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研究課題名（和文）Novel adjuvant method of malignant brain tumor diagnosis and therapy with targeted nanoparticles made of high-Z elements

研究課題名（英文）Novel adjuvant method of malignant brain tumor diagnosis and therapy with targeted nanoparticles made of high-Z elements

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研究成果の概要（和文）：本研究は、ヒアルロン酸（HA）を組み合わせた放射線腫瘍治療実現にむけ、金ナノ粒子（GNPs）を含むホウ素化合物を使用し評価を行った。ホウ素-HA（BHA）はラット実験で腫瘍および健常組織で異なる蓄積を示した。BHAのキレート結合に関しては、試験薬剤の安定性向上のために重要と考えられた。また、加速器ベース中性子源を使用しGNPsとホウ素-フェニルアラニン（BPA）によるホウ素中性子捕捉療法の実験も行い、吸収中性子線量を推定しその有効性を評価した。本手法は、高Z元素を含む合成ホウ素化合物を用いた場合の線量吸収中性子を測定できるほか、同位体走査及び陽電子放出断層撮影法（PET）にも応用可能と考える。

研究成果の概要（英文）：We evaluated the feasibility of hyaluronic acid (HA) to be used as targeting agent for boron compounds, including gold nanoparticles (GNPs), for combined glioma radiotherapy. Boron-HA (BHA) showed different accumulation in tumor and healthy tissues in rats. Security of BHA bonds might be necessary to increase the stability of the compound. We evaluated the efficacy of boron-neutron capture therapy using boron-phenylalanine (BPA) with GNPs used for absorbed neutron dose estimation at the accelerator-based neutron source. Our method allows to determine the absorbed neutron dose using combined boron compounds containing high-Z element and can be implemented in isotope scanning and positron emission tomography (PET).

研究分野：　radiation oncology

キーワード：　glioma　nanoparticles　radiotherapy　radiodiagnosis　BNCT

1. 研究開始当初の背景

(1) Hyaluronic acid (HA) is a polymer widely spread in different organisms, including humans. HA in certain chemical reactions is able to imbed a large number of boron atoms and form a boron-hyaluronic acid (BHA). HA can selectively bind to CD44 receptors, which play a role in tumor invasion and are expressed in different tumor cells, including gliomas [1]. Using HA as a carrier for boron or on the surface of nanoparticles can improve the targeted drug delivery to tumors and reduce irradiation of healthy tissues.

(2) Boron neutron capture therapy (BNCT) has been shown to be effective novel adjuvant radiotherapy for malignant glioma in a number of clinical trials [2]. The use of an accelerator to produce neutrons instead of a nuclear reactor makes it possible to place the treatment facility in medical institutions. In comparison with other radiotherapy methods, the neutron capture nuclear reaction with alpha-particle release takes place within tumor cells, making it impossible to measure the absorbed neutron dose directly. Golden foils can provide only approximate data after gold activation in proximity to the irradiation target by accumulation of radioactive ^{198}Au isotope with a half-life of 2.7 days. We propose using gold nanoparticles in combination with boron to evaluate the neutron dose directly in the irradiated tissue and suggest implementing such an approach in development of new compounds for BNCT.

2. 研究の目的

(1) To evaluate the feasibility of hyaluronic acid to be used as a targeting agent for boron compounds for BNCT as well as prospectively on the surface of nanoparticles for combined glioma radiotherapy.

(2) To evaluate the efficacy of boron-neutron capture therapy method using boron-phenylalanine (BPA) with gold nanoparticles used for absorbed neutron dose estimation at the proton accelerator-based neutron source with the lithium target.

3. 研究の方法

(1) **BHA applicability evaluation.**

BHA synthesis: Solid-state modification of polysaccharide-based polymers was used to synthesize BHA according to the method described elsewhere [3].

ICP-AES: Initial boron concentration, boron accumulation in vitro and in tumor samples was evaluated by inductively coupled plasma atomic emission spectroscopy (ICP-AES; ICP-8100, Shimadzu, Kyoto, Japan).

Tumor cells: C6 and 9L rat glioma cells and V79 Chinese hamster lung fibroblasts were incubated in MEM (M4655; Sigma-Aldrich, MO, USA) supplemented with 10% fetal bovine serum (FBS; JRH Nitirei Bioscience, Tokyo, Japan) and 1% penicillin-streptomycin (Sigma-Aldrich, MO, USA) at 37°C in humidified atmosphere with 5% CO2.

