I-1. PROJECT RESEARCHES

Project 1

PR1

Preclinical studies for applying BNCT to veterinary medicine

M. Suzuki

Institute for Integrated Radiation and Nuclear Science, Kyoto University

Radiation therapy has been incorporated as one means of treating cancer in companion animals. In university hospitals, state-of-the-art radiotherapy equipment which is used in human radiotherapy has been installed. BNCT for head and neck cancer has been performed as an approved treatment modality at medical institutes. To expand the application of BNCT to the veterinary field, the research project has been conducted with the inclusion of veterinary researchers.

In this research project, six research projects were included. Details of the four projects are referred to each progress report.

P1-1. The Basic Study Aimed at performing the BNCT for Canine Malignant Melanoma

Malignant melanoma is one of the most major cancers in dogs. Melanomas that occur in the canine oral cavity are highly invasive and difficult to surgically resect. In addition, they are known to be refractory tumors that are difficult to treat with conventional radiation therapy. Therefore, new treatment strategies are needed. In this study, the survival after the neutron irradiation using the canine malignant melanoma cell lines.

P1-2. Preparation of anti BSH/HER2 Chimeric Bispecific Antibody and Its Estimation of Binding Ability to BSH and HER2

The aim of this study is to create a chimeric bispecific antibody against BSH and HER2 by linking a humanized anti-HER2 antibody scFv to the Fc region of a previously prepared rabbit-canine chimeric anti-BSH antibody. These chimeric bispecific antibodies are expected to contribute to cancer treatment in dogs

P1-3. Development of carborane loading extracellular vesicles as a boron agent for boron neutron capture therapy.

In this study, extracellular vesicles (EVs) which have the important features such as excellent biocompatibility, low immunogenicity, and targeting properties to specific tissue, are investigated as potentially applicable as platforms for drug delivery systems in BNCT.

P1-4. Meg-BPA IL Delivery via Cerebrospinal Fluid Circulation for BNCT in a Rat Glioma Model

The aim of this study is to establish an optimal administration protocol for Meg-BPA IL

via the CSF. Glioma model rats were treated using different CSF-based delivery methods— continuous infusion and bolus injection, varying in BPA concentration—and subsequently subjected to BNCT at the Kyoto University Research Reactor. This approach offers a minimally invasive, effective alternative for boron delivery in BNCT, paving the way for clinical use.

PR1-1

The Basic Study Aimed at perfoeming the BNCT for Canine Malignant Melanoma

S. Yoshihashi, A. Niimi, K. Tsuchimoto, R. Iwasaki[1], A. Uritani, Y. Sakurai[2], M. Suzuki[2]

Graduate School of Science, Kyoto University
[1] Obihiro University of Agriculture and Veterinary Medicine
[2]Institute for Integrated Radiation and Nuclea r Science, Kyoto University

INTRODUCTION: Malignant melanoma is one of the most major cancers in dogs. Melanomas that occur in the canine oral cavity are highly invasive and difficult to surgically resect. In addition, they are known to be refractory tumors that are difficult to treat with conventional radiation therapy. Therefore, new treatment strategies are needed.
BNCT is a therapeutic method that selectively destroys the tumor while leaving normal tissues al-most unharmed by utilizing the nuclear reaction with neutron and boron, which tends to accumulate in the cancer cells. In human malignant melanoma, LAT1, an amino acid transporter of boron com-pound, is shown to be overexpressed in many malignant tumor cells. In this study, the survival after the neutron irradiation using the canine malignant melanoma cell lines.
****

EXPERIMENTS: Two cell lines derived from canine malignant melanoma, primary skin melanoma (CMeC) and metastatic mandibular lymph nodes of oral melanoma cells (LMeC), were used for this study [1]. As boron agent, ^{10}B-p-boronophenylalanine (BPA) was used. Expression of LAT1 has also been found in canine melanoma [2]. CMeC and LMeC were neutron irradiated after incorpo-rating BPA at boron concentrations of 50 ppm and 25 ppm, respectively. Cells after neutron irradiation were cultured for 7 days and cell viability was evaluated using the colo-ny formation assay.
****

