I-1. PROJECT RESEARCHES

Project 4

PR4 Preclinical studies for appling BNCT to veterinary medicine

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Recently, a concept of "One health" is considered important. The concept is a collaborative, multisectional, and transdisciplinary approach – working at the local, regional, national, and global levels – with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment. Due to the commonality of human and animal disease collaboration between medicine and veterinary medicine will improve the heath of both.

In 2020, accelerator-based boron neutron capture therapy (BNCT) for head and neck cancer using was approved by Japanese Ministry of Health, Labor, and Welfare. At two medical institutes, BNCT for head and neck cancer has started under the insurance coverage. Common malignancy encountered in companion animals include head and neck cancer of the dog. BNCT group in Argentina reported the results of BNCT for head and neck cancers in dogs using a research reactor. In Japan, human head and neck cancers have been treated with BNCT as mentioned above. Considering these circumstances, this project was planned to obtain scientific evidence for the adaptation of BNCT to veterinary medicine.

In this research project, six research projects were included. The four researches were planned by veterinarians. Unfortunately, two researches were not carried out. Details of four projects are referred to each progress report. I hope that this project will arouse much interest in BNCT among veterinarians.

P4-1: The Basic Study Aimed at Performing the Boron Neutron Capture Therapy for Canine Osteosarcoma.

In this project, to investigate the possibility of applying BNCT to canine osteosarcoma, LAT-1 expression in canine osteosarcoma cell lines were examined, and the amount of intracellular boron using the BPA was measured. By Western blotting, LAT1 proteins in two osteosarcoma cell lines, POS and HMPOS, were found at approximately the same levels of LMeC and CMec-1 which were canine malignant melanoma and used as a positive control. By incubation with BPA at the concentration of 14, 28, 57 ppm, there were dose-dependent increase in ^{10}B uptake in both osteosarcoma cell lines. These results indicate that BNCT can be applied to canine osteosarcoma.

P4-2: Caninization of Anti BSH Antibody Prepared from Rabbit Lymphocytes

In this study, the complimentary-determining regions (CDRs), which are BSH-binding sequences, from rabbit anti-BSH antibody was identified. A canine anti-BSH antibody was prepared by replacing the regions other than the CDRs with canine sequences.

P4-3: Antibody conjugated BNNT/β-1,3-glucan complex as a boron agent for BNCT.

In this study, as a new boron agent, HER-2 recognizing antibody modified boron nitride nanotube/β-1,3-glucan complex was investigated. In addition, for the conjugation of antibody, protein A mimicking ligand molecules (PAM) were introduced to BGL (PAM-BGL). Conjugation of HER-2 recognizing antibody enhanced cellular uptake of BNNT/BGL in human ovarian cancer cells (SK-OV3 cell). This new novel boron agent has possibility to be applicable for BNCT.

P4-4: Investigation of the relationship between the therapeutic efficacy of boron neutron capture therapy and the persistence of boron in tumors.

In this study, the effect of tumor tissue diversity (stromal volume and blood flow distribution) on BPA residence time and the antitumor effect of BNCT. Two human pancreatic carcinoma cells, Capan-1 containing high stroma and PSN-1 containing low stroma, were used. Accumulation of BPA in the Capan-1 was lower than that in the PSN-1. However, in the preliminary in vivo BNCT study, the same anti-tumor effects were observed in both Capan-1 and PSN-1 tumors. These results may indicate that low boron concentration in the tumor with high stroma has been successfully treated with BNCT. In the further study, using the canine or feline tumor cells with high stroma, relationship between boron accumulation and anti-tumor effect will be investigated.

PR4-1 The Basic Study Aimed at Performing the Boron Neutron Capture Therapy for Canine Osteosarcoma

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INTRODUCTION:

Canine osteosarcoma, which commonly occurs in the extremities, is generally treated with leg amputation followed by the postoperative adjuvant chemotherapy. On the other hand, if surgery is not performed due to comorbidities, distant metastases, or the owner's wishes, radiation therapy can also be a treatment option. However, osteosarcoma is generally resistant to X-rays and carries the risk of damaging surrounding normal tissue for tumor control.

Boron neutron capture therapy (BNCT) is a therapeutic method that selectively destroys the tumor while leaving normal tissues almost unharmed by utilizing the nuclear reaction with neutron and boron, which tends to accumulate in the cancer cells. In human, LAT1, an amino acid transporter that has been found to be particularly involved in the intracellular transport of boron compound, is shown to be overexpressed in many malignant tumor cells.