Cytotoxicity analysis with MTS assay was performed as described previously [4] using Cell Titer 96 AQueous One Solution (Promega, WI, USA).

Animal experiments were approved by the ethics committee of the University of Tsukuba. To form subcutaneous animal tumor models, C6 cells were injected subcutaneously into Wistar rats in 6 locations on the back (n = 3) in amounts of $3x10^6$ cells. After subcutaneous tumors reached 10 mm in diameter, BHA was administered via the tail vein in the amount of 360 μg of boron per rat (ca. 150g). Subcutaneous tumors were removed 15, 30, 45, 60, 90 and 120 minutes after BHA administration and analyzed for boron accumulation by ICP-AES. 9L cells were injected subcutaneously in the flank area of Fisher (n = 8) in amounts of $3x10^6$ cells. 10^5 9L cells were injected at the depth of 4mm using 10μl Hamilton syringe at a special instrumental table for the syringe fixation. After subcutaneous tumors reached 10 mm in diameter and animals showed neurological symptoms specific for brain tumor, BHA was administered via the tail vein in the amount of 720 μg of boron per rat (ca. 300g). Subcutaneous and brain tumors, brain, skin, kidney, liver blood, muscle, and spleen tissues were removed 24, 48, and 72 hours after BHA administration and analyzed for boron accumulation by ICP-AES.

(2) **BNCT with gold nanoparticles.**

T98G tumor cells were cultured in Iscove's modified Dulbecco's minimum essential medium (MEM) (SIGMA 17633 Iscove's DMEM with L-glutamine and 25mH HEPES, without sodium bicarbonate), supplemented with 10% fetal bovine serum (Thermo scientific HyClone SV30160.03 HyClone UK ltd.), maintained at 37˚C in the atmosphere of 5% CO2.

BPA: p-boronophenylalanine was purchased from KATCHEM Ltd. (Praha, Czech Republic). The enrichment of 10B was ≥99.6%. 500mg of BPA was mixed with 1100 mg of fructose, 15 ml of H_2O (Milli-Q water) and 2.7ml of 1M NaOH, neutralized with HCl to pH=7.2. Fructose-BPA final concentration was 1100μg of 10B/mL [5].

Gold nanoparticles were purchased from Winered Chemical, Co. Ltd., (Tokyo, Japan).

Transmission electron microscopy (TEM): The size and localization of GNPs in T98G cells were studied using a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at an accelerating voltage of 120 kV.

Cytotoxicity analysis with MTS assay was performed as described previously [4] using Cell Titer 96 AQueous One Solution (Promega, WI, USA).

ICP-AES: Boron/gold (BPA/GNPs) accumulation in cells was assessed by ICP-AES. To the cells incubated with GNPs the necessary amount of hydrochloric acid was added to obtain Aqua Regia (1HNO3:3HCl) to dissolve gold.

Irradiation and neutron dose estimation by GNPs: T98G cells after incubation with 0, 10, 20 and 40 ppm

of BPA and 50 ppm of gold in GNPs were trypsinized, washed with PBS. The cells were placed in vials with 1 ml of the medium in which they were incubated. T98 cells incubated in BPA only without gold were used as controls. Neutron irradiation of the samples in the organic glass phantom was performed during 2 hours at 2.0 to 2.3 MeV proton energy and 1 to 3 mA proton current, resulting in 300 million events of epithermal neutron generation. The activation of gold in the samples was measured by a gamma spectrometer.

Colony-forming assay (CF-assay) was performed according to the previously reported protocol [6]. Colonies of 50 cells and over were included in the statistical analysis. The statistical significance was evaluated by one-way analysis of variance (ANOVA).

4. 研究成果

(1) **BHA applicability evaluation.** BHA possessed relative toxicity against C6 tumor cells. At 0–225 µg/ml BHA showed the lowest toxicity at the shortest exposition (12 h), and the highest toxicity at the maximum concentration (the lowest cell proliferation rate of 0.784±0.031). Results at 18 h exposition did not differ significantly from that at 12 h (0.712±0.033 at the maximum 225 µg/ml concentration, p=0.051. After 24 h the cell proliferation decreased to 0.493±0.039 at maximum boron concentration and differed significantly from that at 12 and 18 h (p=0.001 and 0.002, respectively). In further experiments 100 µg/ml of BHA was used to assure cell survival. The toxicity against V79 cells did not differ significantly from that against C6 cells (0.493±0.039 vs 0.490±0.008, p=0.902). Traces of boron were found after C6 cells incubation with 100 mg/ml of BHA for 24 h. In C6 animal tumor model boron concentration in tumor tissue decreased gradually within the first 2 h after BHA injection (Fig.1). In 9L glioma model the maximum boron

