Cell cycle of primitive hematopoietic progenitors decelerated in senescent mice is reactively accelerated after 2-Gy whole-body irradiation

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Abstract

Aging is considered to be a functional retardation of continuous xenobiotic responses over a lifetime after the developmental period; thus, the effects of ionizing radiation over a lifetime may be somewhat accounted for by a modifier of aging effects. This study was conducted to evaluate the possible/synergic effects of radiation during aging by determining cell-cycle parameters of hematopoietic stem cells/hematopoietic progenitor cells (HSCs/HPCs), such as the percent of cells in cycling, the generation doubling time, and the cumulative cycling-cell fraction, by bromodeoxyuridine-ultraviolet assay, which enables the determination of their cycling capacity *in vivo*. Colony-forming progenitor cells, such as colony-forming unit (CFU)-granulocyte/macrophage (GM), CFU in the spleen on day 9 (CFU-S9), and CFU-S on day 13 (CFU-S13) for mature, less mature, and immature HPCs, respectively, were evaluated in young and old mice (6 weeks and 21 months of age, respectively) with or without 2-Gy whole-body irradiation and a 4-week recovery period. Then, cell-cycle parameters were evaluated and compared. As a result, the generation doubling time of all types of HPC was prolonged by the irradiation in both young and old mouse groups, except that of CFU-S13 in old mice, which showed acceleration of the cell cycle following the irradiation. In addition, only CFU-S13 in irradiated old mice showed a significant increase in the cumulative cycling-cell-fraction ratio. Significant changes due to the effects of aging and irradiation on HPCs were observed only in the immature HPCs, i.e., the cell cycle of immature HPCs was suppressed by aging without irradiation and was, in contrast, accelerated as the cells recovered from radiation-induced damage. This suggests that the mechanisms of peripheral blood recovery after 2-Gy whole-body irradiation are markedly different between young and old mice, although 21-month-old mice showed almost the same level of recovery as the young mice.

Keywords: Whole-body irradiation, hematopoietic stem cells, cell cycle, bromodeoxyuridine, senescence, mice

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Introduction

A single dose of radiation, at not only a sublethal dose (e.g., 6 Gy), but also a leukemia-inducing dose¹ (e.g., 2 Gy), decreases the number of hematopoietic stem cells/hematopoietic progenitor cells (HSCs/HPCs) over a lifetime and induces accelerated-aging phenotypes.^{2,3} On the other hand, sensitivity to radiation-induced effects (in terms of, e.g., survival, tumorigenesis including leukemia, and suppression of HSCs/HPCs) differs depending on the age at exposure.^{4–7} The sensitivity to radiation-induced effects in old mice (e.g., 21 months of age), is, however, not precisely known yet. Concerning the cell cycle, the effects of ionizing radiation and senescence on the hematopoietic system,

specifically on HSCs/HPCs, are unclear and need to be better defined. In general, depending on the nature of radiation (such as total dose, dose rate, duration, and radiation quality), a single dose of whole-body irradiation has been considered to accelerate the cell cycle of primitive HSCs/HPCs.^{8,9} In contrast, however, nonirradiated old mice show deceleration of the cell cycle of primitive HSCs/HPCs.¹⁰

The cell cycle assay of hematopoietic colony-forming progenitor cells was carried out in this study by continuous bromodeoxyuridine (BrdUrd) incorporation *in vivo* and ultraviolet A (UVA) light irradiation to purge cells that incorporated BrdUrd (BUUV assay). The assay specifically provides various cell-cycle parameters, such as the percent

incorporation of BrdUrd at time zero, generation doubling time, and the cycling-cell fraction accumulated during BrdUrd incorporation, which is in contrast to the dormant cell fraction, in certain HPCs.^{10–12} By this BUUV assay, we evaluated cell kinetic changes with aging¹⁰ and the effects of benzene inhalation.^{12,13} In addition, the effects of various genes related to HSC maintenance were also evaluated using mice with modified genes, e.g., Trp53,^{11,14} aryl hydrocarbon receptor,^{15,16} thioredoxin,^{17,18} and connexin 32^{19–21} genes. This BUUV assay can provide the dynamics of the cell-cycle status with greater accuracy than other methods, such as *in vitro* labeling/cytociding (which may provide an artificially high percent of cycling cells) or limited short-term treatment with thymidine analogs *in vivo* (which may provide only a snapshot of features). Other methods could provide only data corresponding to the percent incorporation of BrdUrd at time zero and sometimes inconsistent results.²²

The aim of this study was to investigate the possible additive/synergic effects of senescence and single-dose whole-body low-linear-energy-transfer (LET)-type irradiation on various cell-cycle parameters of HSC/HPC compartments by BUUV assay. Given that the cell cycle accelerates in young mice after acute irradiation, in this study, we tested the hypothesis that the cell cycle of immature HPCs in old mice, which is suppressed during aging, may be accelerated after irradiation as the cells recover from radiation-induced damage.

