VIII-Ⅱ-1. Project Research

Project 7

PR7 # The Origin of Radiation Carcinogenesis is not DNA Damage

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OBJECTIVES AND RESEARCH SUBJECTS:

It has been believed that the first target of radiation carcinogenesis is DNA. However, this hypothesis is not proved as a main route for carcinogenesis of low dose radiation yet. After analyzing our results of research of malignant cell transformation with low dose radiation during the past 30 years, we came to strongly believe that a radiation cancer-causing primary target is not DNA itself. Recently, several reports including our reports suggested that non-target effects, such as bystander effect and delayed effect, modify cell transformation frequency. From these results, we speculate that non-genomic damage plays an important role in an initial process of cellular malignant transformation. Especially, we speculate that aneuploid may be closely related on the induction of malignant cell transformation by radiation.

Therefore, the aim of this project is focused on elucidation of process of aneuploid formation related to carcinogenesis.

MAIN RESULTS OF THIS PROJECT:

As a result, we found that the intracellular oxidation degree, such as reactive oxidative radicals and long lived radicals, was elevated by high density culture and radiation exposure both in mammalian cells. Specially, long-lived radicals (LLRs) play an important role of genetical effects of radiation. These radicals attack several proteins, such as telomere related protein and centrosome, and destroy their structure. Telomere destabilization induces telomere fusion and makes dicentric chromosome and reason of chromosome instability. In fact, dicentric chromosome is dominant aberration induced by low dose radiation. Radiation induced radicals also attacked centrosome. Centrosome destabilization induces nondisjunction and raises the frequency of aneuploid. In early process of cell transformation, structural aberration of chromosome does not occur, but aneuploid is seen in high frequency. Aneuploid is also induced by deficiency in cell cycle checkpoint at G_2-M. By treatment of vitamin C prevent induction of LLRs, aneuploid, and thymic lymphomas in B6C3F1 mice. Interestingly, the thymic lymphomas in vitamine C treated mice lacked point mutation of *Ikaros*, suggesting a suppression of point mutation by VC

Low dose radiation activated repair capacity of DNA damage in irradiated cells (A-7). Because radiation-induced genomic instability is induced in some fraction of the progeny of a single survived cell, not a single gene mutation but some epigenetic changes may be involved in the initiation of radiation-induced genomic instability. Oxidative stress and altered chromatin structure have been proposed as the mechanisms of perpetuation of radiation-induced genomic instability.

These results suggest a possibility that a main target of radiation carcinogenesis is not DNA, but is centrosome, which are the proteins to constitute chromosomal homeostasis maintenance mechanism (Fig1). If our results are right, “Mutation Theory of Carcinogenesis” is to be wrong. I will suggest a new hypothesis about radiation carcinogenesis, which was named as "protein target theory" by this report.

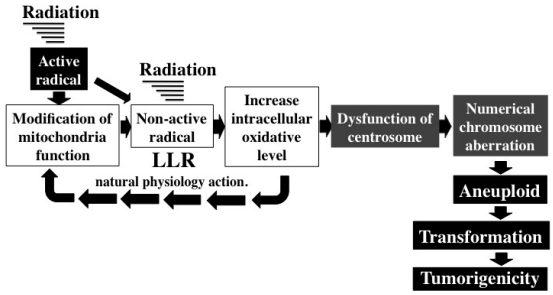

Fig. 1. A new model of radiation carcinogenesis.

採択課題番号 23P7　　DNA を標的としない放射線発がんの仕組みに関する研究　　プロジェクト（京大・原子炉）渡邉正己

PR7-1

Participation of Aneuploidy on Radiation-induced Cellular Malignant Transformation

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INTRODUCTION:

It has been believed that the first target of radiation carcinogenesis is DNA. However, this is not proved for radiation carcinogenesis yet. We discovered that frequency of aneuploid cell was closely related to that of radiation-induced cell transformation and natural cell transformation by high-density cultivation, but gene mutation was not [1-4]. Cell with p53 gene becomes tetraploid, but does not get tumorigenicity. On the other hand, cells without p53 gene function become a triploid easily, and acquires tumorigenicity. Both radiation exposure and high-density cultivation elevated the level of intracellular oxidative radicals [5-7]. These radicals induced centrosome destabilization and produced cells carrying extra centrosome, which promote merotelic attachment of chromosome by altering spindle geometry. Unresolved merotelic attachments can give rise to lagging chromosomes at anaphase. Aneuploidy was seen in high frequency in early process of cell transformation. These results strongly suggest that a main target of carcinogenesis by low dose radiation is not DNA, but is centrosome, which are the proteins to constitute chromosomal homeostasis maintenance mechanism. In addition, this route may be the same as that of natural carcinogenesis.

