Project Research

Project 9

PR9 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 experimental animal tumor cell lines. 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. 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. 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 recovery 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, especially into intratumor hypoxic cells with low uptake capacities.

Hypoxia is suggested to enhance metastasis by increasing 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, nicotinamide, into tumor-bearing mice as a combined treatment with γ-ray irradiation in terms of repressing lung metastasis. 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 hypoxia. 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 irradiation 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 administration of nicotinamide or bevacizumab may be promising for reducing numbers of lung metastases. Consequently, BPA-BNCT in combination with nicotinamide and/or bevacizumab treatment may show a little more potential to reduce the number of metastases. Now, it has been elucidated 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 (27P9-1): 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 (27P9-2): Development of Hypoxic Microenvironment-Oriented ^{10}B-Carriers. (H. Nagasawa, *et al.*)

ARS-3 (27P9-3): Clarification of Mechanism of Radio-Resistance in Cancer Using Optical Imaging at Tissue Level. (H. Harada, *et al.*)

ARS-4 (27P9-4)*: Analysis of Radiation-Induced Cell-Killing Effect in Neutron Capture Reaction. (R. Hirayama, *et al.*)

ARS-5 (27P9-5)*: Transdermal Drug Delivery System using Hyaluronan-Conjugated Liposomes as ^{10}B-Carrier in Boron Neutron Capture Therapy for Melanoma (S. Kasaoka, *et al.*)

ARS-6 (27P9-6) *: Evaluation of Inclusion Complex of Carborane Modified Kojic Acid and Cyclodextrin as ^{10}B-Carrier in Boron Neutron Capture Therapy. (T. Nagasaki, *et al.*)

ARS-7 (27P9-7)*: Molecular Design and Synthesis and Functional Evaluation of Anticancer and Molecular Targeting Agents. (Y. Uto, *et al.*)

ARS-8 (27P9-8)*: Analyzing Biological Effect of BNCT from the Viewpoint of the Changes in Oxygenation Level. (H. Yasui, *et al.*)

ARS-9 (27P9-9): Analyses on the Responsiveness of Malignant Tumors to BNCT. (M. Masutani, *et al.*)

ARS-10 (27P9-10) *: Assay for Tumor Cell Survival and Tumor Growth Delay through Neutron Capture Reaction according to the Changes in Intracellular Concentrations within Solid Tumors of Newly- Developed ^{10}B-Carriers. (K. Nakai, *et al.*)

ARS-11 (27P9-11)*: Antitumor and Metastasis-Reppresing Effect of BNCT on Human Breast and Pancreatic Cancer Cell Lines. (Y. Matsumoto, *et al.*)

(***** Due to the absence of operating our reactor in 2015, no data were obtained, resulting in no reporting here.)

PR9-1 The Effect of *p53 Status* of Tumor Cells on Radiosensitivity of Irradiated Solid Tumors with Accelerated Carbon-ions Compared with γ-rays or Reactor Neutrons

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INTRODUCTION: We examined the characteristics of radio-sensitivity in the total (proliferating (P) plus quiescent) and quiescent (Q) cell populations in solid tumors irradiated with 290 MeV/u accelerated carbon ion beams at varying LET values in a 6-cm spread-out Bragg peak (SOBP) installed at the National Institute of Radiological Sciences (Chiba, Japan) compared with irradiation with ^{60}C γ-rays and reactor thermal and epithermal neutron beams at our institute with our method for selectively detecting the response of Q cells within solid tumors [1,2], using two different tumor cell lines with identical genetic backgrounds except for *p53* status.

MATERIALS AND METHODS: Human head and neck squamous cell carcinoma cells transfected with mutant *TP53* (SAS/*mp53*) or with neo vector (SAS/*neo*) were injected subcutaneously into hind legs of nude mice. Tumor-bearing mice received 5-bromo-2'-deoxyuridine (BrdU) continuously to label all intratumor P cells. They received γ-rays or accelerated carbon-ion beams at a high or reduced dose–rate. Other tumor-bearing mice received reactor thermal or epithermal neutrons at a reduced dose-rate. Immediately or 9 hours after the high dose-rate irradiation (HDRI), or immediately after the reduced dose-rate irradiation (RDRI), the tumor cells were isolated and incubated with a cytokinesis blocker, and the micronucleus (MN) frequency in cells without BrdU labeling (= Q cells) was determined using immunofluorescence staining for BrdU.

