Characterization of the Long-Term Interaction between Intracellular Reactive Oxygen Species and Oxidative DNA Damage in Murine Lin⁻/Sca-1⁺ Cells Exposed to Ionizing Radiation

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Abstract
Mutations in the Sfpi1 gene are essential for the development of radiation-induced acute myeloid leukemia. In this study, we investigated long-term interaction among immature hematopoietic cell number, intracellular reactive oxygen species contents, and oxidative DNA damage frequency after irradiation. Lin⁻/Sca-1⁺ cells were isolated from C3H/HeN mice on days 1 - 400 after 0 - 3 Gy total body irradiation. On days 1 - 7, the number of surviving cells decreased and reached a minimum; however, the number of cells gradually recovered until day 200. Intracellular reactive oxygen species contents significantly increased from day 1 to day 30. In addition, the frequency of oxidative DNA damage tended to increase from day 1 and day 30, and that at day 30 was significantly increased in the 3 Gy group compared with that in the control group. In contrast, decreased cell number, increased intracellular reactive oxygen species content, and decreased oxidative DNA damage frequency were observed on day 400. These results suggested that oxidative DNA damage was involved in intracellular reactive oxygen species generation induced by cell proliferation to compensate for cell death after irradiation.

Keywords
Acute Myeloid Leukemia, Oxidative DNA Damage, Reactive Oxygen Species

1. Introduction
Ionizing radiation has many beneficial applications, including uses in medicine, industry, and research. Exposure to ionizing radiation causes damage to DNA,
proteins, and lipids in mammalian cells induces mitochondria-dependent generation of reactive oxygen species (ROS), with subsequent induction of cell cycle arrest, apoptosis, and stress-related responses, including alterations to gene expression [1] [2] [3]. Radiation contributes to carcinogenesis via a multistage process [4] [5] and/or genomic instability [6] [7]; however, the exact mechanisms are still unclear. Because beneficial applications of radiation are indispensable, elucidation of the mechanisms of radiation-induced carcinogenesis is a socially important issue.

Acute myeloid leukemia (AML) is a representative human disease induced by radiation exposure. Many previous studies have used mouse models to study radiation-induced AML [8]-[19]. These reports have demonstrated that a latency period of more than 1 - 2 years is required [8] [9] [10] [11] [12] and that a deletion of chromosome 2 [13] [14] [15] [16] and a mutation in the Sfp1 gene, also known as the PU.1 gene, on the retained homolog [17] [18] [19] are essential for the pathogenesis of radiation-induced AML. Moreover, radiation-induced deletion is known to occur early, i.e. within 24 h after irradiation [14] [20]. Ban and Kai proposed a mechanism for the pathogenesis of radiation-induced AML using a mathematical model [21]. They suggested that this process may be divided into three steps, as follows: 1) irradiation immediately induces cell death and deletion of chromosome 2; 2) cell proliferation to compensate for dead cells induces hematopoietic stem cell senescence caused by replicative stress; and 3) AML develops because of increased intracellular ROS generation accompanied by cell senescence, resulting in spontaneous point mutations [21]. The Sfp1 mutation found in radiation-induced AML is typically a point mutation at codon 235 of the DNA-binding Ets domain, particularly a C:G to T:A transition at the CpG site [17] [18] [19] [21], which is the most common spontaneous mutation induced by intracellular ROS [21] [22] [23]. However, the detailed mechanisms mediating mutations in the Sfp1 gene, which is an essential factor affecting the development of radiation-induced AML, remain unclear.

Accordingly, we attempted to experimentally verify the mathematical model proposed by Ban and Kai, as a part of elucidating pathogenesis of radiation-induced AML. Practically, in this study, we investigated the long-term interactions among the number of immature hematopoietic cells, levels of intracellular ROS, and frequency of oxidative DNA damage after irradiation in order to elucidate the detailed mechanisms mediating the development of radiation-induced AML.

2. Materials and Methods

2.1. Animals and Gamma-Irradiation

This study was approved by the Committee of Research Ethics and Safety in Oita University of Nursing and Health Sciences (Oita, Japan). All experiments were conducted according to legal regulations in Japan and following the Guideline for Animal Experiments of Oita University of Nursing and Health Sciences.
Male C3H/HeN Jcl mice, as a model mouse for AML, were delivered at 7 weeks of age from the breeding facility of Clea Japan (Tokyo, Japan). At 8 weeks of age, five mice in each group were exposed to 0, 1, and 3 Gy of $^{137}$Cs gamma-rays at a dose rate of 0.72 Gy/min by Gammacell 40 Exactor (Marubeni Utility Service, Tokyo, Japan). All mice were housed in a temperature-controlled room under a 12-h light/dark cycle in a conventional animal breeding facility. The mice received food and water *ad libitum.*

