VIII-Ⅱ-1.　Project Research

Project 11

PR11 Participation of Aneuploidy on Radiation-induced Cellular Malignant Transformation

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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 and delayed effects, 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.

The collaborators and allotted research subjects (S) are as follows;

S-1: Participation of aneuploidy on radiation-induced cellular malignant transformation (M. Watanabe, K. Watanabe, G. Kashino, J. Kumagai, H. Nawata, K. Tano)

S-2: The change of mitochondrial potential by radiation induced bystander effect (G. Kashino, J. Kumagai, M. Watanabe)

S-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 (Y. Li, T. Inoue, M. Watanabe)

S-4: Chromosome instability in radiation- induced mouse lymphomas (A. Nakata, M. Yoshida, M. Akiyama, T. Takabatake, M. Nishimura, T. Imaoka, M. Watanabe, S. Kakinuma, Y. Shimada)

S-5: Effect of numerical chromosome aberrations on early stage of embryo development (H. Tateno, H. Kusakabe, M. Watanabe)

S-6: Direct observation of long-lived radicals for the study of oncogenic pathway (J. Kumagai, K. Mioki, G. Kashino, M. Watanabe)

S-7: Radiation-quality dependent cellular involvement of mitochondrial dysfunction in cancer development and progression (K. Tano, E. Inoue, M. Watanabe)

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 (VC) prevent induction of LLRs, aneuploid, and thymic lymphomas in B6C3F1 mice. Interestingly, the thymic lymphomas in VC 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. If our results are right, “Mutation Theory of Carcinogenesis” is to be wrong. I will suggest a new hypo- thesis about radiation carcinogenesis, which was named as "protein target theory" by this report.

採択課題番号 22P11　　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
（京大・原子炉）渡邉正己

PR11-1 **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]. Recently, several reports including our reports suggested that non-target effects, such as bystander effect and delayed effect, modify cell transformation frequency [2,4]. From these results, we speculate that non-genetic damage plays an important role in an initial process of cellular malignant transformation. However, it is the other important finding that this process is strictly inhibited in a human cell *in vitro* [5]. Therefore, we were searching for an intracellular target related to carcinogenesis in mouse embryo fibroblast (ME), SHE and human embryo fibroblast (HE) cells.

MATERIALS AND METHODS: We cultured normal human cells and rodent cells (ME and SHE) under 20% oxygen conditions, and analyzed mechanism of cellular malignant transformation and chromosomal aberration. Particularly, we squeezed focus for a study to how centric abnormality occurred and dysfunctioned.

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 [5-8]. These radicals attacked centrosome and induced centrosome destabilization. Centrosome destabilization promotes merotelic attachment of chromosome by altering spindle geometry [9,10]. 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 [2,4,5].

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. These serial results support necessity of a review of a LNT hypothesis at a radioprotective point of view.

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採択課題番号 22P11-1　　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
（京大・原子炉）渡邉正己、渡邉喜美子、菓子野元郎、熊谷 純、縄田寿克、田野恵三

PR11-2 The Change of Mitochondrial Potential 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 CHO cells in a culture flask was X-ray irradiated, and then the medium was transferred to other culture flasks in which non-irradiated CHO 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: Chinese Hamster Ovary (CHO) cells 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 4 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 JC-1 staining dye to detect the mitochondrial membrane potential. 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 JC-1. Relative rate of the red fluorescent indicating imported dye were calculated by the fluorescent intensity in each condition. Reactive oxygen species were analyzed by the MitosoxRed dye, which is a marker of superoxide radical (O_2^-) in mitochondria. Slow releasing long lived radicals were detected by Dr. Kumagai in Nagoya Univ. [1]. Inductions of gene mutation were analyzed by HPRT mutation assay.

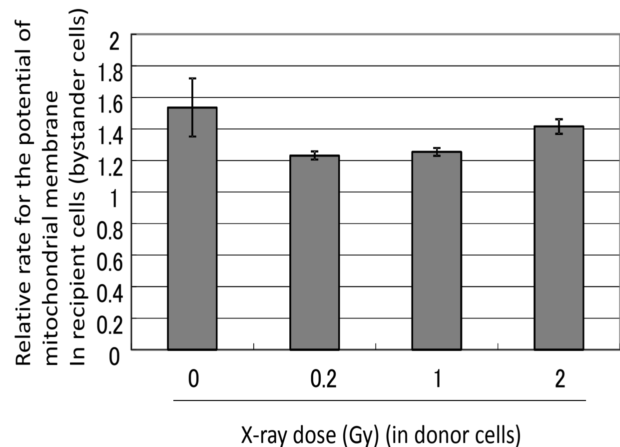

Fig.1. Relative rate for the potential of mitochondrial membrane in cells treated with conditioned medium from irradiated cells. Two hour after treatment, cells were analyzed by the JC-1 assay. Results were indicated by the average of three independent studies.