RESULTS AND DISCUSSION:

Cell with p53 gene becomes tetraploid, but does not get tumorigenicity. On the other hand, cells without p53 gene function become a triploid easily, and acquires tumorigenicity. Both radiation exposure and high-density cultivation elevated the level of intracellular oxidative radicals and long-lived radicals in ME, SHE cells and HE cells. These radicals attacked centrosome and induced centrosome destabilization. Centrosome destabilization promotes merotelic attachment of chromosome by altering spindle geometry. Unresolved merotelic attachments can give rise to lagging chromosomes at anaphase. This is the main route of production of aneuploid cell. We previously have shown that in early process of cell transformation, structural aberration of chromosome does not occur, but aneuploid is seen in high frequency [2,4].

To study the effects of aneuploidy in normal human cells, we generated artificial cells of human primary fibroblast having three chromosome 8 (trisomy 8 cells) by using microcellmediatedchromosome transfer technique. In addition to decreased proliferation, the trisomy 8 cells lost contact inhibition and reproliferated after exhibiting senecence-like characteristics that are typical of transformed cells [8]. Furthermore, the trisomy 8 cells exhibited chromosome instability, and the overall gene expression profile based on microarray analyses was significantly different from that of diploid human primary fibroblasts. Our data suggest that aneuploidy, even a single chromosome gain, can be introduced into normal human cells and causes, in some cases, a partial cancer phenotype due to a disruption in overall gene expression.

These results strongly suggest that a main target of carcinogenesis by low dose radiation is not DNA, but is centrosome, which are the proteins to constitute chromosomal homeostasis maintenance mechanism.

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採択課題番号 23P7-1　　放射線による染色体異数化と細胞がん化の関連性　　プロジェクト
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PR7-2 The Change of Mitochondrial ROS by Radiation Induced Bystander Effect

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INTRODUCTION: The effects of ionizing radiation have been examined in biological model. One of the impacts recently is that some signals are interacted between targeted cells and non-targeted cells. This effect, called as "bystander effect" has impact on risk estimation in lower dose range, because non-targeted cells may be leading to cancer. Some factors from irradiated cells are thought to be effective in non-targeted cells. In this study, culture medium with human cells in a culture flask was X- ray irradiated, and then the medium was transferred to other culture flasks in which non-irradiated cells were plated. It is expected that irradiated cells as donor cells release some soluble bystander factors into the medium, so that the non-irradiated cells as recipient cells may be affected by the factors. The result showed that bystander factors from irradiated cells modulate the membrane potential of mitochondria in non-irradiated cells. This response is thought to be a trigger of following responses such as the induction of reactive oxygen species (ROS), and gene mutation.

EXPERIMENTS: Normal human cells (HE35, HE49, NB1RGB) and tumor cell lines (HeLa, SF126) were used in this study. Cells were cultured in MEM-alpha medium supplemented with 10%FBS. Cells were maintained at 37^0C in a humidified atmosphere with 5% CO_2. Cells were seeded onto T25 flask one day prior to irradiation. Immediately before irradiation, medium was changed and cells were irradiated with 0.5 Gy of X-rays. Cells were incubated for 24 hours following irradiation. The culture medium was filtrated with 0.2 μm filter and transferred to unirradiated cultured cells on T25. Twenty-four hours after transfer, cells were treated with MitosoxRed staining dye to detect the mitochondrial supeoxide (O_2^-) level. After the 30 min treatment, cells were washed with PBS and harvested. Then, suspended cells were analyzed by FACscan to detect the fluorescent intensity of MitosoxRed. In the study of radical scavenger treatment, 0.2 mM Ascorbic acid and 2% DMSO were used. Cells were treated with these scavengers at the same time of medium transfer of conditioned medium.

