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The Combined Effect of Electroporation and Borocaptate in Boron Neutron Capture Therapy for Murine Solid Tumors

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^{10}B-Enriched borocaptate (BSH) was administered intraperitoneally to SCCVII tumor-bearing C3H/He mice. Electroporation (EP) was conducted by using a tweezers-type electrode. The ^{10}B contents in tumors were measured by prompt γ-ray spectrometry. The colony formation assay was applied to investigate the antitumor effects of boron neutron capture therapy (BNCT) and thereby to estimate the intratumor localization of BSH. The ^{10}B concentrations in tumors decreased with time following BSH administration, falling to 5.4(±0.1) ppm at 3 h, whereas EP treatment (3 repetitions) 15 min after BSH injection delayed the clearance of BSH from tumors, and the ^{10}B level remained at 19.4(±0.9) ppm at 3 h. The effect of BNCT increased with the ^{10}B concentration in tumors, and the combination with EP showed a remarkably large cell killing effect even at 3 h after BSH injection. The effect of BNCT, i.e., slope coefficient of the cell survival curve of tumors, without EP was proportional to tumor ^{10}B level (r=0.982), and that of BSH-BNCT combined with EP lay close to the same correlation line. However, tumors subjected to EP after BSH injection did not show high radiosensitivity when irradiated after conversion to a single cell suspension by enzymatic digestion. This indicates that the increase of the BNCT effect by EP was a consequence of enclosure of BSH in the interstitial space of tumor tissue and not within tumor cells. This is different from a previous *in vitro* study. The combination of EP and BNCT may be clinically useful, if a procedure to limit EP to the tumor region becomes available or if an alternative similar method is employed.

Key words: BSH — Electroporation — SCCVII tumors

^{10}B emits an α particle and a recoiling ^{7}Li ion with an average total kinetic energy of 2.34 MeV through ^{10}B(n,α)^{7}Li reaction, and these particles represent high LET radiation. Most of the energy is deposited very locally, because the tracks do not exceed 1 cell diameter (10 μm).[1)] Moreover, the cross section of this reaction is extremely high, 3837 barn (cm^2), in comparison with ^{1}H, ^{12}C, ^{14}N and ^{16}O. Therefore, if a sufficient fluence of thermal neutrons is delivered, tumor cells that selectively accumulate ^{10}B can be destroyed completely with minimal damage to adjacent cells containing no ^{10}B. This principle has been applied to the treatment of malignant glioma and malignant melanoma.[2)] ^{10}B-Enriched borocaptate sodium (BSH: $Na_2B_{12}H_{11}SH$), which does not cross the blood brain barrier (BBB) into normal brain, but accumulates in malignant brain tumors because of their disrupted BBB, is used as an agent for boron neutron capture therapy (BNCT) of malignant glioma.[3)] However, BSH has poor membrane permeability and the accumulation of BSH in tumor cells is low. Moreover, in tumors in other organs, BSH distribution is not selective because of the lack of an appropriate selective barrier such as the BBB.[2)] It has been reported that passage of an electric current across a cell membrane can increase its permeability, and this technique (known as electroporation (EP)) has frequently been applied to introduce drugs into cells.[4)] Because the tracks of α particles and recoiling ^{7}Li ions are very short as described above, the energy deposition in DNA varies depending upon the site of boron neutron capture reaction. Biological effectiveness decreases with the distance between the reaction site and the cell nucleus. This difference in cell killing can be easily detected by means of colony formation assay. In our previous study the tumor cells subjected to EP in the presence of BSH before neutron irradiation exhibited a much higher sensitivity to neutrons than did cells not exposed to EP.[5, 6)] The potential significance of the combination of EP as an approach for improving *in vitro* BSH-BNCT effect is clear. In the present study the influence of EP on the effectiveness of BSH-BNCT was investigated in solid tumors.

