VIII-II-1. Project Research

Project 3

PR3 Biological Effects of Low Dose Radiation

M. Watanabe

Research Reactor Institute, Kyoto University

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.

Therefore, the aim of this project is focused on elucidation of non-genetic factors related to carcinogenesis.

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

ARS-1: Non-DNA Target of Radiation Carcinogenesis
(M. Watanabe, H. Yoshii, H Nawata, G. Kashino, K. Watanabe, and K. Tano)

ARS-2: Low Dose Radiation-Induced Telomeric Instability and Carcinogenesis
(S. Kodama, M. Takebe, K. Shiraishi, and M. Watanabe)

ARS-3: Effect of Vitamin C on Radiation Induced Thymic Lymphomagenesis and *Ikaros* Mutation
(S. Kakinuma, Y. Amasaki, N. Kowatari, K. Yamau- chi, M. Nishimura, T. Imaoka, K. Ariyoshi, G. Kashi- no, M. Watanabe, and Y. Shimada)

ARS-4: Slow-Releasing Long-Lived Radicals for Radiation Carcinogenesis
(J. Kumagai, K. Miura and M. Watanabe)

ARS-5: Aneuploidy Induced by Low Dose Radiation and Carcinogenesis
(A. Yamamoto, M. Imai, R. Matsuno, S. Sawai, L.R. Lhenimi and K. Yamamoto)

ARS-6: Expression profile of cancer-related genes in response to low dose radiation
(K. Suzuki, A. Koga, K. Niwa, K. Sakai, T. Nitta and M. Watanabe)

ARS-7: Biological Responses to Low Dose Radiation
(K. Sakai, and 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 (ARS-1 and 4). 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 (ARS-1, 2 and 5). Telomere destabilization induces telomere fusion and makes dicentric chromosome (ARS-2) and reason of chromosome instability. In fact, dicentric chromosome is dominant aberration induced by low dose radiation (ARS-1 and 2). Radiation induced radicals also attacked centrosome (ARS-1). Centrosome destabi- lization induces nondisjunction and raises the frequency of aneuploid (ARS-1 and 5). In early process of cell transformation, structural aberration of chromosome does not occur, but aneuploid is seen in high frequency (ARS-1). Aneuploid is also induced by deficiency in cell cycle checkpoint at G_2-M (ARS-5). By treatment of vitamin C prevent induction of LLRs, aneuploid, and thymic lymphomas in B6C3F1 mice (ARS-3 and 4). Interestingly, the thymic lymphomas in vitamine C treated mice lacked point mutation of *Ikaros*, suggesting a suppression of point mutation by VC (ARS-3 and 4).

Low dose radiation activated repair capacity of DNA damage in irradiated cells (ARS-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 (ARS-1 and 6).

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 presentation.

PR3-1 Mechanism of Non-Targeted Effects of Radiation

M. Watanabe, H. Yoshii, H. Nawata, G. Kashino, K. Watanabe and K. Tano

Research Reactor Institute, Kyoto University

INTRODUCTION: It has been believed that the first target of radiation carcinogenesis is DNA. However, this hypothesis is not proved for carcinogenesis of low dose radiation directly yet. However, this hypothesis is not proved as route for carcinogenesis of low dose radiation yet. We analyzed our results of research of malignant cell transformation by low dose radiation during the past 30 years and came to strongly believe that a radiation cancer-causing primary target was not DNA itself. One evidence which supports our thought is that transformation frequency in Syrian hamster embryo fibroblast (SHE) cells irradiated with low dose of X-rays is 500〜1,000 times higher than that of somatic mutation [1-3]. This contradicts "the multistage mutation theory" which carcinogenesis produces in accumulation of 3〜5 independent mutations. Transformation frequency should be smaller than independent mutation frequency theoretically.

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 atmospheric (20%) and physiological (hypoxic; 2% and 0.5%) oxygen conditions, and measured cell growth, levels of intracellular oxidative stress, and quantity and function of mitochondria of each.

RESULTS: 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 in ME, SHE cells and HE cells [6,7]. These radicals attack several proteins, such as telomere related protein and centrosome, and destroy their structure [8,9]. Telomere destabilization induces telomere fusion and makes dicentric chromosome. In fact, dicentric chromosome is dominant aberration induced by low dose radiation [10]. Centrosome destabilization induces non-disjunction 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.