RESULTS: The difference in radio-sensitivity between the total (= P + Q) and Q cells after γ-ray irradiation was markedly reduced with reactor neutron beams or carbon ion beams, especially with a higher linear energy transfer (LET) value. Following γ-ray irradiation, SAS/*neo* tumor cells, especially intratumor Q cells, showed a marked reduction in sensitivity due to the recovery from radiation-induced damage, compared with the total or Q cells within SAS/*mp53* tumors that showed little repair capacity [3]. In both total and Q cells within both SAS/*neo* and SAS/*mp53* tumors, carbon-ion beam irradiation, especially with a higher LET, showed little recovery capacity through leaving an interval between HDRI and the assay or decreasing the dose-rate. The recovery from radiation-induced damage after γ-ray irradiation was a *p53*-dependent event, but little recovery was found after carbon-ion beam irradiation. With RDRI, the radiosensitivity to reactor thermal and epithermal neutron beams was slightly higher than that to carbon ion beams.

DISCUSSION: Two major pathways for the repair of potentially lethal DNA dsbs exist in mammalian cells. The non-homologous end-joining (NHEJ) pathway is imprecise, error-prone, and mutagenic, and mutant cell lines lacking key components of this pathway all exhibit impaired kinetics of DNA dsb repair and exquisite radio-sensitivity. Homologous recombination (HR) is a more precise (error-free) repair mechanism and is more important for the repair of dsbs in late-S and G2 when a sister chromatid is available for the recombination reaction. Cell lines with defects in HR also exhibit increased radio-sensitivity and decreased fidelity of repair.

A cellular safeguard against genetic destabilization is activation of the *p53* tumor suppressor protein, which commonly responds to DNA damage signals by inducing apoptosis or regulating the cell cycle including DNA damage repair. As also shown in our previous report, the net MN frequencies in SAS/*neo* tumor cells were lower than those in SAS/*mp53* tumor cells under all conditions ($P < 0.05$), probably resulting from exclusion of a higher number of radiation-induced apoptotic SAS/*neo* cells than SAS/*mp53* cells.

Loss-of-function of *wild-type TP53* can result in loss of the G1/S cell-cycle checkpoint and an increase in HR. As *p53* seems to interact with RAD51, the absence of normal *p53* function is thought to enhance RAD51-mediated DNA pairing activity and HR, due to overexpression of RAD51 out of regulation by normal *p53*. Thus, HR is thought to be a principal mechanism of DNA dsb repair in SAS/*mp53* cells. The very small repair capacity of SAS/*mp53* cells *in vivo* may show that the repair in solid tumors with a mutant *p53* is mainly due to, if anything, the NHEJ rather than HR.

CONCLUSION: For tumor control, including intratumor Q-cell control, accelerated carbon-ion beams, especially with a higher LET, and reactor thermal and epithermal neutron beams were very useful for suppressing the recovery from radiation-induced damage irrespective of *p53* status of tumor cells [4].

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PR9-2 Development of New Membrane Penetrating Boron Carriers Comprised of BSH and Pepducins

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INTRODUCTION: Selective delivery of sufficient quantity of 10B to tumor cells is essential for the success of boron neutron capture therapy (BNCT). Although sodium borocaptate (BSH) has been used clinically as a boron carrier for BNCT, it is impermeable to plasma membrane due to its high hydrophilicity and anionic charges. Recently, we found that pepducin, which are artificial lipopeptides derived from an inner loop domain of G protein-coupled receptors (GPCRs), [1] enabled anionic molecule such as fluorescein to penetrate membrane directly.[2] These findings let us to envisage that an anionic boron cluster can be delivered into cytosol using the pepducin delivery unit.

