Potential Clinical Implication of LDR Hormesis and Adaptive Response: Original Research

Reduction of Delayed Homologous Recombination by Induction of Radioadaptive Response in RaDR-GFP Mice (Yonezawa Effect): An Old Player With a New Role

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Abstract
Radiotherapy (RT) treats cancer effectively using high doses of ionizing radiation (IR) to kill cancer cells and shrink tumors while bearing the risk of developing different side effects, including secondary cancer, which is most concerning for long-term health consequences. Genomic instability (GI) is a characteristic of most cancer cells, and IR-induced GI can manifest as delayed homologous recombination (HR). Radioadaptive response (RAR) is capable of reducing genotoxicity, cell transformation, mutation, and carcinogenesis, but the rational evidence describing its contributions to the reduction of radiation risk, in particular, carcinogenesis, remains fragmented. In this work, to investigate the impact of RAR on high-dose, IR-induced GI measured as delayed HR, the frequency of recombinant cells was comparatively studied under RAR-inducible and -uninducible conditions in the nucleated cells in hematopoietic tissues (bone marrow and spleen) using the Rosa26 Direct Repeat-green fluorescent protein (RaDR-GFP) homozygote mice. Results demonstrated that the frequency of recombinant cells was significantly lower in hematopoietic tissues under RAR-inducible condition. These findings suggest that reduction in delayed HR may be at least a part of the mechanisms underlying decreased carcinogenesis by RAR, and application of RAR would contribute to a more rigorous and scientifically grounded system of radiation protection in RT.

Keywords
adaptive response, ionizing radiation, genomic instability, homologous recombination, rosa26 direct repeat-GFP (RaDR-GFP) mice

Introduction
Radiotherapy (RT) treats cancer effectively using high doses of ionizing radiation (IR) to kill cancer cells and shrink tumors. More than 50% of all patients with cancer need RT as an integral part of treatment at some point of the time. On the other hand, RT bears the risk of developing different side effects including secondary cancer, which is one of the important late side effects causing the great concern for long-term health consequences and impacting on optimal treatment decision-making.¹⁴

Genomic instability (GI), the accumulation of multiple changes to convert a stable genome to an unstable genome, is characterized by varied end points, for example, chromosomal rearrangements and aberrations, amplification of genetic material, micronucleus formation, and gene mutation. The IR is capable of inducing GI in mammalian cells, manifesting as delayed homologous recombination (HR) in vitro and in vivo.

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Radioadaptive response (RAR) is an evolutionary conserved phenomenon in which exposure to a low dose of IR (priming dose) reduces the biological effects of subsequent higher IR doses (challenge dose). Since the discovery of RAR, it has been demonstrated in a variety of biosystems from simple prokaryotes to higher eukaryotes including mammalian animals with end points such as DNA damage, chromosomal aberrations, cell transformation, cell death, and mutation in vitro and prenatal death, malformation, hematopoietic death, and carcinogenesis in vivo. There is a great potential for use of low-dose IR for certain diseases as treatment or prevention and for improved treatment. Of note, RAR is demonstrated in the normal cells but cancer cells including cancer stem cells in vitro and in vivo, revealing its potential application to improve cancer RT by priming the normal tissues to protect multiple organs from the side effects of RT including the secondary cancer. As a fact, RAR is capable of reducing genotoxicity, cell transformation, mutation, and carcinogenesis, while the rational evidence describing its contributions to the reduction in radiation risk, in particular, carcinogenesis, remains fragmented. In addition, as a major concern regarding the application of RAR, it may protect cells initially but predispose surviving cells to increased GI later.

In our previous study, we used the RAR mouse model (Yonezawa Effect) established by Yonezawa and colleagues, which could rescue bone marrow death through induced resistance in the hematopoietic system. We verified and confirmed this model under the setup in our experimental facility and observed the reduction of high-dose, IR-induced GI measured as frequency of micronucleus erythrocytes in bone marrow cells in surviving mice rescued by RAR. In this work, we investigated the impact of RAR on high-dose, IR-induced GI measured as delayed HR. The frequency of recombinant cells was comparatively studied under RAR-inducible and -uninducible conditions in hematopoietic tissues (bone marrow and spleen) using the Rosa26 Direct Repeat-green fluorescent protein (RaDR-GFP) mice. Significantly decreased frequency of recombinant cells under RAR-inducible condition was found when compared to that under RAR-uninducible condition.