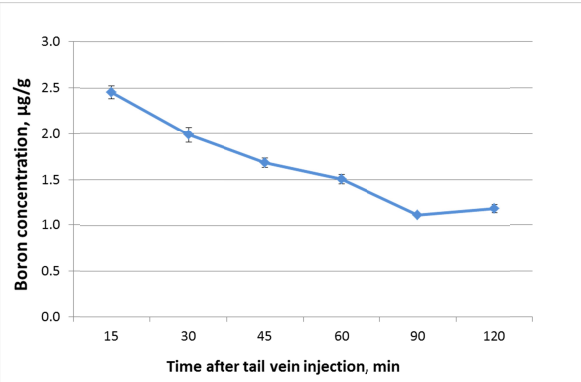

Figure 1. BHA accumulation in subcutaneous C6 glioma models after tail vein injection in Wistar rats (n=3). Values represent means ± SDs.

accumulation was observed in the spleen and blood (Fig.2). Though accumulation in the brain tumor was higher than in the surrounding brain tissue, the specific accumulation could not be reached because of high boron concentration in blood. Modification of the compound to connect boron covalently with the increase in number of boron atoms in BHA and the concentration of BHA in the injected solution might be necessary make the compound more efficacious in further preclinical and clinical evaluations.

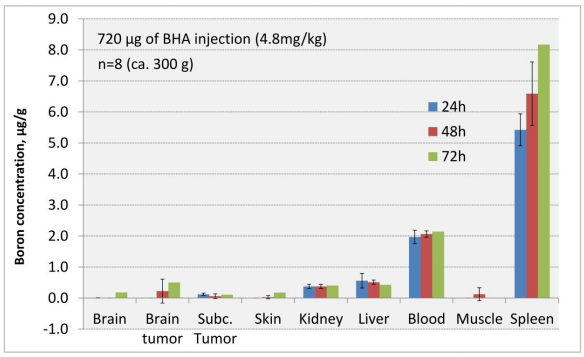

Figure 2. BHA accumulation in 9L glioma bearing Fisher rats after tail vein injection (n=8). Values represent means ± SDs.

(2) **BNCT with gold nanoparticles.** Electronic microscopy could reveal the size and localization on GNPs in tumor cells (Fig.3). The cytotoxicity of BPA

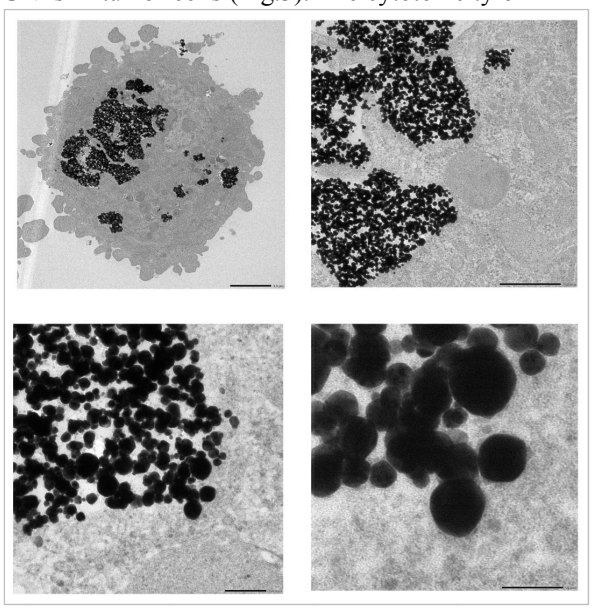

Figure 3. Transmission electron microscopy images of GNPs in T98G cells.

and GNPs was low and didn't influence the cell proliferation significantly (Fig.4, 5). Activation of the

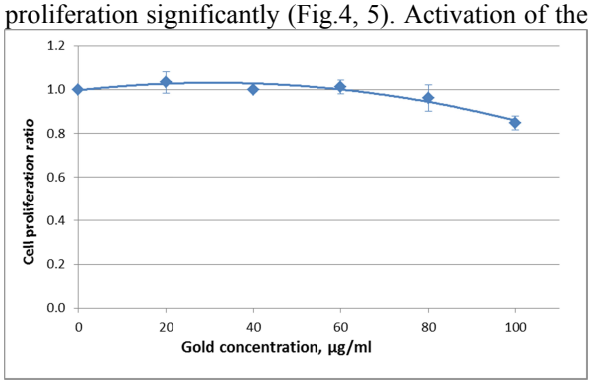

Figure 4. Cytotoxicity of GNPs for T98G glioma cells after 24 h of incubation. The experiments were repeated 3 times. The data represent means ± SDs.

