using a thermal neutron guide installed at the KUR [17]. ^{10}B concentrations were measured twice and the mean values were obtained.

BNCT for oral SCC xenografts in nude mice

Tumor-bearing animals were given 50 μl of 250 ppm B_4C solution intratumorally. Thereafter, tumors were exposed to thermal neutrons. Control tumors were left untreated. Neutron irradiation was delivered via a neutron beam at 5 MW (thermal neutron mode 00-0011) in the KUR. The mouse was held stationary during the irradiation protocol for 20 min in a custom-designed acrylic resin box from which the tumor-bearing legs were pulled out through a narrow slit and fixed with adhesive. A LiF thermoplastic plate with a hole that defined the irradiation field was placed on the animal to expose the tumor-bearing leg only to the neutron beam. Neutron fluence was measured from the radioactivation of gold foil (3 mm in diameter, 0.05 mm thick) placed at the front and back of the tumors [19,20]. The average fluence of thermal neutrons was $7.98×10^{12}$ n/cm^2 at 5 MW. Thermoluminescent dosimeters were used for gamma-ray dosimetry, and the total gamma ray dose was 0.97 Gy after irradiation for 20 min. Thermal neutron fluence was converted to a physical dose using the equation "$D=\Phi(6.93×10-14 ×B+6.78×10-14 ×N)+G$".

where D=physical dose, Φ=neutron fluence (n/cm^2), B=boron concentration (ppm), N=nitrogen concentration (2%), and G=gamma-ray dose (Gy). The experiments performed to examine the antitumor effects of BNCT were started at the same time as neutron irradiation. Tumor length (L) and width (W) were measured every 3-4 days. Tumor volume (V) was determined by the formula: $V=LW^2/2$. Experimental groups included untreated control, neutron only, and B_4C-mediated BNCT groups. Four tumors in each group were used in an experiment. Repeated data were combined to evaluate tumor volumes and survival rates. Experiments using nude mice were

performed with the approval of the Institute of Laboratory Animals, Osaka University Graduate School of Dentistry.

Statistical analysis

All values were expressed as the mean ± SD. ANOVA tests were used to determine the significance of differences in cell viability, the surviving cell fraction, and tumor volume. After significant differences were confirmed, data were analyzed with the unpaired Student's t test. Statistical analyses were performed using the software Statcel3 (OMS, Tokyo, Japan). A value of $P < 0.05$ was considered to be significant.

Results

Effects of B_4C-mediated BNCT on SAS cells

The effects of B_4C-mediated BNCT were examined in cell cultures. When cells were incubated with B_4C particles for 10 min and exposed to neutrons in the presence of B_4C particles, cell viability was decreased to 77% of the control 48 h after the treatment (Figure 1). Neutrons alone decreased cell viability to 96% of the control. A significant difference ($P < 0.01$) was observed between the neutron and B_4C-mediated BNCT groups (Figure 1). SAS cells were incubated with B_4C particles as a control, and no significant changes were noted. When the surviving cell fraction was measured from the formation of colonies after neutron irradiation and B_4C-mediated BNCT, the number of colonies was decreased to 79% and 38 % of the untreated control, respectively (Figure 2). The difference between these groups was significant ($P < 0.01$).
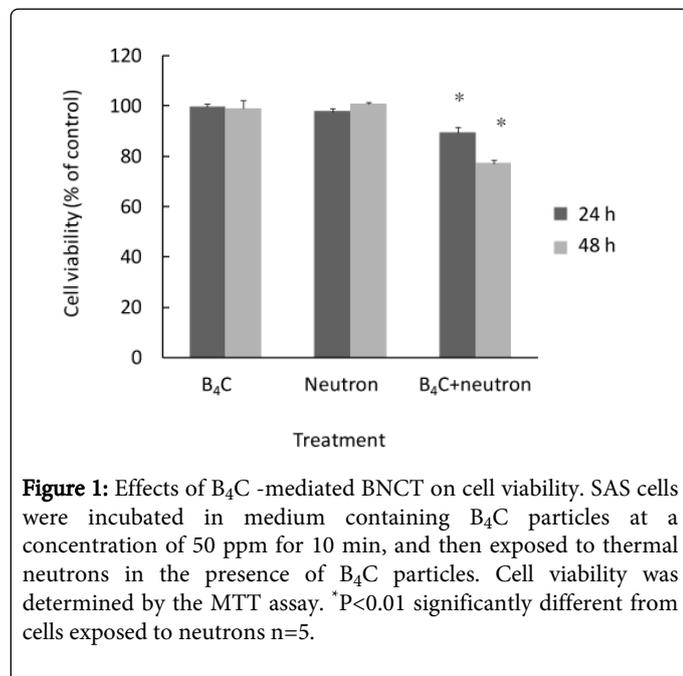