RESULTS: In CHO cells, the mitochondrial membrane potentials were detected by JC-1 analysis, and were reduced by the secreted factors from 4 Gy-irradiated cells. The similar levels of reductions (approximately 20%) were also observed in 0.2–2 Gy-irradiation. Therefore, the bystander response (is meaning that reactions are occurred from irradiated cells toward non-irradiated cells) is saturated at lower does. What is meaning of this response to the destiny of cell? We hypothesized (ROS) should be increased after this response. (ROS) were examined by MitosoxRed analysis, and increased levels of ROS, which is thought to be superoxide radical (O_2^-) in mitochondria, were detected following the reduction of the potential of mitochondrial membrane. The increases in ROS were thought to be leading to the production of "slow releasing long lived radicals" (SRLLRs) [1], which is thought to be a cause of mutagenesis. The treatment of secreted factors from irradiated cells resulted in the induction of gene mutation at *HPRT* locus. These results suggest that secreted factors from irradiated cells can lead to mutagenesis through the change of mitochondrial function.

REFERENCE:
[1] J. Kumagai *et al.*, unpublished result (2011)

採択課題番号 22P11-2　　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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PR11-3 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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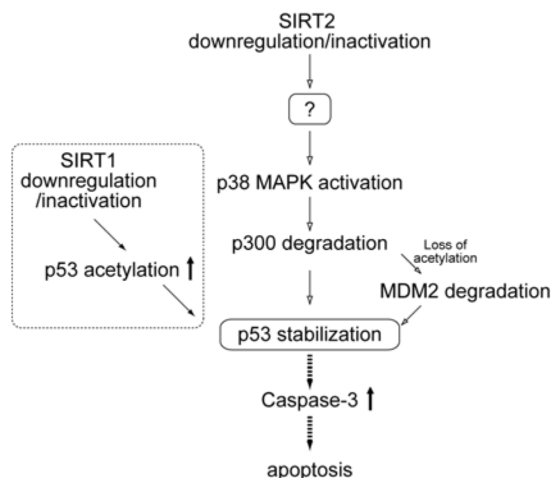
INTRODUCTION: The yeast silent formation regulator protein-2 (sir2) is a NAD+-dependent protein deacetylase, the mammalian homolog of sir2 is the sirtuin family containing 7 members, SIRT1-SIRT7. These sirtuins are involved in gene silencing, cell cycle control, apoptosis, and even energy homeostasis .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 provide evidence 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. 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 22P11-3　新規Ｍ期チェックポイント蛋白 SIRT2 の中心体における　プロジェクト
機能の解明
（鳥取大・医）李 艶沢 （鳥取大・医）井上 敏昭

PR11-4

Effect of Numerical Chromosome Aberrations on Early Stage of Embryo Development

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INTRODUCTION: Chromosome aberrations are the major cause of developmental defects. Previous studies have demonstrated that most of structural chromosome aberrations are originated from spermatozoa, and aneuploidy is exclusively of maternal origin. However, there is a potential risk of generating aneuploidy in early embryos derived from spermatozoa exposed to γ-rays [1]. In the present study, we analyzed the chromosomes of mouse embryos at different cleavage stages of ova fertilized with spermatozoa that had been exposed to γ-rays to comprehensively assess the quantitative change in structural chromosome aberrations, aneuploidy, and mosaicism during early cleavages.

EXPERIMENTS: Mature male mice were exposed to 2 Gy or 4 Gy of ^{137}Cs γ-rays, and their spermatozoa were used to produce embryos via in vitro fertilization (IVF). The metaphase chromosomes were prepared from one-cell, two-cell, and four-cell embryos. In the chromosome preparations from two-cell and four-cell embryos, the separation of the sister blastomeres was precluded by treatment of the embryos with concanavalin A to identify mosaicism. The incidence of embryos with γ-ray-induced structural chromosomal aberrations, aneuploidy, or mosaicism was estimated.