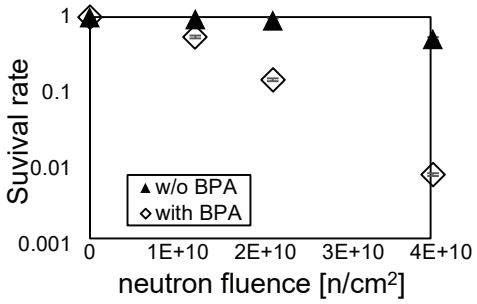

Fig. 1. Comparison of cell survival rate with and without boron for neutron irradiation.

RESULTS: Fig. 1 shows the cell viability against neu-tron fluence of CMeC with and without BPA. This figure shows a clear difference between the cases with and without BPA at neutron fluence above 1 x 10^{12} n/cm^2, in-dicating that BNCT is effective against CMeC. Fig. 2 shows the cell viability to absorbed dose of CMeC with BNCT and gamma irradiation. This figure shows that BNCT resulted in lower cell viability than gamma irradia-tion at the same dose. Similar results were obtained for experiment results for LMeC. Furthermore, DNA damage was evaluated by fluorescent immunostaining, which showed more severe damage in BNCT cells than in gam-ma-irradiated cells.

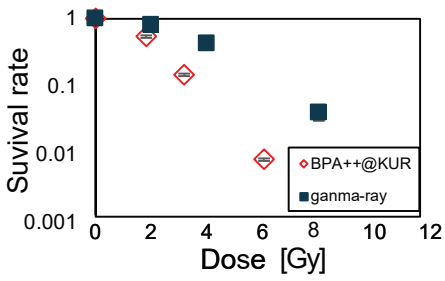

Fig. 2. Comparison of cell survival rate in the case of gamma and neutron irradiation

REFERENCES:

[1] K. Inoue *et al.,* J. Vet. Med. Sci., **66**(11) (2004) 1437-1440.
[2] S. Fukumoto *et al.,* Biochemical and Biophysical Research Communications, 439(1) (2013) 103.

PR1-2

Preparation of anti BSH/HER2 Chimeric Bispecific Antibody and Its Estimation of Binding Ability to BSH and HER2

N. Ueda, T. Kanai, N. Yasukawa, R. Kawasaki[1], Y. Sakurai[2], Y. Sanada[2], N. Kondo[2], M. Suzuki[2], and T. Nagasaki

Graduate School of Engineering, Osaka Metropolitan University
[1] Graduate School of Advanced Science and Engineering, Hiroshima University
[2]Institute for Integrated Radiation and Nuclear Science, Kyoto University

INTRODUCTION: Malignant tumors are the leading cause of death in dogs. Boron Neutron Capture Therapy (BNCT) has attracted attention as a solution to canine cancer. One of the BNCT drugs clinically used is mercaptoundecahydrododecaborate (BSH). Although BSH is a boron cluster with 12 ^{10}B atoms per molecule, resulting in a very high boron atom integration density, It has the disadvantage of no tumor cell selectivity. HER2 has a structure similar to the epidermal growth factor receptor and belongs to the EGFR family. It is a glycoprotein of approximately 185 kDa, and known as a cancer antigen that is highly expressed in various types of cancer, including breast cancer and gastric cancer. It has been revealed that HER2 is also overexpressed in canine cancer cells, and a single-chain humanized anti-HER2 antibody (scFv) with a HER2-binding domain has already been developed. In this study, we aimed to create a chimeric bispecific antibody against BSH and HER2 (Figure 1) by linking a humanized anti-HER2 antibody scFv to the Fc region of a previously prepared rabbit-canine chimeric anti-BSH antibody. By applying chimeric bispecific antibodies, we will try to contribute to cancer treatment in dogs.