MATERIALS AND METHODS

Tumor and mice SCCVII tumor cells (mouse squamous cell carcinoma), exponentially growing in Eagle's mini-

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mum essential medium supplemented with 292 mg/liter glutamine and 12.5% fetal calf serum, were inoculated (5×10^4 cells) into the thighs of 8- to 10-week-old male C3H/He mice. About 7 days later, the tumors reached suitable sizes for experiments (mean diameter 7 mm). At present, BNCT is primarily used to treat malignant glioma and malignant melanoma, but to explore the feasibility of applying BNCT for various cancers, and also from the viewpoint that the effects of EP are probably similar in various kinds of tumors, the well-known murine SCCVII tumors were used in the present research, as previously.[5–7)]

Boron compounds and administrations BSH (^{10}B-BSH-Na) was used as a boron compound. BSH was dissolved in physiological saline at a concentration of 6 mg/ml (3.43 mg ^{10}B/ml) and injected into mice intraperitoneally (BSH 75 mg/kg body weight). BSH was purchased from BBI (Boron Biologicals, Inc., Raleigh, NC).

EP and measurement of boron concentration in tissues EP treatment (500 V, 1 ms, 8 pulses) was applied to tumors grown in the thigh by using a Gene Pulsar (Bio-Rad Laboratories, Hercules, CA) and a tweezers-type electrode 15 min after BSH administration. EP treatment was performed under general anesthesia induced by an intraperitoneal injection of Nembutal (50 mg/kg). It was repeated up to three times at an interval of 1 min. Three hours later, the tumors were excised and ^{10}B-concentrations were measured by prompt γ-ray spectrometry using a thermal neutron guide tube installed at Kyoto University Reactor (KUR).

Total nephrectomy to delay the clearance of BSH from tumors It is known that BSH is excreted into the urine, and our previous *in vitro* study indicated that BSH accumulation in tumor cells increased with contact time of the cells with BSH in the culture medium. Therefore, we attempted to delay the clearance of BSH in mice by performing total nephrectomy before BSH administration. The operation was performed under general anesthesia induced with Nembutal.

Thermal neutron irradiation The tumors were irradiated with a thermal neutron beam as follows. The tumors were excised from mice and immediately placed in 2-ml Teflon tubes with a tight screw cap to prevent drying. Thereafter, irradiation with thermal neutrons accompanied with a negligible amount of fast neutrons from the heavy water facility of KUR was started within 10 min after tumor excision. The longest irradiation time was 60 min.

The cadmium ratio of the thermal neutron beam was 148, i.e., a mixed beam of 2×10^9 thermal, 1.4×10^7 epithermal and 2.8×10^6 fast neutrons at the fluence rate base ($cm^{-2}s^{-1}$) produced by the heavy water facility of KUR. The neutron fluence was measured by the spectrometry of γ-rays generated from ^{198}Au produced in the reaction of ^{197}Au(n,γ)^{198}Au, as in a previous study.[7)] Au foils were placed on the front and back of the Teflon tube, and the fluence of neutrons that reached the tumor tissue was taken as the arithmetic mean value.

Colony formation assay Within 5 min after irradiation the procedure of colony formation assay was initiated. The tumors exposed to BNCT were minced with scissors, and single cell suspensions were made by digesting tumor fragments with a mixed solution of 0.05% trypsin and 0.02% EDTA at 37°C for 15 min. An appropriate number of live tumor cells to form 50 to 100 colonies was inoculated onto 60 mm diameter Petri dishes containing complete fresh medium. After incubation for 10 days, the colonies were fixed with ethanol, stained with crystal violet, and counted macroscopically to determine the surviving cell fraction (SF). Tumor tissues kept in Teflon tubes without neutron exposure for the same duration were used as controls for colony formation assay. The plating efficiency of control tumors was around 40%. To obtain SF, the plating efficiency following BNCT was divided by that of control tumors. The best-fitted linear relationship between neutron fluence and SF was determined by the least-squares method.

