I-1. PROJECT RESEARCHES

Project 6

PR6 Analyzing Tumor Microenvironment and Exploiting its Characteristics in Search of Optimizing Cancer Therapy Including Neutron Capture Therapy

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BACKGROUNDS AND PURPOSES: Human solid tumors contain moderately large fractions of quiescent (Q) tumor cells that are out of the cell cycle and stop cell division, but are viable compared with established experi-mental animal tumor cell lines. The presence of Q cells is probably due, in part, to hypoxia and the depletion of nu-trition in the tumor core, which is another consequence of poor vascular supply. As a result, Q cells are viable and clonogenic, but stop cell division. In general, radiation and many DNA-damaging chemotherapeutic agents kill prolif-erating (P) tumor cells more efficiently than Q tumor cells, resulting in many clonogenic Q cells remaining following radiotherapy or chemotherapy. Therefore, it is harder to control Q tumor cells than to control P tumor cells, and many post-radiotherapy recurrent tumors result partly from the regrowth of Q tumor cells that could not be killed by radiotherapy. Similarly, sufficient doses of drugs cannot be distributed into Q tumor cells mainly due to heterogeneous and poor vascularity within solid tumors. Thus, one of the major causes of post-chemotherapy recurrent tumors is an insufficient dose distribution into the Q cell fractions.

With regard to boron neutron capture therapy (BNCT), with ^{10}B-compounds, boronophenylalanine-^{10}B (BPA) increased the sensitivity of the total cells to a greater extent than mercaptoundecahydrododecaborate-^{10}B (BSH). However, the sensitivity of Q cells treated with BPA was lower than that in BSH-treated Q cells. The difference in the sensitivity between the total and Q cells was greater with ^{10}B-compounds, especially with BPA. These findings concerning the difference in sensitivity, including other re-covery and reoxygenation following neutron irradiation after ^{10}B-compound administration were mainly based on the fact that it is difficult to deliver a therapeutic amount of ^{10}B from ^{10}B-carriers throughout the target tumors, es-pecially into intratumor hypoxic cells with low uptake ca-pacities.

Hypoxia is suggested to enhance metastasis by increas-ing genetic instability. Acute, but not chronic, hypoxia was reported to increase the number of macroscopic metastases in mouse lungs. We recently reported the significance of the injection of an acute hypoxia-releasing agent, nicotin-amide, into tumor-bearing mice as a combined treatment with γ-ray irradiation in terms of repressing lung metasta-sis. As the delivered total dose increased with irradiation, the number of macroscopic lung metastases decreased reflecting the decrease in the number of clonogenically viable tumor cells in the primary tumor. The metastasis-repressing effect achieved through a reduction in the number of clonogenic tumor cells by irradiation is much greater than that achieved by releasing tumor cells from acute hy-poxia. On the other hand, more ^{10}B from BPA than from BSH could be distributed into the acute hypoxia-rich total tumor cell population, resulting in a greater decrease in the number of highly clonogenic P tumor cells with BPA-BNCT than with BSH-BNCT and with neutron beam irra-diation only. BPA-BNCT rather than BSH-BNCT has some potential to decrease the number of lung metastases, and an acute hypoxia-releasing treatment such as the ad-ministration of nicotinamide, bevacizumab, wortmannin or thalidomide may be promising for reducing numbers of lung metastases. Consequently, BPA-BNCT in combination with the treatment using these agents may show a little more potential to reduce the number of metastases. Now, it has been elidated that control of the chronic hypoxia-rich Q cell population in the primary solid tumor has the potential to impact the control of local tumors as a whole, and that control of the acute hypoxia-rich total tumor cell population in the primary solid tumor has the potential to impact the control of lung metastases.

The aim of this research project is focused on clarifying and analyzing the characteristics of intratumor microenvironment including hypoxia within malignant solid tumors and optimizing cancer therapeutic modalities, especially radiotherapy including BNCT in the use of newly-developed ^{10}B-carriers based on the revealed findings on intratumor microenvironmental characteristics.

RESEARCH SUBJECTS:

The collaborators and allotted research subjects (ARS) were organized as follows;

ARS-1 (31P6-1): Optimization of Radiation Therapy Including BNCT in terms of the Effect on a Specific Cell Fraction within a Solid Tumor and the Suppressing Effect of Distant Metastasis. (S. Masunaga,*et al.*)

ARS-2 (31P6-2): Development of Hypoxic Microenvironment-Oriented ^{10}B-Carriers. (H. Nagasawa, *et al.*)

ARS-3 (31P6-3)*: Search and Functional Analysis of Novel Genes that Activate HIF-1, and Development into Local Tumor Control. (H. Harada, *et al.*)

ARS-4 (31P6-4): Radiochemical Analysis of Cell Lethality Mechanism in Neutron Capture Reaction. (R. Hirayama, *et al.*)

ARS-5 (31P6-5): Development of Neutron Capture Therapy Using Cell-Membrane Fluidity Recognition Type Novel Boron Hybrid Liposome. (S. Kasaoka, *et al.*)

ARS-6 (31P6-6): Drug Delivery System Aimed at Adaptation to Neutron Capture Therapy for Melanoma. (T. Nagasaki, *et al.*)

ARS-7 (31P6-7): Molecular Design, Synthesis and Functional Evaluation of Hypoxic Cytotoxin Including Boron. (Y. Uto, *et al.*)

ARS-8 (30P8-8): Bystander Effect on Malignant Trait of Tumor Cells by Irradiation. (H. Yasui, *et al.*)

ARS-9 (31P6-9): Analysis of the Response of Malignant Tumor to BNCT. (M. Masutani, *et al.*)

ARS-10 (31P6-10): Cell Survival Test by Neutron Capture Reaction Using Boron Compound and Inhibitory Effect on Tumor Growth. (K. Nakai, *et al.*)

ARS-11 (31P6-11): Multilateral Approach Toward Realization of Next Generation Boron Neutron Capture Therapy. (Y. Matsumoto, *et al.*)

ARS-12 (31P6-12): Analysis of Radiosensitization Effect through Targeting Intratumoral Environmental. (Y. Sanada, *et al.*)

ARS-13 (31P6-13)*: Exploratory Research on the Optimal Administration of ^{10}B Compound Aiming at New Enforcement Method of Neutron Capture Therapy (S. Masunaga *et al.*)

ARS-14 (31P6-14) *: Examination of Cancer Cell Accumulation Property of New Boron Agent (CbaP14). (J. Hiratsuka, *et al.*)

(*There was no allocated time for experiments using reactor facilities during their operation periods of FY 2019.

PR6-1 Effect of a change in reactor power on response of murine solid tumors *in vivo*, especially on that of quiescent tumor cells, in boron neutron capture therapy

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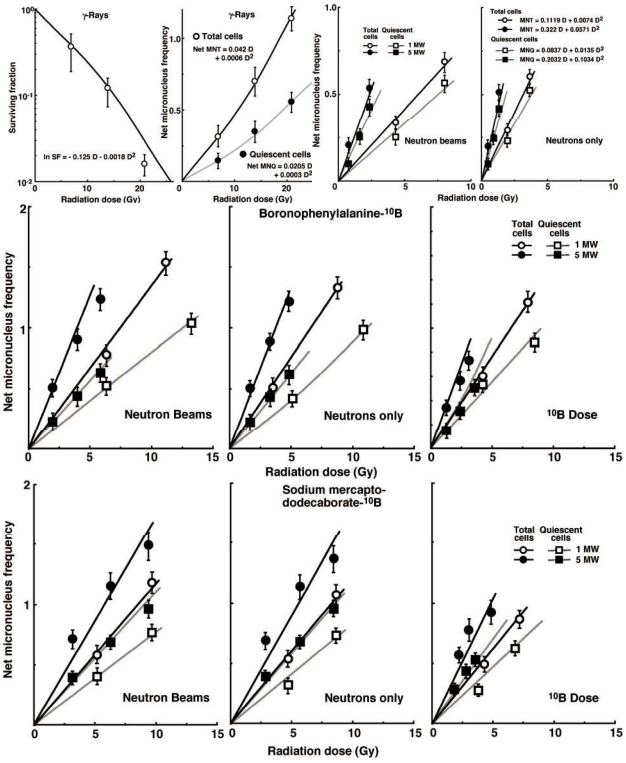
INTRODUCTION: Neutron beams, that are essential for performing BNCT, have been supplied at some research reactors. In Japan, based on legal regulations, more reactor operation personnel have to be in place at the time of high power reactor operation (5 MW), than with low output operation (1 MW). In our reactor, to prevent nuclear fuel from being depleted during the reactor operable period, the output power during reactor operation for research use has been reduced from 5 to 1MW since 2010. However, reactor operation output remains at 5MW at the time of clinical boron neutron capture therapy (BNCT) to prevent prolongation of irradiation time.

