VIII-Ⅱ-1. Project Research

Project 3

PR3

Analyzing Tumor Microenvironment and Exploiting its Characteristics for Controlling Malignant Solid Tumors

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BACKGROUNDS AND PURPOSES: Human solid tumors are thought to 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 experimental animal tumor cell lines that have been employed for various oncology studies [1]. The presence of Q cells is probably due, in part, to hypoxia and the depletion of nutrition in the tumor core, which is another consequence of poor vascular supply [1]. As a result, Q cells are viable and clonogenic, but stop cell division. In general, radiation and many DNA-damaging chemotherapeutic agents kill proliferating (P) tumor cells more efficiently than Q tumor cells, resulting in many clonogenic Q cells remaining following radiotherapy or chemotherapy [1]. 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 cell populations that could not be sufficiently killed by radiotherapy [1]. Further, sufficient doses of drugs cannot be distributed within Q tumor cell populations mainly due to heterogeneous and poor vascular distributions within solid tumors. Thus, one of the major causes of post-chemotherapy recurrent tumors is an insufficient dose distribution in 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 [2]. Q cells showed greater potentially lethal damage repair (PLDR) capacities than the total cells. γ-Ray irradiation and neutron irradiation with BPA induced larger PLDR capacities in each cell population. In contrast, thermal neutron irradiation without the ^{10}B-compound induced the smallest PLDR capacity in both cell populations. The use of the ^{10}B-compound, especially BPA, resulted in an increase in the PLDR capacity in both cell populations, and made the PLDR patterns of the both cell populations look like those induced by γ -ray irradiation [3]. In both the total and Q tumor cells, the hypoxic fractions (HFs) immediately after neutron irradiation increased suddenly. Reoxygenation after each neutron irradiation occurred more rapidly in the total cells than in the Q cells. In both cell populations, reoxygenation appeared to be rapidly induced in the following order: neutron irradiation without ^{10}B-compounds > neutron irradiation following BSH administration > neutron irradiation following BPA administration > γ -ray irradiation [4]. These findings concerning the difference in sensitivity, PLDR and reoxygenation following neutron irradiation after ^{10}B-compound administration were thought to be mainly based on the fact that it is difficult to deliver a therapeutic amount of ^{10}B from currently used ^{10}B-carriers throughout the target tumors, especially into intratumor hypoxic cells with low uptake capacities [5,6].

Therefore, 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 radiation therapy including BNCT in the use of newly-developed ^{10}B-compound based on the revealed findings on intratumor microenvironmental characteristics.

RESEARCH SUBJECTS:

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

ARS-1 (23P3-1): 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,Y. Sakurai, H. Tanaka, Y. Liu, M. Takagaki, N. Kondo and Y. Matsumoto)

ARS-2 (23P3-2): Development of Hypoxic Microenvironment-Oriented ^{10}B-Carriers
(H. Nagasawa, S. Masunaga, K. Okuda, S. Y. Hirayama and T. Harada)

ARS-3 (23P3-3): Clarification of Mechanism of Radio-Resistance in Cancer Using Optical Imaging at Tissue Level
(H. Harada, M. Hiraoka, S. Masunaga, S. Itasaka, M. Ogura and M. Yoshimura)

ARS-4 (23P3-4): Analysis of Radiation-Induced Cell-Killing Effect in Neutron Capture Reaction
(R. Hirayama, S. Masunaga, G. Kashino, Y. Sakurai, H. Tanaka and Y. Matsumoto)

ARS-5 (23P3-5): Transdermal Drug Delivery System using Hyaluronan-Conjugated Liposomes as ^{10}B-Carrier in Boron Neutron Capture Therapy for Melanoma
(S. Kasaoka, K. Hashimoto and S. Masunaga)

ARS-6 (23P3-6): Evaluation of Inclusion Complex of Carborane Modified Kojic Acid and Cyclodextrin as ^{10}B-Carrier in Boron Neutron Capture Therapy
(T. Nagasaki, S. Masunaga, M. Kirihata, H. Azuma, K. Li, U. Shu, Y. Kagoshima, K. Hayashi and R. Kawasaki)

(Underline: Representative at each research group)

REFERENCES:

[1] P. Vaupel, *Semin. Radiat. Oncol.* **14** (2004) 197-275.
[2] S. Masunaga *et al., Int. J. Radiat. Oncol. Biol. Phys.* **41** (1998) 1163-1170.
[3] S. Masunaga *et al.*, *J. Cancer Res. Clin. Oncol.* **125** (1999) 609-614.
[4] S. Masunaga *et al.*, *Int. J. Radiat. Oncol. Biol. Phys.* **44** (1999) 391-398.
[5] S. Masunaga *et al.*, *Radiat. Med.* **24(6)** (2006) 98-107.
[6] S. Masunaga *et al.*, *Clin. Exp. Metastasis* **26** (2009) 693-700.