samples containing 129.8±7.3µg of gold resulted in generation of $4.02\pm0.4\times10^7$ radioactive ^{198}Au isotopes that provided data for neutron dose evaluation for each boron concentration. Experimentally, depending on gold concentration in tumor cells we established the boron dose evaluation equation as follows:

$$D(Gy) = \frac{0.58\gamma(Bq)}{m(g)} \cdot \frac{ppm(B)}{ppm(Au)}$$

To determine the boron dose, we need to measure the activation of gold in the samples (γ, Bq), measure the

sample mass (m, g), and know the concentration of both components in samples (ppm, B, Au).

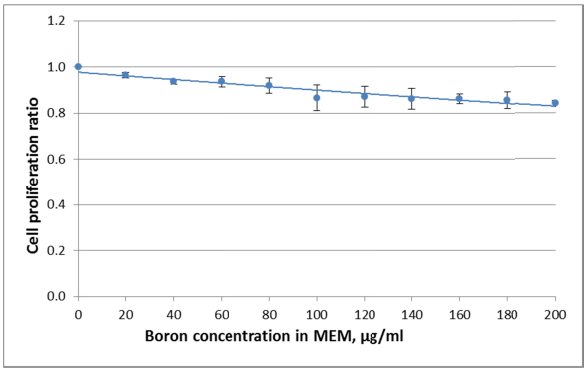

Figure 5. Cytotoxicity of BHA for T98G glioma cells after 24 h of incubation. The experiments were repeated 3 times. The data represent means ± SDs.

CF-assay confirmed the efficacy of boron neutron capture reaction. The efficacy increased after increase of neutrons from 50 to 300 million events (Fig.6,7).

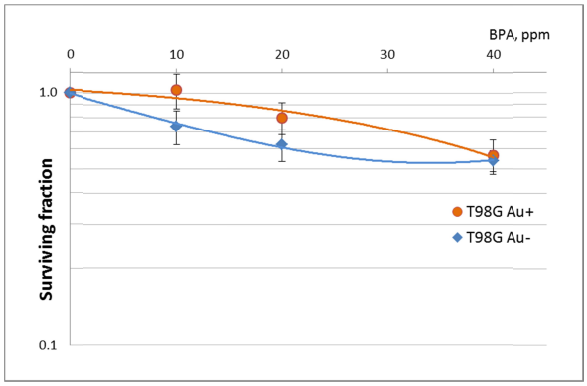

Figure 6. T98G glioma cell colony formation after $5x10^7$ neutrons delivered.

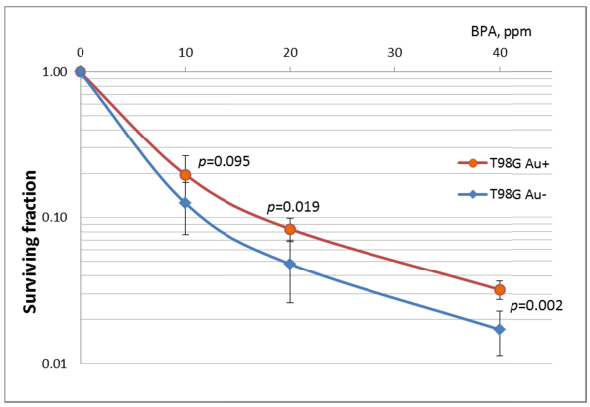

Figure 7. T98G glioma cell colony formation after $3x10^8$ neutrons delivered.

Colony formation in gold-containing samples didn't show significant difference from that without gold at $5x10^7$ neutrons delivered, and was different at $3x10^8$ neutrons, whatmight be related with the impact of gamma and fast neutron radiation shielding by gold nanoparticles. The shielding did not change the overall tendency in tumor cell killing effect by BNCT. The new approach in dosimetry for BNCT that we tested in the current study may allow us to determine the absorbed neutron dose using combined boron compounds containing additional high-Z element. It may open a new perspective in boron compound distribution and treatment efficacy evaluation by adjusting such methods as isotope scanning or positron emission tomography (PET).

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6. 研究組織

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