DISCUSSION: These results suggest a possibility that a main target of radiation carcinogenesis is not DNA, but is centromere and 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 hypothesis about radiation carcinogenesis, which was named as "Protein Target Theory".

REFERENCES:

[1] Watanabe M, Horikawa M, Nikaido O, *Radiat. Res.,* **98** (1984) 274-283.

[2] Watanabe M, Suzuki K, Kodama S, *Cancer Res.,* **50(3)** (1990) 760-765.

[3] Watanabe M, Suzuki K, *Mutat. Res.,* **249(1)** (1991) 71-80.

[4] Suzuki K, Yasuda N, Suzuki F, Nikaido O, Watanabe M, *Int. J. Cancer,* **44(6)** (1989) 1057-1061.

[5] Kodama S, Mori I, Roy K, Yang Z, Suzuki K, Watanabe M, *Radiat. Res.,* **155 (1 Pt 2)** (2001) 254-262.

[6] Yoshii H, Watanabe M, J. Radiat. Res., **50** (2009) 127-137.

[7] Harada T, Kashino G, Suzuki K, Matsuda N, Kodama S, Watanabe M, Int. J. Radiat. Biol., **379** (2009) 809-814.

[8] Urushibara A, Kodama S, Suzuki K, Desa M.B., Suzuki F, Tsutsui T, Watanabe M, *Biochem. Biophys. Res. Commun.,* **313(4)** (2004) 1037-1043.

[9] Undarmaa B, Kodama S, Suzuki K, Niwa O, Watanabe M, *Biochem. Biophys. Res. Commun.,* **315(1)** (2004) 51-58.

[10] Roy K, Kodama S, Suzuki K, Fukase F, Watanabe M, *Radiat. Res.,* **154(6)** (2000) 659-666.

採択課題番号 20P3-1　低線量放射線による非標的影響発現メカニズムに関する研究　プロジェクト（京大・原子炉）渡邉正己（京大院・理）吉居華子、吉川智裕

PR3-2 Low Dose Radiation-Induced Telomeric Instability and Carcinogenesis

S. Kodama, M. Tanabe, K. Shiraishi and M. Watanabe[1]

Organization for University-Industry-Government Cooperation, Osaka Prefecture University
[1]Research Reactor Institute, Kyoto University

INTRODUCTION: Chromosomal instability is a hallmark of most cancer cells. However, the biological significance of chromosome aberrations differs in types of cancer [1]. In hematological malignancies and soft tissue sarcomas, most of which are common in childhood cancers, non-random chromosome translocations have a crucial role in the initial steps in carcinogenesis as a driving force in developing cancers. In contrast, in epithelial cancers, most of which are common in adulthood cancers, chromosome aberrations are random, and the clonal expansion of cells with a specific translocation is rare [2]. Therefore, the role and mechanism of chromosomal instability in carcinogenesis still remain poorly understood. In the last KURRI Progress Report 2007, we reported the study of chromosome aberrations in mouse cells transferred with unirradiated and irradiated human chromosomes 6 and 8, and concluded that the chromosome instability could be transmitted via the directly irradiated chromosome into the progeny of unirradiated cells. To confirm this conclusion further, in the present study, we transferred an irradiated human chromosome 8 in unirradiated mouse recipient cells and examined the stability of an irradiated human chromosome 8 by fluorescence *in situ* hybridization.

MATERIALS AND METHODS:
Cells and Cell Culture: Mouse A9 cells containing a single copy of human chromosome 8 were used as chromosome donors. Mouse m5S cells established from embryonic skin fibroblasts were used as recipient cells.
Chromosome Transfer: The method for microcell-mediated chromosome transfer was described previously [3].
X-irradiation: The donor cells were irradiated with 4 Gy of X-rays using a soft X-ray generator (OM-B205; OH-Mic, Tokyo) operating at 70 kVp and 5 mA with a 0.5 mm Al filter at a dose rate of 0.596 Gy/min.
Chromosome Samples: Metaphases were harvested by the treatment with 60 ng/ml Colcemid for 30 min, treated with hypotonic KCl (0.075 M) solution for 25 min, and fixed in fixative (methanol:acetic acid, 3:1). The mitotic cell suspensions were dropped onto a slide glass and dried for 24 h at room temperature.
Fluorescence In Situ Hybridization: The stability of the human chromosomes 8 was investigated by fluorescence *in situ* hybridization (FISH) as described previously [3].

