VIII-II-1. Project Research

Project 10

PR10 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 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, boronophentlalanine-^{10}B (BPA) increased the sensitivity of the total cells to a greater extent than sodium borocaptate-^{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 two 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 injection > 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 analyzing the characteristics of intratumor microenvironment including hypoxia within malignant solid tumors and optimizing cancer therapy, 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) are as follows;

ARS-1 (21P10-1): 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. Matsumoto, Y. Sakurai, H. Tanaka, G. Kashino, Y. Liu, M. Takagaki and K. Nagata)

ARS-2 (21P10-2): Development of Hypoxic Microenvironment-Oriented Boron-10 Carriers
(H. Nagasawa, S. Masunaga, K. Okuda, S. Ueda and S. Kimura)

ARS-3 (21P10-3): Clarification of Mechanism of Radio-Resistance in Cancer Using Optical Imaging at Tissue Level
(H. Harada, M. Hiraoka, S. Masunaga, K. Shibuya and S. Itasaka)

ARS-4 (21P10-4): Analysis of Radiation-Induced Cell-Killing Effect in Neutron Capture Reaction
(R. Hirayama, S. Masunaga, G. Kashino, Y. Matsumoto, A. Uzawa, Y. Sakurai and H. Tanaka)
Unfortunately, they cannot report the results this fiscal year due to the absence of our reactor operation.

(Underline: Representative at each research group)

REFERENCES:

[1] A. J. Kogel. ed, Semin. Rdiat. Oncol., **14** (2004).
[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.*, Int. J. Hyperthermia **22(4)** (2006) 287-299.

採択課題番号 21P10　　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

PR10-1 Manipulating Hypoxia in Solid Tumors Has the Potential to Influence Lung Metastases *in vivo*

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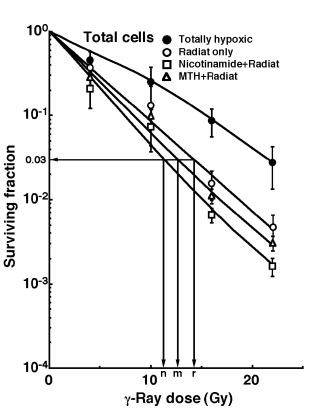
BACKGROUNDS AND PURPOSES: Many cells in solid tumors are quiescent *in situ* but are still clonogenic [1]. Quiescent (Q) tumor cells are more resistant to low-LET radiation because of their larger hypoxic fraction and greater capacity to recover from radiation-induced DNA damage than proliferating (P) tumor cells. Our original method for selectively detecting the response of intratumor Q cells [1] has made it possible to evaluate the usefulness of various modalities for cancer therapy in terms of effectiveness against intratumor Q cell populations. Based on the characteristics of the response of intratumor Q cells to various DNA-damaging treatments, more effective and useful treatment modalities for local tumor control can be developed.

Meanwhile, metastasis is a leading cause of cancer deaths and involves a complex, multi-step progress by which tumor cells disseminate to distant sites to establish discontinuous secondary colonies. Recently, it was reported that acute and cyclic but not chronic hypoxia significantly increased the number of spontaneous lung metastases in mice by a factor of about 2, and that this effect was due to the effect of the acute hypoxia treatment on the primary tumor and was not due to other potential effects of the treatment such as damage to the ling epithelium [2]. Based on this report, in the current *in vivo* study, the significance of the loading of an acute-hypoxia releasing agent, nicotinamide, into tumor-bearing mice as a combined treatment with gamma-ray irradiation was examined in terms of lung metastases compared with mild temperature hyperthermia (MTH), which also supposedly has the potential to manipulate intratumor hypoxia [1]. In addition, concerning a local tumor response to gamma-ray irradiation with or without nicotinamide or MTH, the effect not only on the total (= P + Q) tumor cell population but also on the Q cell population was also evaluated using our original method for detecting the response of intratumor Q cells [1].

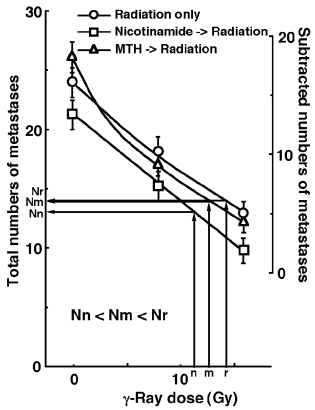
MATERIALS AND METHODS: B16-BL6 melanoma tumor-bearing C57BL/6 mice were continuously given 5-bromo-2'-deoxyuridine (BrdU) to label all P cells. They received gamma-ray irradiation following loading with the acute hypoxia-releasing agent nicotinamide or local hyperthermia at mild temperatures (MTH). Immediately after the irradiation, cells from some tumors were isolated and incubated with a cytokinesis blocker. The sensitivity of quiescent (Q) cells was assessed in terms of the micronucleus frequency using immunofluorescence staining for BrdU. That of the total (= P + Q) tumor cell population was determined from BrdU non-treated tumors. In other tumor-bearing mice, 17 days after irradiation, macroscopic lung metastases were enumerated.