We analyzed the changes in the values of relative biological effectiveness (RBE) for neutron-only irradiation and compound biological effectiveness (CBE) factors for employed ^{10}B-carriers according to the value of a power of the operated reactor [1,2]. The neutron capture reaction was performed with two kinds of ^{10}B-carriers, boronophenylalanine-^{10}B (BPA, C9H12^{10}BNO4) or sodium mercaptoundecahydrododecaborate-^{10}B (sodium borocaptate-10B, BSH, Na2^{10}B12H11SH). Regarding local tumor response, the effect not only on the total [proliferating (P) + quiescent (Q)] tumor cell population, but also on the Q cell population, was evaluated using our original method for selectively detecting the response of Q cells in solid tumors [3].

MATERIALS AND METHODS: SCC VII tumor-bearing mice continuously received 5-bromo-2'-deoxyuridine (BrdU) to label all P tumor cells, and were treated with BPA or BSH. After reactor neutron beam irradiation at a power of 1 or 5MW with an identical beam spectrum, cells from tumors were isolated and incubated with a cytokinesis blocker. The responses of BrdU-unlabeled Q and total (P + Q) tumor cells were assessed based on the frequencies of micronucleation using immunofluorescence staining for BrdU.

RESULTS: After neutron irradiation with or without ^{10}B-carrier, radio-sensitivity was reduced by decreasing reactor power in both cells, especially in Q cells and af-ter irradiation with BPA. The values of RBE and CBE were larger at a power of 5MW and in Q cells than at a power of 1MW and in total cells, respectively. The sensitivity difference between total and Q cells was widened when combined with ^{10}B-carrier, especially with BPA, and through decreasing reactor power.

CONCLUSION: In actual clinical situations, it takes approximately 5 times longer time at a power of 1MW than 5MW to carry out one session of BNCT. Patients cannot move their body at the optimum treatment position all through the treatment time for BNCT. Thus, it is very reasonable that reactor power was maintained at 5MW for BNCT even after the power during reactor operation for research use was reduced to 1 MW. Consequently, taking these findings obtained in the current *in vivo* studies and clinical realities into consideration, 5MW is more useful, convenient, appropriate, advantageous and practical than 1MW as a reactor operation power in clinical BNCT [4].



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[4] S. Masunaga *et al., Int. J. Radiat. Biol.,* **95** (2019) 635-645.

PR6-2 Development of new amino acid-type boron carriers for BNCT

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INTRODUCTION: In malignant tumor cells, there is a significant increase in the demand for amino acids to maintain rapid growth and energy metabolism, and in particular such cancer cells show glutamine addiction.

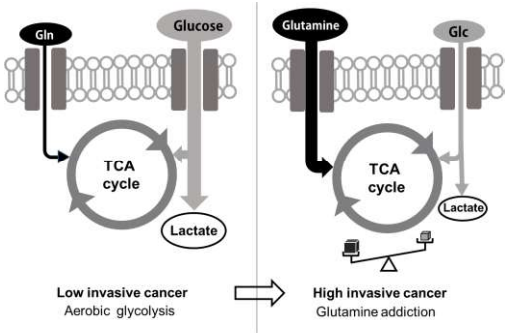
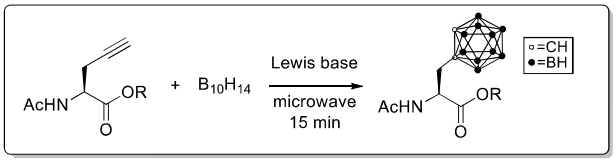


Fig. 1. Metabolic shift in malignant tumor cells.

Thus, such tumor cells overexpress transporters (ASCT2 and LAT1) that transport glutamine and neutral amino acids with aromatic or branched side chains. In this study, we designed and synthesized boron cluster-containing amino acid derivatives for the purpose of developing boron carriers that can efficiently capture ^{10}B atoms in tumors using tumor-specific amino acid transporters, focusing on glutamine addiction in cancer.

EXPERIMENTS AND RESULTS: First, utilizing boron clusters as unique hydrophobic pharmacophores, we synthesized carborane($C_2B_{10}H_{12}$)-containing amino acid derivatives L-*o*-carboranylalanine (**1**) and *O*-(*o*-carboran-1-ylmethyl) -L-tyrosine (**2**), which mimic the transport substrate of LAT1. They were efficiently obtained from the corresponding amino acid alkynes and decaboran in a short step using our microwave-assisted reaction, with total yields of 53% and 46%, respectively. Rm values of all compounds were examined by TLC method (RP-8 modified silica gel TLC, 20 mM phosphate buffer (pH 7.2) : methanol = 7:3) as hydrophobic parameter.



Next, the uptake of boron atoms was evaluated after treatment of T98G with these compounds and L-boronophenylalanine(BPA). Intracellular boron uptake was measured by ICP-AES after 20 h of treatment with 10 μg ^{10}B/mL of the boron carriers. As a result, it was found that compound **1** (41 ng $^{10}B/10^6$ cells) and **2** (206 ng $^{10}B/10^6$ cells) showed significantly higher uptake than BPA (23 ng $^{10}B/10^6$ cells).

To evaluate neutron sensitizing ability of the compounds, T98G cells were treated with 10 μg ^{10}B/mL boron carriers for 24 h. Then the cells were washed with PBS, suspended in serum containing medium and aliquoted into Teflon tubes for irradiation. Cells were irradiated using the neutron beam at the Heavy Water Facility of the Kyoto University Research Reactor (KUR) operated at 1 MW power output. The survival rates of the irradiated cells were determined using conventional colony assays. The D_{10} of BNCT was calculated from survival curve shown in Fig. 2.

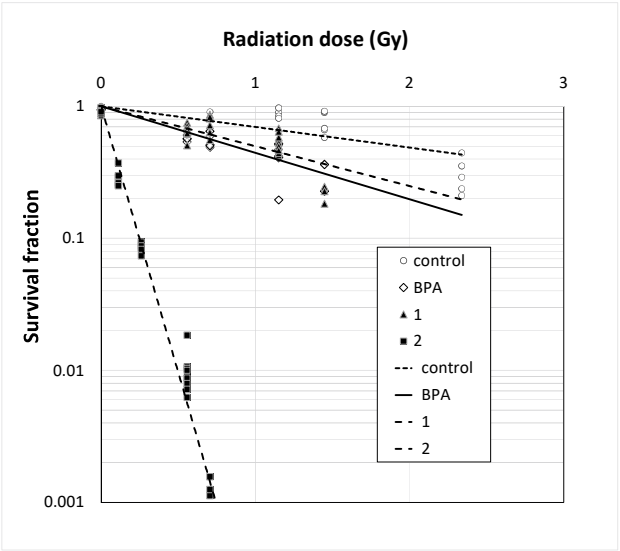


Fig. 2. Survival fraction of T98G cells treated with compound **1**, **2** and BPA and irradiated by mixed-neutron bean for BNCT.

