Delivery of Sodium Borocaptate (BSH) to Oral Cancer by Transferrin-PEG-Liposome, for Boron Neutron Capture Therapy (BNCT)

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ABSTRACT

Sodium borocaptate (BSH) is a boron compound used in clinical Boron Neutron Capture Therapy (BNCT).

BSH is accumulated in cancer tissues in concentrations proportional to its blood concentration, but it is rarely actively taken up by itself. Therefore, a novel drug delivery system (DDS) is required. Here, we devised a method to accumulate BSH selectively in tumors in mice, using transferrin-conjugated polyethylene glycol liposome (TF-PEG-liposome).

MATERIALS and METHODS: The tumor-bearing mice were intravenously injected with one of the three boron compounds (Bare BSH, PEG-liposome-BSH, TF-PEG-liposome-BSH).

After 24, 48, 72h, the boron concentration was measured by inductively coupled plasma-atomic emission spectrometry.

RESULTS: Blood levels of boron were high in the PEG-liposome-BSH group and TF-PEG-liposome-BSH group (TF group) in comparison with the Bare BSH group at 24h after. But, at 48h after blood levels of boron were reduced in all three groups. Boron concentrations in tongue tissue were low in all treatment groups. The TF group exhibited higher boron concentrations in tumor tissues compared to the other groups at 24 and 48h.

CONCLUSION: The TF-PEG-liposome increased the boron concentration of tumor selectively and, furthermore, extended retention time. These results suggest that TF-PEG-liposome has the potential to improve tumor boron delivery in BNCT.
INTRODUCTION

T1- and T2-stage oral cancers are frequently treated with surgery. However, after primary therapy is administered, progressive and recurrent cancers are difficult to be cured, even with modern combined therapies with surgery, such as radiotherapy, or chemotherapy. Without some kind of new treatment strategy, improvement in treatment outcome would be limited.

We initiated Boron Neutron Capture Therapy (BNCT), from 2005 for patients with recurrent oral cancer and inoperable cervical lymph node metastasis. This treatment exhibited excellent clinical characteristics and is marked local control effects. Additionally, in cases for which cancer is not completely eliminated, BNCT helps to decrease pain and bleeding and improves eating functions, thereby improving patient’s quality of life, i.e., the vital prognosis [1,2,3].

When the stable boron isotope Boron10 (10B) captures a neutron, it divides into a high-energy alpha particle and a lithium recoiled nucleus, the particle sizes of which are approximately 9 μm and 4 μm, respectively; roughly the size of cancer cells. These short pathlengths can attribute the cytotoxic effects only in 10B-containing cells. BNCT is based on this reaction. When the boron can be incorporated into only cancer cells, it can be possible to selectively destroy these cells alone.

For successful BNCT, a large quantity of boron atoms must be taken up selectively in tumor cells. Ideally, a boron concentration of 15-30 μg/g is required in a tumor, and the ratio of boron level in the tumor to the circumference of normal tissue (T/N ratio) should be 5 or more [4,5,6].

Boronophenylalanine (BPA) and sodium borocaptate (BSH) are boron compounds used in clinical BNCT. BPA is an analog of phenylalanine, which accumulates at high concentrations in rapidly dividing cells [7]. In oral cancer, tumor cells readily take up BPA, leading to clinically satisfactory outcomes. When BPA uptake is low, BSH is used in combination with BPA [1]. BSH, which contains 12 boron atoms per molecule, is accumulated in cancer tissues in concentrations proportional to its blood concentration, but it is rarely actively taken up by itself [6,8,9]. Therefore, a novel drug delivery system (DDS) is necessary for selective and efficient transfer of BSH to cancer cells requires. We evaluated the ability of liposomes modified with polyethylene glycol (PEG-liposomes), known clinically as Stealth® liposomes, and PEG-liposomes conjugated to transferrin (TF-PEG-liposomes) to facilitate selective boron delivery to tumor cells [10]. Liposomes are useful as drug carriers because they can encapsulate pharmaceutical agents. However, liposomes are taken up by reticuloendothelial systems (RES) containing many mononuclear phagocytes.

PEG-liposomes encapsulate a large amount of boron and achieve high blood boron levels by preventing uptake by RES.

Transferrin is a glycoprotein that is present in plasma and is involved in the transport of iron into cells. Cancer cell express more transferrin receptors on the surface compared to normal cells.

TF-PEG-liposomes as the DDS targets a transferrin receptor.

Therefore it is expected that TF-PEG-liposomes deliver much boron compounds selectively in a cancer cell.