RESULTS: In the total cells, a more marked enhancement in sensitivity was observed with nicotinamide than MTH. In Q cells, MTH combination induced a more marked enhancement than nicotinamide. Both nicotinamide and MTH reduced the size of the hypoxic fraction in the two cell populations, especially nicotinamide in the total cells and MTH in Q cells. Without gamma-ray irradiation, nicotinamide loading tended to decrease the number of lung metastases. With gamma-ray irradiation, nicotinamide loading and MTH, especially the former, reduced the number of metastases more than gamma-ray irradiation only (Figs. 1 and 2).

CONCLUSION: Hypoxia manipulation in solid tumors has the potential to influence lung metastases. Notably, acute hypoxia-releasing nicotinamide may be promising for reducing the number of lung metastases [3].



(Fig. 1) Initial response of local tumors



(Fig. 2) Numbers of metastases

REFERENCES:

[1] S. Masunaga *et al.*, J. Radiat. Res., **43** (2002) 11-25.
[2] B. A. Cairns *et al.*, Cancer. Res., **61**(2001) 8903-8908.
[3] S. Masunaga *et al.*, Clin. Exp. Metastasis, **26** (2009) 693-700.

プロジェクト採択 21P10-1　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 BNCT を含む放射線治療の最適化　プロジェクト

（京大・原子炉）増永慎一郎、櫻井良憲、田中裕基、菓子野元郎、劉　勇
（放医研）松本孔貴、（藍野学院短期大）高垣政雄、（石切生喜病院放）永田憲司

PR10-2 Development of New Tumor Targeting ^{10}B-carrier: Boron Cluster-Cyclic RGD Pentapeptide Conjugates for Boron Neutron Capture Therapy

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INTRODUCTION: A neutron capture reaction in boron [^{10}B(n, α) ^{7}Li] is, in principle, very effective in destroying tumors selectively, providing that sufficient amount of ^{10}B can be accumulated in the target tumor. However one of the major limitations in boron neutron capture therapy (BNCT) is insufficient and heterogeneous delivery of ^{10}B from currently clinical-used ^{10}B carriers, such as sodium mercaptoundecahydrododecaborate-^{10}B (BSH) and L-4-boronophenylalanine-^{10}B (BPA), into the target tumors. To augment the efficiency of 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. The $\alpha_v\beta_3$ integrin is expressed on proliferating vascular endothelial cells such as those present in growing tumors, as well as on tumor cells of various origins. In this report, various boron cluster-cyclic RGD pentapeptide conjugates were designed and synthesized, and we analyzed their biodistribution and pharmacokinetics in a xenograft model to evaluate their usefulness in BNCT.

EXPERIMENTS: Chemistry: The cyclo(-Arg-Gly-Asp-D-Phe-Lys-) {*c*[RGDfK]} was synthesized as a tumor targeting moiety by Fmoc solid-phase method and conjugated to various boron clusters, such as *o*-carborane and BSH, through alkyl amide linker chain. **Biological evaluation:** The cytotoxic effect of RGD-conjugates for 24 h on U87MG, SCCVII and HCT116 was assessed by the crystal violet staining method. Binding affinity of all compounds to integrin $\alpha_v\beta_3$ was evaluated using cell adhesion assay on U87MG to vitronectin coated plates. **Biodistribution:** Biodistribution studies were carried out using C3H/He female mice bearing SCCVII tumors on both hind legs. At various time points after intravenous or intraperitoneal administration of boron carriers dissolved in10% HP-β-cyclodextrin (pH 7), tumors and some organs were collected and boron concentration was measured by ICP-AES.

