DETERMINATION OF LIPOSOMAL BORON BIODISTRIBUTION IN TUMOR BEARING MICE BY USING NEUTRON CAPTURE AUTORADIOGRAPHY

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ABSTRACT

It is necessary to accumulate the $^{10}$B atoms selectively to the tumor cells for effective boron neutron capture therapy (BNCT). In order to achieve accurate measurements of $^{10}$B concentrations in biological samples, we employ a technique of neutron capture autoradiography (NCAR) of the sliced whole body samples of tumor bearing mice using CR-39 plastic track detectors. The CR-39 detectors attached with samples were exposed to thermal neutrons in the thermal column of the TRIGA II reactor at the Institute for Atomic Energy, Rikkyo University. We obtained NCAR images for mice injected intravenously by $^{10}$B-polyethylene-glycol (PEG) binding liposome or $^{10}$B-bare liposome. The $^{10}$B concentrations in the tumor tissue of mice were estimated by means of alpha and lithium track density measurements. In this study, we increased the accumulation of $^{10}$B atoms in the tumor tissues by binding PEG chains to the surface of liposome, which increase the retension in the blood flow and escape the phagocytosis by reticulo-endothelial systems. Therefore, $^{10}$B-PEG liposome is a candidate for an effective $^{10}$B carrier in BNCT.

1 INTRODUCTION

The cytotoxic effect of BNCT is due to a nuclear reaction between $^{10}$B and thermal neutrons ($^{10}$B + $^1n \rightarrow ^3\text{Li} + ^4\text{He} + 2.31$ MeV (93.7 %) / 2.79 MeV (6.3 %)). The resultant lithium ions and α particles are high linear energy transfer (LET) particles which give high biological effect. Their short range in tissue (5 - 9 µm) restricts radiation damage to those cells in which boron atoms are located at the time of neutron irradiation.
Liposomes can contain a large amount of $^{10}$B compound, which can be delivered to tumor cells. We have reported that $^{10}$B atoms delivered by immunoliposomes are cytotoxic to human pancreatic carcinoma cells (AsPC-1) with thermal neutron irradiation in vitro [1], and intratumoral injection of boronated immunoliposomes can increase the retention of $^{10}$B atoms in tumor cells, and suppress tumor growth in vivo under thermal neutron irradiation [2].

In this study, we prepared the polyethylene-glycol binding single unilamellar liposomes (PEG-liposome) and transferrin conjugated PEG-liposome (TF-PEG-liposome) as the effective $^{10}$B carrier to avoid the phagocytosis by the reticuloendothelial system RES. The accurate measurement of $^{10}$B distributions in biological samples with a sensitivity in the ppm range is essential for evaluating the various boron-containing compounds for BNCT. We performed a technique of neutron capture autoradiography using Solid State Nuclear Track detectors (SSNTDs) to qualitatively determine the $^{10}$B biodistribution in whole body samples of mice.

2 MATERIALS & METHODS

2.1 Chemicals
Sodium salt of undecahydro-mercaptocloso-dodecaborate ($\text{Na}_2^{10}\text{B}_{12}\text{H}_{11}\text{SH}$) was obtained by Wako Chemical Co. Ltd. (Tokyo, Japan). Dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC) and distearoyl phosphatidylethanolamine (DSPE) were kindly donated by NOF Corp., Tokyo. NOF also provided a monomethoxy polyethyleneglycol succinimidyl succinate (PEG-OSu) of average molecular weight 2000. Cholesterol (CH) and triethylamine were purchased from Wako Pure Chemical, Osaka. An amphiphatic PEG (DSPE-PEG) was synthesized systematically by combination of DSPE with PEG-Osu [3].

2.2 Target Tumor Cells and Mice
The human pancreatic carcinoma cell line AsPC-1 was obtained from Dainihon Seiyaku Co. Ltd. (Osaka, Japan). AsPC-1 was maintained in RPMI 1640 medium (Hazleton Biologics Inc., Kansas, USA) supplemented with 10 % fetal calf serum (Cell Culture Laboratories, Ohio, USA) and 100 µg ml$^{-1}$ kanamycin. All cultures were incubated in high moisture air with 5 % CO$_2$ at 37°C. Male BALB/c $\nu/\nu$ mice were obtained from Nihon SLC (Shizuoka, Japan) and used at 6 to 7 weeks of age. The procedures for tumor implantation and sacrifice of the animals were in accordance with approved guidelines of the Institution’s Animal Ethics Committee.