Materials and Methods

Animals

The Rosa26 Direct Repeat-GFP (RaDR-GFP) mice on the C57BL/6J background were kind gifts from Dr Bevin P. Engelward (Massachusetts Institute of Technology, Cambridge, Massachusetts) via Dr Jac A. Nickoloff (Colorado State University, Fort Collins, Colorado) and maintained in heterozygote background. In the mouse genome, a direct repeat HR substrate is targeted to the ubiquitously expressed Rosa26 locus and HR between 2 truncated enhanced green fluorescent protein (EGFP) expression cassettes can yield a fluorescent signal (Figure 1). Animals bearing homozygote RaDR-GFP background generated as offspring from heterozygote intercrosses were used. They were kept in a specific pathogen-free animal facility until 5-week postpartum and then transferred to a conventional animal facility. C57BL/6J mice aged 5 weeks old were purchased from SLC, Inc (Japan). Animals were kept under a 12-hour light–12-hour dark photoperiod, housed in autoclaved cages with sterilized wood chips, and allowed to standard laboratory chow (MB-1; Funabashi Farm Co, Japan) and acidified water ad libitum. The RaDR-GFP mice were acclimatized to the laboratory conditions 1 week before use. To avoid possible effects from the developmental condition of the animals, 6-week-old mice with a significantly different body weight (more or less than the mean ± 2 SD) were omitted from this study. Based on the preliminary trials, in the present study, at least 6 mice were used in each experimental point. C57BL/6-Tg (CAG-EGFP) mice aged 8 weeks old were purchased from SLC, Inc (Japan). All experimental protocols (Experimental Animal Research Plan No. 14-1014-2, No. 16-1033 and No. 16-2010-4) involving mice were reviewed and approved by The Institutional Animal Care and Use Committee of the National Institute of Radiological Sciences, National Institutes for Quantum and Radiological Science and Technology (QST-NIRS), Japan. The experiments were performed in strict accordance with the QST-NIRS Guidelines for the Care and Use of Laboratory Animals.

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**Figure 1.** Homologous recombination (HR) in the RaDR-GFP substrate giving rise to fluorescence. The substrate (A) consists of 2 EGFP expression cassettes arranged in tandem (large arrows), one with deletion at 5' (Δ5) end and the other at 3' (Δ3) end of the coding sequences. The cell harboring the full-length EGFP-coding sequences (black bars with glowing markers) after HR events, namely, gene conversion (B) and unequal sister chromatid exchange or replication fork repair (C), gives rise to fluorescence. Dotted bars indicate deletions, gray bars stand for EGFP-coding sequences, and white bars represent the CAG promoter and polyadenylation signal sequences.
Irradiation

X-rays were generated with an X-ray machine (Pantak-320S; Shimadzu, Japan) operated at 200 kVp and 20 mA, using a 0.50 mm Al + 0.50 mm Cu filter. An exposure-rate meter (AE-1321M; Applied Engineering Inc, Japan) with an ionization chamber (C-110, 0.6 mL; JARP, Applied Engineering Inc, Japan) was used for the dosimetry. The dose rate for delivering the priming dose and the challenge dose was at about 0.30 Gy/min and 0.90 Gy/min, respectively. The mice held in acryl containers were exposed to total body irradiation (TBI) at room temperature.