Figure 1: Effects of B_4C -mediated BNCT on cell viability. SAS cells were incubated in medium containing B_4C particles at a concentration of 50 ppm for 10 min, and then exposed to thermal neutrons in the presence of B_4C particles. Cell viability was determined by the MTT assay. *$P < 0.01$ significantly different from cells exposed to neutrons n=5.
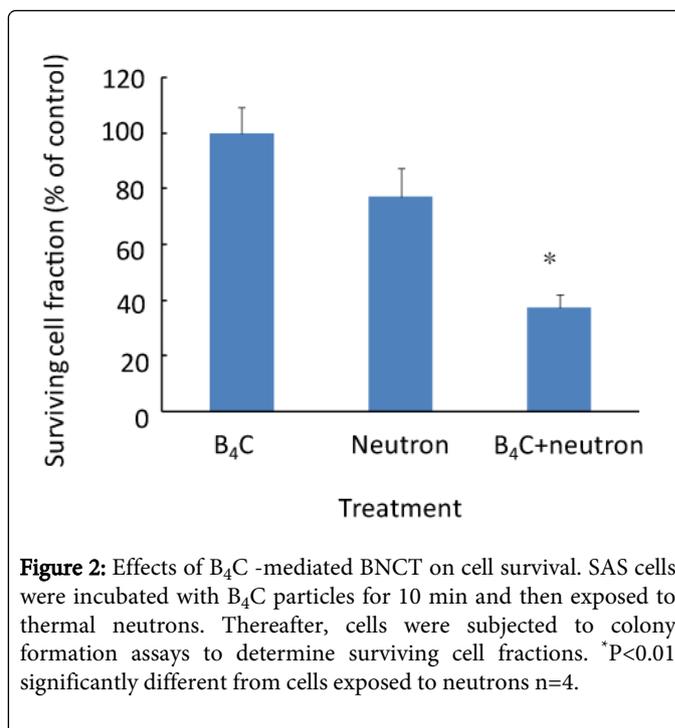

Figure 2: Effects of B_4C -mediated BNCT on cell survival. SAS cells were incubated with B_4C particles for 10 min and then exposed to thermal neutrons. Thereafter, cells were subjected to colony formation assays to determine surviving cell fractions. *$P < 0.01$ significantly different from cells exposed to neutrons n=4.

^{10}B concentrations in oral SCC xenografts in nude mice

Fifty microliters of B_4C solution was injected into nude mouse tumours and the ^{10}B concentration was measured 10 min later. The ^{10}B concentration was 18.57 ppm in the tumour, and was less than 1 ppm in the liver, kidney, and spleen. Using ^{10}B concentrations in tumours, the physical dose in B_4C -mediated BNCT was calculated as 13.06 Gy (Table 1).

Group	^{10}B concentration (ppm)	Physical dose
Neutron	0	2.05
B_4C^+neutron	18.57±2.66	13.06±3.63

Table 1: Boron concentration in tumours and the physical dose.

Effects of B_4C-mediated BNCT on tumour growth in nude mice

We examined whether B_4C particles themselves could affect the growth of nude mouse tumours. Three tumours in nude mice received 50 μl of B_4C solution. The mean tumor volume had increased after 14 days and was 7.2-fold higher than that of the initial volume, while the increase in tumour volume in the control group was 7.7-fold. Therefore, no significant difference was observed between the B_4C particle-treated and control group, which indicated that B_4C particles did not inhibit the growth of tumours.