In this study project, we investigated the possibility of applying BNCT to canine osteosarcoma by examining LAT1 expression in canine osteosarcoma cell lines and by measuring the amount of intracellular boron when using the BPA as boron compound.

EXPERIMENTS: Two cell lines derived from canine osteosarcoma, POS and HMPOS, were used for this study. As boron compound, p-boronophenylalanine (BPA) was prepared at a dose of 30 mg/ml.

Western blotting Cultured cells were collected and lysed with RIPA lysis buffer. After measuring the concentration with Lowry assay, 15 µg/lane of proteins were separated by SDS-PAGE for 70 min. Proteins were transferred onto the PVDF membrane and probed with LAT1 antibody overnight. After being probed with HRP-linked secondary antibodies, bound antibodies were detected with Immobilon Forte Western HRP substrate (EMD Millipore). Two cell lines of LMeC and CMeM-1 derived from canine malignant melanoma and anti-β-actin mouse monoclonal antibody were used as positive and loading control, respectively.

Inductively coupled plasma atomic emission spectrometry (ICP-AES) 1×10^6 cells of POS and HMPOS were co-incubated with BPA for 0.5, 1, 2 and 4 h. The boron-10 (^{10}B) concentrations in BPA solution were adjusted with culture medium to 14, 28 or 57 ppm, respectively. Each sample was digested by heating overnight in nitric acid (60%), then diluted with distilled water and divided into three test tubes. After measuring the boron concentration in these tubes using ULTIMA2 (HORIBA, Ltd., Kyoto, Japan), their average was taken as the amount of the sample and expressed in "ng ^{10}B / 10^6 cells."

RESULTS: Fig. 1 shows the LAT1 expressions in POS and HMPOS by the Western blotting. In both cell lines, LAT1 proteins were found at approximately the same levels of LMeC and CMeC-1.

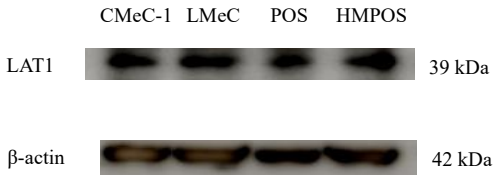

Fig. 1. Expression assessment of LAT1 protein in canine osteosarcoma cell lines of POS and HMPOS by Western blotting.

Fig. 2 shows the intracellular ^{10}B uptake measured by the ICP-AES. In both cell lines, ^{10}B concentrations decreased gradually by increasing the co-incubation time with low concentrations of BPA (14 ppm). On the other hand, co-incubation with BPA at 28 and 57 ppm had little time-dependent changes in^{10}B uptake. Furthermore, by incubation with BPA for 2 h and 4 h, there were dose-dependent increases in ^{10}B uptake in both cell lines.

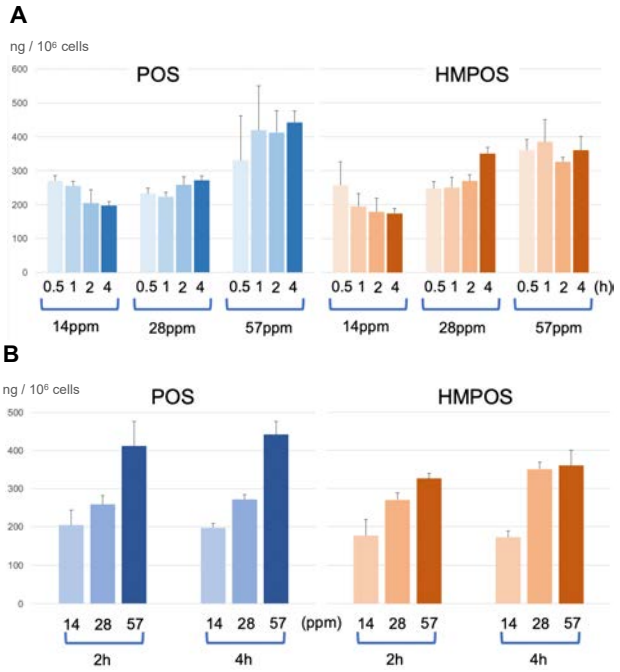

Fig. 2. Intracellular ^{10}B concentrations in POS and HMPOS by ICP-AES. (A) and (B) show the amount of ^{10}B in each cell line by BPA co-incubation time and the concentration, respectively.