2.3 Preparation of PEG binding liposomes containing $^{10}$B-compound
Liposomes were prepared from DPPC/DSPC/CH (7:2:1 molar ratio) and an appropriate amount of DSPE-PEG by the reverse-phase evaporation (REV) method and extrusion method. The lipid mixture (5 mg in total lipids) was dissolved in 600 µl of a chloroform / diethyl ether mixture (1:1, v/v) and 300 µl of 300 mM citric acid (pH 4.0) were added. Liposomes were formed by the REV procedure and extruded more than ten times through polycarbonate filters (Nuclepore, Nomura Science, Tokyo) to control size. One-half ml of 125 mM $^{10}$B-compound ($\text{Na}_2^{10}\text{B}_{12}\text{H}_{11}\text{SH}$, BSH) solution was added to the lipid films, and this admixture was heated at 60°C for 10 min with intermittent vortex mixing and chromatographed on a BioGel A1.5m column (2 cm x 20 cm, Bio-Rad). Uncapsulated $^{10}$B-compound was removed by using centrifugation at 20,000 g. $^{10}$B entrapped bare liposomes were prepared in the same way without polyethylene glycol [3]. The amount of $^{10}$B compound entrapped in liposomes was
determined by the prompt gamma-ray spectrometry at the Research Reactor Institute, Kyoto University [4].

2.4 Experimental Procedure

2.4.1 Tumor injection

AsPC-1 cells (1 x 10^7) were injected subcutaneously into the back of male BALB/c nu/nu mice. The mice were sacrificed 48 and 60 hours after the injection of 10^B entrapped-liposomes (10^B bare-lip., 10^B PEG-lip. or 10^B TF-PEG-lip.).

2.4.2 Preparation of sliced mice samples

The sacrificed mice were frozen at -60° C. Subsequently, the frozen mice were cut sagittally into 40 µm thick slices and put on a mending tape; freeze-dried at -20° C for 2 weeks, and air dried for one more week [5].

2.4.3 Neutron irradiation

The sliced sections were put in close contact with the CR-39 plates (HARZLAS; Fukuv Chemical Industry) using thin adhesive tape. The set of mouse samples were simultaneously exposed in the TRIGA-II reactor of the Rikkyo University (RUR). The neutron flux was 1.0 x 10^8 n/(cm^2 s) and the Cd ratio was 6400. The gamma rays were filtered by a provisional 16 cm thick Pb filter made of lead bricks and the irradiation facility was not optimal regarding the neutron beam intensity and gamma ray background [6].

The thermal neutron fluences varied regarding the objectives of the experiments and were as follows:

- For 10^B concentration measurement : 7 x 10^10 neutrons / cm^2
- For Imaging : 4 x 10^12 neutrons / cm^2

2.4.4 Etching procedure and track analysis

For α-autoradiographic imaging including proton tracks produced by 14N (n, p) reaction, where 14N is the biogenically abundant nuclide, the CR-39 detector plates were etched in a 6.25 N NaOH solution at 70° C for 120 minutes to reveal tracks. The NaOH etching method is commonly used to etch the CR-39 detectors.

The tracks were automatically measured by the TRACOS track analysis system of the J.Stefan Institute [7]. TRACOS is capable of accurate measurements of dimensions, shape, grey level intensity and accurate position of individual tracks on a large CR-39 plate. On the basis of local track densities obtained from positional information of tracks having different origin it is thus possible to make separate digital radiographic images.

3 RESULTS

1.1 Neutron Capture Autoradiography using Track Etch Detectors

In order to examine 10^B-biodistribution in mice, we performed NCAR of sliced mice samples using the CR-39 track etch detectors. Proton and α-tracks were measured by the TRACOS track analysis system. Images of whole body mice by neutron capture autoradiography are shown in Figure 1. Figure 1 shows a whole-body sections of neutron capture radiograph from a set of AsPC-1 pancreatic cancer-bearing mice that have been intravenously given the injection of about 0.7 mg of 10^B-liposome solution. The slices of sacrificed and frozen mice were prepared 60 hours after the injection. It is readily apparent that the tumor contains high level boron until 60 hours after injection. The concentration of boron in tumor after injection of 10^B PEG-lip. and 10^B TF-PEG-lip. was two times higher than that of 10^B bare-lip. at 60 hours. There are also areas within liver which contain high levels of boron.
Proceedings of the International Conference Nuclear Energy in Central Europe, Portorož, Slovenia, Sept. 10-13, 2001

Figure 1: Neutron capture autoradiography of AsPC-1 bearing mouse with intravenous injection of $^{10}$B liposomes. The slices of mice were prepared 60 hours after the injection. (a) $^{10}$B Bare-liposome, (b) $^{10}$B PEG-liposome, (c) $^{10}$B TF-PEG-liposome.

