VIII-Ⅱ-1. Project Research

Project 7

PR7 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 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 (22P7-1): 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, N. Kondo, M. Takagaki and K. Nagata)

ARS-2 (22P7-2): Development of Hypoxic Microenvironment-Oriented ^{10}B-Carriers
(H. Nagasawa, S. Masunaga, K. Okuda, S. and T. Harada)

ARS-3 (22P7-3): Clarification of Mechanism of Radio-Resistance in Cancer Using Optical Imaging at Tissue Level
(H. Harada, M. Hiraoka, K. Shibuya, S. Itasaka and S. Masunaga)

ARS-4 (22P7-4): Analysis of Radiation-Induced Cell-Killing Effect in Neutron Capture Reaction
(R. Hirayama, S. Masunaga, G. Kashino, Y. Sakurai, H. Tanaka and Y. Matsumoto)

(Underline: Representative at each research group)

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採択課題番号 22P7　　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

PR7-1 A Trial to Detect the Radio-Sensitivity of Oxygenated Tumor Cell Fractions in Quiescent Cell Populations within Solid Tumors

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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 and EL4 leukemia cell tumors with a diameter of 1 cm implanted in the hind legs of C3H/He and C57BL/6 mice were 55.1 ± 6.2 (mean ± SD) % and 42.5 ± 5.4 %, respectively. They were 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. In this study, we tried to selectively detect the response of the pimonidazole non-labeled, probably oxygenated cell fraction of the Q cell population by combining our method for selectively detecting the response of Q cells in solid tumors with the method of detecting cell and tissue hypoxia using pimonidazole and MAb1 to pimonidazole.

MATERIALS AND METHODS: C57BL mice bearing EL4 tumors received 5-bromo-2'-deoxyuridine (BrdU) continuously to label all P cells in the tumors. They received gamma-ray irradiation at a high dose-rate or reduced dose-rate one hour after the administration of pimonidazole. The responses of Q and total cell populations were assessed based on frequencies of micronucleation and apoptosis using immunofluorescence staining for BrdU. Meanwhile, the response of pimonidazole unlabeled tumor cell fractions was assessed by apoptosis frequency using immunofluorescence staining for pimonidazole.

RESULTS: The cell fraction not labeled with pimonidazole showed significantly greater radio-sensitivity than the whole tumor cell fraction more remarkably in the Q than total cell population. However, the pimonidazole unlabeled cells showed a significantly clearer decrease in radio-sensitivity through a delayed assay or decrease in irradiation dose-rate than the whole cell fraction, again more markedly in the Q than total cell population.

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].

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採択課題番号 22P7-1　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

（京大・原子炉）増永慎一郎、櫻井良憲、田中浩基、菓子野元郎、劉　勇、近藤夏子
（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

PR7-2 Development of New Tumor Targeting ^{10}B-carrier: Synthesis and Evaluation of Cyclic RGD-Boron Cluster Conjugate, GPU-201

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INTRODUCTION: Boron-containing agents play a key role in successful boron neutron capture therapy (BNCT). 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. 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 conjugates were designed, synthesized, and evaluated for the biodistribution to develop tumor-selective boron carriers. On the other hand, despite the wide application of 1,2-dicarba-*closo*-dodecaboranes (*o*-carboranes), the synthetic methods for functionalized *o*-carboranes are very limited. An efficient synthesis of mono- and di-functionalized *o*-carboranes from the corresponding alkynes was also achieved by employing microwave irradiation.

EXPERIMENTS: Chemistry: Synthesis of cyclic RGD-boron cluster conjugates. 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.

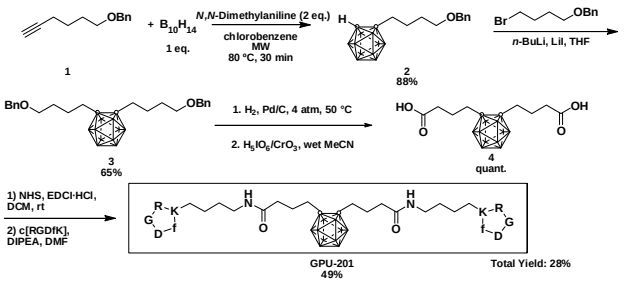
General procedure for synthesis of *o*-carboranes. Acetylene (1 equiv.), $B_{10}H_{14}$ (1-1.5 equiv.), and *N*,*N*-dimethylaniline (2-3 equiv.) in chlorobenzene ware irradiated with microwave at 120 ºC, for 15 min using microwave reactor. After irradiation was completed, the reaction was cooled to room temperature and the residual decaborane was decomposed with small amount of methanol. The reaction mixture was purified by preparative TLC or silica-gel column chromatography.