EXPERIMENTS: PCR was performed using previously reported rabbit-dog chimeric anti-BSH heavy chain expression vector (pCAGEN chBSH HC) [1] as templates to produce 1st PCR products. Using the 1st PCR product as a template and linker sequence containing primer, 2nd PCR was performed to produce a linker sequence-containing 2nd PCR product. The 2nd PCR product was digested with restriction enzymes Nhe I and Not I and ligated to pCAGEN chBSH HC to afford linker-added chBSH heavy chain expression vector (pCAGEN chBSH HC linker). On the other hand, PCR was performed using gifted human anti-HER2 scFv-Fc expression vector (pCAGEN h4D5 scFv-Fc) as templates to produce 1'st PCR products. The 1'st PCR product was digested with restriction enzymes XhoI and Not I and ligated to pCAGEN chBSH HC linker, which had been previously digested with the same enzymes, to create a rabbit-dog chimeric anti-BSH/HER2 bispecific antibody heavy chain expression vector (pCAGEN chBSH h4D5scFv HC). Using ExpiCHO cells as a host, pCAGEN chBSH h4D5scFv HC and previously prepared light chain expression vectors (pCAGEN chBSH LC) [1] were cotransfected to produce rabbit-dog chimeric anti-BSH/HER2 bispecific antibody (cBhs: chBSH h4D5scFv). To confirm the simultaneous binding ability to BSH and HER2, sandwich ELISA between BSH-modified albumin (BEB) and mouse Fc-fused canine Her2 was performed using HRP-labeled secondary anti-mouse IgG antibody. (Fig. 2).

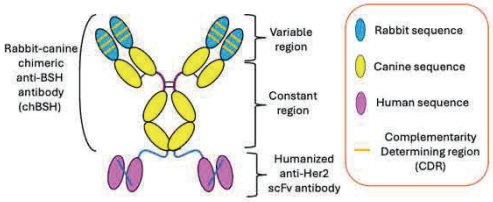

Fig. 1. Format of chimeric anti BSH/HER2 bispesific antibody (cBhs: chBSH h4D5scFv).

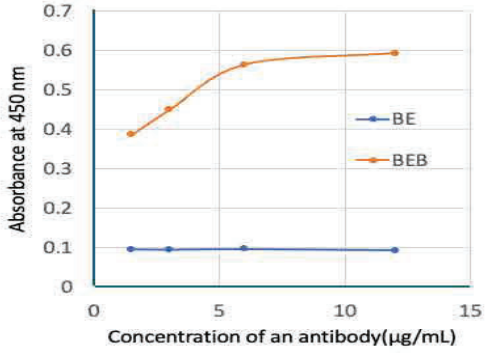

Fig. 2. Evaluation of simultaneous binding ability to BSH and HER2 with cBhs.

RESULTS: The amount of bispecific antibody produced per a liter of culture medium was 15.1 mg. The ELISA results are shown in Fig.2. As the concentration of antibody increased, the absorbance value at 450 nm also increased with immobilized BSH-modified BSA (BEB). Therefore, it was confirmed that cBhs simultaneously binds to BSH and HER2. Evaluation of cBhs as a BNCT boron delivery system is currently progressing by BNCT toward tumor-bearing mice using HER2-expressing cells.

REFERENCES:

[1] N. Ueda *et al.,* KURNS Prog. Rep., (2024) R5P2-2.