RESULTS: The rate of diploid one-cell embryos that reached the first cleavage metaphase was 100% in the 2 Gy group and 99.4% in the 4 Gy group. The high developmental capacity of embryos after γ-irradiation was maintained at two-cell stage [2 Gy: 98.9%, 4 Gy: 98.2%]. The percentage of four-cell embryos, in which all the sister blastomeres reached metaphase, was still high in the 2 Gy group (96.4%) and the 4 Gy group (93.7%). The exposure of spermatozoa to 2 Gy and 4 Gy γ-rays caused structural chromosome aberrations in 25.9% and 35.7% of the resultant one-cell embryos, respectively (Fig. 1). At two-cell embryonic stage, the incidence of structural chromosome aberrations was 17.4% in the 2 Gy group and 27.1% in the 4 Gy group. At the four-cell embryonic stage, the incidence of embryos with γ-ray-induced structural chromosome aberrations was similar to that at the one-cell stage. The incidence of aneuploidy was high in two-cell and four-cell embryos after both doses of γ-rays. The incidence of mosaicism increased in dose- and embryonic stage-dependent manners. Anaphase lag, and the degeneration and nondisjunction of the aberrant chromosomes were frequently observed in aneuploid and mosaic embryos (Fig. 2). Thus, structural chromosome aberrations of sperm origin are unstable in their behavior and structure during cleavage, and therefore cause secondary aneuploidy and mosaicism in the cleaving embryo. These chromosome aberrations survive early stage of development. Heritable risk of numerical chromosome aberrations should be considered in embryos derived from spermatozoa after irradiation.

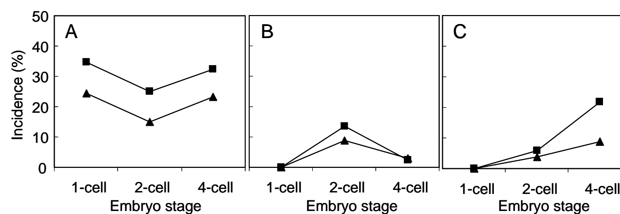

Fig. 1. Net incidences of structural chromosome aberrations (A), aneuploidy (B) and mosaicism (C) in early embryos derived from spermatozoa after 2 Gy (—▲—) and 4 Gy (—■—).

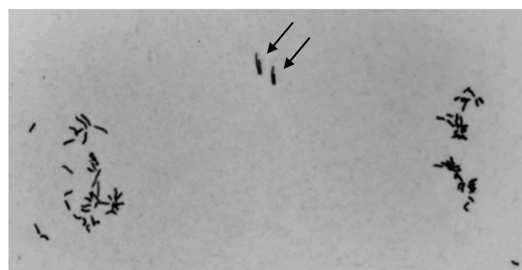

Fig. 2. Chromosome preparation of two-cell embryo showing two anaphase lagging chromosomes (arrows) in a cleavage furrow.

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採択課題番号 22P11-4　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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PR11-5 Chromosome Instability in Radiation-induced Mouse Lymphomas

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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 [3-5]. However, information on the risk assessment of secondary cancer is insufficient up to date.

Mouse thymic lymphomas (TLs) can be reproducibly induced by radiation and chemicals, and have been used for the characterization of genes 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 [6]. 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, in the previous reports on radiation-induced TLs [7-9]. On the other hand, trisomy for chromosome 15 was most frequently identified in both spontaneous and carcinogen-induced lymphomas, and, thus, appeared to be a specific change associated with the development of TLs irrespective of the causations[6].

In order to analyze the genetic events associated with lymphomagenesis by irradiation of a heavy-ion carbon beams, chromosome analysis was performed on TLs induced by carbon beams in C57BL/6 and C3H mice, which have marked difference in susceptibility to radiation lymphomagenesis [10].

EXPERIMENTS:

We used the following mouse strains: C57BL/6(B6) (CLEA Ltd., Yokohama, Japan) and C3H (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 in C57BL/6 that interstitial deletion of chromosome 11 and chromosome translocations were more prominent in carbon ion-induced TLs than in X-ray-induced ones. The incidence of polyploidy also was higher for carbon induced TLs. Unexpectedly, trisomy of chromosome 15, which is characteristic for this tumor type, was not observed. In contrast to C57BL/6, trisomy of chromosome 15 was frequent but interstitial deletion of chromosome 11 and translocations were less in TLs from C3H mice. These results indicate that there are carbon ion-associated chromosome aberrations, and that the genetic background of host animals has a profound influence on the type of chromosome abnormality.

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採択課題番号 22P11-5 インビボ発がんにおける染色体異数化 プロジェクト
（放医研・発達期）島田義也、（京大･原子炉）菓子野元郎、渡邉正己

PR11-6 Direct Observation of Long-lived Radicals for the Study of Oncogenic Pathway

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INTRODUCTION: Ionizing radiation-induced bystander effects have been recognized that non-irradiated cells received some bystander factors from irradiated cells via gap junctions and/or culture medium express biological responses similar to radiation biological effects such as mutation, chromosomal aberration, and so on. For this reason, bystander effects must be very important process for inducing carcinogenesis. In this study culture medium with CHO cells in a culture flask was X- or γ-ray irradiated, and then the medium was transferred to other culture flasks in which non-irradiated CHO 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. We have succeeded in detecting bystander responses as elevations of both levels of long-lived radicals (LLRs) and the point mutation frequency in recipient CHO cells.