採択課題番号 23P3 腫瘍内微小環境解析とその特性利用による悪性腫瘍制御の試み　プロジェクト（京大・原子炉）増永慎一郎

PR3-1 Radiosensitivity and Capacity to Recover from Radiation-Induced Damage in Oxygenated Intratumor Quiescent Cell Population Depend on *p53* Status

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BACKGROUNDS AND PURPOSES: Using our method for selectively detecting the response of quiescent (Q) cells in solid tumors to DNA-damaging treatment including conventional irradiation and chemotherapeutic agents, the following characteristics of Q cells in murine solid tumors were clarified: Q tumor cells are more radio- and chemo-resistant than the total (proliferating (P) + Q) tumor cell population; Q cells have a greater capacity to recover from potentially lethal damage than the total cell population; and Q cell populations include a larger hypoxic fraction (HF) than total cell populations. Further, it was also indicated that the clonogenicity of Q cells is lower than that of P cells, and that the HF of Q cells is largely comprised of a diffusion-limited chronically HF with a smaller perfusion-limited acutely HF [1,2].
Meanwhile, the Q cell population in solid tumors has never been shown to be fully hypoxic. Actually, the sizes of HFs of Q cell populations in SCC VII squamous cell carcinomas with a diameter of 1 cm implanted in the hind legs of C3H/He mice were 55.1 ± 6.2 (mean ± SD) %. The size was significantly less than 100 %. This means that the Q cell population undoubtedly includes oxygenated tumor cells [3].
A few years ago, the detection of hypoxic cells in both tissues and cell cultures universally became possible using pimonidazole, substituted 2-nitroimidazole, and a mouse IgG1 monoclonal antibody (MAb1) to stable covalent adducts formed through reductive activation of pimonidazole in hypoxic cells. Further, the genetic and functional status of the *p53* gene is thought to be an important factor in guiding therapeutic strategies for cancer patients. In the present study, the radio-sensitivity of the pimonidazole non-labeled cell fraction of the Q cell populations within *p53*-wild and –mutated type tumors, following γ-ray irradiation at both a high dose rate (HDR) and a reduced dose rate (RDR), was determined.

MATERIALS AND METHODS: Human head and neck squamous cell carcinoma cells transfected with mutant *TP53* (SAS/mp53), or with neo vector as a control (SAS/neo), were inoculated subcutaneously into left hind legs of Balb/cA nude mice. The tumor-bearing mice received 5-bromo-2'-deoxyuridine (BrdU) continuously to label all intratumor P cells. Tumors were irradiated with γ-rays at a high dose-rate or a reduced dose-rate at 1 h after the administration of pimonidazole. The responses of Q and total cell populations were evaluated with the frequencies of micronucleation and apoptosis using immunofluorescence staining for BrdU. The response of pimonidazole unlabeled tumor cell fractions was assessed with apoptosis frequency using immunofluorescence staining for pimonidazole.

RESULTS: The pimonidazole-unlabeled tumor cell fraction showed significantly enhanced radio-sensitivity compared with the whole tumor cell fraction more remarkably in Q cells and *p53*-mutated tumors than total cells and *p53*-wild type tumors, respectively. However, a significantly greater decrease in radio-sensitivity in the pimonidazole-unlabeled than the whole cell fraction, evaluated using a delayed assay or a decrease in radiation dose rate, was more clearly observed in Q cells and *p53*-wild type tumors than total cells and *p53*-mutated type tumors, respectively. Concerning the whole tumor cell fraction, the Q cells showed significantly greater radio-resistance and recovery capacity from radiation-induced damage than the total cells both in *p53*-wild and *p53*-mutated type tumors.