RESULTS

The ^{10}B concentration in tumors decreased with time following BSH administration, i.e., the values at 15, 30 min and 3 h were 25.7(±1.1), 14.3(±1.5) and 5.4(±0.1) ppm, respectively (Fig. 1). However, the application of EP treatment 15 min after dosing of BSH delayed the clearance of BSH from tumors (Fig. 1). Furthermore, the ^{10}B levels in tumors at 3 h after BSH injection increased with the repetition of EP treatment, i.e., 8.9(±0.6), 16.3(±2.8), 19.4(±0.9) ppm for one, two and three EP treatments, respectively (Fig. 1).

The surviving cell fraction decreased exponentially with increasing neutron fluence, and addition of BSH significantly enhanced the cell killing effect of NCT on tumors in a ^{10}B concentration-dependent manner, i.e., the slope coefficients of the cell survival curves were 0.171 ($\pm0.0112)\times10^{-12}$, 0.370($\pm0.0101)\times10^{-12}$, 0.715(±0.0525) $\times10^{-12}$ and 1.606($\pm0.0369)\times10^{-12}$ cm^2s for neutron irradiation alone, and combined with 5.4, 14.3 and 25.7 ppm ^{10}B, respectively (Fig. 2 and Table I). The EP destroyed tumor cells and the live cell yield decreased to the level of 71%(±6.8%) of the control tumors. However, EP alone did not increase the sensitivity of tumors to neutrons, as observed in cultured cells (data not shown). BNCT in combination with EP showed a marked cell killing effect even at 3 h after BSH injection and EP, i.e., the slope coefficient was 0.891(±0.0571) (Fig. 2 and Table I).

The slope coefficients of cell survival curves showed a linear relationship with boron-10 levels in tumors (Fig. 3, r=0.982). The coefficient of cell survival curves in BSH-BNCT combined with EP lay close to the same linear

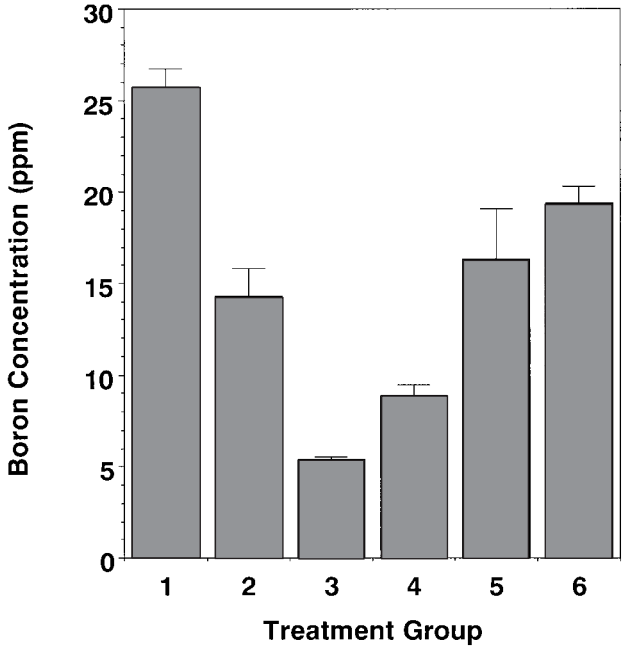

Fig. 1. ^{10}B concentrations in tumors following BSH (100 mg/kg) administration with or without electroporation (EP). Treatment groups 1, 2 and 3 were used to determine the ^{10}B concentrations 15, 30 min and 3 h after BSH administration without EP, respectively. Treatment groups 4, 5 and 6 received one, two and three repetitions of EP, respectively. Each data point with vertical line represents the mean value and standard deviation in six to ten tumors.