To determine the effects of B_4C-mediatd BNCT on tumour growth, tumour-bearing animals received an intratumoral injection of B_4C solution followed by neutron irradiation. The experimental groups were as follows: untreated control, neutron only, and B_4C -mediated BNCT. No significant differences were observed in body weight among these groups during the experimental period (Figure 3). Tumours continued to grow and were 5,867 mm^3 in control animals

42 days after the start of the experiment. When tumours were subjected to B_4C-mediated BNCT, tumour volume decreased from 7 days after neutron irradiation. A significant difference was observed between the B_4C-mediated BNCT and neutron only groups 42 days after BNCT ($P<0.01$) (Figure 4).
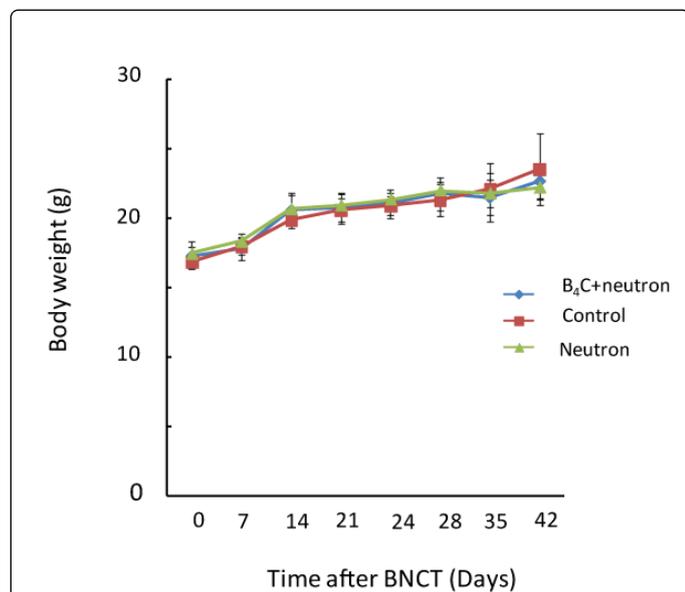

Figure 3: Effects of B_4C-mediated BNCT on body weight. Nude mice carrying SAS tumors received 50 µl of 250 ppm B_4C solution intratumorally. Ten minutes later, tumors were exposed to thermal neutrons. The experimental groups were as follows: untreated control, neutron only, and B_4C-mediated BNCT. After BNCT, body weight was measured during the experiment n=5.
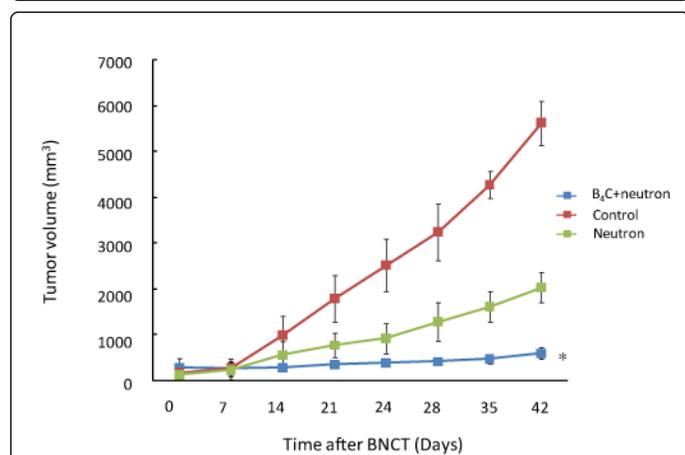

Figure 4: Effects of B_4C-mediated BNCT on tumour volume. Nude mice carrying SAS tumours were treated as described in Figure 3. After BNCT, tumour size was measured and tumour volume was determined. *$P<0.01$ significantly different from neutrons only n=5.

The skin on tumours had a normal appearance during the experiment following B_4C-mediated BNCT. No ulceration was observed in any experimental animal until the extensive growth of tumours and necrotic changes occurred.

Discussion

Tumour cells that retain [10]B are destroyed by the capture of neutrons and production of short-path length particles. BPA is expected to be incorporated into tumour cells by specific uptake mechanisms involving LAT-1 [3,4,21,22]. BSH is not actively incorporated into tumour cells, but enters cells by a concentration gradient. The path lengths of the high LET particles produced by BNCT are in the range of a cell diameter. Therefore, the distance from the boron compound to the cell nucleus is critical.