EXPERIMENTS: Initially 7 x 10^6 of normal Chinese Hamster Ovary (CHO) cells for donor of soluble bystander factor were introduced to a T175 flask with 35 ml of D-MEM with 10% FBS in a cell incubator for 24 h at 37 °C in a humidified atmosphere with 5% CO_2 and 21 % O_2 conditions. After the incubation, the medium in the flask was exchanged to the 100 mL of new culture medium after washing the flask with 10 mL of PBS two times. The flask was then γ-ray irradiated (4 Gy) and was placed in the incubator for 24 h. We denote the medium in the flask at 24 h after irradiation as "bystander medium". Other five flasks initially having 3.5 x 10^6 of normal CHO cells for recipient cells (bystander cells) were incubated for 48 h with the culture medium. After the incubation, the medium in each flask was exchanged to the 18 mL of the bystander medium. The bystander medium was introduced to each flask through a membrane filter, and the flasks were stored in the incubator for 6 or 24 h. After the incubation, the recipient cells were harvested and moved into a Suprasil quartz tube for ESR measurement.

RESULTS: Levels of LLRs in the recipient cells are plotted in Fig. 1. When the bystander medium was treated to the donor cells for 6 h, no difference on the levels of LLRs can be observed in the recipient cells with the bystander medium without irradiation (control) and the irradiated bystander medium. When the bystander medium was transferred to the flasks of recipient cells and treated for 24 h, significant increase as +19% ($p < 0.001$) can be detected in the recipient cells. It should be noted that the significant increase in the levels of super oxide can be found in recipient cells at 6 h after the medium transfer from the flask of 4 Gy irradiated donor cells, so that increase in the levels of LLRs could slowly induced after the increase in the levels of super oxide. Significant difference can be observed between 6 and 24 h treated cells with non-irradiated bystander medium. It could be speculated LLRs may be slightly produced in the recipient cells near confluent condition. Addition of ascorbate (AA: 1 mM) at the medium transfer and treated for 24 h to the recipient cells reduced the levels of LLRs as control level, however, that of *N*-acetylcysteine (0.5 mM; NAC) did not. These phenomena of LLRs completely related to the induction of point mutation. AA suppressed the induction of point mutation by the medium transfer in significant level, but NAC did not. These results indicate that LLRs induced in the recipient cells are likely to be responsible for mutation induction.

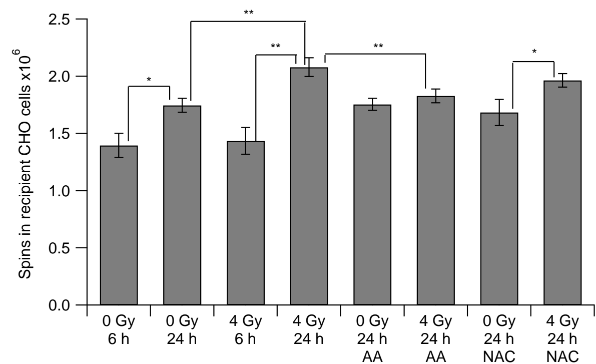

Fig. 1. Levels of LLRs in CHO recipient cells measured by ESR at 77 K. The medium in the flask of donor cells was γ-ray irradiared (0 or 4 Gy) and were transferred to the flask of donor cells after removing pre-existed medium. The bystander medium was treated for 6 or 24 h. AA (*L*-ascorbic acid; 1.0 mM) or NAC (*N*-acetylcysteine; 0.5 mM) were added to the bystander medium and treated for 24 h. * : $p < 0.05$; ** : $p < 0.01$. Values are mean ±S.D.

REFERENCES:

[1] K. Kashino *et al.*, unpublished result (2010).