Table 1. Intracellular uptake of ^{10}B atom, Rm values, D_{10} values and the enhancement ratios of boron carriers.

Compound	^{10}B uptake (^{10}B ng/10^6 cells)	Rm value	D_{10} value (Gy)	enhancement ratio
control	nd	—	6.41	1.00
BPA	23	nd	3.31	1.94
1	41	0.302	2.85	2.25
2	203	1.09	0.25	25.8

In summary, Compound **2** showed approximately 9-fold higher intracellular ^{10}B uptake than BPA. In addition, compound **2** enhanced the inhibition of cell survival of T98G cells irradiated with thermal neutrons by 13-fold compared to BPA. These results suggest that **2** is a promising candidate for new boron carrier. We are now synthesizing more diverse derivatives and investigating the structure-activity relationship analysis in BNCT.

PR6-3 Molecular mechanism underlying HISP2-mediated radioresistance of hypoxic tumor cells

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INTRODUCTION: Cancer cells acquire 2-3 times more radioresistance under hypoxia compared to normoxia, which causes poor prognosis, e.g. poor overall survival and poor local control, of patients with more hypoxic regions in their tumor tissues [1]. Although the radioresistance is known to be enhanced by biological mechanisms, at least in part, in a hypoxia-inducible factor 1 (HIF-1)-dependent manner, further studies are needed for better understanding of the nature of hypoxic cell radioresistance [2, 3].

EXPERIMENTS & RESULTS:
We conducted a DNA microarray analysis to explore novel genes whose expression are induced under hypoxic conditions. We found that not only expression but also secretion of one of the genes is markedly upregulated upon hypoxia; and therefore, we designated it as hypoxia-inducible secretory protein 2 (HISP2). The hypoxia-dependent increase in the HISP2 mRNA levels was abrogated by a transcription inhibitor, actinomycin D, suggesting that HISP2 were upregulated under hypoxia at transcription initiation levels. A loss-of-function study found that the hypoxia-dependent increase in the HISP-2 mRNA levels was significantly abrogated by silencing of HIF-1β, but not by that of HIF-1α and HIF-2α, suggested that the hypoxia-mediated expression is dependent on HIF-1β. A reporter gene assay with the use of an expression vector for EGFP-53BP1M fusion protein demonstrated that HISP2-overexpression decreased the number of DNA double strand breaks after irradiation. Clonogenic cell survival assay showed that cancer cells acquired radioresistance when transfected with HISP2-overexpression vector. We preliminary found that the radioresistance was dependent on EGFR and Nrf2. All of these data suggested that HISP2 proteins are secreted under hypoxic conditions in a HIF-1-independent but HIF-1β-dependent manner and decrease the number of double-strand break after irradiation, leading to radioresistance of cancer cells in an autocrine fashion. In combination with our recent findings about HIF-1- and hypoxia-mediated malignant phenotypes and therapy resistance of cancer cells [4-6], we elucidated one aspect of biological mechanism behind radioresistance of cancer cells.

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PR6-4 OH radicals from the indirect actions of neutron beam induce cell killing

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INTRODUCTION: Excellent dose distribution in a cell of neutron capture reaction of boron atom induces high relative biological effectiveness. These phenomena are commonly assumed to be an interaction between cells and low energy heavy particles (α and Li) resulting from the boron atom fis-sions in the cells. However, there has been little study done concerning the action of the particles on living cells. We have investigated contributions of indirect actions of radiation in cell killing by heavy ions with radical scavenger that selectively reduces the indirect action [1].

Therefore, it is important that how these mechanisms can be made to clear through a thorough basic research in boron neutron capture therapy is urgently discussed. The main object of this year is to make clear the contribution of indirect action of neutron beam to cell killing using mammalian cells.

EXPERIMENTS: We used HSGc-C5 (JCRB1070,here after call as HSG) cells. HSG cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) under humidified air with 5% CO_2 at 37˚C. The cells were suspended at a density of about 3×10^5 cells/ml. The cells in Polypropylene tubes were irradiated at the remodeled heavy water facility at the KURRI.

Total fluencies of thermal neutron, epithermal neutron and fast neutron were measured by means of gold foil activation analysis. The gamma ray dose including secondary gamma rays was measured with a thermo luminescence dosimeter. Boron concentrations in the cells were taken to be equivalent to those in the medium as reported previously [2].

After irradiation, cells were seeded in triplicate onto 100 mm (Φ) culture dishes at densities to give approximately 100 colonies per dish. After 14 days of incubation, the colonies were fixed with 10 % formalin solution and stained with 1 % methylene blue in water. Colonies consisting of more than 50 surviving cells were scored.

The contribution of indirect action on cell killing can be estimated from the maximum degree of protection by dimethylsulfoxide (DMSO) [1], which suppresses indirect action by quenching OH radicals without affecting the direct action of neutron beam on cell killing.

RESULTS: The surviving fractions of HSG cells irradiated with 5.85 Gy dose initially increased with increasing DMSO concentration and then plateaued at higher concentrations (Fig.1A). Degrees of protection (DP: $(\ln SF_0 - \ln SF_x)/\ln SF_0$) defined was calculated and plotted as a function of the DMSO concentration (Fig. 1B). Since the curve were upper concave in nature, DP curve was approached saturation as the concentration of DMSO increased. The maximum DP (i.e., saturation level) for DMSO concentrations was 0.29. Namely, the OH radical-mediated indirect action contributions to cell killing by neutron beam was 29%.

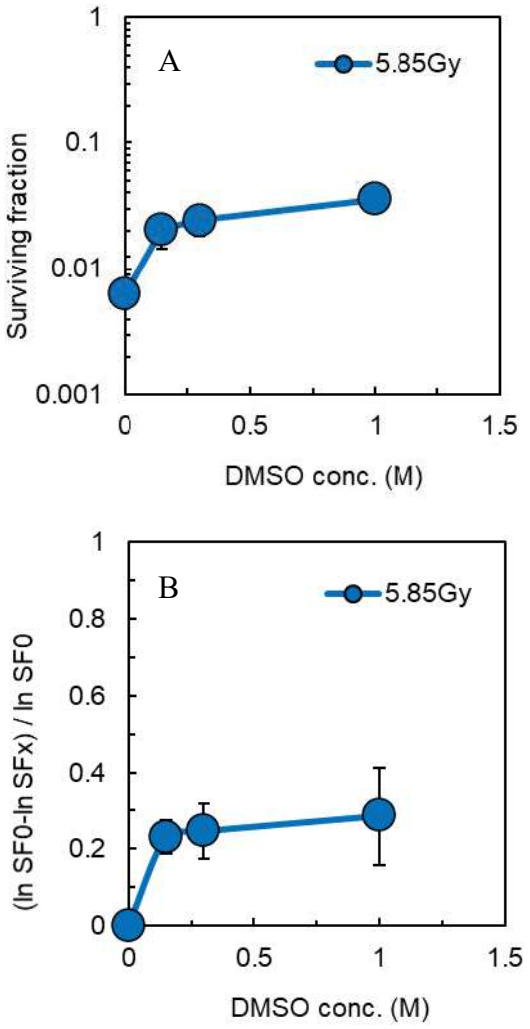

Fig. 1. Effects of DMSO on the survival of HSG cells after exposure to neutron beam. (A) HSG cell survival irradiated with neutron beam in the presence of various concentration of DMSO. (B) Degrees of protections were determined from panel A. The curve was fitted by MichaelisMenten kinetics. The error bars represent the standard errors.