Materials and methods

Mice

Two groups of C57BL/6CrSlc male mice, 6 weeks and 21 months of age, respectively, were used as donor mice. For the old group, 34 mice used in this study were maintained in an environmentally controlled specific-pathogen-free laboratory animal facility at the Tokyo Metropolitan Institute of Gerontology (TMIG) until 1 month before the study was initiated. Healthy-looking mice were selected from a group of mice among the aging animal stock maintained at TMIG. The mice of the stock were originally purchased from Japan SLC, Inc. (Hamamatsu, Japan) at 4 weeks of age every 3 months. When a gross neoplasm was observed in a mouse during the harvesting of bone marrow cells, the mouse was eliminated from analysis. For the young control group, 36 of the 6-week-old C57BL/6CrSlc male mice purchased from Japan SLC, Inc. were used. Five hundred and seventy-six 8-week-old C57BL/6Ncrj male mice purchased from Charles River Laboratories (Yokohama, Japan) were used as recipients in the assay of colony-forming units in the spleen (CFU-S). This subline of mice was used because our preliminary study indicated that they were more radioresistant and hence more suitable than C57BL/6CrSlc as the recipients. At each observation time point, 10 mice for CFU-S on day 9 (CFU-S9) analysis and 14 mice for CFU-S on day 13 (CFU-S13) analysis were used. All the donor and recipient mice were maintained in environmentally controlled specific-pathogen-free rooms supplied with air from a high-efficiency particulate air (HEPA) filter under a 12-h

light/12-h dark cycle in a laboratory animal facility of the National Institute of Health Sciences (NIHS). Sterilized acidified water (pH 4) and food pellets (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) were provided *ad libitum* and all the equipment and supplies, including cages, water bottles, and wooden chips used for bedding, were sterilized. The animal facility was approved by the Health Science Center for Accreditation of Laboratory Animal Care (HSCAA), Japan (from 21 March 2009 valid until 24 March 2017: approval numbers 08-001, 11-001, and 14-001). All the experimental protocols involving the laboratory mice used in this series of studies were reviewed and approved by the Interdisciplinary Monitoring Committee for Proper Animal Use and Welfare of Experimental Animals, a peer review panel established at NIHS with the experimental code #110-2007.

Whole-body irradiation of donor mice or recipient mice for spleen colony assay

The ¹³⁷Cs-gamma irradiator (Gamma Cell 40, CSR, Toronto, Canada) at NIHS was used for the whole-body irradiation of mice at a dose rate of 0.95 Gy/min with a 0.5-mm aluminum-copper filter. Twelve mice were irradiated simultaneously in a round flat plastic cage divided into 12. For spleen colony assay, the Till and McCulloch method was used.²³ Among the 240 mice used for CFU-S9 analysis and 336 mice for CFU-S13 analysis, 7 (2.9%) and 16 (4.8%) mice were moribund or dead before or on the day of analysis, respectively. All the groups had one or none of such mice, and data from such mice were eliminated from the analysis.

Peripheral blood count

To collect peripheral blood from the submandibular sinuses, a Goldenrod animal bleeding lancet (Medipoint, Inc., Mineola, NY, USA) was used. The number of peripheral blood cells was determined using a Coulter counter (Sysmex K-4500; Sysmex Co., Kobe, Japan) as described elsewhere.²

BrdUrd-ultraviolet-light cytocide assay

The BrdUrd (Sigma, St. Louis, MO)-labeled bone marrow cells from the femur of each donor mouse, purged with UVA light to evaluate the kinetics of hematopoietic colony-forming progenitor cells, were employed as described elsewhere.^{10–12} Accordingly, the percent mortality of BrdUrd-incorporating HPCs purged with UVA light corresponds to the percent fraction of cells that had incorporated BrdUrd. Figure 1 shows the experimental design. BrdUrd treatment was started 4 weeks after the whole-body irradiation when all the peripheral blood parameters had recovered. The durations of the treatment were set as 1 h, and 1, 2, 3, 5, and 12 days.