Mouse Model for Radiation-Induced AR

The RAR mouse model for rescue of bone marrow death (Yonezawa Effect) established by Yonezawa and colleagues was adopted, verified, and confirmed in C57BL/6J mice under the experimental conditions in our research facilities. It was applied to the present work using X-rays and RaDR-GFP mice. The timing for delivery of the priming dose and challenge dose was on postnatal age of 6 and 8 weeks of the mice, respectively. A dose of 0.50 Gy was used as the priming dose to verify the existence of RAR in the 30-day survival test and to investigate the frequency of recombinant cells in the nucleated cells in hematopoietic tissues under RAR-inducible and -uninducible conditions. For the challenge dose, based on the preliminary trials, 2 doses, namely, a lethal dose at 6.00 Gy and a sublethal dose at 5.75 Gy, were used in the 30-day survival test. A non-lethal dose at 4.00 Gy was used in the study on the frequency of recombinant cells. The TBI treatment of mice with both the priming dose and the challenge dose was defined as RAR-inducible condition and with the challenge dose alone as RAR-uninducible condition.

Enumeration of Recombinant Cells

To comparatively evaluate the homologous recombination frequency in the nucleated cells in the bone marrow and spleen in mice under experimental conditions capable of inducing RAR (receiving both the priming dose of 0.50 Gy X-rays and the challenge dose of 4.00 Gy X-rays) or incapable of inducing RAR (receiving only the challenge dose of 4.00 Gy X-rays), the recombinant cells (GFP-positive cells) in the nucleated bone marrow cells and splenocytes were analyzed by flow cytometry. In brief, the mice were euthanized by CO2 asphyxiation the following day after the 30-day survival test, and the femurs and spleens were collected. Single-cell suspensions of dissociated bone marrow tissues and spleens in phosphate-buffered saline free from calcium and magnesium ions (PBS (−); Wako Pure Chemical Industries, Ltd, Japan) were filtered through a 40-µm cell strainer (Corning Inc, Corning, New York) after treating with Tris-buffered ammonium chloride for the lysis of red blood cells and washing with PBS (−), and then the nucleated bone marrow cells and splenocytes were fixed with 1% paraformaldehyde phosphate buffer solution (Wako Pure Chemical Industries) at 4°C. Before analysis with flow cytometry, the cells were washed once with PBS (−), resuspended in Opti-MEM (Life Technologies, Carlsbad, California), and filtered through a 35-µm cell strainer (Corning Inc). The cell suspensions were gated using forward and side scatter and analyzed on a BD FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey) with an excitation laser at 48 nm, emission filters to measure green fluorescence (530 nm), and autofluorescence (580 nm). The cells from C57BL/6-Tg (CAG-EGFP) mice constitutively expressing EGFP and wild-type C57BL/6J mice with no EGFP expression were used, respectively, as a positive control and a negative control for flow cytometry. Cells with significantly higher levels of fluorescence at 530 nm than 580 nm were judged as recombinant cells, namely, RaDR-GFP-positive cells with “green fluorescence.” Six to 12 animals were used per experimental point, and for each sample at least 1 million cells were analyzed. The frequency of recombinant cells was expressed as the number of GFP-positive cell parts per million of nucleated bone marrow cells or splenocytes.

Statistical Analysis

Statistical evaluation of the data was done using the χ² test for the 30-day survival and Student t test for the recombinant cell frequency. The statistical significance was assigned to P < .05.

Results

Validation of RAR in RaDR-GFP Mice Using a Sublethal Dose as the Challenge Dose

Reproducibility of the RAR mouse model (Yonezawa Effect) in RaDR-GFP mice using 30-day survival test as the end point was verified and confirmed. Based on the preliminary trial, no statistically significant gender difference was found in the survival rate in the animals receiving the same treatment; thus, analysis of the data pooled from all animals of both genders in each group was done. Under the RAR-inducible condition, TBI of the animals was performed with a priming dose of 0.50 Gy X-rays at postnatal 6 weeks followed by a challenge dose of 5.75 Gy X-rays at postnatal 8 weeks. Under the RAR-uninducible condition, only the challenge dose of 5.75 Gy X-rays was delivered to the mice at postnatal 8 weeks. Results showed that administration of the priming dose significantly increased the survival rate of the mouse from 29.5% to 98.4% (Figure 2) and clearly indicated that RAR was demonstrated in our experimental setup.