However, we previously examined the effects of BPA and BSH on oral SCC cells in the presence or absence of these compounds with exposure to neutrons, and demonstrated that an extracellular as well as an intracellular [10]B compound increased the cytotoxicity of BNCT in the cell culture [17]. Although the incorporation of B_4C particles into tumour cells is not expected because of the grain size and no modification for targeting, extracellular boron-rich B_4C particles may capture neutrons and destroy tumour cells. To examine this possibility, we performed BNCT in the presence of B_4C particles and found that cell viability was decreased even though the B_4C particle itself did not affect the growth of SCC cells. A significant difference was observed between the groups treated with and without B_4C particles. Colony formation by oral SCC cells was also suppressed by B_4C-mediated BNCT. Thus, it was concluded that B_4C particles acted as a boron compound for BNCT in cell cultures.

We recently examined the effects of BPA-mediated BNCT on tumours in nude mouse, in which 250 µg BPA was administrated intraperitoneally into tumour-bearing mice. Two hours later, the [10]B concentration in tumours was 10.21 ppm and the physical dose was 8.15 Gy. Neutron irradiation to these tumours decreased the tumor volume to 74% that of tumours treated neutron irradiation only after 42 days [20]. Since the systemic administration of B_4C particles was considered to be inappropriate, we used B_4C particles intratumorally at a concentration of 250 ppm; the calculated [10]B concentration was 38.94 ppm. After injecting 50 µl B_4C solutions, the measured intratumoral [10]B concentration was 18.57 ppm. It is likely that B_4C particles were retained in the intercellular space of the injected tumours. Based on the [10]B concentration, the physical dose was calculated to be 13.06 Gy. As expected from the attained [10]B concentration, the tumor volume was decreased to 38% that of tumors treated with neutron irradiation only after 42 days. The difference between B_4C-mediated BNCT and neutron groups was significant. Thus, the intratumoral injection of B_4C particles achieved a [10]B concentration that was sufficient to suppress tumor growth, as observed by BPA-mediated BNCT in which BPA was administrated intraperitoneally. It is likely that boron-rich B_4C particles localized adjacent to tumor cells may capture neutrons and produce high LET particles to affect cell nucleus.

The effects of B_4C-mediated BNCT are related to the distribution of the injected B_4C particles. The amount of B_4C particles in 50 µl solution was 12.5 µg. In NCT with Gd nanoparticles, a total of 2.4 mg of Gd-nanoCP200 was injected into tumours [12]. "In a non-invasive RF field-generating system", VX2 tumours were injected with 500 µg of single-walled carbon nanotubes to destroy tumours [7]. Mice were administrated 50 µg of gold nanoflowers intratumorally, followed by irradiation with continuous wave near-infrared light to heat the tumours [10]. Although insoluble agents were retained at the injected site of tumours, this was similar to interstitial radiation therapy using gold seeds. No harmful materials can be kept in the body in exchange for tumour regression. Therefore, 12.5 µg of B_4C particles was used in

the present study to induce a significant antitumor effect. A high ^{10}B concentration was achieved in tumours, but not in the liver, kidney, or spleen. Body weight was also unaffected during the experiment. Thus, the side effects of B_4C-mediated BNCT appear to be minimal.

If B_4C particles are distributed between the epithelial cells of the covering skin, B_4C-mediated BNCT may affect the viability of skin as well as tumours [20]. However, we did not observe any skin defects after BNCT in the present study. Although aberrant tumour blood vessels allow B_4C particles to reach the tumour environment, B_4C particles do not accumulate in the intracellular space of a normal epithelium following an intratumoral injection. Therefore, skin is not damaged by BNCT.

Conclusions

We demonstrated that B_4C particles in combination with neutron irradiation decreased cell viability in cell cultures. The growth of oral SCC xenografts in nude mice was more significantly suppressed with B_4C-mediated BNCT than with neutron irradiation alone. Therefore, B_4C particles may be used locally as a ^{10}B compound in the treatment of oral SCC cells by BNCT.

The authors declare that they have no competing interests.

Acknowledgments

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