PR1-3

Development of carborane loading extracellular vesicles as a boron agent for boron neutron capture therapy

R. Kawasaki,[1] A. Oshige,[1] K. Yamana,[1] Y. Miura,[1] T. Saito,[1] Y. Sanada,[2] Y. Sakurai,[2] H. Tanaka,[2] M. Suzuki,[2] M. Sadakane,[1] Y. Hattori,[3] M. Kirihata,[3] R. Mizuta,[4] Y. Sasaki,[4] K. Akiyoshi,[5] S. Miyaki,[6] T. Nagasaki,[7] and A. Ikeda[1]

[1] *Applied Chemistry Program, Department of Advanced Science and Engineering, Graduate School of Advanced Science and Engineering, Hiroshima University*

[2] *Institute for Integrated Radiation an d Nuclear Science, Kyoto University*
[3] *Research center for BNCT, Osaka Metropolitan University*
[4] *Department of Polymer Chemistry, Graduate School of Engineering, Kyoto University*
[5] *Graduate School of Medicine, Kyoto University*
[6] *Department of Orthopedic Surgery, Graduate School of Biomedical and Health Sciences, Hiroshima University* [7] *Department of Applied Chemistry and Bioengineering, Graduate School of Engineering, Osaka Metropolitan University*

INTRODUCTION: With excellent biocompatibility, low immunogenicity, and targeting properties to specific tissue, extracellular vesicles (EVs) are potentially applicable as platforms for drug delivery systems. EVs can provide nanospace to encapsulate both hydrophilic and hydrophobic pharmaceuticals as well as lipid-based nanoparticles. Today, pre- and post-loading methods are the option in encapsulating drugs toward EVs, which is essential technique to utilize EVs as a delivery platform. Though post-loading technique are attractive due to their excellent efficiencies, the methods require external stimuli such as heating, sonication, and electric fields, which are potentially disrupt EVs structure and defunctionalize EVs. Moreover, in encapsulation of small drugs with high hydrophobicity, usage of organic solvent which can be mixed with water like dimethyl sulfoxide and ethanol are indispensable, which are also potentially harmful to EVs. For these perspectives, we have developed hydrophobic drug loading technique for EVs via exchanging reaction based on supramolecular chemistry strategy without using organic solvents. We employed this system for hydrophobic boron cluster molecule, carborane, and demonstrated the performance of BNCT.

RESULTS AND DISCUSSION: As the model EVs, we isolated EVs from milk via ultracentrifugation and the purified EVs exhibited representative properties found in EVs. Exchanging reaction of carborane from cyclodextrin complex to EVs were tracked by measuring H NMR spectra as the molecular dynamics of carborane changes drastically via exchanging reaction. As a result, the loading of carborane to EVs finished within 15 min and carboranes are highly condensed within lipid membrane, which is advantageous in increasing the efficiencies to achieve boron neutron capture reaction. We further addressed uniformity in loading drugs by single particle analysis using imaging flow cytometry and current method can introduce cargo molecules to EVs with high uniformity and without leaving any EVs empty.

We next investigated the performance of EVs trapping carborane as a boron agent for BNCT, and L-BPA/fructose complex and carborane loading liposomes are employed as control. As a result, EVs systems exhibited the highest BNCT activity against colon carcinoma cell line (Colon26) among these three systems. Moreover, our systems efficiently accumulated in tumor tissue mainly via EPR effect even in tumor bearing mice model which was established by transplantation of Colon26 cells to mice. For their excellent deliverability of boron agent to tumor tissues, thermal neutron irradiation to the mice treated with carborane loading EVs enabled to eliminate cancer cells in tumor tissue, compared to the other systems. For these results, our systems are potentially applicable as a boron agent for BNCT.

REFERENCES:

[1] R. Kawasaki *et al.,* ACS Applied Materials and Interfaces, **16** (2024) 47137-47149.