DISSCUSSION: In recent years, the concept of cancer stem cells (CSCs) has ignited a great deal of interest because of the potential clinical implications associated with these cells. In part, these cells are thought to exist in a patho-physiological microenvironment where hypoxia, low pH, and nutrient deprivation occur. In addition, within the tumor microenvironment, significant heterogeneity, both spatial and temporal, also occurs. The tumor microenvironment has very similar conditions to the intratumor area where dividing tumor cells become quiescent. Further, a subset of CSCs is thought to be non-dividing quiescent cells. Thus, we tried to clarify the radio-biological characteristics of this sub-population of the intratumor Q cell population, hoping to contribute to CSC research.
One mechanism of CSC resistance to cytotoxic treatment was reported to result from an enhanced DNA repair capacity. Here, the pimonidazole unlabeled cell fraction among Q cells showed a much greater repair capacity than the whole Q cell population even if the repair capacity was significantly larger in the whole Q cell population than the total tumor cell population as a whole. In other words, from the viewpoint of not only quiescent status but also enhanced DNA repair capacity, the characteristics of the pimonidazole unlabeled cell fraction in the Q cell population was found to be very similar to those of CSCs.
Although there was similarity between the pimonidazole unlabeled Q cell fraction and the CSCs in terms of quiescent status and enhanced repair capacity, CSCs are thought to exist under rather hypoxic conditions. Therefore, we would like to further analyze the characteristics of the intratumor Q cell population in connection with those of CSCs, including the use of human tumor cell lines, in future [4,5].

REFERENCES:
[1] S. Masunaga *et al.*, J. Radiat. Res. **43** (2002) 11-25.
[2] N. Hamada *et al.*, J. Radiat. Res. **51** (2010) 365-383.
[3] S. Masunaga *et al.*, Int. J. Radiat. Oncol. Biol Phys. **49** (2001) 1361-1368.
[4] S. Masunaga *et al.* Radiat. Res. **174** (2010) 459-466.
[5] S. Masunaga *et al.* World J. Oncol. **2** (2011), 1-9.

採択課題番号 23P3-1　腫瘍内各特定細胞集団の制御及び転移抑制効果をも加味した プロジェクト BNCT を含む放射線治療の最適化

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PR3-2 Evaluating the Usefulness of a Novel Tumor Targeting ^{10}B-carrier, GPU-201 in Boron Neutron Capture Therapy

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INTRODUCTION: Boron-containing agents play a key role in successful boron neutron capture therapy (BNCT). We developed new ^{10}B carriers containing the Arg-Gly-Asp (RGD) motif which is the minimum recognition element for the $\alpha_v\beta_3$ integrin. Integrin $\alpha_v\beta_3$ is an attractive target for antitumor drug delivery because of its specific expression in proliferating endothelial and tumor cells of various origins. Therefore, icosahedral boron cluster-Arg-Gly-Asp (RGD) peptide conjugate, GPU-201(Fig. 1) was developed as a tumor-selective boron carrier.[1] We evaluated the usefulness of GPU-201 in BNCT.[2]

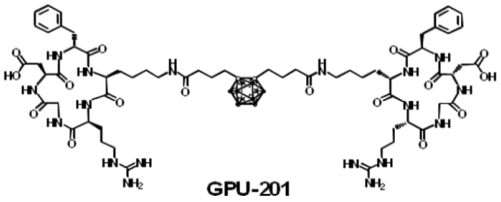

Figure 1

EXPERIMENTS: Chemistry: Synthesis of GPU-201: The cyclo(-Arg-Gly-Asp-D-Phe-Lys-) {*c*[RGDfK]} was synthesized by Fmoc solid-phase method and conjugated to ^{10}B cluster 1,2-dicarba-*closo*-dodecaborane-^{10}B through alkyl amide linker chain.

Enhancement of BNCT and γ-ray effect by GPU-201: Mercaptododecaborate-^{10}B (BSH) dissolved in physiological saline and BSH and GPU-201 dissolved with 10% HP-β-cyclodextrin (HP-β-CD) (pH 7) as a solubilizing and dispersing agent were intraperitoneally administered to SCC VII tumor-bearing mice. Then, the ^{10}B concentrations in the tumors and normal tissues were measured by γ-ray spectrometry. Subsequently, tumor-bearing mice were continuously given 5-bromo-2'-deoxyuridine (BrdU) to label all proliferating (P) cells in the tumors, then treated with GPU-201, BSH-CD, or BSH. Immediately after reactor neutron beam or γ-ray irradiation, during which intratumor ^{10}B concentrations were kept at levels similar to each other, cells from some tumors were isolated and incubated with a cytokinesis blocker. The responses of the Q and total (= P + Q) cell populations were assessed based on the frequency of micronuclei using immunofluorescence staining for BrdU.