RESULTS AND DISCUSSION: *Stability of an Unirradiated Human Chromosome*: We transferred an unirradiated human chromosomes 8 into the unirradiated mouse m5S cells by microcell fusion, isolated the microcell hybrids, and then examined the stability of the transferred human chromosomes 8 in two microcell hybrids using whole chromosome painting-FISH (WCP-FISH). No structural abnormalities were detected in two microcell hybrids, indicating an unirradiated human chromosome was structurally stable in mouse m5S cells. However, as observed in the previous study, most (89%) of one of two microcell hybrids had two copies of human chromosome 8. Combined with the result in the last report, five out of seven (71%) microcell hybrids transferred with human chromosome 8 showed an extra copy of chromosome 8. This indicates that a human chromosome 8 tends to double in mouse recipient cells and that radiation effect is not involved in the increase of copy number of chromosome 8.
Stability of an Irradiated Human Chromosome: We examined the stability of X-irradiated human chromosome 8 in eight microcell hybrids. In contrast to the microcell hybrids transferred with the unirradiated chromosome 8, three (4X8-3, 5X8-7, and 5X8-16) out of eight microcell hybrids transferred with the irradiated human chromosome 8 exhibited more than three different types of aberrations and those cells with the aberrations consisted of more than 11% of total cell populations. This confirms the previous our result and indicates that the irradiated chromosome is unstable as compared with the unirradiated chromosome, suggesting that radiation-induced lesions that trigger subsequent rearrangements involving the irradiated chromosome remain at long times after exposure. The copy number of the irradiated human chromosome 8 increased in five out of eight microcell hybrids, further indicating that the increase of the copy number occurred independently of radiation exposure.
Conclusions: This study confirms the previous our conclusion that chromosomal instability can be transmitted via the irradiated chromosome to the clonal progeny of unirradiated cells. This suggests that the irradiated chromosome itself enhances the potential for genomic rearrangement, and this promotes genomic instability possibly involved in radiation carcinogenesis. Further study is needed to clarify the involvement of telomeric instability in inducing chromosome instability observed in the present study.

REFERENCES:
[1] F. Mitelman *et al*., Nat. Rev. Cancer, **7** (2007) 233-245.
[2] R. A. DePinho, Nature, **408** (2000) 248-254.
[3] N. Mukaida *et al*., Radiat. Res., **167** (2007) 675-681.

採択課題番号 20P3-2　低線量放射線によるテロメア不安定化と発がん　　プロジェクト
（大阪府立大・産学官連携機構）児玉靖司、白石一乗（京大・原子炉）渡邉正己

PR3-3 Effect of Vitamin C on Radiation Induced Thymic Lymphomagenesis and *Ikaros* Mutation

S. Kakinuma, Y. Amasaki, N. Kowatari, K. Yamauchi, M. Nishimura, T. Imaoka, K. Ariyoshi[4], G. Kashino, M. Watanabe[1] and Y. Shimada

Experimental Radiobiology for Children's Health Research Group, National Institute of Radiological Sciences
[1]Research Reactor Institute, Kyoto University

INTRODUCTION: The DNA damage of radiation is caused by both direct action and indirect action. Indirect action is mediated by not only short-lived but also long-lived radicals. The generation of radiation-induced long-lived radicals in cells is reported to be suppressed by addition of vitamin C (VC)[1)]. Furthermore, post-treatment of VC on irradiated cells reduces the frequency of mutations and morphological transformations, suggesting long-lived radicals may be involved in these biological phenomena[2)]. The reduction of point mutation by the post-treatment of VC was also found following neutron irradiation[3)] and high-LET carbon-ion irradiation[4)]. However, the data on the role of VC and long-lived radicals in radiation carcinogenesis *in vivo* are still insufficient.

Mouse thymic lymphomas (TL) can be reproducibly induced by exposure to ionizing radiation or chemical carcinogens. This animal model is considered useful in the search for characterization of genes involved in the development of human acute T-lymphoblastic leukemia. In X-ray induced TL, we previously found a unique locus with a high frequency of loss of heterozygosity (LOH) (50%) in the centromeric region of chromosome 11[5)]. In contrast, LOH at this locus was very rarely observed in *N*-ethyl-*N*-nitrosourea (ENU)-induced or spontaneously developing lymphomas, suggesting that it was a radiation-associated molecular change. In this LOH locus, we mapped *Ikaros*, which is a transcription factor that plays a critical role in both lineage commitment and differentiation of lymphocytes[6)]. The molecular analysis of Ikaros in the X-ray-induced TL was caused by several alterations; null expression, splicing alteration, point mutation and small insertion/deletion, most of which were associated with LOH[7,8)]. Further, chromosomal analysis showed that LOH at *Ikaros* locus was generated by intra-chromosomal deletion[9)]. In addition, spectrum of K*ras* mutation differed between X-ray-induced and ENU-induced-TL[10,11)].