Validation of RAR in RaDR-GFP Mice Using a Lethal Dose as the Challenge Dose

Thirty-day survival test was performed to validate rescue efficacy of RAR against a lethal dose in RaDR-GFP female mice. Under RAR-inducible condition (TBI with a priming dose of 0.50 Gy X-rays at postnatal 6 weeks followed by a challenge...
dose of 6.00 Gy X-rays at postnatal 8 weeks), the survival rate was 38.9%. On the other hand, under RAR-uninducible condition (TBI with only the challenge dose of 6.00 Gy X-rays at postnatal 8 weeks), all the animals died within 20 days after exposure. The increase in the survival rate was statistically significant (Figure 3). The results clearly showed that under RAR inducible condition, still more than one-third of the animals could survive from a lethal dose indicating the high efficacy of RAR for rescuing the animals from high dose-induced bone marrow death.

Validation of the Frequency of Recombinant Cells in the Nucleated Bone Marrow Cells in RaDR-GFP Mice

Frequency of recombinant cells measured as GFP-positive cells and expressed in number of per million of nucleated bone marrow cells was flow cytometrically studied in each experimental group in male RaDR-GFP mice (Figure 4). “Control group” was sham-treated with IR, “0.50-Gy group” was irradiated with only a priming dose of 0.50 Gy X-rays at postnatal 6 weeks. Animals in the “4.00-Gy group” were irradiated with only a challenge dose of 4.00 Gy X-rays at postnatal 8 weeks. Animals in the “0.50 + 4.00-Gy group” were primed with a dose of 0.50 Gy X-rays at postnatal 6 weeks and then followed by a challenge dose of 4.00 Gy X-rays at postnatal 8 weeks. The number of animals in the control group, the 0.50-Gy group, the 4.00-Gy group, and the 0.50 + 4.00-Gy group were 8, 8, 11, and 11, respectively. One asterisk (*) symbolizes statistically significant differences ($P < .05$) between the 2 groups that were compared.
8.60 ± 3.80, 12.7 ± 7.50, 17.0 ± 17.9, and 7.00 ± 6.00, respectively. There was a statistically significant increase in the frequency of recombinant cells for the “4.00-Gy group” compared to the “control group.” On the other hand, there was a statistically significant decrease in the frequency of recombinant cells in the “0.50 + 4.00-Gy group” compared to the “4.00-Gy group.” There was no statistical difference in the frequency of recombinant cells between the “control group” and the “0.50+4.00-Gy group.” These results clearly showed that increased frequency of recombinant cells was observed under RAR-uninducible condition, decreased frequency of recombinant cells was confirmed under RAR-inducible condition, and induction of RAR could significantly reduce the frequency of recombinant cells in the “0.50+4.00-Gy group” to a low level similar to that in the “0.50-Gy group.”

Discussion

Radiotherapy, as a critical and inseparable component of comprehensive treatment, is one of the more cost-effective cancer therapeutic modalities on a global scale. On the other hand, IR protection is a critical issue in many fields including cancer RT, where IR is a double-edged sword having a well-established role in the cancer treatment while bearing the risk of inducing secondary cancer. Despite important scientific and technological advances in radiation delivery, RT still induces irreversible side effects on the normal tissues surrounding the tumor, thus making the tolerance dose of the normal tissue a major dose-limiting factor and also resulting in the increased secondary cancer risk. Research and development of the intervention strategies to protect the normal tissues from the detrimental effects of radiation exposure at high doses remain a significant unmet medical need and an attractive goal as well.

The maintenance of genomic stability by DNA repair mechanisms is essential for cellular integrity to prevent errors during DNA replication. Unrepaired DNA damage and acquired genomic alteration due to genotoxic stress and carcinogen insults such as IR could generate GI. The GI could provide the cell a shorter cell cycle and/or an advantage of bypassing intracellular and immunological control systems, cause an accumulation of chromosomal aberrations and mutations, and give cancerous cells an advantage in both growth and malignant transformation, being responsible for various clinical phenotypes including carcinogenesis. As one of the critical DNA repair pathways, HR is usually error free, but it could sometimes result in cancer-promoting mutations. In fact, IR could directly induce HR, where it may mainly function by preventing misrepaired DNA double-strand breaks and broken replication forks to decrease the mutation load of the cells and thus reduce GI and carcinogenesis. On the other hand, IR-induced GI played a critical role in carcinogenesis and increased delayed HR is one of the phenomena of IR-induced GI.