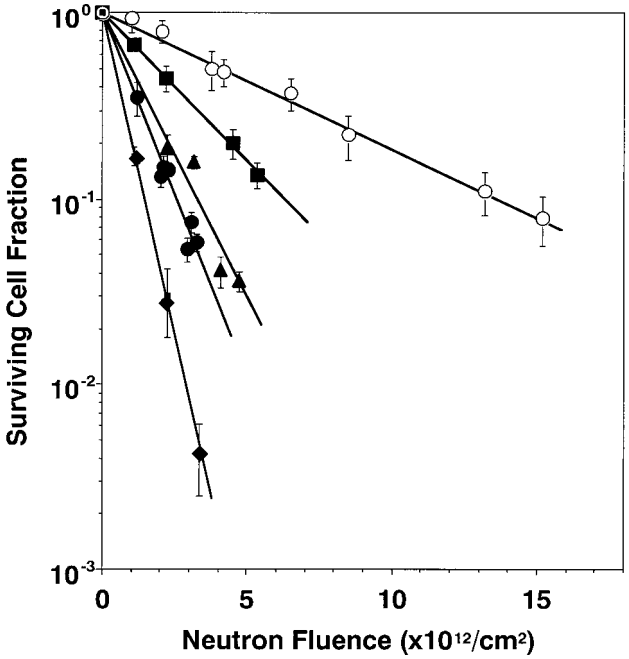

Fig. 2. The relations between neutron fluence and surviving cell fraction in tumors that received NCT under various conditions. ○ NCT alone, ■ NCT at tumor ^{10}B content of 5.4 ppm, ▲ NCT at tumor ^{10}B content of 14.3 ppm, ● NCT at tumor ^{10}B content of 19.4 ppm and EP, ◆ NCT at tumor ^{10}B content of 25.7 ppm. Data points and vertical lines represent mean and SD values of six to ten tumors in two independent experiments.

Table I. Parameters of Cell Survival Curves of SCCVII Tumor Cells Following BSH-BNCT under Various Conditions

Treatment group	$-\ln SF=C+\alpha\phi$	
	C	$\alpha\pm$SD ($\times10^{-12}$)
1) Neutrons alone	−0.0378	0.171±0.0112
2) EP (–), 5.4 ppm	−0.0110	0.370±0.0101
3) EP (–), 14.3 ppm	−0.0419	0.715±0.0525
4) EP (–), 25.7 ppm	−0.0321	1.606±0.0369
5) EP (+), 19.4 ppm	0.0219	0.891±0.0571
6) Nephrect., EP (–), 31.5 ppm, single cells	−0.0163	0.278±0.0135
7) EP (+), 19.4 ppm, single cells	−0.0150	0.330±0.0157

ϕ, neutron fluence; Nephrect., nephrectomy; EP, electroporation.

regression line (Fig. 3). In our previous study, the effect of BNCT on cultured cells increased with increasing incubation time of the cells in BSH containing medium before neutron exposure. In the present study, we increased the contact time of tumors with BSH by total nephrectomy. The boron-10 levels in tumors were undetectable in mice without total nephrectomy. After this operation, boron-10 concentration in tumors was maintained at a high level, i.e., 31.5(±2.3) ppm even 14 h after BSH administration. However, the tumor cells in these mice did not show high radiosensitivity when they were irradiated in the form of a single cell suspension obtained by digesting the tumors with 0.05% trypsin (slope coefficient: 0.278±0.0135) (Fig. 4 and Table I). A similar phenomenon was observed in tumors that received EP combined with BSH-BNCT (slope coefficient: 0.330±0.0157) (Fig. 4 and Table I).