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PR6-5 Intracellular Delivery of Membrane-fluidity Sensitive Boron Liposomes with Tumor-specific Cell Penetrating Peptides

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INTRODUCTION: There are many reports that membranes in cancer cells are relatively more fluid compared to healthy cells. Higher membrane fluidity in cancer cells closely relates to their invasive potential, proliferation, and metastatic ability [1]. Liposomes composed of dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenedodecylether were found to inhibit the growth of human promyelocytic leukemia cells without using any drugs [2]. Cell-penetrating peptides (CPPs) have been studied for their capacity to translocate across the lipid membrane of several cell types. 17-amino acid cell penetrating peptide (BR2) was found to have cancer-specificity without toxicity to normal cells [3]. In this study, we have developed a novel boron delivery system using the membrane-fluidity sensitive boron liposomes (MFSBLs) composed of DMPC and borocaptate (BSH)-conjugated chemical compounds with cell penetrating peptides (BR2) for boron neutron capture therapy.

EXPERIMENTS: Octadecylamine and 1,2-dimyristoyl-sn-glycero-3-phosphorylethanola mine were conjugated with BSH using the opti-mal hetero-crosslinking agents for boron com-pounds. BR2-conjugated MFSBLs composed of DMPC, polyoxyethylenedodecylether, boron com-pounds and BR2 at mole ratios of 8:0.9:1.1:0.1 were prepared by sonication method in 5% glucose so-lution at 45°C. The diameter of MFSBLs was measured with a light scattering spectrometer. The boron concentra-tion was measured by inductively coupled plasma atomic emission spectrometry. B16F10 murine melanoma cells and human fibroblast cells were incubated with 2 ppm of ^{10}B at 37°C for 14 hours before neutron irradiation. The cells were rinsed twice in PBS and suspended in fresh medium. After neutron irradiation the cells were plated into plastic Petri dishes 60 mm in diameter at 200 cells per dish. They were incubated for an additional 7 days to allow colony formation.

RESULTS: Tumor-specific CPPs (BR2) conjugated MFSBLs had a mean diameter of 78.5 nm. High encapsulation efficiency value from 48% to 74% of ^{10}B in MFSBLs were obtained. BRs-conjugated MFSBLs had high stability (92-97%) in the retention of ^{10}B during storage at 4°C for 4 weeks. All borocaptate-loaded formulations had low cytotoxic effects in human fibroblast cells. BR2-conjugated were efficiently fused to melanoma cells, but were inefficiently fused to human fibroblast cells. As shown in Fig. 1, BR2-conjugated MFSBLs showed higher suppression of growth of murine and human melanoma cells than BSH solution. This result suggested novel BR2-conjugated MFSBLs is useful for ^{10}B carrier on BNCT for melanoma.

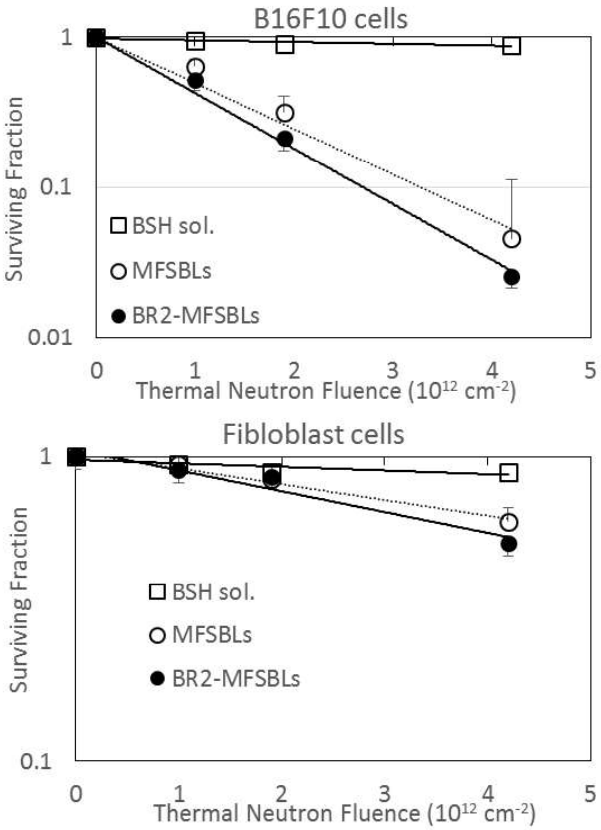

Fig. 1. Suppression of the colony formation of B16F10 cells and fibroblast cells after in vitro BNCT.

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PR6-6 Preparation and Characterization of a Novel Bispecific Antibody That Targets Her2 and BSH for Boron Neutron Capture Therapy

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INTRODUCTION: Boron neutron capture therapy (BNCT) is a cancer treatment that specifically kills cancer cells with the energy of particle beams generated by a nuclear reaction between boron (^{10}B) and thermal neutrons. The key to successful BNCT is how to selectively deliver boron atoms into cancer cells. However, conventional boron drugs are poor in water solubility, tumor selectivity, and accumulation. Therefore, the development of effective boron delivery system is serious issue for the spread of BNCT. In this study, we developed novel bispecific antibodies against BSH, a water-soluble boron drug with a high boron content, and Her2 that is overexpressed on the surface of cancer cells. Moreover, we aim to construct a boron drug delivery system with high tumor selectivity and tumor accumulation by using it.

EXPERIMENTS: Rabbits were immunized with BSH-modified KLH, anti-BSH antibody-producing lymphocytes were obtained by the single cell method using ASONE Cell Picking System, and anti-BSH antibody was successfully produced genetically (Fig. 1A). The prepared antibody was confirmed for its binding ability to BSH by the ELISA method. In addition, colon 26 cells internalized with BSH-modified BSA were immunostained to evaluate the function of the anti-BSH antibody.

A bispecific antibody light chain expression vector was constructed by linking the prepared rabbit anti-BSH antibody light chain variable region gene with a human antibody CL region gene. n addition, the rabbit anti-BSH antibody heavy chain variable region gene was incorporated into an expression vector containing the Her2 binding site gene that had been successfully constructed to construct a bispecific antibody heavy chain expression vector (Fig. 1B). Bispecific antibodies against BSH and Her2 were prepared using the prepared bispecific antibody expression vector, and the binding ability to BSH was confirmed by ELISA evaluation, and the binding ability to Her2 was confirmed by flow cytometry evaluation.

RESULTS: First, we succeeded in producing an anti-BSH antibody using rabbits and decoded the gene sequence. By using the ELISA evaluation with BSH-modified BSA immobilized, it was confirmed that the prepared bispecific antibody binds to BSH (Fig. 2). In the flow cytometric evaluation, SKBR3 cells as Her2 positive cells and NIH3T3 cells as Her2 negative cells were used. The binding could be confirmed only when the prepared bispecific antibody treated with SKBR3 cells. From this, the affinity for Her2 was confirmed (Fig. 3).

These results suggest that the prepared bispecific antibody against BSH and Her2 is expected as a potent boron drug delivery tool.

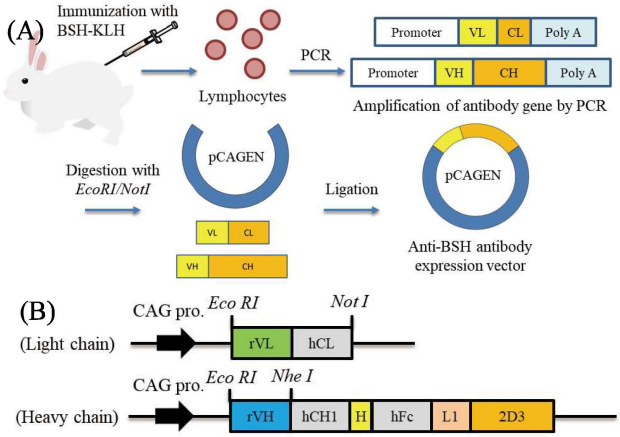

Fig. 1. (A) Construction of anti-BSH antibody expression vector (B) Sequences of light and heavy chains.