The RAR is well established in varied biosystems, being capable of reducing the risk of late adverse effects of IR exposures, in particular, reduction in late genomic toxicity/GI and carcinogenesis. Although it still remains uncertain and in a vague proposition as to whether RAR will have any utility in establishing risks of IR to humans, keen interest has been shown to RAR. As approaches directing toward reducing IR-induced GI are in hope of controlling the initiation and
progress of the secondary cancer in RT, induction of RAR, as a promising radiation countermeasure, is expected to be applied for clinical trials such as priming the normal tissue with low dose of IR before performing radiotherapy. A better understanding of RAR is needed to know to which extent low-dose IR might be beneficial in humans. The RAR could induce protective effects through mechanisms critical to life such as reduction in cell death, chromosomal aberrations, mutations, GI, and malignant transformation in vitro, and enhancement of antioxidative capacities, increase in DNA repair capacity, and increase in radioresistance in the hematopoietic tissue in vivo.

To gain insight into the genomic stability in vivo after treatment with RAR-inducible and -uninducible conditions, using the RAR mouse model (Yonezawa Effect), we confirmed that RAR could reduce late genomic toxicity/GI with 2 end points at different levels: in our previous study, reduction of micronuclei in the erythrocytes in bone marrow in the surviving mice rescued by RAR, and in the present work, reduction in delayed HR induced by a challenge high dose in the mice treated under RAR-inducible condition. These results suggest at least in part the mechanisms underlying RAR-induced decrease in carcinogenesis. These findings also imply the potential for applying RAR to protect normal tissues from IR during RT.

The mouse RAR model (Yonezawa Effect) is a well-designed system in investigating the late health effect in vivo such as GI and carcinogenesis. There are 2 different phenotypes of RAR in this model that involved different mechanisms: The first phenotype could be induced 2 weeks after a 0.30 to 0.50 Gy priming IR with the involvement of the induced radioresistance in blood-forming tissues, and the second phenotype could be induced 2 months after a 0.05 to 0.10 Gy priming exposure with the interaction between blood-forming tissue and the central nervous system. Interestingly, partial exposure to priming IR of trunk or head is also effective for induction of RAR. The model for the first phenotype was applied to the present study. Compared to most of the existing models, applying RaDR-GFP mice to this system further adds technology advantages to the mechanistic research, for example, the approach to quantification of recombinant cells in vivo opens new doors to RAR studies, in particular, giving rise to a more opportunity for understanding the underlying molecular mechanisms linking to RAR-induced reduction of carcinogenesis. In addition to that, the assay of recombinant cells has the advantage over time and labor-intensive cytogenetic analysis, as it is capable of detecting and quantifying recombinant cells in situ within an intact organ; thus, this system could be used to study the clonal expansion of a single cell as well as to reveal the location and cell types that have undergone HR. For example, in mouse spleen as subpopulations of splenocytes responded differently to IR at low dose and high dose, this system makes it possible to study the susceptibility of different cell types to IR-induced GI under RAR-inducible and -uninducible conditions. By using this system, it is expected to reveal the relationship among different end points for GI leading to a breakthrough in understanding of the molecular mechanisms underlying RAR, for example, the micronuclei formation reposted in our previous work and delayed hyper-recombination observed in the present study.

To modify the radioresistance of both the cancer cells and the surrounding normal tissues, triggering the mechanisms to selectively increase the radioresistance of the normal tissues and sensitize the cancer cells is of great importance. Approaches such as development of radioprotective agents, pharmaceutical gene regulating, and intervening dietary habit, psychological stress and life style were proposed, but the efficacy of these approaches is still largely uncertain.

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