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 in 10% HP-β-cyclodextrin (pH 7), tumors and some organs were collected and boron concentration was measured by ICP-AES.

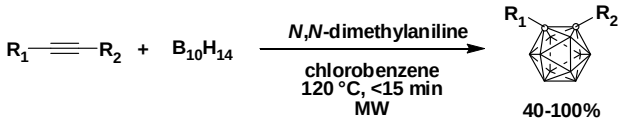
RESULTS: All RGD conjugates had high *in vitro* integrin $\alpha_v\beta_3$-binding affinity, and showed dose-dependent inhibition of cell adhesion with IC_{50} values of 0.19-2.66 μM in a vitronectin mediated cell adhesion assay on U87MG cells and SCCVII cells. By analyses of *in vivo* biodistribution, RGD dimer-boron clusters had higher tumor uptake and slower clearance than that of monomeric RGD conjugates. In particular, GPU-201 should be a promising candidate due to its dose dependent-tumor uptake and significantly longer tumor retention than BSH [1].

We carried out scale-up synthesis of GPU-201 for evaluation of its radio-sensitizing effect under thermal neutrons beam exposure. The synthesis of GPU-201 from 5-hexyn-1-ol (1) was performed in good yield using a new microwave-accelerated procedure to obtain *o*-carborane (2) as shown in Scheme 1.



Scheme 1

An efficient synthesis of mono- and di-functionalized *o*-carboranes from the corresponding alkynes was achieved by employing microwave irradiation. This method is tolerant of aryl and alkyl acetylenes with various substituents. Compared with conventional thermal reaction, the present microwave promoted formation of *o*-carborane provides fast and efficient access to a wide range of *o*-carboranes (unpublished data).



Scheme 2

Conclusion: We synthesized the cyclic RGD-boron cluster conjugates to develop tumor-selective boron carriers for BNCT. All RGD conjugates had high *in vitro* integrin $\alpha_v\beta_3$-binding affinity. By analyses of *in vivo* biodistribution, GPU-201 should be a promising candidate due to its dose dependent-tumor uptake and significantly longer tumor retention than BSH. We shall evaluate its radio-sensitizing effect under thermal neutrons beam exposure in future studies. We also have developed a novel synthetic method for rapid preparation of aryl or alkyl *o*-carboranes bearing various substituents by utilizing microwave irradiation technique.

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採択課題番号 22P7-2　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（岐阜薬大・薬化学）原田友宏、木村禎亮、奥田健介、永澤秀子（京大・原子炉）増永慎一郎

PR7-3 HIF-1-Dependent Cellular Dynamics behind Tumor Recurrence after Radiation Therapy

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INTRODUCTION: Hypoxia is a typical microenvironment for solid tumors and is strongly associated with malignant phenotypes and radioresistance of tumors. Hypoxia mediates many of its effects through the induction of the transcription factor hypoxia-inducible factor 1 (HIF-1). However, the relationship of the dynamics of hypoxic tumor cells and the function of HIF-1 to tumor recurrence after radiation therapy remain unexplained. By tracking the postirradiation fate of cells which were in hypoxic regions at the time of radiation, here we demonstrate the HIF-1-dependence of tumor cell relocation leading to tumor recurrence.

EXPERIMENTS: We previously constructed a HIF-1-dependent promoter, which induces gene expression under the control of five tandem repeat of hypoxia-responsive elements (HRE) [1-4]. By combining the HIF-1-dependent promoter with a Cre-ER^{T2}/loxP site-specific recombination system, we developed a novel strategy to intentionally tag hypoxic cells in a tumor xenograft with luciferase proteins at a specific time after tamoxifen administration. Human cervical epithelial adenocarcinoma cells (HeLa) were stably introduced with this HIF-1-dependent tagging system, and transplanted into an immune-deficient nude mouse. The resultant tumor-bearing mice were used in the present study. Gammacell 40 Exactor (MDS Nordion International) was used to treat the tumor-bearing mice with 25 Gy of ^{137}Cs γ-ray radiation. The tumor-bearing mouse was subjected to optical imaging after being injected with a substrate for luciferase, luciferin. The dynamics and fate of the tagged cells were tracked as luciferase bioluminescence.