### 2.2. Collection and Purification of Lin$^{-}$/Sca-1$^{+}$ Cells as Hematopoietic Stem/Progenitor Cells

Mice were sacrificed under deep anesthesia at 0, 1, 7, 30, 90, 200, and 400 days after exposure to 0, 1, and 3 Gy total body irradiation (105 mice in total). Briefly, mononuclear cells were isolated from the bilateral femur by flushing with phosphate-buffered saline (PBS). The cells were then processed for lineage-negative (Lin$^{-}$) cell enrichment according to the manufacturer’s instructions. An EasySep Mouse Hematopoietic Progenitor Cell Enrichment Kit (Veritas Corporation, Tokyo, Japan) was used for the negative selection of Lin cells. The cells were then processed for Sca-1$^{+}$ cell enrichment according to the manufacturer’s instructions. An EasySep Mouse SCA1 Positive Selection Kit (Veritas Corporation) was used for the positive selection of Sca-1$^{+}$ cells. Using the procedure described above, Lin$^{-}$/Sca-1$^{+}$ cells were obtained; although this population is heterogeneous, this isolated Lin$^{-}$/Sca-1$^{+}$-enriched cell population was composed of hematopoietic stem and progenitor cells.

### 2.3. Number of Survival Cells

After isolation of Lin$^{-}$/Sca-1$^{+}$ cells, some of the cells for each condition were harvested, and the viable cells were stained and counted using Burker-Turk-solution (Nacalai Tesque, Kyoto, Japan) under a microscope. The relative value normalized to the control value was calculated as the ratio of the number of gamma-irradiated cells to the number of non-irradiated cells at the same time point.

### 2.4. Intracellular ROS Contents

The intracellular ROS contents in Lin$^{-}$/Sca-1$^{+}$ cells were analyzed using CellROX Green Oxidative Stress Reagents (Life Technologies, Carlsbad, CA, USA) with a Tali Image-Based Cytometer (Life Technologies). Briefly, after irradiation and breeding for the indicated period, the mice were sacrificed, and Lin$^{-}$/Sca-1$^{+}$ cells were isolated from the femur. These cells were stained with 5 μM CellROX Green Oxidative Stress Reagent for 30 min at 37°C in the dark. After labeling, unincorporated CellROX Green Oxidative Stress Reagent was removed by three washes with PBS. Each sample was resuspended in PBS and analyzed using an image-based cytometer. For each experiment, unstained cells were used as a negative control.
2.5. Total DNA Extraction

Total DNA was extracted from Lin-/Sca-1+ cells (five samples for each dose). Total DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Bothell, WA, USA) according to the manufacturer’s instructions, and the DNA concentration was determined using a fluorometer (Qubit 3.0 Fluorometer; Life Technologies) and Qubit dsDNA HS Assay Kit (Life Technologies).

2.6. Oxidative DNA Damage

Oxidative DNA damage was investigated using an Oxiselect Oxidative DNA Damage ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer’s instructions. This kit contained a competitive enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of 8-hydroxyguanosine (8-OHdG). Briefly, after irradiation and breeding for the indicated period, the mice were sacrificed, and Lin-/Sca-1+ cells were isolated from the femur. Total DNA extracted from these cells was digested, and the mixture was centrifuged. The supernatant was collected and applied for the ELISA. Five microliters of the supernatant or standard were added to each well of 8-OHdG conjugate-coated plates. The plate was incubated for 10 min at room temperature on an orbital shaker. After incubation, diluted anti-8-OHdG antibodies were added (5 μL/well), and plates were incubated for 60 min at room temperature on an orbital shaker. All wells were then washed and stripped three times, and excess wash buffer was completely removed. Next, diluted secondary antibody-enzyme conjugate was added (100 μL/well), and plates were incubated for 60 min at room temperature on an orbital shaker. After incubation, all wells were washed and stripped four times. Subsequently, substrate solution was added (100 μL/well), and plates were incubated for 5 min at room temperature. Then, stop solution was added (100 μL/well). Finally, the absorbance of each well was read using a microplate reader (Tecan Japan Co., Ltd., Kanagawa, Japan) at 450 nm.

2.7. Statistical Analysis

The differences among the non-irradiated and irradiated groups were analyzed by multiple comparison tests, such as Steel’s test. Statistical analysis was performed using Excel 2007 (Microsoft, WA, USA) with the add-in software Statcel 3 (OMS, Saitama, Japan). Statistically significant differences were defined at a significance level of 5%.