To clarify an involvement of long-lived radical in radiation carcinogenesis, we examined the effect of post-treatment of VC on induction of TL and the mutation of *Ikaros* and K*ras* genes.

EXPERIMENTS: Four-weeks old female B6C3F1 mice were exposed to X-rays (1.4 Gy per week) for 4 consecutive weeks. VC derivative sodium-L-ascobyl-2-phosohate was then administered as anti-radical agent (100 mg / kg). Four experimental groups were set, (i) irradiation alone (X), (ii) irradiation followed by VC just after each X-irradiation (X+VC4), (iii) irradiation with additional VC treatment for subsequent three months (X+VC13) and (iv) (X+VC4) with additional VC treatment for subsequent three months (X+VC16), each group contained 40 mice. The control groups, which were treated with saline instead of VC solution, were also set (X+Saline4, X+Saline13, X+Saline16, 20 mice each). In total, 260 mice were used in this study. We have analyzed lifespan of mice, latency of TL induction and LOH at chromosome 11 in the TLs. Also, the point mutation, and the protein expression of both tumor suppressor gene *Ikaros* and oncogene K*ras* have been studied. To determine the accumulation of point mutation after radiation exposure, *gpt-delta* mice, which are transgenic mice for mutation analysis, was also irradiated as described above.

RESULTS: Latent period of TL induction was not significantly different among the groups, suggesting VC has little effect for tumor induction at least in this experimental protocol. Frequency of LOH among the groups, X+VC4 (45%:10/22), X+VC13 (59%:13/22), X+VC16 (38%:8/21), and X+Saline (61%:19/31) were not significantly different. However, the results suggest that clearance of long-lived radicals by VC treatment immediately after irradiation might reduce LOH generation.

The molecular analysis in the mutation of *Ikaros*, *Kras* and *gpt-delta* gene in the stored lymphomas induced in this year has been investigated.

REFERENCES:

[1] S. Koyama *et al.*, Mut. Res., **421** (1998) 45-54.
[2] J. Kumagai *et al.*, Radiat. Res., **160** (2003) 95-102.
[3] Y. Kinashi *et al.*, Applied. Radiat. Isotopes., **61** (2004) 929-932.
[4] C.A. Waldren *et al.*, Mut. Res., **551** (2004) 255-265.
[5] Y. Shimada *et al.*, Radiat. Res., **154** (2000) 293-300.
[6] K. Georgopoulos *et al.*, Science, **258** (1992) 808-812.
[7] S. Kakinuma *et al.*, Radiat. Res., **157** (2002) 331-340.
[8] S. Kakinuma *et al.*, Mut. Res., **572** (2005) 132-141.
[9] M. Yoshida *et al.*, Cance. Genet. Cytogenet., **179** (2007) 1-10.
[10] Y. Shimada *et al.*, Int. J. Radiat. Biol., **77** (2001) 465-473.
[11] Y. Shimada *et al.*, Int. J. Radiat. Biol., **79** (2003) 423-430.

採択課題番号 20P3-3　　低線放射線の生体影響に関する研究　　プロジェクト
（放医研・発達期）柿沼志津子、有吉健太郎、島田義也、山田　裕（京大・原子炉）渡邉正己

PR3-4 Slow-Releasing Long-Lived Radicals for Radiation Carcinogenesis

J. Kumagai, K. Miura and M. Watanabe[1]

Graduate School of Engineering, Nagoya University
[1]Research Reactor Institute, Kyoto University

INTRODUCTION: Bystander effects are one of the most interesting topics in radiation biology because non-irradiated cells have been suffered to show radiation effects by some signals from irradiated cells indirectly. [1-2] Recently, it becomes very clear that there are soluble bystander factor in culture medium induced from irradiated cells. Bystander factor can cause some aberrations to non-irradiated recipient cells like radiation effects.

In this study, we have directly measured electron spin resonance (ESR) spectra of the recipient cells and confirmed that levels of slow releasing long-lived radicals (SRLLRs) in the cells increase by medium mediated bystander effect, which is consistent to the induction of point mutation.