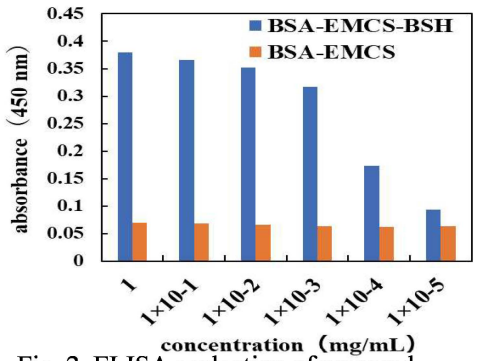

Fig. 2. ELISA evaluation of prepared bispecific antibodies against BSH and Her2.

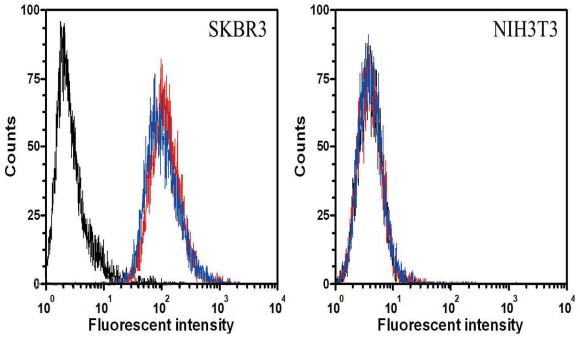

Fig. 3. Flow cytometric evaluation of prepared bispecific antibodies against BSH and Her2.

REFERENCE

[1] K. Ono *et al.*, *Int.l J.s Radiation Oncol. Biol. Phys.*, **43** (1999)431.

PR6-7 **Development of novel BPA-Tirapazamine hybrid BNCT agent targeting hypoxic tumor cells**

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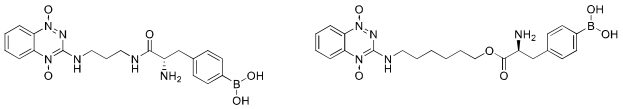
INTRODUCTION:

Hypoxia is a ubiquitous environment of cancer, it has a resistance to anticancer drugs and X ray, and accelerated infiltration / metastasis is a problem. Boron neutron capture therapy (BNCT), a kind of radiotherapy, is a treatment method utilizing neutron capture reaction in tumor cells by accumulating ^{10}B in tumor tissue. Hybrid molecules of Tirapazamine (TPZ), as a hypoxic cytotoxin and the neutron scavenger *p*-borono-L-phenylalanine (BPA) are useful as selective neutron scavengers for hypoxic tumors. Since boron neutron capture therapy depends on ^{10}B concentration, it is expected to provide an effective therapeutic effect for hypoxic cancer.

In this study, UTX-117 with amide linkage of TPZ and BPA and UTX-118 with ester linkage of TPZ and BPA were designed and synthesized, and antitumor activity and neutron sensitizing activity were evaluated. UTX-118 exhibited hypoxia selective cytotoxicity. Further, UTX-118 showed neutron sensitizing activity.

EXPERIMENTS:

Tirapazamine derivatives and BPA were condensed to synthesize UTX-117 and UTX-118 (Fig.1). Using HeLa cells, the uptake amount of UTX-117 and UTX-118 was measured by ICP-AES, and the intracellular boron concentration was calculated. The IC_{50} values of UTX-117 and UTX-118 were calculated by WST-1 assay. After adding ^{10}B enriched UTX-117, ^{10}B enriched UTX-118 and ^{10}B enriched BPA-F (>99.5% ^{10}B) to HeLa cells, neutrons were irradiated to calculate cell viability.



UTX-117 UTX-118

Fig.1

RESULTS:

The uptake test of UTX-117, UTX-118 and BPA-F was performed using HeLa cells (Fig. 2). The boron concentration required for BNCT is 1.0×10^9 atoms / cell.[1] since the maximum uptake of UTX-118 was approximately 1.0×10^9 atoms / cell, a neutron sensitization effect can be expected for UTX-118. On the other hand, UTX-117 was below the detection limit of ICP-AES.

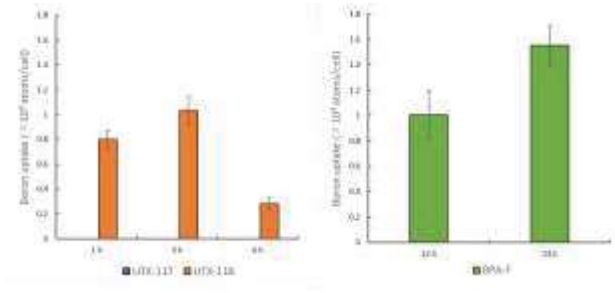


Fig.2

Toxicity test was performed by WST-1 assay using HeLa cells. As shown in Table 1, UTX-118 exhibited hypoxia-selective toxicity. The low toxicity of UTX-117 may be due to low uptake.

	Normoxia	Hypoxia	N/H ratio
UTX-117	>1000 μM	299 μM	-
UTX-118	24.4 μM	7.17 μM	3.40
TPZ	346 μM	21.1 μM	16.4

N/H ratio=the IC_{50} value of Normoxia / the IC_{50} value of Hypoxia

Table. 1

Neutron sensitizing activity of ^{10}B enriched UTX-117, ^{10}B enriched UTX-118 and ^{10}B enriched BPA-F was evaluated using HeLa cells (Fig. 3). ^{10}B enriched UTX-118 showed the neutron sensitizing activity (sensitization ratio: 4.15 compared to the neutron irradiation control group). Since the sensitization ratio of ^{10}B enriched BPA-F was 3.27, ^{10}B enriched UTX-118 showed higher neutron sensitization activity than ^{10}B enriched BPA-F.

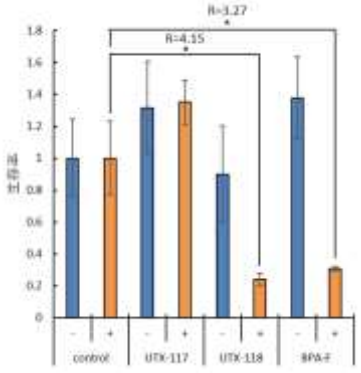


Fig.3. +:Irradiation, -:Non-irradiation

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PR6-8 The tumor invasion enhanced by the conditioned-medium after X-irradiation

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INTRODUCTION: In cancer studies, it has been suggested that irradiation sometimes enhances invasion of tumor cells. Wild-Bode et al. reported that sublethal dose of γ-ray irradiation enhanced invasiveness of human glioblastoma, and the enhancement was accompanied by the activation and/or up-regulation of integrin and matrix metalloproteinase (MMP) contributed to cellular adhe-sion and degradation of extracellular matrix (ECM) [1]. Fujita et al. also reported that X-ray irradiation enhanced cellular invasive ability via up-regulation of MMP in some human pancreatic tumors [2]. Including these re-ports, a number of studies suggested that ECM degrada-tion activity was involved in the irradiation-enhanced invasiveness of tumor cells. However. although there are many studies that estimate effects of direct irradiation on tumor cell invasiveness, not so many reports did by-stander effects of irradiated tumor cell conditioned me-dium (CM) on it. Furthermore. the same effect of high LET radiation therapy such as boron neutron capture reaction (BNCT) has not been reported. In this study, we conducted the experiments to examine effects of X-irradiated tumor cell CM on cellular invasiveness in breast cancer and lung cancer-derived cells, prior to BNCT challenge. In 2018, using human breast adenocarcinoma MDA-MB-231 cells and human lung adenocarcinoma A549 cells, we reported the X-ray irradiated ce1l-derived CM promotes cell infiltration ability even if taken into consideration cell proliferation activity [3]. In this year, we examined the expressions of growth factors such as Epidermal growth factor (EGF), Vascular endothelial growth factor (VEGF) and Transforming Growth Factor-β1 (TGF-β1) in X-irradiated MDA-MB-231 cells.