採択課題番号 22P11-6　　遅発性長寿命ラジカルの直接観測による　　プロジェクト
放射線発がん過程の研究
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PR11-7 Involvement of Mitochondrial Dysfunction in Cancer Development and Progression

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INTRODUCTION: Superoxide dismutase (SOD) is an antioxidant protein converting superoxide to hydrogen peroxide to avoid intracellular oxidative stress. In vertebrate cells, SOD2 is present in the mitochondrial matrix and SOD1 is present in the cytoplasm, nucleus as well as mitochondrial inter-membrane space. Oxidative stress and mitochondrial dysfunction has been considered to be involved in cancer development and progression. To investigate the roles of either SOD1 or SOD2 to quality control of mitochondria, we have conditionally disrupted SOD1, SOD2 gene respectively in DT40 cells. SOD1 depleted cells showed lethal phenotype while depletion of SOD2 showed mild growth defect. In both depleted cells, mitochondrial superoxide levels were significantly increased in concomitant with overall intracellular oxidative stress. Our intriguing finding in this study is that ascorbic acid completely off-set deficiencies caused by depletion either SOD1 or SOD2 including reduction of mitochondrial superoxide levels and intracellular oxidative stress. These data suggested that either SOD1 or SOD2 has important roles of keep superoxide levels in mitochondria and reduced intracellular oxidative stress.

EXPERIMENTS: Chicken DT40 cells 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 SOD1- and SOD2-knock-out DT40 cells were established as described previously (1,2). Intracellular generation of ROS was detected by using BES-So-AM (Wako Pure Chemical Industries Ltd.), a highly specific fluorescent probe for superoxide. Mitochondrial superoxide level was measured by flow cytometry (FACS) after staining with the mitochondrial superoxide-specific dye MitoSOX red (Invitrogen). Mitochondrial membrane potential was measured using JC-1 (Molecular Probes, USA). Intracellular oxidative stress was measured based on the intracellular peroxide-dependent oxidation of DCFH-DA (Molecular Probes, USA).

RESULTS: Because SOD2 is exclusively localized in mitochondrial matrix, amounts of superoxide in mitochondrial were determined by using analysis of Mito-SOX red oxidation. Expectedly, mitochondrial super oxide levels in SOD2 depleted cells were higher than that in the SOD2 expressing cells. Elevated levels of mitochondrial superoxide were also found in SOD1 depleted cells. These data suggested that leaking of superoxide from either mitochondria matrix (SOD2) of inter-membrane

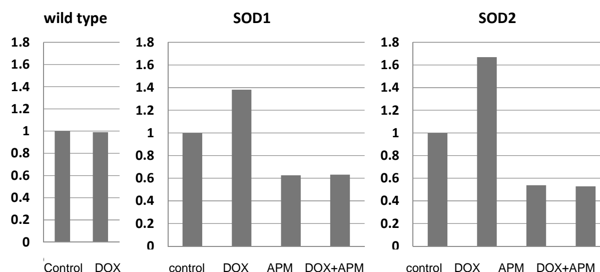

Fig. 1. Intracelluer reactive oxygen spacies level (DCFH Assay 1). Intracellular ROS levels were increased upon deple-tion of either SOD1 or SDO2. APM completely com-pensated for depletion of SOD1 or SOD2 with regard to the increase intracellular ROS levels.

space (SOD1) caused the elevation of mitochondrial superoxide levels. Ascorbic acid worked properly to reduce the amounts of superoxide in mitochondria. It was assumed that increased levels of overall oxidative levels would be elevated followed by increasing amounts of superoxide in SOD1 or SOD2 depleted sells. To confirm so, we stained SOD1 depleted or SOD2 depleted cells with DCFH, cell-permeable fluorescence dye reacting to a broad spectrum of cellular reactive oxygen species reflected by intra-cellular oxidative stress. Both SOD1- and SOD2-depletion caused an increase of oxidative stress. In the presence of ascorbic acid, it was hardly detected the increase of oxidative stress in either SOD1 depleted or SOD2 depleted cells. The mitochondrial membrane potential (ΔΨ) is an important criterion of mitochondrial function. To determine the impact of the elevated mitochondrial superoxide levels in SOD2 depleted cells, the mitochondrial membrane potential (ΔΨ) was analyzed by using a unique cationic dye, JC-1. Abnormal mitochondrial permeability up-transitions were observed in SOD2 depleted cells. Abnormal mitochondrial membrane potential observed in SOD2 depleted cells was completely recovered in the presence of ascorbic acid. While significant elevation of mitochondrial superoxide levels in SOD1 depleted cells, there were no significant difference of mitochondrial membrane potential in SOD1 depleted cells. Because SOD1 presents in the inter-membrane space of mitochondria, these SOD1 fraction is sufficient to keep normal inter-membrane potential or cytoplasmic-SOD1 activity completely clean up superoxide to prevent mitochondrial dysfunction. Above the results so far suggested that mitochondrial SOD has critical functions of mitochondrial quality and ascorbic acid works completely mimic for either SOD1 or SOD1.

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