In this study, we designed and synthesized novel pepducin-boron cluster hybrid compounds as boron carriers for BNCT and evaluated the intracellular delivery of them.

MOLECULAR DESIGN: As shown in Fig. 1, we designed new hybrid molecules comprising pepducin (Pep) as a vehicle, and boron cluster (BS) as a cargo through an appropriate linker. When this molecule is internalized into the cytosol, intracellular glutathione can cleave the disulfide bond (SS) to release the boron cluster cargo into the cytosol. 10B atoms are accumulated in the cells due to hydrophilicity of the anionic property.

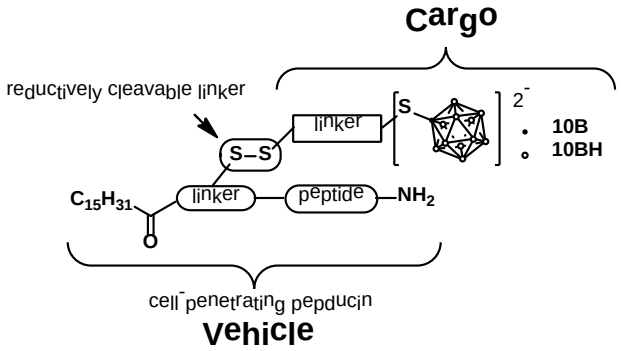
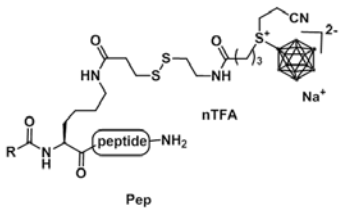


Fig. 1 Design of membrane penetrating boron carriers

METHODS: To explore the structural requirements of membrane penetrating boron carrier for intracellular uptake, we evaluated the sequence and length of peptide and structure of lipid moiety. Various lipidated peptides were prepared by solid-phase synthesis and then combined with BSH through an appropriate liker to afford the boron carrier. T98G cells were treated with the boron carriers (10 μM) at 37 °C for various time, then, washed with PBS three times, and dissolved in 200 μL HNO_3 for 1 h. The boron concentrations of the extracts were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES: HORIBA JOVIN YBON ULTIMA2) detected at 249.773 nm.

RESULTS AND DISCUSSION: Various lipopeptide -BSH hybrids were synthesized (Table 1). Compound 13Pep showed the highest boron concentration in cells

Table 1 Structures of boron carriers



Pep	R	sequence
7Pep	$C_{15}H_{31}$	KKSRALF
13Pep	$C_{15}H_{31}$	AVANRSKKSRALF
Ac7pep	CH_3	KKSRALF
Ac13pep	CH_3	AVANRSKKSRALF
10Pep	$C_{15}H_{31}$	AVANRSKKSR

among the test compounds examined by ICP-AES. The boron concentration increased over time from 2h to 12 h. (Fig. 2). These data suggested that the palmitoyl tail and

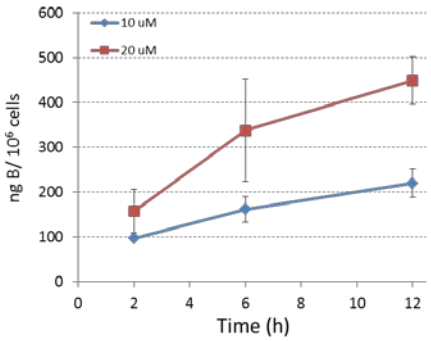


Fig. 2 Time course of the intracellular uptake of 10B by the use of boron carrier 13Pep

hydrophobic residues at C-terminal end are necessary for the intracellular penetration.

We are performing the further optimization of peptides and linker structures to promote the intracellular accumulation.