Statistical analyses

Data were processed for statistical analyses to obtain the mean and standard deviation (SD), which were subjected to Student's *t*-test and/or multiple comparison by the

Figure 1 Experimental design indicating the duration of BrdUrd treatment and observation time points: The groups of mice irradiated at 6 weeks of age (top) and 21 months of age (bottom) are shown for comparison. Each donor mouse was randomly assigned to one of the two groups with or without whole-body irradiation (2 Gy). Then, these four groups (young and old, with and without whole-body irradiation) were subdivided into 6 groups, according to the duration of BrdUrd treatment (1 h, and 1, 2, 3, 5, and 12 days). For BUUV assay, pooled bone marrow cells from mice in each group were used. BrdUrd: bromodeoxyuridine; IR: irradiation

Dunnett test to determine the statistical significance of difference using Microsoft Office Excel 2007 (Microsoft, Redmond, WA). Differences were considered significant at $p < 0.05$.

Results

Hematological parameter recovery 4 weeks after whole-body irradiation

Body weight and the majority of other hematological parameters of the irradiated groups, 4 weeks after 2-Gy whole-body irradiation, was nearly identical to those of the age-matched nonirradiated control groups irrespective of the age of mice (Figure 2a–g), except for the primitive hematopoietic colony-forming progenitor cells, CFU-S9, and CFU-S13 (Figure 2h and i). The numbers of CFU-S9 and CFU-S13 in the irradiated groups were significantly lower than those in the age-matched nonirradiated control groups. The irradiation-induced decrease in the number of CFU-S13 was nearly identical between the young ($78.9 \pm 16.3\%$) and old ($80.1 \pm 28.4\%$) groups, whereas that in the number of CFU-S9 was significantly different between the young and old groups ($57.8 \pm 11.7\%$ vs. $77.2 \pm 16.3\%$, $p < 0.05$).

Hematopoietic colony-forming progenitor cell kinetics in young mice with or without whole-body irradiation

Figure 3 shows sequential changes in percent incorporation of BrdUrd in each type of colony-forming progenitor cells, i.e., mature HPCs (CFU-GM), less mature HPCs (CFU-S9), and immature HPCs (CFU-S13), as a function of the duration of BrdUrd treatment in the young groups with or without 2-Gy whole-body irradiation 4 weeks prior to the start of BrdUrd treatment. All the types of HPC of the non-irradiated control groups showed curves compatible with

previous observations,^{10,11} i.e., the percent incorporation of BrdUrd increased logarithmically with BrdUrd treatment duration and reached a plateau on day 3 or 5. Compared with the nonirradiated control groups, the irradiated groups showed a significantly higher percent BrdUrd incorporation at 1 h but flatter slopes of the extrapolation curves in the log phase of increasing ratio. The latter-noted change in kinetics corresponded to the generation doubling time and a significantly lower level of the plateau except for CFU-S13.

Hematopoietic colony-forming progenitor cell kinetics in old mice with or without whole-body irradiation

Figure 4 shows sequential changes in percent incorporation of BrdUrd in each type of colony-forming progenitor cell, as a function of the duration of BrdUrd treatment in the old groups with or without 2-Gy whole-body irradiation 4 weeks prior to the start of BrdUrd treatment. Compared with the nonirradiated control groups, the irradiated groups showed HPC-differentiation-stage-specific changes as follows: (a) the slope of the extrapolation curve in the log phase of increasing ratio is flatter for CFU-GM and CFU-S9 but steeper for CFU-S13; (b) the plateau level is significantly lower for CFU-GM but higher for CFU-S13, (c) the percent incorporation of BrdUrd at 1 h is not significantly different among all three types of HPCs. Accordingly, the cell cycle of mature HPCs, specifically CFU-GM, was suppressed, whereas that of immature CFU-S13 was accelerated in old mice following irradiation.

Comparison of cell kinetics among young and old groups with or without whole-body irradiation

To compare the cell kinetics among all types of HPCs in the young and old groups with or without whole-body

Figure 2 Hematological parameters 4 weeks after whole-body irradiation. All the parameters were identical between the same age-matched pairs with and without irradiation, except for CFU-S9 (h) and CFU-S13 (i). (a) Body weight, (b) Weight of the spleen, (c) Number of femoral bone marrow cells, (d–f) Numbers of cells in peripheral blood: white blood cells (d), red blood cells (e), and platelets (f), (g–i). Numbers of hematopoietic colony-forming progenitors: CFU-GM (g), CFU-S9 (h), and CFU-S13 (i). (Each bar indicates an average value. Vertical bars indicate standard deviations. * $p < 0.05$). CFU: colony-forming unit; CFU-GM: CFU-granulomacrophage; CFU-S9: CFU in the spleen on day 9; CFU-S13: CFU in the spleen on day 13

irradiation, parameters related to cell kinetics were calculated from the extrapolation curves and average values of the percent incorporation of BrdUrd in the log and plateau phases, respectively. The generation doubling time (the slope of the extrapolation curves, Figure 5a), the percent incorporation of BrdUrd at time zero (the Y intercept of the extrapolation curves, Figure 5b), and the cumulative cycling fraction (the ratio of the plateau level of irradiated groups to that of the nonirradiated control, Figure 5c) are tabulated with the coefficients for the extrapolation curves and plateau levels (average with SDs) in Supplemental Table 1.