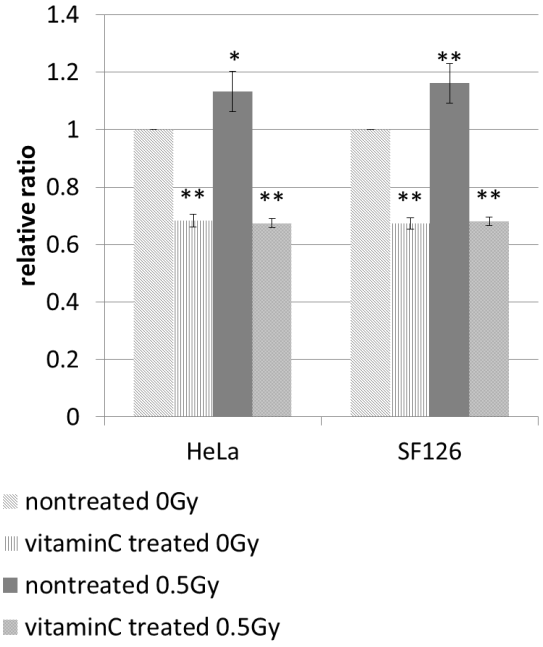

Fig.1 Relative ratio of fluorescent values for MitosoxRed in two human tumor cell lines (HeLa and SF126). Twenty-four hours after irradiation, conditioned medium were transferred and then ROS levels were measured at 24 h after transfer by MitosoxRed method. Ascorbic acid (0.2 mM) was used for the purpose of relief of ROS in mitochondria.

RESULTS: In all normal human cells and human tumor cells, the mitochondrial ROS were detected by MitosoxRed method, and levels were increased by the secreted factors from 0.5Gy-irradiated cells. Therefore, the bystander response (is meaning that reactions are occurred from irradiated cells toward non-irradiated cells) is occurred in both normal cells and tumor cells, and mitochondira may be key organ in this response. Also, these responses were suppressed by the treatment of ascorbic acid, but not DMSO. It is possible that ascorbic acid can be incorporated into mitochondria, therefore ascorbic acid is effective scavenger for bystander responses. We hypothesized reactive oxygen species (ROS) should be increased after the signal activation through secreted factor from irradiated cells. We need to clarify the signal pathway. The increases of ROS were thought to be leading to the production of "slow releasing long lived radicals" (SRLLRs), which is thought to be a cause of mutagenesis. Our results suggest that secreted factors from irradiated cells can lead to mutagenesis through the change of mitochondrial function inn both normal and tumor cells.

採択課題番号 23P7-2　　染色体異数化誘導へのバイスタンダー効果の影響　　プロジェクト
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PR7-3 **SIRT2, a Mitotic Checkpoint Protein, as a Novel Target for Cancer Therapy: SIRT2 Down-regulation in HeLa can Induce p53 Accumulation via p38 MAPK Activation-dependent p300 Decrease, Eventually Leading to Apoptosis**

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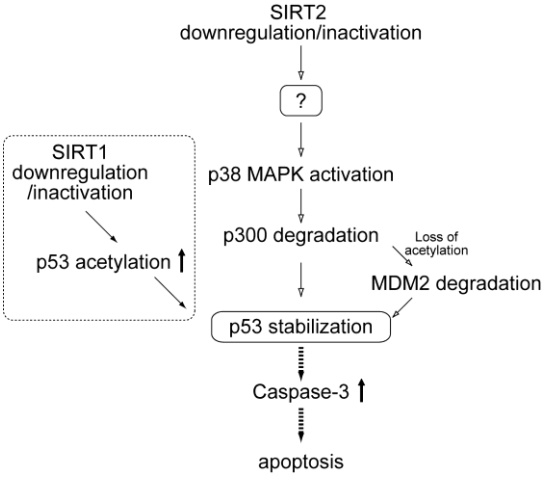
INTRODUCTION: The yeast silent formation regulator protein-2 (sir2) is a NAD+-dependent protein deacetylase, the mammalian homolog of sir2 is the sirtuin family containing 7 members, SIRT1-SIRT7. These sirtuins are involved in gene silencing, cell cycle control, apoptosis, and even energy homeostasis. In our previous study mentioned above, we observed that SIRT2 downregulation confers resistance to mitotic cell death from the spindle checkpoint in the presence of microtubule inhibitors in HCT116 cells, a mitotic checkpoint-proficient cancer cell line used for studying checkpoints. However, in some cancer cell lines, we observed that siRNA-mediated SIRT2 knockdown causes massive cell death even in the absence of microtubule inhibitors. This raises the possibility that SIRT2 inhibition may be a target for the killing of cancer.

EXPERIMENTS: We sought to obtain evidences for the molecular mechanism through which SIRT2 inhibition is involved in tumor cytotoxicity in the present study. Several human cell lines were transfected with siRNA of SIRT2 or negative control siRNA. Transfection of siRNA to SIRT2 resulted in significantly decreased cell numbers in HeLa, hiMSC, HT1080, 293T, and CC1cell lines, but not in normal cells such as TIG-1 cells. The most prominent case was HeLa cells, while thus, we used HeLa cells for further study to delineate the mechanism by which SIRT2 downregulation leads to suppression of colony formation.

RESULTS: The apoptosis was caused by p53 accumulation, which is mediated by p38 MAPK activation-dependent degradation of p300 and the subsequent MDM2 degradation. Consistent with the observation, the sensitivity of SIRT2 siRNA among cancer cell lines reflects the p53 status.