CONCLUSION: Our results indicate that BNCT can be applied to canine osteosarcoma because the sufficient amount of ^{10}B uptake was confirmed in this study. It is necessary to perform the *in vitro* or *in vivo* irradiation assessment compared with X-ray and neutron beam without BPA to verify the anti-tumor effect considering the current results.

PR4-2 Caninization of Anti BSH Antibody Prepared from Rabbit Lymphocytes

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INTRODUCTION: Recently, tumor diseases have increased in dogs as they have aged due to the development of veterinary medicine and therapy [1]. However, 30% of the causes of death are malignant tumors. Three major therapies such as surgical treatment, drug therapy, and radiation therapy, are used, but in many cases, there are limitations due to the burden on the dog's body, side effects, and therapeutic effects. Boron neutron capture therapy (BNCT) is expected as a new therapeutic strategy [2].

One of the boron agents used in BNCT is BSH. BSH is a boron cluster with a unique icosahedral structure, with twelve ^{10}B atoms per molecule. It has also the advantage on extremely high water-solubility and low toxicity.

In our previous research, rabbit anti-BSH antibody have been prepared [3]. In this study, we identified the complementarity-determining regions (CDRs), which are BSH-binding sequences, from this antibody, and prepared a canine anti-BSH antibody (caBSH IgG) by replacing the regions other than the CDRs with canine sequences.

EXPERIMENTS: We identified the CDRs from the rabbit anti-BSH antibody, and synthesized DNA by caninizing the other sequences. Using canonized DNA sequences, the antibody gene was amplified by PCR. Antibody genes were incorporated into plasmids by restriction enzyme treatment and ligation to construct caninized anti-BSH heavy and light chain expression vectors. A caninized anti-BSH antibody (caBSH IgG) was prepared by co-transfecting heavy and light chain expression vectors into Chinese hamster ovary-derived ExpiCHO cells. After seven days culture, the supernatant was purified using a protein A column and analyzed by SDS-PAGE.

An antigen (BSH-modified BSA) was immobilized, and ELISA was performed using an HRP-labeled secondary antibody to evaluate the antigen-binding ability of the produced antibody.

RESULTS: caBSH IgG was purified from the culture supernatant using a protein A column. As a result of SDS-PAGE analysis, although the purity was low, bands at approximately 27 kDa (light chain) and approximately 50 kDa (heavy chain) were detected, confirming that the caninized anti-BSH antibody (caBSH IgG) had been successfully produced. In addition, it was found that the antibody production amount per 1 L of medium was 0.94 mg, which was very low efficiency. It is necessary to improve the production efficiency in order to use caBSH IgG *in vivo* experiments in the future.

As shown in Fig. 1, when the antibody prepared were analyzed by ELISA, the absorbance at 450 nm increased with increasing antibody concentration. Therefore, caBSH IgG is considered to have the ability to bind to BSH.

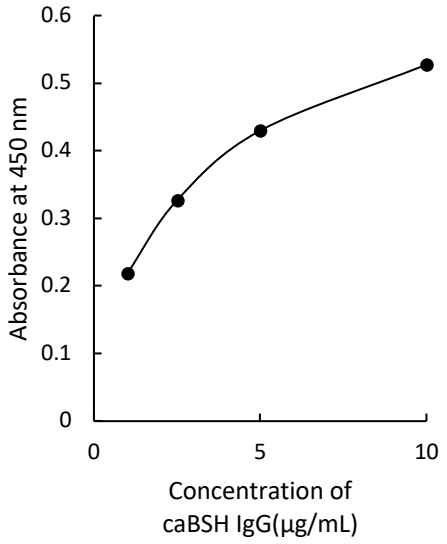

Fig. 1. Evaluation of antigen binding ability of caBSH IgG using ELISA.

In the future, we aim to improve the expression efficiency of caBSH IgG and also attempt to prepare a chimerized anti-BSH antibody that is expected to have high expression efficiency. After success of developing a mass production system for canine or chimerized antibodies, bispecific antibodies with cancer antigen Her2 will be created and evaluated as canine BNCT drugs.

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[3] unpublished data.