RESULTS: In the evaluation of time course of the ^{10}B concentration in tumor and organs, the retention of ^{10}B from BSH-CD and GPU-201 was similar except in blood, where the concentration of ^{10}B from GPU-201 was higher for longer. We also demonstrated that the BSH formed an inclusion complex with β-CD by NMR study. The stoichiometry and association constant of the complex were estimated by using ^{1}H-NMR techniques. These analysis showed that the stoichiometric ratio was 1 : 1 and the K_a value was 229.8 M^{-1}. The complex of BSH with HP-β-CD is effective for sustainable localization in tumor. Based on the findings concerning these ^{10}B biodistribution patterns, irradiation was started from 60 min and 30 min after the intraperitoneal administration of BSH-CD or GPU-201 at a dose of 0.75 μmole・g^{-1} and BSH at a dose of 1.2 μmole・g^{-1}, respectively.

Under neutron beam irradiation, the sensitivity of tumor cells was increased with any ^{10}B-carrier, however GPU-201 and BSH- CD showed a greater rdiosensitizing effect than BSH. In contrast, no reliable radio- sensitizing effect was obtained with any ^{10}B-carrier under γ-ray irradiation. (Fig.1) There was no significant difference of tumor growth 14 days after

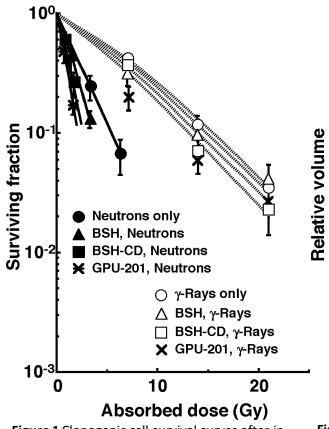

Figure 1 Clonogenic cell survival curves after *in vivo* irradiation

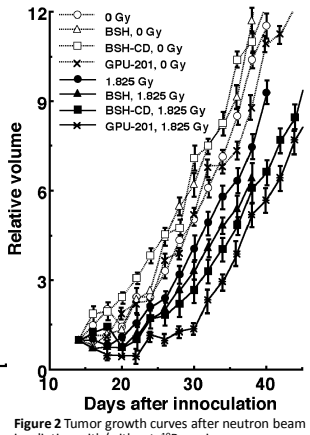

Figure 2 Tumor growth curves after neutron beam irradiation with/without ^{10}B-carriers.

the tumor cell inoculation without irradiation, with or without a ^{10}B-carrier. With irradiation at a physically absorbed dose of 1.825 Gy, the period required was significantly prolonged compared with no irradiation ($p < 0.05$), except for neutrons only without a ^{10}B-carrier, and the treatments ranked in the following order; without a ^{10}B-carrier < with BSH < with BSH-CD < GPU-201(Fig. 2).

Conclusion: A novel ^{10}B-carrier conjugated with an integrin-binding RGD peptide (GPU-201) that sensitized tumor cells more markedly than conventional ^{10}B-carriers may be a promising candidate for use in BNCT. However, its toxicity needs to be tested further.

REFERENCES:

[1]S. Kimura *et al.*, *Bioorg. Med. Chem.*, **19** (2011) 1721-1728.
[2]S. Masunaga *et al.*, *World J. Oncol.*, in press (2012)

採択課題番号 23P3-2　低酸素微小環境を標的とするボロンキャリアの開発　　プロジェクト
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PR3-3 A Biological Mechanism behind the Radioresistance of Hypoxic Tumor Cells

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INTRODUCTION: Because of the limited distance molecular oxygen can diffuse, most malignant tumors grow individually as a conglomerate of so-called 'micro tumor cords', in each of which a blood vessel is surrounded by well-oxygenated (normoxic), then oxygen-insufficient (chronic hypoxic), and finally oxygen-depleted (anoxic/necrotic) cancer cells [1-3]. Immunohistochemical analysis with antibodies against a well-known hypoxia marker, pimonidazole, and against a transcriptional factor HIF-1α recently revealed that HIF-1α-positive cells are not necessarily stained with pimonidazole. Namely, in some types of malignant tumors, chronic hypoxia can be histologically categorized into 2 sectors; HIF-1α$^+$/pimonidazole$^-$ (herein: HIF-1$^+$ regions) and pimonidazole$^+$/HIF-1α$^-$ (herein: pimonidazole$^+$ regions). Tumor hypoxia and HIF-1 have long been associated with tumor radioresistance and tumor recurrence after radiation therapy; however detailed molecular mechanisms behind them are largely unknown. To address these questions, we performed following two basic researches.