Sirtuin inhibitors are emerging as antitumor drugs and this function has been ascribed to the inhibition of SIRT1, the most well-characterized sirtuin that deacetylases p53 to promote cell survival and also binds to other proteins in response to genotoxic stress. The present study suggests that SIRT2 can be a novel molecular target for cancer therapy and provides a molecular basis for the efficacy of SIRT2 for future cancer therapy.



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採択課題番号 23P7-3　　新規 M 期チェックポイント蛋白 SIRT2 の中心体における機能の解明　　プロジェクト

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PR7-4 Chromosome Instability in Radiation-induced Mouse Lymphomas: Study II

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INTRODUCTION:

Irradiation with heavy-ion beams is one of the effective cancer radiotherapy because of its good conformity to tumor shape, high relative biological effectiveness (RBE), smaller independency of oxygen concentration and, thereby, high local control rates. In order to develop more efficient and safer protocol with less side effects, it is important to know the acute and late biological responses of both cancer cells and normal tissues to heavy ion beams. Generally, heavy ions give large relative biological effectiveness (RBEs) for cell killing and induction of mutations and chromosome aberrations. The high frequency of the intrachromomal and complex-type chromosomal exchange is reported characteristic for the cells treated with carbon ions than for those treated with X-rays [1]. Chromosome instability, which can be seen in the progeny of irradiated cells after several cell divisions, is also manifested by carbon ions more efficiently than X rays [2]. Several studies documented experimental data on the induction of cancers by heavy ions using in vitro and in vivo model [3-6].

Mouse thymic lymphomas (TLs) can be reproducibly induced by radiation, and have been used for the characterization of genes and chromosomes involved in the development of radiation lymphomagenesis. Cytogenetic studies have reported interstitial deletion of chromosome 11, translocation with deletion of the distal region in chromosome 12 and trisomy of chromosome 15 in radiation-induced lymphomas [7] . The former two chromosome aberrations are consistent with frequent loss of heterozygosity on chromosomes 11 and 12, where tumor suppressor genes, Ikaros and Bcl11b, are mapped, respectively [8-10]. On the other hand, trisomy for chromosome 15 was most frequently identified in both spontaneous and carcinogen-induced lymphomas [7].

Age is an important factor that influences the carcinogenic effect of radiation. Generally, the younger the age of subject is, the larger the cancer risk is. It is well known that childhood thyroid cancers have frequent RET-PTC conversion whereas adult type thyroid cancers have B-RAF point mutation. However, information on the distinct molecular changes in cancers after early life exposure and those after adulthood exposure are still insufficient.

In order to identify the age-dependent genetic events during lymphomagenesis by heavy-ion carbon beam irradiation, karyotypes was compared in TLs induced by carbon beams in B6C3F1 mice, which were irradiated at either one week of age or four weeks of age.

EXPERIMENTS:

We used the B6C3F1 mice at the age of one or four weeks of age (Charles River Japan Inc., Yokohama). TLs were induced by whole-body irradiation with carbon ions (1.2 or 1.6 Gy) once a week for four consecutive weeks. Chromosomes from the enlarged thymus were prepared for analysis by short-term culture or direct methods. To confirm chromosome loss, painting and BAC FISH techniques were used in several cases.

RESULTS:

We first found that the incidence of aneuploidy, especially trisomy of chromosome 15, was high for carbon induced TLs in both age group. It is of note that small deletion in centrometic region and structural change in chromosome X was more characteristic to TLs from mice irradiated at one week of age. Karyotype and array CGH analysis indicated high frequency of loss of Bcl11b in TL form mice irradiated at one week of age (6/7; 86%), compared to adult TLs (4/8; 50%). These results indicate that there are age-associated chromosome aberrations in TLs induced by carbon ions.

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採択課題番号 23P7-5　　インビボ発がんにおける染色体異数化　　プロジェクト

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PR7-5 Involvement of Quality Control of Organela in Cancer Development and Progression

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INTRODUCTION: Peroxiredoxins (Prdxs) are a family of peroxidases currently known to possess six mammalian isoforms. Although their roles in cellular redox regulation and antioxidant protection are distinct, all of them convert hydrogen peroxide to water and oxygen. Among PRDX family proteins, PRDX1 is present in the cytoplasm and PRDX2 is present in the mitochondria. Both of them work as a molecular chaperone to reduce an intermolecular disulfide bond within other proteins besides metabolizing hydrogen peroxide. To investigate the roles of PRDX1and 2 to maintain normal intracellular oxygen environment, in this study we have generated conditional *PRDX1*$^{-/-}$ DT40 cells in which human PRDX1 trans-gene was expressed under control of the tetracycline inducible promoter. PDRX1 depleted cells showed lethal phenotype and the depletion of PRDX1 resulted in the accumulation of intracellular oxidative stress. The lethality and increased of oxidative stress of PRDX1 depleted cells was completely suppressed by 2-mercaptethanol. Our data, in contrast to SOD1 or SOD2, suggested that PRDX1 has roles not only as antioxidant but also as coupled thiol-redox-dependent pathway in cells.