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PR9-3 LY6E; a Conductor of Malignant Tumor Growth through Modulation of the PTEN/PI3K/Akt/HIF-1 Axis

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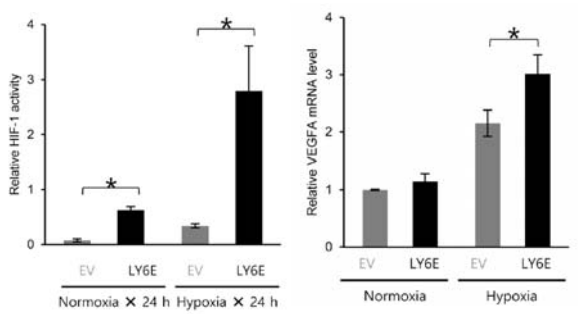
INTRODUCTION: Accumulating evidence has shown that hypoxia-inducible factor 1 (HIF-1) plays critical roles in radioresistance of cancer cells and tumor recurrence after radiation therapy and eventually causes death among cancer patients. Clinical studies have demonstrated consistent data that HIF-1 could be used as an adverse prognostic factor for not only local tumor recurrence but also distant tumor metastasis in cancer patients. These findings justify targeting HIF-1 for cancer therapies [1,2].

HIF-1, a heterodimeric transcription factor com-posed of an α-subunit (HIF-1α) and a β-subunit (HIF-1α), its activity is known to depend on the expression levels of HIF-1α protein. Under normoxic conditions, HIF-1α protein is actively degraded through the hydroxylation- and subsequent ubiquitination-mediated proteolysis reactions. On the contrary, HIF-1α becomes active under hypoxic conditions because of the inactivation of the hydroxylases, and then, interacts with its binding partner, HIF-1β. Resultant heterodimer, HIF-1, binds to its cognate enhancer sequence, the hypoxia-responsive element (HRE), and induces transcriptions of various genes related to the escape from hypoxia (invasion and metastasis of cancer cells) as well as the improvement of oxy-gen-availability (angiogenesis) and adaptation of cellular metabolism to hypoxia (metabolic reprogramming).

In order to explore novel genes which are responsible for the HIF-1-mediated tumor malignant progression, we recently established a new genetic screening method and found that overexpression of lymphocyte antigen 6 complex, locus E (LY6E) is responsible for the activation of HIF-1. In the present study, we analyzed both the molecular mechanisms underlying the LY6E-mediated activation of HIF-1 and the involvement of LY6E-HIF-1 axis in malignant progression of cancers.

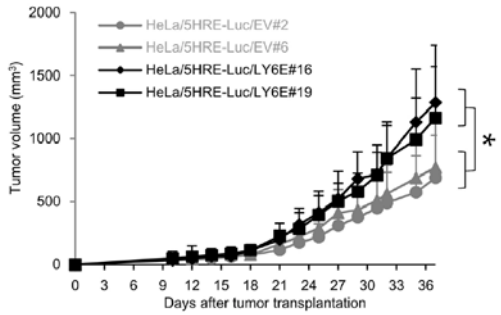
Fig. 1



EXPERIMENTS & RESULTS: Forced expression of LY6E using a plasmid-based expression vector for LY6E increased HIF-1α gene expression principally at the transcription level (Fig. 1).

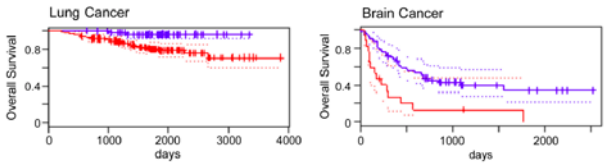
This, in turn, led to the expression of the pro-angiogenic factors, VEGFA and PDGFB, through decreases in the expression levels of PTEN mRNA and subsequent activation of the PI3K/Akt pathway. The LY6E-HIF-1 axis functioned to increase tumor blood vessel density and promoted tumor growth in immune-deficient mice (Fig. 2).

Fig. 2



LY6E expression levels were significantly higher in human breast cancers than in normal breast tissues, and were strongly associated with the poor prognoses of various cancer patients (Fig. 3). Moreover we found that LY6E induced radioresistance of cancer cells via the activation of HIF-1.