EXPERIMENTS: Chinese Hamster Ovary (CHO) cells as donor of bystander factor were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum and gamma-ray irradiated (1 Gy). The medium in the flasks were exchanged to new ones at 24 h after irradiation and kept for 24 h to be “bystander medium” including bystander factor. Other CHO cells as recipient were then exposed to the bystander medium for 24 h, harvested and introduced to an ESR quartz tube. ESR spectra of recipient cells were measured at 77 K.

RESULTS: Fig. 1 shows the levels of radicals in the recipient cells. The levels of radicals in the recipient cells increased in 20% by the medium transfer. The increase could not be detected when medium was irradiated without donor cells. The increased radicals are denoted as slow releasing long-lived radicals (SRLLRs) those are increased by the bystander factor produced from irradiated donor CHO cells. Increase in mutation frequency for four times were observed by exposing to bystander medium, so that SRLLRs might be related to mutation induction.

A myxothiazol (Myx.) for blocking electron transportation in mitochondria [3] was treated to the culture medium of donor cells for 2 h before irradiation (0.5 μM), a new medium was replaced in the flasks of donor cells and irradiated in 1 Gy. The irradiated one as bystander medium was exposed to recipient cells for 24 h. Increase in

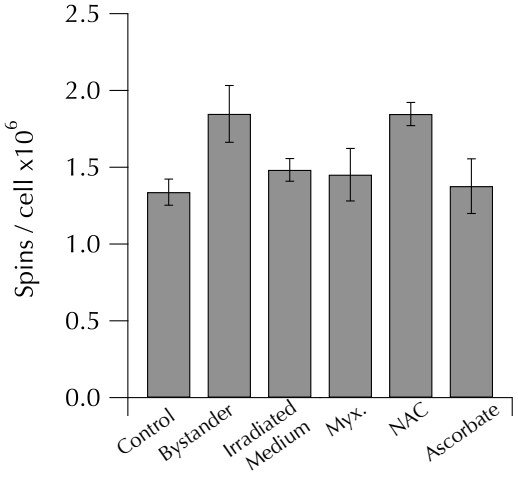

Fig. 1. Concentration of radicals in the recipient CHO cells with individual treatments.

the levels of SRLLRs in CHO recipient cells could not be detected by the treatment of myxothiazol. It indicates that dysfunctional mitochondria in irradiated donor cells produce the bystander factor to induce bystander effects.

When a *N*-acetylcysteine (NAC) and an ascorbate were added to the bystander medium just before exposing to recipient cells in 5 and 1 mM, respectively, levels of SRLLRs in the recipient cells did not decrease in the case of NAC but in ascorbate. This is also consistent with mutation analysis of $HPRT^-$ locus that the NAC treatment could not reduce the mutation frequency but ascorbate could [4]. These results indicate that SRLLRs might be responsible for the induction of mutation which caused by bystander factor. It is very interesting why ascorbate can reduce mutation and levels of SRLLRs simultaneously but NAC cannot although both chemicals have similar function and ability of reducing oxidative chemicals such as radicals. Bystander effect through irradiated medium with cells as donors of bystander factor might be related to radiation carcinogenesis, and the mechanisms must be elucidated as our future work.

REFERENCES:

[1] G. Kashino*, et al.,* Mutat. Res., **619** (2007)**,** 134-138.
[2] K. Prise M., Occupational Medicine-Oxford, **56** (2006) 156-161.
[3] Thierbach G. *et al.,* Biochim. Biophys. Acta,, **638** (1981) 282-289.
[4] G. Kashino, Private communication.

採択課題番号 20P3-4　　放射線発がんにおける長寿命ラジカルの関与　　プロジェクト
（名大院・工）熊谷　純、三浦和人、白坂公宏、清水裕太（京大・原子炉）渡邉正己

PR3-5 Aneuploidy Induced by Low Dose Radiation and Carcinogenesis

A. Yamamoto, M. Imai, R. Matsuno, S. Sawai, L.R. Lhenimi and K. Yamamoto

Graduate School of Life Sciences, Tohoku University

INTRODUCTION: It has been predicted that mis-segregation during cell divisions could result to chromosome instability (CIN), also known as aneuploidy, leading cancer. Human cancer cells frequently have abnormal chromosome complements suggesting that aneuploidy may cause cancer. On the other hand, the concept that cancer develops through the accumulation of gene mutations of protooncogenes and/or tumor-suppressor genes is widely accepted. Thus, whether aneuploidy is a contributing factor, or merely a consequence of tumorigenesused is has long been debated.