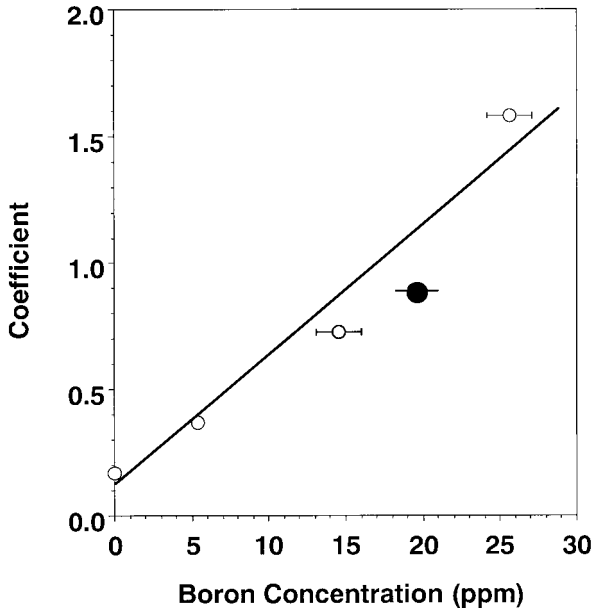

Fig. 3. The relation between boron concentration in tumors and slope coefficient of cell survival curves. Closed symbol represents the tumors exposed to EP combined with BSH-BNCT. Data in the table are plotted in Fig. 3.

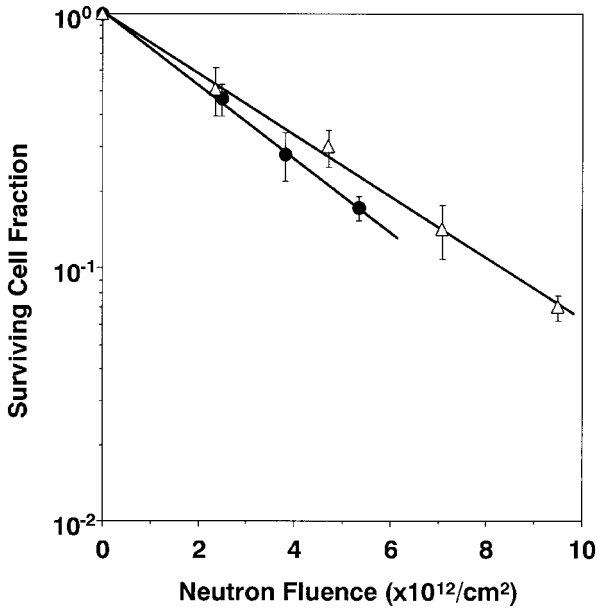

Fig. 4. The relation between neutron fluence and surviving cell fraction of tumors that were converted to a single cell suspension by using an enzyme cocktail before irradiation. ● EP and BSH administration before digestion of tumors into single cells, ^{10}B content in tumors was 19.4 ppm. △ Total nephrectomy and BSH administration before digestion of tumors into single cells, ^{10}B content in tumors was 31.5 ppm. Data points and vertical lines represent mean and SD values of six to ten tumors in two independent experiments.

DISCUSSION

BNCT permits the application of a high radiation dose to tumors, if the boron compound is selectively accumulated in tumors. However, clinical studies so far performed have not provided sufficient data to prove its superiority to conventional treatments. This is attributable to the insufficiently selective accumulation of boron compounds in tumors and poor penetration of thermal neutrons into tissues. BSH has 12 B-10 atoms in the molecule and is a very efficient carrier of B-10. However, the selectivity of accumulation depends upon the BBB in the brain and, therefore, its utility is limited to malignant glioma. We designed this study for two reasons, i.e., to increase the selectivity of accumulation in tumors without the help of the BBB and consequently to extend the applicability of BSH in BNCT, and BNCT itself.

Some investigators have found that BSH accumulates in tumor cells at higher concentrations than in the blood, i.e., the tumor/blood ratio is 1.3–1.46.[7, 8] However, others reported that the tumor/blood ratio of boron concentration does not exceed unity in clinical cases.[9–11] Furthermore, almost complete loss of the BSH-BNCT effect occurs upon washing cells, even after 24-h preincubation.[12] In our previous study, the membrane permeability to BSH and the accumulation in the cells were confirmed, but the potency was low.[5, 6] That is, the BNCT effect increased with preincubation time of cells with BSH and did not disappear even after washing. The study also indicated that BSH in combination with EP remarkably enhanced the neutron-sensitivity of the cells in comparison with control cells or the cells received preincubation treatment alone in the BSH containing medium.[5, 6] Washing of the cells did not block this enhancement by EP.[5, 6]