EXPERIMENTS:

1. A Molecular Mechanism behind Tumor Recurrence after Radiation Therapy: 【Purpose & Methods】 To directly examine the involvement of hypoxia in tumor recurrence, we developed a novel cell-tagging strategy mediated by a hypoxia-inducible *Cre-loxP* system. We tracked the post-irradiation fate of the cells which existed in perinecrotic regions at the time of radiation [4]. **【Results】** Although the perinecrotic/Pimonidazole$^+$ cells were originally HIF-1-negative, they acquired HIF-1 activity after surviving radiation, which triggered their translocation toward tumor blood vessels [4]. HIF-1 inhibitors suppressed the translocation and decreased the incidence of post-irradiation tumor recurrence [4]. **【Conclusions】** For the first time, our data unveil the HIF-1-dependent cellular dynamics during postirradiation tumor recurrence and provide a rational basis for targeting HIF-1 after radiation therapy [4].

2. A Biological Mechanism behind Radioresistance of Perinecrotic Hypoxic Tumor Cells: 【Purpose & Methods】 Accumulated evidence has suggested that some intracellular and extracellular factors, such as oxygen-availability, transcriptional activity of HIF-1 and cell cycle status, have been suggested to affect radioresistance of cancer cells. We next analyzed mechanistic and spatio-temporal relationships among them in highly heterogeneous tumor microenvironments in order to elucidate a mechanism behind the predominant survival of perinecrotic tumor cells after radiation. **【Results】** Experiments *in vitro* demonstrated that a decrease in the glucose concentration reduced the transcriptional activity of HIF-1 and expression of a downstream gene for the cell cycle regulator $p27^{Kip1}$ even under hypoxic conditions. Then, the proportion of cells in radioresistant S-phase increased, whereas those in radiosensitive G_1-phase decreased, significantly. Immunohistochemical analyses showed that cancer cells in perinecrotic hypoxic regions, which should be under low glucose conditions, expressed little HIF-1α; and therefore, were mainly in S-phase and less damaged by radiation treatment. Continuous administration of glucagon, which increased blood glucose concentration and resultantly improved glucose-availability in perinecrotic hypoxic regions, induced HIF-1α expression and increased radiation-induced DNA damage. **【Conclusions】** Cancer cells in perinecrotic regions, which would be under low glucose and hypoxic conditions, acquire radioresistance by decreasing the level of both HIF-1 activity and $p27^{Kip1}$ expression and adjusting their cell cycle to the radioresistant S-phase [5].

REFERENCES:

[1] *Harada H. Gene Therapy Strategy for Tumor Hypoxia. *Targets in Gene Therapy*. **11**: 185-200. 2011.
[2] Yeom CJ, Zeng L, Zhu Y, Hiraoka M, *Harada H. Strategies to assess hypoxic/HIF-1-active cancer cells for the development of innovative radiation therapy. *Cancers*. **3**: 3610-3631. 2011.
[3] *Harada H. How can we overcome tumor hypoxia in radiation therapy? *J Radiat Res*. **52**: 545-556. 2011.
[4] *Harada H, Inoue M, Itasaka S, Hirota K, Morinibu A, Shinomiya K, Zeng L, Ou G, Zhu Y, Yoshimura M, McKenna WG, Muschel RJ, Hiraoka M. Cancer cells that survive radiation therapy acquire HIF-1 activity and translocate toward tumor blood vessels. *Nat Commun*. in press.
[5] Zhu Y, Zhao T, Itasaka S, Zeng L, Yeom CJ, Hirota K, Suzuki K, Morinibu A, Shinomiya K, Ou G, Toshimura M, Hiraoka M, *Harada H. Involvement of Decreased Hypoxia-Inducible Factor 1 Activity and Resultant G_1-S Cell Cycle Transition in Radioresistance of Perinecrotic Tumor Cells. *Oncogene*. in pres

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採択課題番号 23P3-3　　腫瘍組織レベルの光イメージングで迫る　　プロジェクト

癌の放射線抵抗性機構の解明

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PR3-4 An Influence of Boron Neutron Capture Reaction on HSG Cell Inactivation

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INTRODUCTION: Excellent dose distribution of neutron capture reaction of boron atom induces high relative biological effectiveness (RBE) and the low oxygen enhancement ratio (OER). These phenomena are commonly assumed to be an interaction between cells and low energy heavy particles (α and Li) resulting from the boron atom fissions 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 [1-3].