Here, we described a novel method to transfer higher boron concentration selectively in tumors in mice using TF-PEG-liposomes as the DDS.

MATERIALS and METHODS

1. Cell culture

The present study used SAS cells from a human oral squamous cell carcinoma-derived cell line (HSRRB, Osaka, Japan). Cells were cultured in medium containing 45% DMEM (Dulbecco’s Modified Eagle’s Medium), 45% F-12 (Ham’s F-12 Medium), 10% FBS (fetal bovine serum), antibiotics (hereafter DMEM/F-12) and incubated in 5% CO2 at 37°C.

2. Generation of tumor-bearing mice

After detaching oral squamous carcinoma SAS cells from tissue culture plates with trypsin ethylenediaminetetraacetic acid, we adjusted the concentration to 6 × 10^6 cells per 0.1 ml. Five-week-old male BALB/cAcl-nu mice (CLEA Japan, Inc.) weighing 20 g were anesthetized by inhalation. The mice were subcutaneously injected on the dorsal side with SAS, 6 × 10^4 cells and were subjected to experiments when the major axis of the tumor became 10 mm (Figure 1).
Delivery of BSH to oral cancer by transferrin

4. Preparation of liposomal BSH

BSH was purchased from Katchem, Ltd. (Czechoslovakia). Plain liposomal BSH was prepared by reverse phase evaporation. Briefly, the aqueous phase was a 150 mM BSH solution. The lipids, distearoyl phosphatidylcholine and cholesterol, were completely dissolved at a 2:1 ratio (m/m) in the organic phase (di-isopropyl ether: CHCl3 = 1:1, v/v). The two phases were then mixed together. Subsequently, the solution was sonicated for 60 s using a probe-type sonicator, and a satisfactory emulsion was formed. Following this, the emulsion was immediately placed in a 60°C hot water bath and the organic solvent was completely distilled away using a rotary evaporator. Plain liposomal BSH was obtained by size fractionation, using an extruder to press the emulsion 20 times through a membrane with a pore diameter of 100 nm.

5. Preparation of PEG-liposome-BSH

PEG-liposomal BSH was prepared using the micelle transfer method, whereby plain liposomal BSH was encapsulated in distearoyl phosphatidylethanolamine (DSPE)-PEG micelles. To form the micelles, 6 mol% (of total lipid mass) of DSPE-PEG was completely dissolved in 30 mM HEPES buffer (pH 7.4). Micelles were mixed with plain liposomal BSH, incubated at 4°C for 24 hours with gentle shaking, and then encapsulated in liposomal membranes. Following incubation, the solution was subjected to ultracentrifugation (300,000xg, 4°C) three times in order to remove DSPE-PEG micelles. The resulting solution was PEG-liposomal BSH.

6. Preparation of Tf-PEG-liposomal BSH

As was done to obtain PEG-liposomal BSH, Tf-PEG-liposomal BSH was prepared using the micelle transfer method. We dissolved 1 mol% (of total lipid mass) of DSPE-PEG-NHS (N-hydroxysulfosuccinimide) in 30 mM HEPES buffer (pH 7.4), and immediately added transferrin to the solution. This mixture was then incubated for 1 hour at room temperature to yield DSPE-PEG-Tf micelles. These micelles were mixed with plain liposomal BSH and incubated at 4°C for 24 hours with gentle shaking. Next, 5 mol% DSPE-PEG was added to the liposomes, and the solution was incubated again at 4°C for 24 hours. Following incubation, the solution was subjected to ultracentrifugation (300,000xg, 4°C) three times in order to remove any unencapsulated micelles. The resulting solution was TF-PEG-1liposomal BSH.

7. Measurement of boron concentrations

The boron concentration was measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) using a dual monochromatic ICP emission analysis system (P-5200, Hitachi Corp.). ICP-AES uses high-energy plasma generated from argon by high voltage to heat samples to 10000°C. Elements are identified by the wavelength and intensity of the emitted light spectrum when activated atoms or molecules return to their ground state. This method is capable of measurements down to the ppb level. Boron levels were determined at 249.773 nm, a wavelength considered specific to boron, using calibration curves produced by standard boron solutions. Results were expressed as boron concentration per tissue mass.