To understand whether aneuploidy has a causal role in tumor formation, we made a usage of *Saccharomyces cerevisiae* and HCT116 human cell. We investigated the effect of chemicals that were tested negative in Ames system and positive in tumor formation or positive in Ames system and negative in tumor formation. In general, the presence of Ames system negative carcinogen suggests the absence of gene mutation for tumorigenesis. We observed that Ames test negative carcinogens can cause G2/M arrest and aneuploidy, on the other hand, Ames test positive non-carcinogens can cause gene mutation and recombination but not aneuploidy. Thus, we argue that aneuploidy is a contributing factor for carcinogenesis.

EXPERIMENTS: We constructed genetic system to measure aneuploidy in yeast [1]. Cell cycle progression was measured by FACS analysis. Budding or spindle morphology was observed by microscopic analysis. Western blotting was performed to understand the signals for aneuploidy formation. The human colon cancer cell line HCT116 was used to measure aneuploidy [2]. Immunofluorescence microscopy was used to measure the amount of DNA damage, and the activation of signal proteins. Cell cycle progression was measured by FACScan analysis. Western blotting was performed to understand cleavage, and phosphorylation of signal proteins.

RESULTS: As a representative of Ames test negative carcinogens, we used phenyl hydroquinone (PHQ), which is a hepatic derivative of fungicide o-phenyl phenol. Using yeast system [3], we demonstrate that PHQ can arrest the cell cycle at the G2/M transition as a result of the stabilization of Swe1 (Wee1 homolog), probably leading to inactivation of the Cdc28 (Cdk1/Cdc2 homolog). Furthermore, Hog1 (p38 MAPK homolog) was robustly phosphorylated by PHQ, which can stabilize Swe1. On the other hand, Chk1 and Rad53 were not phosphorylated by PHQ, indicating that Mec1/Tel1 DNA damage checkpoint was not functional. Mutation of *swe1* and *hog1* abolished the PHQ-induced arrest at the G2/M transition and became resistant to PHQ lethality and aneuploidy formation. These data suggest that PHQ-induced G2/M transition checkpoint which is activated by the Hog1-Swe1 pathway plays a role in the formation of aneuploidy.

To further delineate the mechanism of action of PHQ, we examined its effect on human cells [2]. Treatment of the colon cancer cell line HCT116 with 0-150 mM PHQ caused a concentration-dependent inhibition of growth, accumulation of cells having G2/M DNA content, and increase in the mitotic index, suggesting delay at metaphase to anaphase transition. Moreover, a dose-dependent increase in apoptotic cells was observed. Finally, a high frequency of aneuploid cells was found. No increase in γ-H2AX foci was observed. The results show that PHQ does induce mitotic arrest, apoptosis and aneuploidy in the absence of DNA damage. We next demonstrated, using histone H3 antibody, that PHQ caused G2/M delay. G2/M delay, but not metaphase/anaphase delay, is directly correlated with aneuploidy, since p53-null cell lines did not show G2/M delay and not aneuploidy. Thus, even in the absence of DNA damage, PHQ activates p53 phosphorylation, causes G2/M delay which is probably the signal for centrosome amplification, leading to aneuploidy.

REFERENCES:

[1] Y. Daigaku *et al*., Mutat. Res., **556** (2004) 183-191.
[2] M. Imai *et al.*, (2009) Gene Genet. Systems, **84** (2009) 73-79.
[3] A. Yamamoto *et al*., FEBS J., **275** (2008) 5733-5744.

採択課題番号20P3-5　　低線量放射線による染色体異数化と発がん　　プロジェクト
（東北大院・生命）今井　勝、山本和生（京大・原子炉）渡邉正己

PR3-6 Expression Profile of Cancer-Related Genes in Response to Low Dose Radiation

K. Suzuki, A. Koga[1], K. Niwa[1], K. Sakai[1], T. Nitta[1] and M. Watanabe[2]

Graduate School of Biomedical Sciences Nagasaki University
[1]Research and Development Center for Higher Education, Nagasaki University
[2]Research Reactor Institute, Kyoto University