3. Results

3.1. Number of Surviving Lin-/Sca-1+ Cells Exposed to Gamma-Irradiation

First, we examined cell survival/death of Lin-/Sca-1+ cells isolated from the bilateral femurs of mice after 1 or 3 Gy total body gamma-irradiation. The results showed that the number of viable cells was immediately decreased after irradiation with 1 or 3 Gy and reached a minimum value at day 3 or day 1 after irradiation.
tion, respectively (Figure 1), with viable gamma-irradiated cells decreasing to 73% ± 35% or 27% ± 5% that of control non-irradiated cells, respectively. Subsequently, the number of viable cells gradually increased, returning to the value of the control at day 200 after irradiation.

However, cells exposed to 3 Gy significantly decreased again by about 50% compared with non-irradiated cells on day 400 after irradiation.

3.2. Intracellular ROS Contents in Surviving Lin−/Sca-1+ Cells Exposed to Gamma-Irradiation

Intracellular ROS contents were significantly elevated, reaching a maximum value in Lin−/Sca-1+ cells on day 1 after gamma-irradiation of about twice that of control cells (Figure 2). Although this significant increase in ROS was observed until day 7, intracellular ROS levels were significantly decreased on day 90, when the surviving fraction equalized with that of the non-irradiated control in the 3 Gy-irradiated group. In contrast, intracellular ROS was significantly increased on day 30 in the 1 Gy-irradiated group. The generation of ROS gradually decreased to the level of the non-irradiated control by day 200 and significantly increased again on day 400 after irradiation in irradiated Lin−/Sca-1+ cells.

3.3. Detection of Oxidative DNA Damage in Surviving Lin−/Sca-1+ Cells Exposed to Gamma-Irradiation

DNA is the most biologically significant target of oxidative attack, and continuous oxidative damage to DNA is a significant contributor to the age-related development of several cancers. Among numerous types of oxidative DNA damage, the formation of 8-OHdG is a ubiquitous marker of oxidative stress targeting DNA [24]. Therefore, we next analyzed the levels of oxidative DNA damage in Lin−/Sca-1+ cells isolated from the bilateral femurs of mice after 1 or 3 Gy total body gamma-irradiation (Figure 3).

Compared with non-irradiated cells, the relative fluorescence intensity of 8-OHdG tended to increase in irradiated Lin−/Sca-1+ cells on day 1 after gamma-irradiation, although this difference was not statistically significant. Subsequently, 8-OHdG levels were elevated again in both irradiated groups on day 30 after irradiation, particularly in the 3 Gy group. Importantly, this elevation was observed at the time point of cell survival recovery to the control level. In contrast, 8-OHdG levels were significantly decreased in the 3 Gy group on day 400 after irradiation.

4. Discussion

In the present study, we aimed to elucidate the detailed mechanisms mediating mutations including Sfpi1 gene, which are essential for the development of radiation-induced AML, by investigating the long-term interactions among the number of immature hematopoietic cells, generation of intracellular ROS, and frequency of oxidative DNA damage after irradiation. Gamma-irradiation decreased
Figure 1. Number of survival Lin−/Sca-1+ cells after gamma-irradiation. Viable cells were stained using Burker-Turk-solution and counted under a microscope. The relative value normalized to the control value was calculated as the ratio of the number of gamma-irradiated cells to the number of non-irradiated cells at the same time. The filled symbols show means, and unfilled symbols show the values of individual samples. *Statistically significant differences, defined as a significance level of 5%, by Steel’s test.

Figure 2. Intracellular ROS contents in Lin−/Sca-1+ cells after gamma-irradiation. Detection of intracellular ROS in the Lin−/Sca-1+ cells was performed using CellROX Green Oxidative Stress Reagent and an image-based cytometer. The relative value normalized to the control value was calculated as the ratio of the fluorescence intensity of gamma-irradiated cells to that of non-irradiated cells at the same time. The filled symbols show means, and unfilled symbols show the values of individual samples. *Statistically significant differences, defined as a significance level of 5%, by Steel’s test.
the number of Lin−/Sca-1+ cells isolated from C3H/HeN mice on day 1 in the 3 Gy group and day 7 in the 1 Gy group. Furthermore, the number of surviving cells gradually recovered until days 90 and 200, respectively, and intracellular ROS contents significantly increased from day 1 to day 30 in both irradiated groups. In addition, the frequency of oxidative DNA damage tended to increase in both irradiated groups on days 1 and 30. However, on day 400, a significant decrease in the number of cells, significant increase in intracellular ROS contents, and significant decrease in the frequency of oxidative DNA damage were observed in the 3 Gy group, and similar trends were observed in the 1 Gy group. These findings provided important insights into the effects of gamma-irradiation on carcinogenesis, particularly AML.