PR1-4

Meg-BPA IL Delivery via Cerebrospinal Fluid Circulation for BNCT in a Rat Glioma Model

S. Kusaka, M, Shirakawa[1], N.Voulgaris, K. Onishi[2], S. Saito[2], I. Murata, T. Takata[3], M. Suzuki[3]

Graduate School of Engineering, Osaka University
[1] *Neuron Therapy Research Center, Okayama University*
[2] *Graduate School of Medicine, Osaka University*
[3] *Institute for Integrated Radiation and Nuclear Science, Kyoto University*

INTRODUCTION: In our previous study, we demonstrated that boron neutron capture therapy (BNCT) using cerebrospinal fluid (CSF)-based delivery of boronophenylalanine (BPA) achieved comparable therapeutic efficacy to the conventional intravenous (IV) route, despite using less than one-thirtieth of the BPA dose [1]. To further enhance the efficiency of CSF drug delivery, we developed a novel ionic liquid (IL) formulation of BPA using meglumine (Meg-BPA IL), enabling stable delivery at concentrations ranging from 2 to 10 times the standard concentration (1400 ppm). [2]. In the present study, we aimed to establish an optimal administration protocol for Meg-BPA IL via the CSF. Glioma model rats were treated using different CSF-based delivery methods— continuous infusion and bolus injection, varying in BPA concentration—and subsequently subjected to BNCT at the Kyoto University Research Reactor. This approach offers a minimally invasive, effective alternative for boron delivery in BNCT, paving the way for clinical use.

****

EXPERIMENTS: The C6 rat glioma models were supplied for the present experiment 10 days after implantation. Eight rats were divided into three groups. Rats A–C received 4200 ppm continuous infusion, while rats D–F received 8400 ppm continuous infusion. Rats G and H were administered a bolus injection of Meg-BPA IL (4200 ppm) via CSF. All CSF administrations were performed via the cisterna magna. All rats were irradiated for 20 minutes at a 5 MW reactor using a heavy water neutron irradiation facility, with an average fluence of 3.8×10^{12} neutrons/cm^2. After the thermal neutron irradiation, all rats were kept under the same conditions for 14 days. MRI was used to evaluate the changes in tumor volume and overall therapeutic effects of BNCT.

****

RESULTS: Rats receiving 8400 ppm Meg-BPA IL via CSF (rats D–F) died within 30 minutes, suggesting potential toxicity related to osmotic stress or high concentration. In contrast, 4200 ppm infusion showed a trend of smaller tumor volume changes post-BNCT compared to bolus injection. (Figs. 1 and 2). Further studies are required to determine statistical significance.

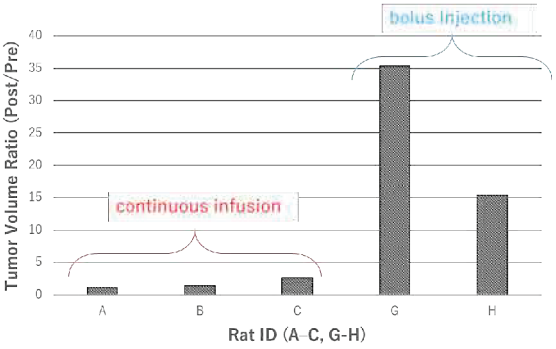

Fig. 1. Relative tumor volume in CSF infusion (A–C)

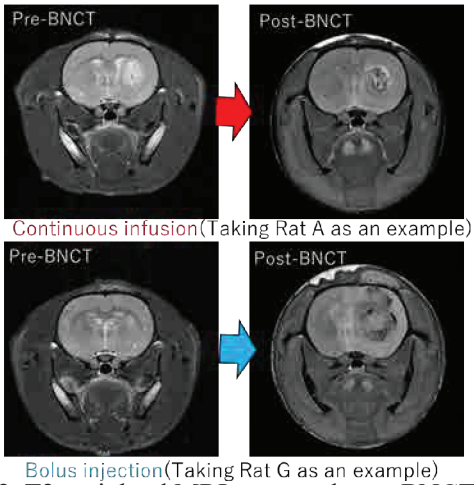

Fig.2. T2-weighted MRIs pre- and post-BNCT. Top: continuous infusion; Bottom: bolus injection.

REFERENCES:

[1] S. Kusaka *et al.,* Cells, **13**(19) (2024) 1-14.
[2] M. Shirakawa *et al.,* Patent No. 7525095.