PR4-3 Antibody conjugated BNNT/β-1,3-glucan complex as a boron agent for BNCT

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INTRODUCTION: With high specificity and strong affinity, antibodies are powerful means to endow nanomaterials with targeting properties. For instance, antibody-drug complexes, which are covalently conjugated with strong toxin such as auristatin E are clinically available anti-cancer agents due to their therapeutic benefits. However, there are several issues in delivery including limitation in loading moieties, linker stability, and leakage of drugs, which can lead undesirable severe side effects on patients. For these points of views, combination of antibody with noninvasive modalities are ideal in achieving cancer therapy. Today, photodynamic immunotherapy, which is achieved by antibody conjugated photosensitizers, enabled to kill cancer cells without any harmful side effects. Here, photodynamic therapy requires several days-incubation under dark condition to avoid side effect, that is known as photosensitivity, after treatment. Here, boron neutron capture therapy (BNCT), which is achieved by nucleic reaction between boron atom and thermal neutron, is more noninvasive because boron atom does not affect on cell viability.

In this study, we demonstrated HER-2 targeted BNCT using HER-2 recognizing antibody modified boron nitride nanotube/β-1,3-glucan complex (BNNT/BGL complex, Figure 1). BNNT has been expected as a boron agent with one-dimensional morphology and large boron content in each nanorod and β-1,3-glucan is a polysaccharide with one-dimensional morphology comprising triple helix structure, which enables to trap hydrophobic compounds within their cavity. Previously, we reported facile preparation method using mechanochemical approaches for water dispersible inclusion complex of carbon nanotube, which is structural analogue of BNNT, with BGL without forming precipitate in aqueous media [1]. These advantages encouraged us to develop water dispersible BNNT/BGL complex as a boron agent for BNCT.

RESULTS: BNNT/BGL complex was prepared by high-speed vibration milling, which is based on mechanochemical approach, and the resulting mixture was extracted with water. The morphology of the complex obtained were observed by transmission electron microscopy. In case of without staining condition, bundle like structure of BNNT were found and their length were less than 200 nm, which is corresponding to the size for enhanced permeation and retention effect. In this work, we synthesized protein A mimicking ligand molecules (PAM) for the conjugation of antibody because mechanochemical system can disrupt conventional crosslinker for the modification of proteins including alkyne *via* click reaction and maleimide *via* Michael addition. The PAM were introduced to BGL (PAM-BGL). After preparation of BNNT/PAM-BGL complex, HER-2 recognizing antibodies were introduced *via* molecular recognition of PAM. We next investigated HER-2 selective BNCT activity of HER-2 antibody conjugated BNNT/PAM-BGL complex *in vitro*. Here, we employed human ovarian cancer cells (SK-OV3 cell) as HER-2 overexpressed cell line. Conjugation of HER-2 recognizing antibody enhanced cellular uptake of BNNT/BGL. Moreover, pre-treatment with anti HER-2 antibody could suppress the cellular uptake amount of boron agent. These results clearly indicate that the modified antibody work as pilot molecules for cancer targeting. The targeting properties in tumor accumulation could enhance BNCT activity toward SK-OV3 cells and the activity using HER-2 antibody conjugated BNNT/PAM-BGL complex was much higher than that using commercially available boron agent, L-BPA. These results clearly indicate that current system is potentially applicable for BNCT.

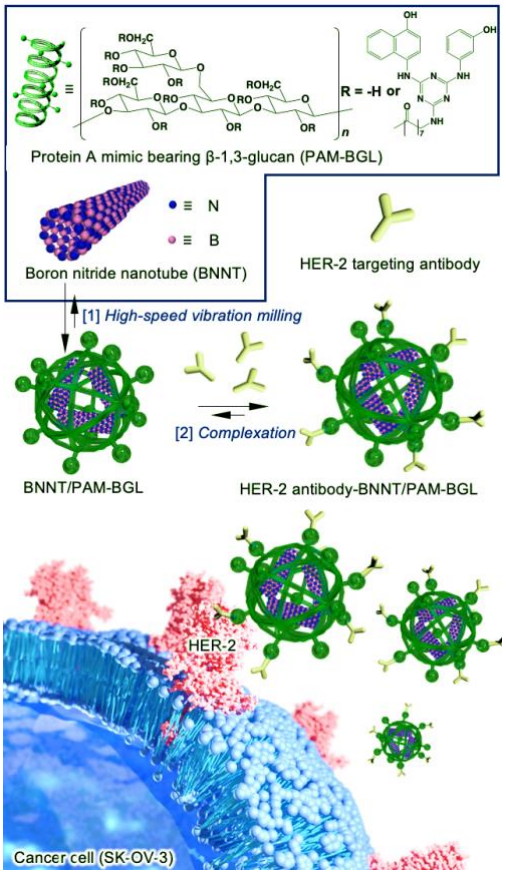

Fig. 1. BNCT using BNNT/BGL complex

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PR4-4 Investigation of the relationship between the therapeutic efficacy of boron neutron capture therapy and the persistence of boron in tumors