Few researchers have investigated the long-term effects of radiation on hematopoietic stem/progenitor cells. However, several short- and/or moderate-term studies have been published, with study periods of up to 60 days. Wang et al. reported that Lin−/Sca-1+/c-Kit+ cells exhibited an immediate transient decrease after irradiation and then gradually recovered via proliferation to compensate for the observed cell death [25]. Additionally, they demonstrated that the multipotency and self-renewal abilities of Lin−/Sca-1+/c-Kit+ cells were both attenuated by proliferation after irradiation [25] [26]. Moreover, persistent intracel-
Intracellular ROS induced by radiation resulted in premature senescence of hematopoietic cells [25] [26] [27] [28]. Additionally, increased intracellular ROS contents and/or premature senescence of hematopoietic cells resulted in radiation-induced residual bone marrow suppression [26] [29] [30]. These results suggested that to compensate for radiation-induced cell death, proliferation accompanied by increased intracellular ROS accelerated the premature senescence of immature hematopoietic cells and resulted in radiation-induced residual bone marrow suppression. Our results indicated that compensation for transient radiation-induced cell death via cell proliferation resulted in intermittent increases in intracellular ROS until day 200, consistent with previous reports.

Previous studies have shown that mutations in the Sfpi1 gene found in radiation-induced AML are primarily C:G to T:A transition mutations [17] [18] [19] [21], which are the most common type of spontaneous mutation induced by intracellular ROS [21] [22] [23]. The results of a mathematical model developed by Ban and Kai supported these findings [21]. In addition, Chung et al. demonstrated that the mutation frequency increased significantly by exposure to oxidative stress [31]. Therefore, these findings indicated that spontaneous ROS caused point mutations, including mutations in Sfpi1. In fact, Wang et al. and Phazhansamy et al. reported that ionizing radiation significantly increased oxidative DNA stress on day 30 [2] [32], supporting our current results. Indeed, our findings suggested that transitions promoting the development of radiation-induced AML occurred on days 1 and 30, when increased DNA damage and intracellular ROS contents were observed. Therefore, we concluded that the period from days 1 to 30 was important for clarifying the mechanisms mediating the development of radiation-induced AML. Further studies are needed to fully elucidate these mechanisms.

In contrast, our results revealed that gamma-irradiation decreased the number of cells, increased intracellular ROS contents, and decreased the frequency of 8-OHdG-positive cells on day 400 in both groups. Although we assumed that oxidative DNA damage would accumulate in mice with radiation-induced AML, these results were contrary to our expectations. Previous reports have demonstrated that ionizing radiation accelerates cell senescence and results in residual bone marrow suppression [25] [26] [27] [28] [29]. Additionally, the frequency of DNA double-strand breaks (DSBs) related to accumulation of ROS was increased in senescent cells [26]. Indeed, our colleagues investigated the frequency of DSBs in mouse bone marrow cells on day 400 using 53BP1 immunofluorescence staining and found a 1.3-fold increase in the 1 Gy group and a 1.2-fold increase in the 3 Gy group compared with that in the non-irradiated control (data not shown). These results suggested that cell senescence related to persistent ROS decreased the number of immature hematopoietic cells by increasing the frequency of DSBs. Moehrle et al. demonstrated that hematopoietic stem cells acquired apoptosis in order to recover from point mutations caused by DNA damage [33]. Accordingly, immature hematopoietic cells may have been exhausted after irradiation in mice that did not develop radiation-induced AML,
and immature hematopoietic cells containing mutations including 8-OHdG may have been relatively easy to eliminate after irradiation. However, further studies are required to verify these assertions.

In conclusion, our current findings suggested that oxidative DNA damage, which is essential for the pathogenesis of radiation-induced AML, was involved in intracellular ROS generated by cell proliferation to compensate for cell death. However, this study was performed under restricted conditions, such as only 1 and 3 Gy of X-irradiation and five samples in each group. Also, to minimize the number of animals in this study, we cannot use a large number of animals resulting none of the mice actually developed radiation-induced AML, and further studies with more mice are needed to obtain AML model mice. Additionally, these limitations may be overcome through development of mathematical models and/or novel experimental methods. In any case, our findings provide important insights into the mechanisms of radiation-induced AML and with being valuable for later more detailed studies.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References


man Cancers. *Nature*, **396**, 643-649. [https://doi.org/10.1038/25292](https://doi.org/10.1038/25292)


Ageing in Radiation-Induced Murine Leukaemia. *British Journal of Cancer*, **101**, 363-371. [https://doi.org/10.1038/sj.bjc.6605135](https://doi.org/10.1038/sj.bjc.6605135)