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 disclose an influence of boron neutron capture reaction on HSG cell inactivation.

EXPERIMENTS: Human salivary gland tumor (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 HSG cells were incubated with 25 µg/ml ^{10}B-para-boronophenylalanine (BPA) for 3 h prior to irradiations. The cells were suspended at a density of about 7×10^5 cells/ml. The cells in Polypropylene tubes (NUNC) 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 [4]. The total absorbed dose resulting from thermal neutron irradiation was calculated by the sum of the absorbed doses mainly from the ^{1}H(n,g)^{2}D, ^{14}N(n,r)^{14}C and ^{10}B(n,a)^{7}Li reactions according to Kobayashi's model [5].

After irradiation, cells were seeded in triplicate onto 60 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 survival curves were fitted by the single-hit model: $SF = \exp(-\alpha \cdot D)$. SF and D are the surviving fraction and the dose, respectively.

RESULTS: The survival curves for neutrons is shown in Fig. 1. The D_{10} value for with and without BPA were 1.62 and 2.79, respectively. The D_{10} value of X-rays was 4.70 Gy. The RBE values for with and without BPA were 1.68 and 2.90, respectively. The survival curves of the coefficient of determination (R^2) were above 0.9. We will retest and add the dose points. These results suggest that he large RBE was induced by low energy heavy particles (α and Li) from boron neutron capture reaction.

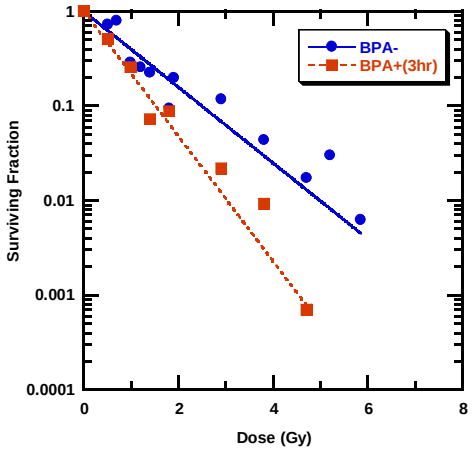

Fig. 1. Survival curves for HSG cells exposed to neutrons in the absence (circle) and presence (square) of 25 µg/ml BPA. The survival parameters were calculated from the data by a curve fitting using: $SF = \exp(-\alpha \cdot D)$.

REFERENCES:

[1] A. Ito *et al.*, Radiat. Res., **165** (2006) 703-712.
[2] R. Hirayama *et al.*, Radiat. Res., **171** (2009) 212-218.
[3] R. Hirayama *et al.*, Radiat. Phys. Chem., **78** (2009) 1175-1178.
[4] H. Fukuda *et al.*, Int. J. Radiat. Biol., **51** (1987) 167-175.
[5] T. Kobayashi *et al.*, Radiat. Res., **91** (1982) 77-94.

採択課題番号 23P3-4　中性子捕捉反応における細胞致死機構の放射線作用解析　プロジェクト
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PR3-5 **Boron-conjugated hyaluronan nanoparticles for Tumor Targeting in Boron Neutron Capture Therapy**

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INTRODUCTION: Hyaluronan is a candidate for active targeting ligand to melanoma, many of which overexpress the hyaluronan receptors CD44 and RHAMM [1]. In this study, we have developed a novel boron delivery system for BNCT using hyaluronan conjugated with borocaptate (BSH). This BSH-bearing hyaluronan nanoparticles (B-HA-NPs) are expected to deliver boron atoms into melanoma cells by receptor-mediated endocytosis for antitumor application in BNCT. Transdermal drug delivery system has been accepted as potential non-invasive route of drug administration, with advantages of sustained therapeutic action and better patient compliance, though, its prevalent use is restricted due to excellent impervious nature of skin. Thus, many approaches have been attempted to perturb skin barrier and enhance the transdermal delivery of drug [2]. The major approaches for enhancing transdermal delivery are physical enhancers (ultrasound, iontophoresis and electroporation), vesicles, particulate systems (liposome, niosome, transfersome, microemulsion, solid lipid nanoparticle) and chemical enhancers. Electroporation (EP) is a physical method that enhances delivery of molecules to tissues in vivo. In this study, a unique nanoparticle-EP-mediated approach has been developed for dermal and intracellular delivery of ^{10}B into melanocytic tumors present in skin to retard melanoma.