1.2 Detection of $^{10}$B concentration in tumor

AsPC-1-bearing mice ($n = 3$) were given i. v. injection with 0.5 ml of solution of $^{10}$B PEG-lip., $^{10}$B bare-lip. or $^{10}$B TF-PEG-lip. The mean concentration of $^{10}$B PEG-lip., $^{10}$B bare-lip. or $^{10}$B TF-PEG-lip. were 1249 ppm, 1500 ppm, 1536 ppm, respectively.

The mice were sacrificed at 48 hrs after injection and the calculation of $^{10}$B concentration in tumor or liver was performed with the track density of tissues.

Using the TRACOS system, the $^{10}$B accumulations were estimated. They are shown in Table 1. In tumor, optimum $^{10}$B accumulations were confirmed to be 13 ppm after injection of $^{10}$B PEG-lip. and $^{10}$B TF-PEG-lip., and be 9 ppm 48 hours after injection of $^{10}$B bare-lip. There is a possibility to escape the uptake of reticuloendothelial systems (RES) in the liver.
using PEG-lip. and $^{10}$B TF-PEG-lip. The boron contents in tumor have continued keeping effective value by retention due to the circulation with PEG 48 hours after injection. Our data indicated that the selective accumulation of $^{10}$B atoms in tumor was achieved by using $^{10}$B PEG-lip. and $^{10}$B TF-PEG-lip.

Table 1. $^{10}$B concentration in tumor & liver 48 hours after injection of $^{10}$B PEG-liposome / $^{10}$B TF-PEG-liposome / $^{10}$B Bare-liposome on AsPC-1 bearing mice.

<table>
<thead>
<tr>
<th></th>
<th>Tumor [ppm]</th>
<th>Liver [ppm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non treated</td>
<td>2.1±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>BSH</td>
<td>2.8±0.1</td>
<td>5.1±0.2</td>
</tr>
<tr>
<td>$^{10}$B Bare-liposome</td>
<td>9.0±0.29</td>
<td>80.1±0.9</td>
</tr>
<tr>
<td>$^{10}$B PEG-liposome</td>
<td>14.3±0.8</td>
<td>44.5±1.9</td>
</tr>
<tr>
<td>$^{10}$B TF-PEG-liposome</td>
<td>11.7±0.6</td>
<td>36.9±0.8</td>
</tr>
</tbody>
</table>

4 CONCLUSIONS

In this study, we prepared the polyethylene-glycol binding liposomes (PEG-liposome) and transferrin (TF) pendant type PEG-liposomes (TF-PEG-liposomes) as the effective $^{10}$B carrier to avoid the phagocytosis by RES. We have reported that the $^{10}$B-PEG-liposome and $^{10}$B-TF-PEG-liposome have the possibility of retention to the tumor cells and providing sufficient $^{10}$B atoms for the BNCT by systemic injections. Intravenous injection of $^{10}$B-PEG-liposome and $^{10}$B-TF-PEG-liposome inhibited tumor cell growth with thermal neutron irradiation in vivo.

We performed a technique of neutron capture autoradiography using Solid State Nuclear Track detectors (SSNTDs) to qualitatively determine the $^{10}$B biodistribution in whole body samples of mice. $^{10}$B accumulation in the tumor mass can be continued in the effective boron range until 60 hours after intravenous injection of $^{10}$B-PEG-liposome or $^{10}$B-TF-PEG-liposome by SSNTDs. The imaging with NaOH method is effective to identify the position of tumor, and can also illustrate organs in the whole body section by means of proton tracks which show weaker contrast than the α track image [6]. Computing automatic image analysis system (TRACOS) is capable of accurate measurements of dimensions, shape, grey level intensity and accurate position of individual tracks on a large CR-39 plate [7]. Using TRACOS, we will be able to calculate the accurate $^{10}$B concentrations in tumors and other organs according to the time course after injection of the $^{10}$B compounds, and evaluate the potential usefulness of $^{10}$B compounds for BNCT. Neutron Capture Auto-radiography using SSNTDs and TRACOS are also useful in the drug delivery systems.
The measurement of $^{10}$B distributions in biological samples with a sensitivity in the ppm range is essential for evaluating the potential usefulness of various boron-containing compounds for BNCT.

$^{10}$B accumulations in the tumor vary depending on the boron delivery system. We found out lower levels $^{10}$B accumulation in the central part of tumors than in the outer part [6]. It is necessary to supply the boron atoms homogeneously into the tumors for effective BNCT. The study of the microdosimetry of $^{10}$B atoms is ongoing, and CR-39 radiography using track counting will be possible to determine the micro- or fine structure, i.e. micro-autoradiography, of $^{10}$B distribution in the tumor.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan (No. 11691202 and No. 11557092 to Hironobu Yanagie).

REFERENCES