RESULTS

Boron concentrations in blood

At 24 h after administration of the boron compounds, the mean boron concentration in the blood was 3.71 μg/g in mice that received bare BSH (BSH group), 140.2 μg/g in mice that received PEG-liposome-BSH (PEG group), and 41.1 μg/g in mice that received TF-PEG-liposome-BSH (Tf group), respectively. The boron concentrations decreased markedly over time for the PEG and Tf groups. At 48 h after administration, those were 2.30 μg/g for the BSH group, 16.5 μg/g for the PEG group, and 25.7 μg/g for the Tf group, respectively. At 72 h after administration, bo-
Boron levels had decreased to 0.92 μg/g for the BSH group, 6.53 μg/g for the PEG group, and 3.27 μg/g for the Tf group, respectively, which were not significantly different. At 24 h after administration of the boron compounds, PEG group and Tf group were high compared with BSH group. (P<0.05). (Figure 2)

Boron concentration in tongue tissue

Boron concentrations in non-tumor tongue tissue were low in all treatment groups at each time point. No significant difference was observed among groups or time points (Figure 3).

Boron concentration in tumor tissue

At 24 and 48 h after administration, the Tf group exhibited higher boron concentrations in tumor tissues compared to the BSH and PEG groups. Mean boron concentration in the Tf group was 23.3 μg/g at 24 h and 23.2 μg/g at 48 h, respectively. In contrast, the boron concentration in the PEG group was only 6.04 μg/g at 24 h and 5.93 μg/g at 48 h, respectively. (p<0.05)(Figure 4)

Figure 2  Time course of boron concentrations in blood
(◊ : Bare-BSH, ■ : PEG-liposome-BSH, ▲ : Tf-PEG-liposome-BSH)
At 24 h after administration of the boron compounds, PEG-liposome-BSH and Tf-PEG-liposome-BSH were high. (P<0.05 compared with BSH) But they decreased sufficiently at 48,72h.

Figure 3  Time course of boron concentration in tongue tissue
(◊ : Bare-BSH, ■ : PEG-liposome-BSH, ▲ : Tf-PEG-liposome-BSH)
Boron concentrations in non-tumor tongue tissue were low in all treatment groups at each time point.
DISCUSSION

The neutrons used in BNCT are thermal neutrons in which the mean energy has been reduced to 0.025 eV through a sufficiently thick layer of heavy water. Low-energy thermal neutrons exert weak cytotoxic effects and are readily absorbed by atomic nuclei. Among the elements of the human body, the neutron capture cross-sectional area is 1.81 barn for nitrogen (1 barn = 10^{-28} cm²). In contrast, the neutron capture cross-sectional area is 3837 barn for 10B, indicating that it is extremely likely to capture neutrons. The range of a 10B atomic nucleus that has captured a neutron splits into a 9-μm α particle, a 4-μm 7Li recoil nucleus, and a γ-ray. The α particle and 7Li recoil nucleus are high linear energy transfer particle rays, which impart to their surroundings high energies of 1.47 and 0.84 MeV during their pathlengths of 9 and 4 μm, respectively. BNCT is based on this nuclear reaction. If boron compounds accumulated only in cancer cells, it would become possible to destroy only cancer cells without damaging normal cells [11]. Therefore, the key point for effective BNCT is selective accumulation of high concentrations of 10B by target cancer cells. An ideal intra-tumor boron concentration appears to be 15 to 30 μg/g [4,5,6]. Currently, drip infusions of BPA alone or combined with BSH are delivered before irradiation [1]. Generally T/N ratio by BPA alone is generally sufficiently high in oral cancer [12]. Also we obtained satisfactory results with the combination of BPA and BSH even in the cases the T/N ratio by BPA alone is low and treatment effectiveness cannot be expected [1]. Actually Miyatake et al. have also reported the effectiveness of BPA combined with BSH in BNCT for malignant gliomas [13].

By the way, new boron compounds designed for BNCT must be efficiently and selectively taken up by cancer cells to eliminate adverse effects. However, only BPA and BSH are currently available for clinical use. Thus, it is necessary to develop new DDS to introduce boron atoms into cancer cells more selectively at higher concentrations.

Liposomes, discovered by Bangham et al [14], are cell-like vesicles consisting of a phospholipid bilayer. They have low antigenicity and toxicity and are useful as drug or gene carriers because they can encapsulate pharmaceutical agents. Vascular permeability is increased in cancer tissues, which allows polymers such as liposomes to leak from blood vessels into tumor tissues. Furthermore, the lymphatic is undeveloped; therefore, liposomes that have accumulated in tumor tissue are difficult to discharge. This ability to accumulate at higher concentrations in tumor tissues than in normal tissues is called as the enhanced permeability and retention (EPR) effect [15]. For passive targeting with EPR, drugs must be maintained at high blood concentrations for extended periods. However, liposomes are taken up by reticuloendothelial systems (RES) containing many mononuclear phagocytes. Attaching amphipathic PEG to the surface of a liposome increases its hydrophilicity and