EXPERIMENTS: BSH was dissolved in acetone and added to N-Succinimidyl 6-Maleimidobenzoate with triethylamine catalysis. The mixture was incubated at room temperature for 2 h under argon. Sodium hyaluronate was dissolved in deionized water. To this solution, the highly acidic ion exchange resin, Dowex-100, was added, and the slurry was stirred for eight hours. The acidic solution was neutralized with 0.2M tetrabutylammonium hydroxide, forming a quaternary ammonium salt of hyaluronate and the tetrabutylammonium group (HA-TBA). The solution was frozen and lyophilized to yield the dry product. The HA-TBA was dissolved in DMSO and added to BSH derivative was of conjugation with diethylamine catalysis. The mixture was incubated at room temperature for 48 h under argon. The solution was dialyzed and lyophilized to yield the dry product.

B16F10 murine melanoma cells were pre-incubated with 25 ppm of B-HA-NPs, B-Folate-conjugates and BSH solution for 6 hours at 37℃ before neutron irradiation. The cells were rinsed twice in PBS and suspended in fresh medium. After 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: Novel BSH-bearing hyaluronan nanoparticles were obtained by the self-assembly in water. The critical micellar concentration of B-HA-NPs (diameter: 90-180 nm) was in the range of 300-800 mg/L determined by the fluorescence probe technique using pyrene. B-HA-NPs had high stability (90-97%) in the retention of ^{10}B during storage at 4-37 ℃. All boborocaptate-loaded formulations had no cytotoxic effects. B-HA-NPs were readily bound to melanoma cells, and were internalized by receptor-mediated endocytosis. As shown in Fig. 1, survival of the washed cells pre-incubated with B-HA-NPs was lowest compared to survival of control cells that were pre-incubated with B-PEG-liposomes, B-HA-liposomes, B-FA-conjugates and BSH solution. This result suggested cytotoxicity depended on internalization of ^{10}B.

We are now ongoing to investigate the therapeutic potential of EP-mediated approach for transcutaneous BSH delivery in a three-dimensional skin reconstruction model of melanoma.

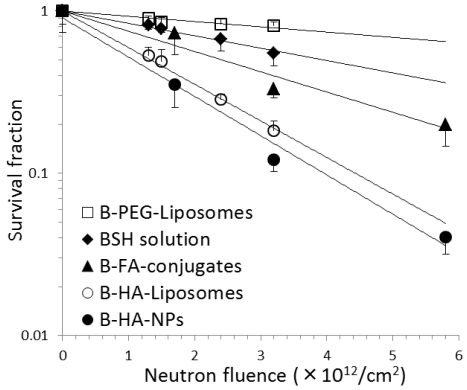

Fig. 1. Suppression of the colony formation of B16F10 cells after in vitro BNCT. Values are means±S.D.

REFERENCES:

[1] D. Peer and R. Magalit, Neoplasia, **6** (2004)343-353
[2] M. Nino et al., Dermatol. Online J. **16**(2010)

採択課題番号 23P3-5 腫瘍内微小環境の解析とその利用による悪性腫瘍制御の試み プロジェクト
（広国大・薬）笠岡　敏、橋本佳奈（京大・原子炉）増永慎一郎

PR3-6 Kojic Acid-Appended Carorane/Hydroxypropyl-β-Cyclodextrin Complex: A Novel Boron Carrier for Melanoma Targeting

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INTRODUCTION: Metastatic melanoma remains s highly lethal cancer. Boron neutron capture therapy (BNCT) has been attracted great deal of attention as a potentially useful modality for this disease [1]. The delivery of ^{10}B compounds deeply inside the tumor cells and, possibly, close the nucleus is also an important requirement in order to improve the efficacy of BNCT. Kojic acid is well known to work as an excellent whitening agent for melanocytes by a strong tyrosinase inhibition [2]. This fact suggests that kojic acid possess a specific affinity for melanocytes. In this paper, we evaluate kojic acid-appended o-carborane (CKA) as BNCT boron agent for melanoma.