INTRODUCTION: Ionizing radiation induces various types of genes, whose products are involved in regulation of cellular response to radiation. Among which, p53 protein is the most well-studied protein, whose function is now recognized as tumor suppressor. In response to ionizing radiation, p53 protein is stabilized and activated as a transcription factor. It binds to DNA sequences, which collectively called p53 responsive elements, and the binding of p53 protein to its responsive elements secondary modifies the local structure of chromatin by recruitment of histone acetylase, such as p300/CBP. As a result, a group of genes is either up-regulated or down-regulated. Interestingly, the genes up-regulated include those related to cell cycle regulation. The most studied gene up-regulated is $p21^{WAF1/Cip1}$. The $p21^{WAF1/Cip1}$ gene was identified as the gene regulated by the wild-type p53 protein, but, it also independently isolated as a gene, whose transcription was increased in senescent cells. Subsequently, it turns out to be clear that $p21^{WAF1/Cip1}$ protein is a small protein inhibitor for cyclin-dependent protein kinases (Cdks). It specifically binds to Cyclin D/Cdk4 and Cyclin D/Cdk6 as well as Cyclin E/Cdk2. Because these cdks phosphorylate RB protein to liberate E2F, which is the transcription factor essential for G1 to S transition, inhibition of these kinases results in cell cycle arrest. Thus, p53- $p21^{WAF1/Cip1}$ axis is indispensable for cellular response to ionizing radiation, particularly in cells exposed to low dose ionizing radiation.

Recent evidences have presented that normal human cells are able to response to very low-dose ionizing radiation. Cellular responses, such as adaptive response and low-dose hyper sensitivity, are the representations of such responses to low-dose ionizing radiation. So far, several studies have shown that p53- $p21^{WAF1/Cip1}$ axis can respond to low-dose radiation exposure, however, unsolved question is how cells can sense intracellular changes caused by such low absorption of energy. Thus, it can be hypothesized that there is a kind of a mechanism amplifying DNA damage response. The purpose of this study is to identify the upstream mechanism to p53 that amplify DNA damage signal in normal human cells.

EXPERIMENTS: Normal human diploid cells were cultured in MEM medium supplemented with 10% fetal bovine serum. The cells were cultured in T25 flasks in order to maintain exponential growth. The cells were irradiated with X-rays at a dose rate of 1 Gy/min.

To perform immunofluorescence, cells were cultured on 22 mm x 22 mm cover slips in 35 mm culture dishes, and incubated for 24 hours. After irradiation, cells were washed with PBS twice, and fixed in 4% formalin for 10 min at room temperature. The cells were then permeabilized with 0.5% Tween-20 for 5 min on ice. DNA double strand breaks were detected using monoclonal antibody against phosphorylated ATM at serine 1981. The primary antibody is mixed in TBS-DT, and the cells were incubated with the primary antibody for 2 hours. Then, the primary antibody was detected by incubating cells with anti-mouse IgG antibody labeled with Alexa488. The samples were examined under a flurescence microscope, and the digital images were obtained by a software (FW4000).

RESULTS: After irradiation, phosphorylated ATM forms discrete foci in the nucleus. The number of phosphorylated ATM foci was correspond to that of estimated number of DNA double strand breaks, indicating that phosphorylated ATM foci are formed at DNA breaks. Interestingly, the size of phosphorylated ATM foci was dramatically changed after irradiation, the initial foci were detected immediately after irradiation, but its size was less than 0.1 um. Thirty min after irradiation, it became to up to 1 um. Such changes in foci size indicate an amplification of DNA damage signal, therefore, total amount of phosphorylated ATM molecules were measured by using SOID (Sum of integrated density of fluorescence). As the result, it became clear that the number of phosphorylated ATM molecules were increasing after irradiation. Using a specific inhibitor for ATM kinase activity, we found that subsequent activation of ATM requires ATM kinase activity itself. Thus, it can be concluded that DNA double strand break repair process secondary induces change in the chromatin structure, which lead to a secondary activation of ATM.

In order to prove that amplification is essential for DNA damage response in cells exposed to low-dose radiation, p53 activation was determined by examining phosphorylation of p53 at serine 15. We confirmed that without amplification p53 could not receive enough signal to be activated as a transcription factor. Thus, amplification of DNA damage signal is a essential process, which must be an acquired mechanism evolved in cells evolved in an environment with natural low-dose radiation.