RESULTS: The *c*[RGDfK] was obtained in quantitative yield by solid phase synthesis. The structures of RGD-peptide conjugate though various linkers with *o*-carborane (**GPU-49, 189, 201**) and with BSH (**GPU-51, 80, 176, 180, 192**) were shown in Fig. 1. The IC_{50} values of cytotoxicity on U87MG, SCCVII and HCT116 cells of all compounds except for **GPU-49** were more than 100 μM. To evaluate binding affinity to integrin $\alpha_v\beta_3$, cell adhesion assay was carried out on U87MG. The results showed that all of RGD conjugates suppressed attachment of U87MG to virtonectin significantly, while non-RGD (random) peptide analog, **GPU-205** did not show any inhibition. Dimeric RGD-peptides analogs (**GPU-189, 201, 180**) showed one digit smaller IC_{50} value in this assay than those of monomeric RGD analogs (**GPU-49** and **51**).

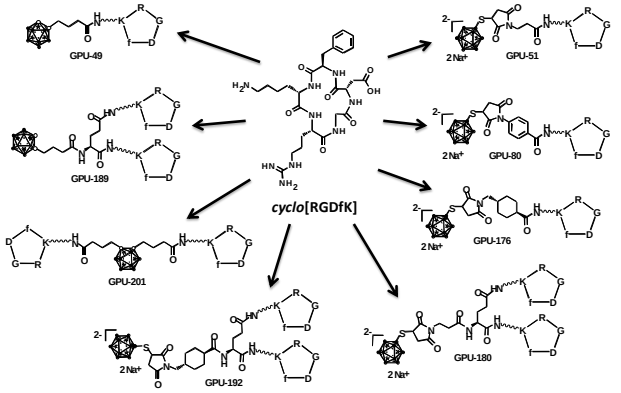

Fig. 1. Structures of *c*[RGDfK]-boron cluster conjugates.

As shown in Fig. 2, dimeric RGD conjugates showed higher boron concentrations in tumor than those of monomeric RGD conjugates. Among dimeric RGD conjugates, *o*-carborane derivative, **GPU-201** delivered to tumor most effectively 30 and 60 min after *i.v.* administration. The tumor-to-blood ratio was nearly twice larger than those of BSH from 15 to 60 min after *i.v.* injection. In the case of *i.p.* injection of **GPU-201**, boron concentrations in tumor from 30 to 120 min after injection were one third less than that of *i.v.* injection at a dose of 0.075 mmol/kg. However, the tumor boron concentration in tumor increased dose-dependently. Maximum boron concentration was more than 8 ppm in tumor at 120 min after *i.p.* injection at a dose of 0.75 mmol/kg of **GPU-201**.

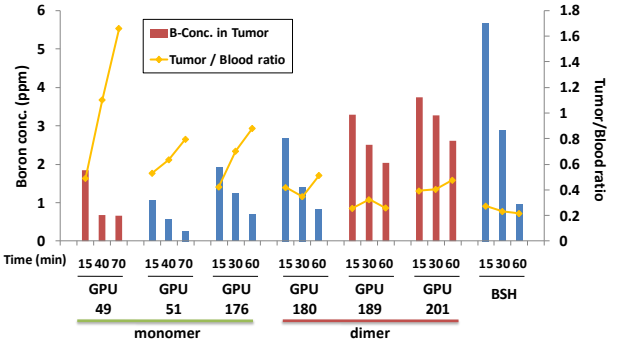

Fig. 2. Tumor distribution of RGD-boron conjugates after intravenous injection at a dose of 0.075 mmol/kg.

CONCLUSION: We synthesized boron cluster (*o*-carborane or BSH)-RGD cyclic pentapeptide conjugates for BNCT. The boron carriers inhibited cell adhesion to vitronectin in a concentration-dependent manner. Dimeric RGD analogs showed higher inhibitory effect of integrin-dependent cell adhesion and tumor delivery *in vivo* than those of monomeric RGD analogs. Among all, dimeric RGD *o*-carborane conjugate, **GPU-201** would be a most promising candidate of boron carrier for BNCT

採択課題番号 21P10-2　　腫瘍選択的ボロンキャリアの開発に関する研究　　プロジェクト
（岐阜薬大・薬化学）木村禎亮、上田　聡、奥田健介、永澤秀子（京大・原子炉）増永慎一郎

PR10-3 Optical imaging of Intratumor HIF-1 Activity to Optimize Treatment Regimen for the Combination of a HIF-1 Inhibitor with Radiation Therapy

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BACKGROUND & PURPOSE: Tumor cells in which the transcriptional activity of hypoxia-inducible factor 1 (HIF-1) is extremely high are recognized as potential targets in cancer therapy because HIF-1 has been strongly associated with not only tumor angiogenesis, invasion, metastasis but also poor prognosis after radiation therapy. Here, we performed a series of optical imaging experiments to clarify the postirradiation changes in intratumor HIF-1 activity. Based on the information, we intended to optimize the protocol for the combination of a HIF-1 inhibitor, YC-1, with radiation therapy.