EXPERIMENTS: Since CKA has some disadvantages water-insoluble compound, water-soluble complex of CKA with hydroxypropyl-β-cyclodextrin (CKA/HPβ-CD, Fig. 1) was prepared. B16BL6 murine melanoma cells and colon 26 murine colon carcinoma cells were grown in RPMI and DMM mediums, respectively, containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin at 37°C with CO_2 atmosphere. In order to evaluate the influence of kojic acid (KA) on internalization of CKA complex, cells were treated in the presence of 10 mM kojic acid. Boron concentration was measured by ICP-AES (Vista-MPX, Seiko instruments Inc.). Intracellular Trafficking of CKA/HPβ-CD was estimated by immuno-fluorescent method with anti-BSH antibody and Zenon® immunolabeling technology (Invitrogen).

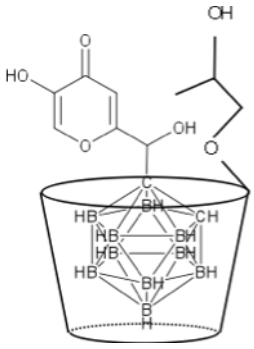

Fig. 1. Water-soluble CKA/HPβ-CD complex.

RESULTS AND DISCUSSION: Inclusion rate of obtained water-soluble complex was calculated as 88% by ^{1}H-NMR and UV spectroscopy. Evaluation of *in vitro* uptake of boron containing CKA/HPβ-CD complex was carried out with B16BL6 and colon 26 cells (Fig. 2A). The 50 times higher uptake of boron by B16BL6 cells was observed than that of colon 26 cells. Inhibition of boron uptake by free kojic acid was confirmed with B16BL6 cells (Fig. 2B). The boron uptake was suppressed one-fourth in the presence of excess kojic acid. It seems that CKA has melanoma affinity based on possessing of kojic acid moiety although detail mechanism is unclear yet. In B16BL6 cells, CKA was interestingly localized in the nucleus. Morrison *et al*. reported that nuclear localization of boron drug increased tumor-killing effect by BNCT [3]. Kojic acid-appended carborane (CKA) is promising to be an efficient boron agent toward melanoma BNCT.

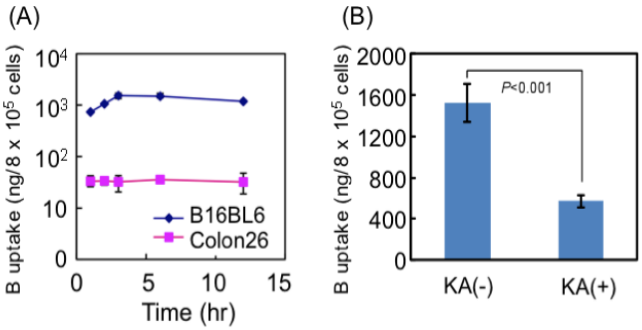

Fig. 2. *In vitro* cellular uptake of CKA by melanoma and colon carcinoma cell lines (A). Inhibition experiment of CKA uptake by kojic acid (B).

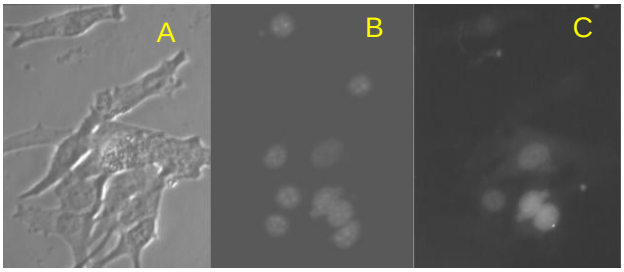

Fig. 3. Intracellular distribution of CKA (transmission, [A]; nucleus stained with DAPI [B]; CKA [C]).

REFERENCES:

[1] H. Fukuda *et al.*, Radiat. Res., **138** (1994) 435-442.
[2] Y. Mishima, Pigment Cell Res., **14** (2001) 47-70.
[3] G.H. Morrison *et al.*, Biol. Cell, **74** (1992) 105-108.

採択課題番号 23P3-6　メラノーマ中性子捕捉療法への適応を目指した薬剤送達システムに関する研究　　プロジェクト

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