採択課題番号 20P3-6　　低線量放射線による発がん関連遺伝子発現の特徴　　プロジェクト
（長崎大院・医歯薬学総合）鈴木啓司、山内基弘、岡　泰由（京大・原子炉）渡邉正己
（長崎大・大教センター）古賀掲維、丹羽量久、坂井一也、新田高士

PR3-7 Biological Responses to Low Dose Radiation

K. Sakai and M. Watanabe[1]

Research Center for Radiation Protection, National Institute of Radiological Sciences
[1]Research Reactor Institute, Kyoto University

INTRODUCTION: From the viewpoint of radiological protection, it has been assumed that radiation-induced cancer risk increases in a proportional manner to dose, no matter how low the dose is. This assumption was based on an extrapolation of data obtained at higher dose range to lower dose range where actual experimental data had been limited. However, recent advances in radiation biology have revealed that biological system responds to low level radiation; typical examples are genomic instability, bystander effects, and adaptive responses. These responses/effects would possess a potential to affect the risk from low dose radiation; the risk may be lower or higher than that expected from the linear extrapolation.

EXPERIMENTAL DATA:

Genomic Instability

If cancer is caused by accumulation of multiple somatic mutations, the frequency of carcinogenesis shown as malignant transformation of a cell should be less than the frequency of mutation. As a matter of fact, however, the frequency of the malignant transformation is more frequent than that of mutagenesis (see ref. 1 for review). This strongly suggests that some system which is responsible to keep genetic information stable is affected in the irradiated cells.

Bystander Effects

At a low flux of alpha-particles, not only hit cells, but also non-hit cells located close to the hit cells showed some types of chromosome aberrations [2]. This phenomenon is called bystander effects. Mechanistic studies have suggested at least two types of signal transfer are involved; signal transduction via “gap junction”, cellular structures connecting two adjacent cells, signaling through some types of molecules secreted from the hit cells [3].

Adaptive Responses

Adaptive response have been defined as radioresistance, induced by pre-irradiation with small dose, against larger doses given after certain period of interval. The first report on the adaptive response was the reduced X-ray-induced chromosom aberration after the treatment with ^{3}H-thymidin [4]. In addition to the chromosome aberrations, the endpoints reported include micronuclei [5], malignant transformation [6], and cell survival [7]. The induction of radioresistance by a small dose of radiation was observed also at whole body level [8]. Half a Gy of conditioning dose given 2 weeks before the lethal dose increased the survival of ICR mice significantly.

The adaptive response has also been observed with the endpoints of malformation [9], and radiation-induced [10, 11] or carcinogen-induced [12] tumorigenesis.

DISCUSSION: The genomic instability has been thought to increase the risk at low dose area. The bystander effects enlarge the target of radiation to increase the risk at low dose area. The adaptive response, on the other hand, certainly works in a protective way against radiation damage, suggesting lower risk at low dose range than currently predicted based on the linear extrapolation. Although there have been intensive discussion on whether these effects/responses really affect the low dose risk, they have not been taken into account in the radiation protection system. Epidemiological studies do not have enough resolution to determine the shape of relationship between dose and risk around and below 100 mSv [13]. To reduce the uncertainty in the estimation of risk from low dose radiation we need to understand the impact of these events on tumorigenic effects, as well as their mechanisms and/or genetic background.

REFERENCES:

[1] M. Watanabe, Radioisotopes, **32** (1983) 249-259.
[2] H. Nagasawa and J. B. Little, Cancer Res., **52** (1992) 6394-6396.
[3] W.F. Morgan and M. B. Sowa, Mutat. Res., **616** (2007) 159-164.
[4] G. Olivieri *et al*., Science, **223** (1984) 594-597.
[5]E. J. Broome *et al*., Radiat. Res., **158** (2002) 181-186.
[6] E. I. Azzam *et al*., Radiat. Res., **138** (1994) S28-31.
[7] J. L. Schwartz *et al*., Int. J. Radiat. Biol., **83** (2007) 153-159.
[8] M. Yonezawa *et al.*, Mutat. Res., **358** (1996) 237-243.
[9] B. Wang *et al*., Radiat. Res., **154** (2000) 277-282.
[10] P. Dange *et al*., J. Environ. Pathol. Toxicol. Oncol., **26** (2007) 273-279.
[11] Y. Ina *et al*., Radiat. Res. ,**163** (2005)153-158.
[12] K. Sakai *et al*., Int. J. Low Radiat., **1** (2003) 142-146.
[13] D.L. Preston *et al*, Radiat. Res., **85** (2003) 4-12.

採択課題番号 20P3-7　　低線量放射線に対する生体応答反応　　プロジェクト
（放医研・放射線防護研究センター）酒井一夫（京大・原子炉）渡邉正己