**Figure 4** Time course of boron concentration in tumor tissue

- : Bare-BSH, ■ : PEG-liposome-BSH, ▲ : TF-PEG-liposome-BSH

At 24 and 48 h, the TF-PEG-liposome-BSH exhibited higher boron concentration in tumor tissues compared to the Bare-BSH and PEG-liposome-BSH groups. (+P<0.05)
sterically prevents interactions with complement system proteins. Because phagocytosis is triggered by particles with highly hydrophobic surfaces, the increased hydrophilicity of PEG-liposomes makes them less likely to be taken up by macrophages, which subsequently prolongs the time of PEG-liposomes remaining in the blood [16,17,18,19,20]. Thus retaining boron compounds within vessels of tumor tissues for extended periods improves the success of passive targeting by EPR effects.

In cancer treatment, active targeting DDSs are ideal in addition to passive targeting. These methods involve specific binding between drugs and tumor cells. Active DDS uses ligands that bind receptors specifically expressed on the surface of target cells as well as monoclonal antibodies that recognize surface receptors. Generally following ligands are used for specific targeting, such as epidermal growth factor, folic acid, and transferrin (Tf). Tf is a glycoprotein synthesized in the liver that is present in plasma (2.5 mg/ml) and is involved in the transport of iron into cells. Every Tf molecule bound to two Fe^{3+} ions (Fe^{3+}-Tf) binds specifically with the Tf receptor (TIR) on the cell surface, where it is encapsulated in a coated vesicle to be taken up by endocytosis. The Fe^{3+}-Tf is then translocated to the endosome. Under the acidic conditions (pH 4-5) of the endosome, the iron affinity of Tf decreases and Fe^{3+} is released. Fe^{3+-free} Tf (Apo-Tf) binds the TIR, and both are excocytosed and re-transported to the surface of the plasma membrane. In the weak alkaline environment outside the cell, Apo-Tf breaks free of the TIR, and recombines with Fe^{3+} to deliver it into the cell [21]. The TIR is strongly expressed on the surface of cancer cells, by encapsulating BSH in Tf-bound PEG-liposomes, boron can be selectively targeted to cancer cells [21, 22,23,24,25,26].

Our study showed high blood levels of boron in the PEG and Tf groups in comparison with the BSH group at 24 h after administration. Boron uptake by RES was circumvented by coating the liposomal surface with PEG, allowing high concentrations of boron to contact the tumor cells and facilitating passive targeting. Blood levels of boron were reduced in all three groups after 48 h, but this is advantageous for BNCT. Neutron irradiation when blood boron concentration is still high risks of damaging vascular endothelial cells, and thereby causing serious complications should be considered [27]. Although blood boron levels were reduced 48 h after boron administration in the Tf group, a high boron concentration was still maintained in the tumors, which allows BNCT to kill tumor cells while reducing the damaging effects on vascular endothelial cells.

The tumor boron concentration in the PEG group was only 6.04 µg/g at 24 h, which was lower than that of the Tf group. In addition, the tumor concentration declined in the PEG group to 5.93 µg/g at 48 h and 1.32 µg/g at 72 h after administration, respectively. In contrast, the Tf group showed a high concentration of 23.3 µg/g at 24 h and maintained a high level of 23.2 µg/g even at 48 h after administration. PEG-liposomes encapsulate a large amount of boron and achieve high blood boron levels by preventing uptake by RES, using the EPR effect to boost concentrations of boron higher than that of the BSH group. But active targeting to the tumor TIR enabled the Tf-PEG-liposome to further boost the tumor boron concentration and extend retention time.

Although TIR is expressed to some extent on the surface of non-tumor cells, in the present study, the boron concentration in the tongue was extremely low and did not differ among treatment groups. Boron concentrations do not increase in normal oral mucosal tissues even if BNCT is performed with Tf-PEG-liposomes.

To efficiently destroy cancer cells with BNCT, the location of boron atom is important. With equivalent amounts of boron in the tumor, the cytotoxic effect is greater if boron is located inside cells rather than distributed uniformly in the interstitial fluid of the cancer tissue. The reaction between Tf and TIR, a mechanism for regulating iron metabolism by intracellular uptake of iron through endocytosis, may be utilized to deliver boron near the cell nuclei.

CONCLUSION

The Tf-PEG-liposome increased the boron concentration of oral squamous carcinoma cells selectively and, furthermore, extended retention time. These results suggest that Tf-PEG-liposome has the potential to improve tumor boron delivery in BNCT.

REFERENCES

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