EXPERIMENTS: The expression of growth factors was evaluated using real-time PCR. Cells were seeded on 60 mm dishes (1×10^6 cells/dish), and incubated with RPMI1640/10% FBS overnight. After washing by PBS, 2 ml of serum-free RPMI1640 medium was added to the dishes followed by 0 or 4 Gy X-irradiation immediately. After 0-24 h incubation, the cells were collected with time. Total RNA of the cells was extracted by SV Total RNA Isolation System (Promega, Madison, WI.) according to the manufacture's instruction. RNA concentration was measured by DU 800 (BECKMAN COULTER, CA.). Reverse transcription was performed by Reverse Transcription System (Promega) according to the manufacture's instruction. Real-time PCR was performed by LightCycler® Nano System (Roche Diagnostics GmbH, Mannheim, Germany) and FastStart Essential DNA Green Master (Roche) according to the manufacture's instruction. GAPDH was used as an internal control. Amount of mRNA was expressed as relative values which set that of non-treated cells to 1.

RESULTS: Since the CM derived from X-ray-irradiated cells promoted the cell invasion ability [3], it was considered that some bystander factor that promotes the cell-infiltrated ability exists in the CM derived from X-irradiated cells. Therefore, using MDA-MB-231 cells, which showed a large effect of promoting invasion ability by CM derived from X-ray-irradiated cells, the expression of mRNA for several proteins related to invasion ability in non-irradiated and 4 Gy-irradiated cells was measured using real-time PCR method. As shown in Fig. 1., the mRNA levels of TGF-β and VEGF were not increased by at least 24 hours in 4 Gy-irradiated cells compared to non-irradiated cells. On the other hand, the amount of EGF mRNA was significantly increased in the 4 Gy-irradiated cells with a peak at 18 hours after X-irradiation. This result suggested that EGF acts as a bystander factor to promote the cell invasion ability.

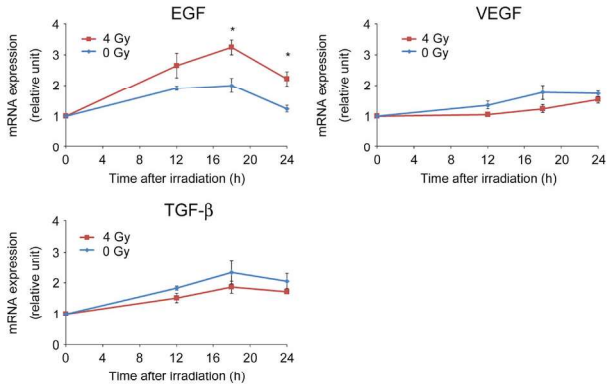

Fig. 1. The time-course of the mRNA expression of EGF, VEGF, and TGF-β in X-irradiated MDA-MB231 cells. *; $p < 0.05$ *vs.* non-irradiated cells.

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[1] C. Wild-Bode *et al.*, Cancer Res., **61** (2001) 2744-50.
[2] M. Fujita *et al.*, Cancer Sci., **102** (2011) 729-8.
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PR6-9 Analysis of the response of tumor tissue and cells to BNCT

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INTRODUCTION: Boron neutron capture therapy (BNCT) is based on nuclear reactions and preferential killing of cancer cells. The reaction is occurred between thermal neutron and boron-10 and causes alpha particle and lithium nuclei in a short length. For the clinical trials, boron compounds such as ^{10}B-boronophenylalanine (BPA) have been used in nuclear reactor or accelerator-based BNCT systems, which have been developed recently. Boron neutron capture reaction (BNCR) efficiently introduces DNA damages, however, it is still difficult to measure or calculate the irradiated dose. This is because BNCT dose is composed by various factors including boron uptake in tumors and irradiated thermal neutron fluence, and furthermore, these factors are variable depending on the irradiation system. We previously observed extensive DNA damage as gamma-H2AX foci, and poly(ADP-ribose) level in the rat lymphosarcoma model after BNCR[1]. We also performed comprehensive analysis of proteome for human squamous carcinoma SAS cells after BNCR[2]. The results suggested that the changes in the particular protein levels may be involved in the early response of BNCT. In this study, we investigated the changes and the dynamics of genes and proteins after BNCR or neutron beam irradiation in comparison with the gamma-ray irradiation.

EXPERIMENTS: We used human squamous cell line SAS, human melanoma A375 cells, and mouse melanoma B16 cells. BALB/c nude male mice of 5 weeks old were used for xenograft models after subcutaneous injection of tumor cells. Approximately ten days before irradiation, SAS and A375 cells were subcutaneously transplanted at hind legs of mice. For BNCR experiment, ^{10}B-BPA fructose complex (BPA) were used as a boron compound, and neutron irradiations at KUR reactor was operated at 1 MW. Irradiation experiments were carried out on July 31, September 4, November 12 of 2019. Cells were irradiated 2 hrs after incubation in the presence or absence of BPA at 25 ppm concentration of boron. Local irradiations to mouse legs were operated using ^{6}LiF containing thermal neutron shield approximately 30 min after intraperitoneal administration.
We used gold foil activation analysis for the measurement of thermal neutron fluences and thermoluminescence dosimeter (TLD) for the measurement of the γ-ray doses including secondary γ-ray. Gold foils are placed on the surface and backside of the cell vials. Total absorbed dose calculation was carried out using the flux-to-dose conversion factor by the sum of the absorbed doses resulting from ^{1}H(n, γ)^{2}D, ^{14}N(n, p)^{14}C, and ^{10}B(n, α)^{7}Li reactions, as previously described. To analyze the acute cellular responses including factors and metabolites, cell culture supernatants were filtrated and analyzed 6 and 24 hrs after irradiation. RNA and proteins were also isolated and RNA expression levels were validated using real-time PCR and protein levels were analyzed by western blot and ELISA.

RESULTS: The irradiated thermal neutron fluence and total doses (Gy-Eq) are exemplified in Table 1. According to the previous microarray analysis in SAS, we analyzed gene expression profiles of *CSF2* gene, that encodes GM-CSF, *GM-CSF receptor*, genes in NF-κB-dependent pathway. We observed the increase of *CSF2* mRNA and its protein level in culture supernatant after the therapeutic dose irradiation. Changes in NF-κB-dependent pathway was also observed within 24 hrs. The results suggest that roles of GM-CSF cascade and NF-κB-dependent pathway in cellular response after BNCT.

Table 1. Irradiated neutron fluence and doses on cells.

Irradiation time [min]	Position	Thermal neutron [/cm²]	Thermal neutron [Gy]	Epi-thermal neutron [Gy]	Fast neutron [Gy]	Gamma-ray [Gy]	Total dose [Gy-Eq]
20	S, U	1.9E+12	0.25	0.027	0.19	0.25	4.21
	B, U	2.0E+12	0.26	0.028	0.19	0.25	4.48
	S, C	1.9E+12	0.26	0.027	0.19	0.25	4.22
	B, C	1.2E+12	0.16	0.017	0.12	0.25	2.83
	S, L	1.6E+12	0.21	0.022	0.16	0.25	3.63
	B, L	1.4E+12	0.18	0.02	0.14	0.25	3.09
13	S, C	1.2E+12	0.17	0.018	0.12	0.14	2.78
	B, C	9.0E+11	0.12	0.013	0.089	0.14	2.05
7	S, U	7.5E+11	0.099	0.011	0.074	0.11	1.67
	B, U	6.8E+11	0.091	0.0097	0.067	0.11	1.53
	S, C	5.0E+11	0.067	0.0071	0.049	0.11	1.16
	B, C	5.7E+11	0.077	0.0082	0.057	0.11	1.33
	S, L	7.3E+11	0.098	0.01	0.072	0.11	1.64
	B, L	6.0E+11	0.08	0.0085	0.059	0.11	1.39
4	S, C	4.5E+11	0.06	0.0064	0.045	0.066	1.03
	B, C	2.6E+11	0.035	0.0037	0.026	0.066	0.61
2	S, C	2.0E+11	0.027	0.0029	0.02	0.039	0.46
	B, C	1.4E+11	0.018	0.0019	0.013	0.039	0.32
60	S, L	6.3E+12	0.84	0.089	0.62	0.9	14.15
	B, L	6.5E+12	0.87	0.092	0.64	0.9	14.50
	S, R	3.8E+12	0.5	0.053	0.37	0.9	8.80
	B, R	3.2E+12	0.43	0.045	0.32	0.9	7.70
	B, R	3.7E+12	0.5	0.053	0.37	0.9	8.80
10	S, L	1.1E+12	0.14	0.015	0.1	0.17	2.38
	B, L	1.0E+12	0.13	0.014	0.099	0.17	2.27
	S, R	5.9E+11	0.079	0.0084	0.059	0.17	1.42
	B, R	6.1E+11	0.081	0.0087	0.06	0.17	1.45