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Introduction

When BNCT is performed, the decision on the indication for BNCT depends on the ratio of tumor uptake of 18FBPA to normal tissue (T/N ratio) in 18F-BPA-PET scans. However, it has recently been reported that prolonged residence time of BPA in tumors contributes to improved therapeutic efficacy of BNCT.[1)] Therefore, BPA uptake alone may not be sufficient to predict therapeutic efficacy. Therefore, in this study, we investigate the effects of tumor tissue diversity (stromal volume and blood flow distribution) on BPA residence time and the antitumor effect of BNCT.

Experiments

Tumor-bearing mice: we used pancreatic carcinoma cells with high stroma (Capan-1) and pancreatic carcinoma cells with low stroma (PSN-1). Each cell was inoculated in the thigh of a nude mouse and used in the experiment when it reached a certain size.

Measurement of boron concentration in tumor tissue: 250 ng/kg of BPA was administered subcutaneously to the mice created, and tissue was sampled at regular intervals after administration, and the amount of BPA uptake was measured by ICP-AES.

Evaluation of the effect of BNCT on tumor growth inhibition: 250 ng/kg of BPA was administered subcutaneously to the mice, and the tumor volume was measured periodically by neutron irradiation at 1 hour after the administration.

Result

The changes of the boron concentration in the tumor tissue periodically after BPA administration are shown in Fig. 1.

Capan-1 and PSN-1 inoculated mice were performed BNCT and the subsequent changes in tumor volume are shown in Fig. 2. Tumor growth of both cell lines tended to be suppressed in the BNCT group.

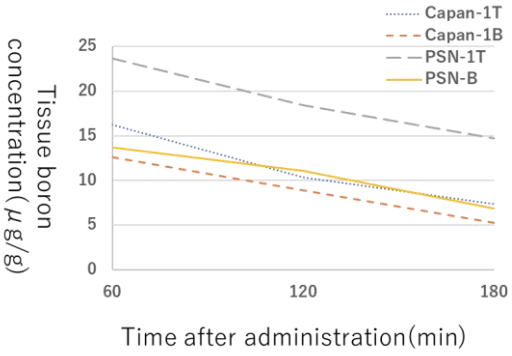

Fig.1. The boron concentration in the tumor tissue after BPA administration.

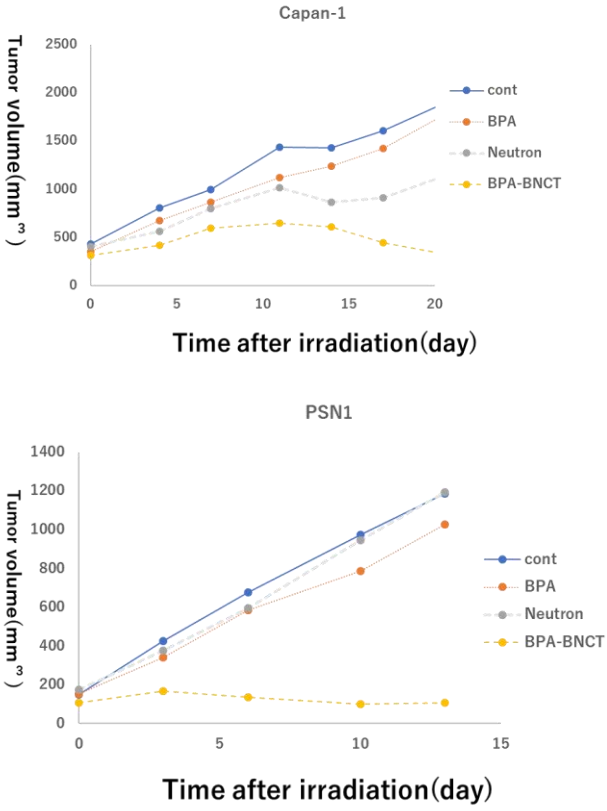

Fig.2. Growth curve of subcutaneous implantation tumor volume.

Reference

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