RESULTS: The optical imaging experiment confirmed that tamoxifen injection induced luciferase bioluminescence in the tumor xenograft. Immunohistochemical analysis with a hypoxia marker Pimonidazole and luciferase antibody confirmed that the Pimonidazole-positive hypoxic tumor cells were indeed tagged with luciferase 2 days after the tamoxifen administration as we desired. We applied 25 Gy of ^{137}Cs γ-ray radiation therapy just after tagging hypoxic tumor cells with luciferase in order to analyze the fate of the tagged cells during tumor recurrence after radiation therapy. We confirmed that, after such a radiation treatment, the tumor xenograft dramatically regressed and became non-palpable once, but later recurred. The optical imaging experiment revealed that bioluminescent intensity in recurrent tumor was significantly higher compared to that in the primary tumor. Flow cytometric analyses using the primary and recurrent tumors revealed that luciferase-positive cells predominantly exist in the recurrent tumor, and they occupied about 60% of the recurrent tumor. Immunohistochemical analyses revealed that tumor cells which were in hypoxic regions at the timing of radiation migrated to tumor blood vessel during the recurrence after radiation therapy. The migration was almost completely inhibited by the pharmacological HIF-1 inhibitor.

CONCLUSION: For the first time, we provide direct evidence obtained *in vivo* that hypoxic/peri-necrotic tumor cells predominantly survive radiation therapy, migrate to tumor blood vessels through activation of HIF-1, and consequently cause tumor recurrence. Our findings provide rational basis for combining radiation therapy with hypoxia- and HIF-1-targeting therapies.

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採択課題番号 22P7-3　　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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PR7-4 Inactivation of Human Salivary Gland Tumor Cells by Neutron Bemas

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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 a relationship between radiation dose and 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 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 parameters were calculated by fitting the curve with the single-hit model: $SF = \exp(-\alpha \cdot D)$. SF and D are the surviving fraction and the dose, respectively.

RESULTS: The survival curve for neutrons is shown in Fig. 1. The D_{10} value was at 2.46, α parameter was 0.936 Gy^{-1}. The RBE value was 2.27 for the neutron calculated using data of cobalt-60 gamma rays [6]. However, the survival curve of the coefficient of determination (R^2) was 0.81. We will retest and add the dose points.

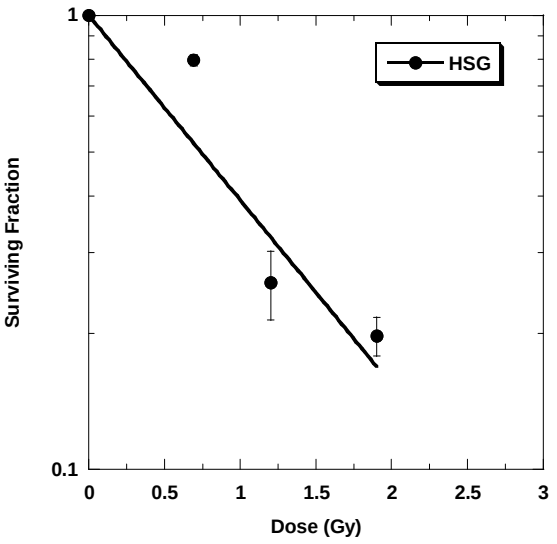

Fig. 1. Survival curve for HSG cells exposed to neutrons. The survival parameters were calculated from the data by a curve fitting using: $SF = \exp(-\alpha \cdot D)$. The symbols and bars are the mean ± SD from at three independent samples.

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採択課題番号 22P7-4　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
（放医研・次世代）平山亮一、松本孔貴、古澤佳也（京大・原子炉）増永慎一郎、櫻井良憲
田中浩基（大分大・医）菓子野元郎