Irradiation was carried out in cell vials on September 4th 2019. S, surface; B, back-side; U, upper position; C, center position; L,left position; R, right position. Total dose was calculated for the presence of 25 ppm boron concentration.

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[2] A. Sato *et al.,* Appl. Rad. Iso., 106 (2015) 213-219.

PR6-10 The contribution of blood boron-neutron reaction to subcutaneous tumor growth suppression was equal to that of neutrons irradiation only group

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INTRODUCTION:

Boron Neutron Capture Therapy (BNCT) is a particle radiation therapy for malignant diseases[1]. However, Boron distribution of extra-cellular fluid or interstitial tumor tissue during the neutron irradiation and radiobiological effect of extracellular born neutron reaction is still unclear. In the previous studies, we have focused on intra-cellular boron concentration and average boron concentrations of tumor tissue. There has intrinsic heterogeneity. The goal of this study is, to clarify a role of extra-cellular/peri-tumoral boron neutron reaction in BNCT.

EXPERIMENTS:

Materials: Boron agent was designed and synthesized by Dr. M. Shirakawa[2]. The concept of this boron particle was that has only blood retention and has not pass through the vascular endothelium. The diameter of the liposome was 400nm, and pegylated, containing BSH solutions. Boron concentration of these solution were about 1400ppm.

Cell Lines: CT-26 murine colon cancer cell lines were cultured in D-MEM supplemented with 10% fetal bovine serum and maintained at 37℃ in a humidified atmosphere with 5% CO_2. After trypsinized and counted, cells were suspended in culture medium.

Tumor models: 4w female bulb/c mice were used. $1.0x10^7$cells were implanted to rt. thigh subcutaneously.

Boron Neutron Reaction:

PEG liposome was administrated via tail vain injection 1hr before neutron irradiation(n=4). Thermal neutron dose was $2.2x10^{12}$ n/cm^2. At KUR, 50min of thermal mode irradiation was performed, Gamma ray dose was 0.18 to 0.32Gy. As a control, Neutron irradiation only group(n=5) and no treatment group(n=6) were also analyzed. Tumor progression were measured by tumor size.

RESULTS and DISCUSSIONS: As shown in Fig. 1, the contribution of PEG liposomal boron-neutron reaction to subcutaneous tumor growth suppression was equal to that of neutrons irradiation only group. This liposome was large sized, and pegylated to have a characteristics of blood retention. It means that boron neutron reaction in the blood vessels were not contribute to tumor suppression. Therefore, it is considered that the boron compound distributed in the stroma or tumor cells during 1hour exerts an effect. To clarify this inference, the detailed boron distribution of intra and extra tumor cells, and interstitial peritumoral tissue should be examined. And also, it is suggested that BNCT is effective for tumor histopathology with early distribution of boron compounds in tumor cells.

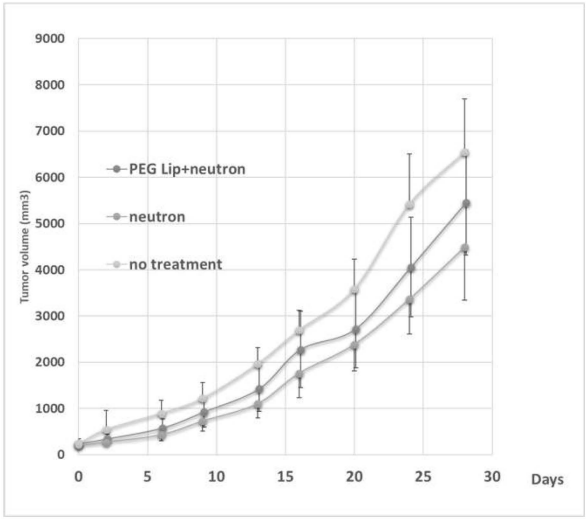

Fig. 1 Subcutaneous CT-26 Tumor volume after Boron containing PEG liposome administration and neutron irradiation. Three groups of animals underwent the irradiation treatment at KUR. The first group (irradiated and i.v. PEG liposome group, n=4) received PEG liposome a dose of 250mg^{10}B/kg, i.v. via tail vein 1 hour before neutron exposure. The second mice group (irradiated only group, n=5) received neutron exposure only. The third group (no treatment, n=6), six mice were followed up as non-treatment group.

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PR6-11 Cell killing effect of BNCT with novel boron compound SMA glucosamine complex

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INTRODUCTION: Tumor vessels usually showed a special characteristic such as active angiogenesis, incomplete anatomy from normal tissue vasculature, highly occluded or embolized, or high or low vessel density compared with normal vessels. Therefore, the vascular permeability of the polymer compound is selectively promoted. Nanoparticles can remain in tumor tissue for extended periods of days to weeks, in contrast to the behavior of small compounds. The enhanced permeability and retention (EPR) effect can be used for tumor-selective delivery of nanopharmaceuticals, leading to avoidance of side effects and enhanced therapeutic effect. Boron neutron capture therapy (BNCT) has been known for more than 60 years, and its basic mechanism of action depends on α rays emitted from 10B when neutrons collide with ^{10}B atoms. A major challenge in the BNCT area is that most of the ^{10}B compounds used for BNCT are low molecular weight compounds such as f-BPA and BSH, and they do not produce EPR effects, so they do not selectively accumulate in solid tumor. We developed a synthetic polymer (SMA, styrene-co-maleic acid) -conjugated glucosamine via amide bond, which was then complexed with boric acid, thus SMA-Glc-NH_2-borate (SGB) showed 10-30 nm in hydrodynamic size and contains about 6-7% boron (w/w). SGB was also shown to stay in circulation for long time (> 6h), and exhibited good tumor selectivity (Tumor/blood ratio: 5-10). Thus, it will be ideal to use for BNCT. The effect of this drug on BNCT was verified, the difference with existing low molecular weight compounds was clarified, and its usefulness was evaluated.

EXPERIMENTS: The following endpoints were examined. 1) Synthesis of SMA polymer, SMA-glucosamine conjugate (SG) and SG-borate complex (SGB), 2) Particle size measurement by UV spectrum method (XYZ-001) and dynamic light scattering method (ELSZ-2000ZS), 3) Zeta potential measurement, 4) Shape and size measurement of nanoparticles by phase contrast electron microscopy (TEM), 5) Quantification of boron containing SGB complex by IPC-MS, 6) Boric acid (BA) from SGB by carmine method Correlation between release amount and pH, 7) Stability of SGB complex in serum, 8) Toxicity evaluation by C26 and S180 tumor-bearing mice, 9) Pharmacokinetics of SGB complex by ICP-MS, 10) Hypoxia treatment Glycolytic changes and X-ray sensitivity after administration of BA, SG and SGB to cells, 11) Cell killing effect by combined use of BA and SGB with thermal neutrons

SAS cells were treated with BPA for 2 hours and BA, SG and SGB complex for 24 hours before irradiation. Cells were collected, placed in a cryotube (FG-CRY-05S), and irradiated with a neutron beam (1 MW) in the reactor. Thermal neutron fluence and γ-dose were measured by activation analysis of gold foil (diameter 3 mm, thickness 0.05 mm) and thermoluminescence dosimeter, respectively. The total absorbed dose due to neutrons was calculated as the sum of the individual absorbed doses, which are mainly the result of $^{1}H(n, c)^{2}D$, $^{14}N(n, p)^{14}C$ and $^{10}B(n, a)^{7}Li$ reactions. The fluences of thermal neutrons and epithermal neutrons were $2.32x10^{12}\pm1.94x10^{11}$ (n/cm^2) and $4.09x10^{11}\pm3.38x10^{10}$ (n/cm^2), respectively.