Fig. 3



Our results characterized LY6E as a novel conductor of tumor growth and tumor radioresistance through its modulation of the PTEN/PI3K/Akt/HIF-1 axis and demonstrated the validity of targeting this pathway for cancer therapy.

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PR7-2 Response of Squamous Cell Carcinoma SAS Cells after BNCR

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INTRODUCTION: Boron neutron capture reaction (BNCR) efficiently introduces DNA double strand breaks in the cells [2], however, tumor cell killing is affected by various factors [3] including the uptake of boron compounds. Heterogeneiety of cancer cells in the tumor tissues, such as the presence of cancer stem-like cells and hypoxic cells, may potentially cause resistant populations to the boron neutron capture therapy (BNCT) [1]. It is therefore important to analyze the responses of the cells to boron neutron capture reaction (BNCR) in various conditions. We have performed comprehensive analysis of mRNA expressions and proteome of human squamous carcinoma SAS cells after BNCR [4]. Changes in the protein levels involved in the various functions, such as endoplasmic reticulum, DNA repair, and RNA processing were observed within 24 hrs after neutron-beam irradiation. We also showed that BNCR induced generation of fragments from endoplasmic reticulum-localized lymphoid-restricted protein (LRMP). The elucidation of biological significance of fragmentation of LRMP is further necessary.

EXPERIMENTS: Because the neutron-beam irradiation with KUR nuclear reactor was not carried out during FY2015, we analyzed the irradiated cell samples prepared before. We used the cell extract of human oral squamous cancer SAS cells [1] to perform the mRNA expression and proteome analysis. Previously, ^{10}B-boronophenylalanine (^{10}B-BPA)-fructose solution was prepared as described [5]. SAS cells were suspended in the polypropyrene vials and incubated 2 hrs with or without 25 ppm of ^{10}B-BPA [5]. After 6 and 24 hrs irradiation operated at 1 MW in the KUR facility, cells were separated to supernatant and cell pellet. Proteome analysis was performed using two dimensional polyacrylamide gel electrophoresis and mass spectrometry. Thermal neutron fluence and gamma-ray dose was measured with thermaluminescence dosimeter. These physical radiation doses were measured with the kind help of Drs. Yoshinori Sakurai and Hiroki Tanaka of KUR. ^{10}B concentration was measured by prompt-gamma ray analysis (PGA) as described elsewhere.

RESULTS: For the analysis of RNA and protein dynamics, we used therapeutic dose conditions. The irradiated total dose of BNCR-treated SAS cells was about 17.5 Gy and that of neutron beam-irradiated SAS cells was about 4.0 Gy. Cell lysates of SAS cells with BPA (BNCR-sample) or without BPA (control) harvested 6 and 24 hrs post-irradiation were analyzed. Twenty-four hours after irradiation, apoptosis was observed as a major cell death and cleavage of caspase-9, caspase-3 and PARP-1 was observed. The sign of necrosis was also observed at this time point. The expression analysis of mRNA demonstrated dynamic changes of various genes related to inflammation and immune responses, and transcription. In the proteomic analysis [4], the peptide sequences from twenty-two spots have been determined by MALDI-TOF/MS from the twenty-nine spots that showed changes in the intensities between BNCR-sample and control.

We observed that proteins involved in the vesicle regulation, mRNA processing, transcription, rRNA processing, GTPase activity, ribosome biogenesis, DNA replication, and respiratory electron transport chain showed dynamic changes. Notably, lymphoid-restricted membrane protein (LRMP) and steroid hormone receptor (ERR1) were detected in multiple spots of different molecular mass. LRMP/ Jaw1 is known to be present at the cytoplasmic face of the ER, but its function has not been well understood [6]. We observed fragmentation of LRMP in the grafted lymphosarcoma of the rat 20 hrs after BNCR [4].

We further analyzed the LRMP in BNCR-sample and control by western blotting. Truncated LRMP of around 22 kDa was induced 24 hrs after BNCR-irradiation. The study for the biological significance of truncated LRMP in BNCR-induced cell death is ongoing.

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