EXPERIMENTS: Chicken DT40 used in this study were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% chicken serum, and kanamycin at 39°C under 5% CO_2. Conditional PRDX1-knock-out DT40 cells were established and the expression of transgenic human PRDX1 is suppressed by treatment with 100 ng/ ml doxycycline (Dox). To assay the effect of various antioxidants for cell growth, cells cultured in the presence or absence of Dox for the indicated periods were treated with 200 µM L-ascorbic acid phosphate magnesium salt, 5 mM N-acetyl-L-cystein (NAC), 50µM 6-hydroxy-2,5, 7,8-tetramethylchroman- 2-carboxylic acid (Trolox), 250 µM 1,2-Dihydroxy-3,5-benzenedisulfonic acid (Tiron,) or 50µM 2-mercaptoethano (EtSH,). Generation of growth curves has been described previously [1,2]. Intracellular oxidative stress was measured based on the intracellular peroxide-dependent oxidation of DCFH-DA (Molecular Probes, USA).

RESULTS: Our previous work showed that SOD1 was essential for cell viability and SOD2 was not essential, but was required for normal cell growth [1,2]. Our intriguing observation from previous work is that lethality observed in *SOD1*$^{-/-}$ cells was completely rescued in the presence of ascorbic acid, L-ascorbic acid phosphate magnesium salt [2]. PDRX1 depleted cells also showed lethal phenotype. In contrast to lethality SOD1 depleted cells, lethality of PRDX depleted cells was completely suppressed by 2-mercaptethanol (Fig. 1). Because PRDX1 is exclusively localized in cytoplasm, it was assumed that increased levels of overall oxidative levels would be elevated followed by depletion of PRDX1. To confirm so, the amounts of hydrogen peroxide in cytoplasm were determined by staining PRDX1 depleted cells with DCFH. DCFH is cell-permeable fluorescence dye reacting to a broad spectrum of cellular reactive oxygen species reflected by intra-cellular oxidative stress. As shown in Fig. 2, PRDX1-depletion caused an increase of oxidative stress. In the presence of 2-mercaptethanol, it was hardly detected the increase of oxidative stress in PRDX1depleted cells. In contrast to SOD1 depleted cells, an elevation of sister chromatid exchange (SCE) frequency was not seen in PRDX1 depleted cells.

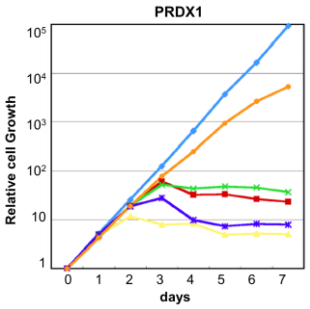

Fig. 1. PDRX1 depleted cells showed lethal phenotype. In contrast to SOD1 or SOD2 depleted cells, 2-mercaptethanol completely suppressed lethality of PRDX1 depleted cells.

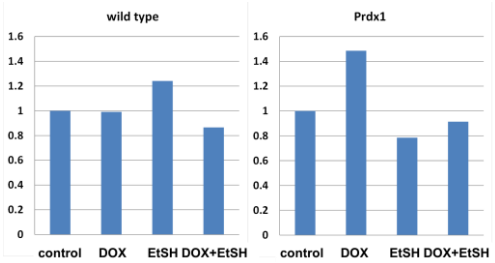

Fig. 2. Intracellular ROS levels were increased upon depletion of Prdx1. 2-mercaptethanol completely compensated with regard to the increase intracellular ROS levels.

The results revealed that PRDX1 was essential for viability and that depletion of PRDX1did not increase DNA damages, suggesting that hydrogen peroxide is accumulated cytoplasm in PRDX1depleted cells might not impact on genome. Our data suggested that PRDX1 has roles for coupled thiol-redox-dependent pathway in addition to roles of antioxidant in cells.

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採択課題番号 23P7-7　細胞内オルガネラの質的管理機構不全の発癌過程への関与　プロジェクト
（京大・原子炉）田野恵三、渡邉正己
（東北大・薬）井上絵里