RESULTS: The SGB complex showed rapid uptake into tumor cells, liberating free boric acid at weakly acidic pH (5-7) of tumor tissue or cytosol. The free boric acid competed with phosphate for phosphorylation of glucose and inhibited tumor growth by inhibiting the tumor glycolysis system. It was found that the 15 mg/kg SGB complex significantly suppressed tumor growth compared with the untreated control group for S180 and C26 tumors and was not toxic during the experimental period. SGB also suppressed the enhanced glycolysis by hypoxia treatment (Fig. 1A-B) and decreased the enhanced radioresistance (Fig. 1C). Furthermore, it was revealed that SGB induces cell death after BNCT extremely efficiently as compared with the amount of ^{10}B contained (Fig. 2).

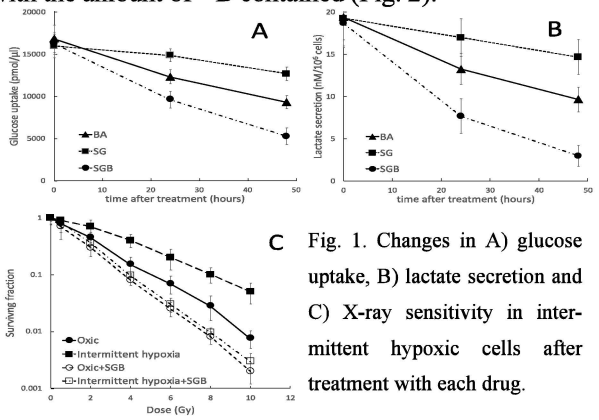

Fig. 1. Changes in A) glucose uptake, B) lactate secretion and C) X-ray sensitivity in intermittent hypoxic cells after treatment with each drug.

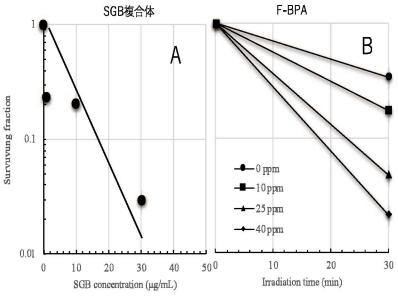

Fig. 2. Cell killing effect by A) SGB complex and B) f-BPA treatment with neutron irradiation.

PR6-12 Attempts to sensitize tumor cells by exploiting the tumor microenvironment

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INTRODUCTION: Hypoxia and glucose deprivation have been suggested to play important roles in resistance to radiation [1]. Attempts to sensitize tumor cells by exploiting the tumor microenvironment have been studied. A major mediator of the cellular hypoxic response, hypoxia inducible factor 1 (HIF-1), is a potential target for cancer therapy, because it transcriptionally regulates a number of genes, including those involved in glucose metabolism, angiogenesis and resistance to chemotherapy and radiation therapy [2]. We previously reported that the disruption of Hif-1α enhanced the sensitivity of murine squamous cell carcinoma (SCC-VII) cells to gamma-ray [3]. In the present study, we investigated whether the disruption of Hif-1α affects the radiosensitivity of SCC-VII cells to the boron neutron capture reaction (BNCR). Previous studies reported that HIF-1 is likely involved in DNA damage and DNA repair. We first examined the extent of DNA damage after exposure to ionizing radiation or neutron beams. Furthermore, in order to determine the intracellular ^{10}B levels in tumor cells, neutron-induced autoradiography was performed.

EXPERIMENTS: In order mark the sites of DNA damage signaling, we established SCC-VII cells expressing EGFP -fused 53BP1 fragment (SCC-VII-BP1 cells). SCC-VII-BP1 cells and SCC-VII-BP1 Hif-1α-deficient (ΔHif-1α) cell suspensions were exposed to gamma-rays (Co-60 Gamma-ray Irradiation Facility) or neutron beam (KUR Heavy Water Facility). The irradiated cells were seeded onto coverslips, and fixed with 4% formaldehyde. The intracellular localization of the EGFP-BP1 proteins was monitored by fluorescence microscopy.
In order to estimate the uptake of boron 10 (^{10}B), neutron autoradiography was performed using CR-39 detec-tors. Cells grown under normoxia or hypoxia on CR-39 were treated with BPA (60 ppm), and then fixed. CR-39 detectors were exposed to thermal neutrons at a fluency of 3×10^{12} n・cm^{-2} (60 min), and etched in 6 N NaOH solution at 70℃ for 45 min. The etched pits were counted using light microscope.

RESULTS:
53BP1 is an early participant in the DNA damage response, and a fusion protein consisting of 53BP1 and GFP (GFP-53BP1) is used as a marker of the DNA damage response. In this study, we established SCC-VII cells expressing EGFP-fused 53BP1 fragment, and counted the number of BP1 foci formed in response to ionizing radiation- or neutron beams-induced DNA damage. We first tested whether the BP1 foci can be detected in the SCC-VII-BP1 cells. It was found that the number of foci after gamma irradiation was formed in a dose dependent manner (Fig 1A). Next, SCC-VII-BP1 cells and SCC-VII-BP1 ΔHif-1α cells were cultured under normoxic or hypoxic condition, incubated with BPA, and then exposed to neutron beams. As shown in Fig. 1B, fewer 53BP1 foci occurred in SCC-VII-BP1 (hypoxia) cells than in SCC-VII-BP1 (normoxia) cells. On the other hand, the number of foci did not differ significantly in SCC-VII-BP1 ΔHif-1α cells (hypoxia and normoxia).

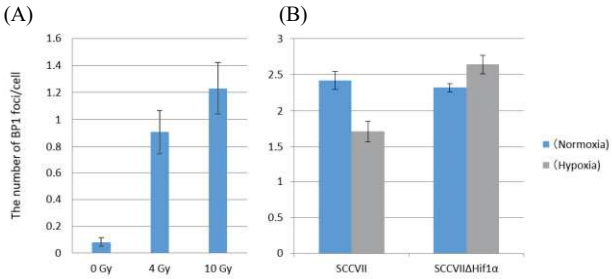

Figure. 1: (A) The number of BP1 foci 60 min after gamma irradiation. (B) The number of BP1 foci 30 min after neutron beam irradiation. The cells were exposed to neutrons at a fluency of 4.0×10^{11} n・cm^{-2}.

We also examined whether the disruption of Hif-1α affects the uptake of BPA by calculating he average tracks on CR-39 per cell. The average tracks per cell were 3.5 tracks for SCC-VII (normoxia) cells and 1.8 tracks for SCC-VII (hypoxia) cells, suggesting that BPA was accumulated in SCC-VII (normoxia) cells more than in SCC-VII (hypoxia) cells. The average tracks per cell were 4.31 tracks for SCC-VII ΔHif-1α (normoxia) cells and 4.72 tracks for SCC-VII ΔHif-1α (hypoxia) cells. The results indicate that, if Hif-1α is disrupted, there was little difference in ^{10}B concentration in normoxia-treated and hypoxia-treated cells.

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