Based upon the above findings, we have examined the effect of EP on BSH-BNCT to *in vivo* tumors. The EP was remarkably effective in maintaining the boron-10 levels in tumors. A boron-10 concentration of 19.4(±0.9) ppm was achieved in the tumor even at 3 h after EP (3 times), whereas the boron-10 concentration in tumors without EP was 5.4(±0.1) ppm (Fig. 1). This finding suggests that the EP trapped BSH in tumor tissues, although it was not clear whether BSH entered into the tumor cells or accumulated in the interstitial space. When the EP treatment was repeated, boron-10 levels in tumors increased with the repetition of EP (Fig. 1). These findings are quite similar to those obtained in cultured cells in our previous study.[6]

The radiosensitivity of tumors to neutrons was enhanced with increasing boron-10 level (Fig. 2), and the slope coefficients of cell survival curves exhibited a linear relationship to boron-10 levels (Fig. 3). This finding is reasonable,

because the radiation dose to tumor cells, especially high LET components from $^{10}B(n,\alpha)^{7}Li$ reaction, increases proportionally with boron-10 level. The slope coefficient of the cell survival curve of tumors that received EP with administration of BSH was also close to the same regression line (Fig. 3). This is different from the finding observed in our previous studies using an *in vitro* system. In the *in vitro* experiments the cells which received EP in the presence of BSH showed a higher sensitivity to neutrons in comparison with that of cells in the culture medium containing BSH without EP, suggesting that BSH was efficiently introduced into tumor cells by EP.[5, 6] Comparison of the present *in vivo* results with previous *in vitro* results implies that BSH trapped in tumor tissue by EP is mainly located in the interstitial space.

The difference in location of BSH between tumors and cultured tumor cells after EP was clearly demonstrated by examining the neutron sensitivity of tumor cells in a single cell suspension prepared from tumors that had received BSH and EP. The sensitivity of tumor cells to neutrons was substantially lost when tumors were digested to give a single cell suspension (Fig. 2, Fig. 4 and Table I). However, in *in vitro* study, trypsinization and centrifugation to remove BSH did not decrease the neutron sensitivity of the cells.[6] This difference is attributable to the difference in BSH location. Macroscopic hemorrhage was observed in tumors with EP when the tumor fragments were minced to form single cell suspensions. Based upon this finding and the above data, it is thought that EP destroyed tumor cells and tumor vasculatures, and consequently inhibited the clearance of BSH from tumors, thereby maintaining the boron-10 content at a high level. In the *in vitro* study, it appeared that prolonged contact time of tumors with BSH might enhance its intracellular accumulation. However, the *in vivo* study showed no effect of prolongation of the contact time of cells with BSH (Fig. 4). This indicates that behavior of BSH is considerably different in cultured cells and in tumors.

In this study, a tweezers-type electrode was used for EP. Therefore, the surrounding normal tissues (skin and muscle) also received the effects of EP, and high levels of boron-10 concentration were observed (data not presented). Markedly enhanced accumulation of BSH by EP has been reported in an experimental rat brain tumor model. However, the accumulation of BSH in surrounding normal brain was also noticed, as observed in our study, when a similar type of electrode was employed for EP.[13] It was also reported that EP selectively increased the accumulation of boron-10 in the same experimental brain tumors when it was combined with boronated porphyrin (BOPP).[13] This suggests that selectivity of boron-10 accumulation in tumors with EP may depend on the boron compound employed. The tweezers-type electrode used in this study is very limited in its application, i.e., depending on tumor volume and location. To overcome this shortcoming and to achieve a differential increase in BSH concentration in tumors compared with surrounding normal tissues, a different type of electrode, such as needle-type electrodes that can be inserted into the tumor lesions, may have to be used. Such an electrode is probably applicable to various tumors, like brachytherapy. As an alternative to EP, shock waves might be effective to increase accumulation of BSH in tumors because the action of a shock wave is similar to EP.[14, 15] Moreover, shock waves can be focused onto lesions deeply situated in the body. We are planning to investigate the effect of BSH plus shock waves using SCCVII tumor models.

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