METHODS: Human cervical epithelial adenocarcinoma cells (HeLa) were stably transfected with a HIF-1-dependent reporter gene, *5HRE-ODD-luc*, which expresses luciferase protein under the control of a HIF-1-dependent promoter [1]. The cells were transplanted into an immune-deficient nude mouse, and the resultant tumor xenograft was treated with 5 Gy of ^{137}Cs γ-ray radiation using a Gammacell 40 Exactor (MDS Nordion International) [2,3]. The tumor-bearing mouse was subjected to optical imaging after being injected with a substrate for luciferase, luciferin. The intratumor HIF-1 activity was visualized in real-time as the luciferase bioluminescence [2-4].

RESULTS: The optical imaging experiment demonstrated that the level of intratumor HIF-1 activity dramatically decreased and reached a minimum at 6 hours after 5 Gy of γ-ray irradiation (Fig. 1) [2]. After that, HIF-1 activity increased and reached a plateau at 18-24 hours postirradiation [2,3], although the timing and duration of the activation seemed to depend on both the dose of radiation and the cell line (unpublished data). Immunohistochemical analysis using HIF-1α antibody revealed that the two phases of the change were mainly dependent on the decrease and increase in HIF-1α protein at 6 and 24 hours after radiation, respectively, at the border between normoxic/viable regions and necrotic regions [2,3].

We next examined the influence of radiation-induced alteration of tumor microenvironments on the regulation of HIF-1 activity. It has been reported that the distribution of oxygen from tumor blood vessels to hypoxic tumor cells is dramatically improved after radiation therapy as a result of the death of well-oxygenated tumor cells and a subsequent decrease in oxygen consumption in these cells. We found that, under such reoxygenated conditions, PHD-VHL-dependent destruction of HIF-1α protein was responsible for the transient down-regulation of intratumor HIF-1 activity 6 h after 5 Gy of γ-ray radiation [2]. On the other hand, we found the importance of radiation-induced increase of glucose-availability as well as reoxygenation in the activation of HIF-1 24 h after radiation. The increase in the availability of oxygen and glucose led to activation of the Akt-mTOR pathway at the edges of viable regions after radiation therapy, and promotes HIF-1α's translation in these regions [3].

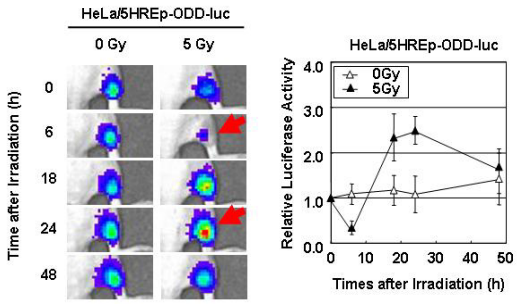

Fig. 1. Optical imaging of Intratumoral HIF-1 Activity as HIF-1-dependent luciferase bioluminescence after 0 or 5 Gy of γ-irradiation (left). Changes in HIF-1 activity (right).

Based on these findings, we intended to optimize treatment protocol for the combination of a HIF-1 inhibitor, YC-1, and radiation therapy. When YC-1 was administered just after the completion of 5Gy of ionizing radiation, the radiation-induced activation of HIF-1 was almost completely suppressed, leading to a significant increase in the number of apoptotic endothelial cells and a decrease in tumor microvessel density [2]. Consequently, tumor growth was significantly delayed by the combination compared to radiation therapy alone [2]. On the other hand, when YC-1 was given prior to radiation, inhibition of HIF-1 activity resulted in a significant decrease in tumor micro vessel density and increase in the radioresistant hypoxic fraction in the tumor. Therefore, the YC-1-first protocol suppressed rather than enhanced the therapeutic effect of radiation.

REFERENCES:

[1] H. Harada *et al*., Biochem. Biophys. Res. Commun., **360** (2007) 791-6.
[2] H. Harada *et al.*, Br. J. Cancer., **100** (2009) 747-57.
[3] H. Harada *et al*., J. Biol. Chem., **284** (2009) 5332-42.
[4] H. Harada *et al*., Oncogene. **26** (2007) 7508-16.

採択 21P10-3 腫瘍組織レベルの光イメージングで迫る癌の放射線耐性機構の解明　プロジェクト

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