Among the nonirradiated young control groups, these parameters changed in a differentiation-stage-dependent manner, as these HPCs differentiated from the immature to

mature stages, i.e., more mature HPCs had a longer generation doubling time, a higher percent incorporation of BrdUrd at time zero, and a larger cycling fraction. Interestingly, the comparison of the radiation effects between the irradiated young and old groups showed almost the same tendencies in all the parameters except those for CFU-S13. The parameters for CFU-S13 in the irradiated old group showed opposite trends, i.e., a shorter generation doubling time and a slightly lower percent incorporation of BrdUrd at time zero but a larger cycling-cell fraction.

Discussion

In this study, the cell kinetic analysis revealed that the mechanisms underlying peripheral blood recovery-processes after

Figure 3 Percent incorporation of BrdUrd in hematopoietic colony-forming progenitors sequentially obtained from femoral bone marrow cells of mice in the young groups, with or without 2-Gy whole-body irradiation at 6 weeks of age, 4–6 weeks prior to the analysis. See Figure 1. (Each symbol indicates an average value. Vertical bars indicate standard deviations. The 1-h BrdUrd-treated groups (leftmost symbols in each panel) show a statistically significant difference ($p < 0.05$) between the groups with and without irradiation by multiple comparison by the Dunnett test.). BrdUrd: bromodeoxyuridine; IR: irradiation;

2-Gy whole-body irradiation are different between the young and old mice, although the 21-month-old mice showed almost the same level of recovery as the young mice. In the case of the young mice, an enlarged cycling fraction at time zero but a prolonged generation doubling time and a lower plateau level, i.e., a smaller cumulative cycling fraction, were observed (Figures 3 and 5). Interestingly, such effects induced by radiation are similar to those induced by senescence in CFU-S13 (Figures 3 and 4, Supplemental Figure 1), suggesting that radiation accelerates aging. Previous reports indicated that the radiation-dose-dependent activation of the ATM/Chk2/p53 pathway, which is related to DNA damage and repair,²⁴ and the suppression of the PI3K/AKT pathway, which is related to cell proliferation,²⁵ occur simultaneously 4 weeks after 0.6- and 3-Gy whole-body irradiation, as shown by

Figure 4 Percent incorporation of BrdUrd in hematopoietic colony-forming progenitors sequentially obtained from femoral bone marrow cells of mice in the old groups, with or without 2-Gy whole-body irradiation at 21 months of age, 4–6 weeks prior to the analysis. See Figure 1. (Each symbol indicates an average value. Vertical bars indicate standard deviations.). BrdUrd: bromodeoxyuridine; IR: irradiation

microarray analysis (Supplemental Figure 2),^{26,27} suggesting that apoptotic signals operate in young mice. Accordingly, although the number of peripheral blood cells in irradiated mice did not change compared with that in the nonirradiated controls, the cell cycle of HPCs following irradiation appeared to be accelerated simultaneously with somewhat impaired, i.e., ineffective hematopoiesis in young mice.

The cumulative cycling fraction of immature CFU-S13 of the old nonirradiated control group was smaller than that of the young nonirradiated control group (Supplemental Figure 1) as previously observed.¹⁰ Moreover, it was reported that beta-catenin, cyclin D1, and PiK3r1 appeared to be downregulated in bone marrow cells of aged mice,² suggesting that hematopoiesis in the old group was suppressed in the steady state, specifically that in immature HPCs. In addition, although there was no significant

Figure 5 Parameters calculated from the extrapolation curves in Figures 3 and 4 in log phase (a and b) and plateau phase (c) (Supplemental Table 1). (a) The generation doubling time of each group was calculated from the slope of the log phase curve. Again, only CFU-S13 in old mice showed the opposite trend, i.e., the generation doubling time of the group without IR was longer than that with IR. (b) The percent incorporation of BrdUrd at time zero, i.e., the Y-intercept value in the log phase indicates the cycling-cell fraction at a certain time. Only CFU-S13 in old mice showed the opposite trend, i.e., the percent incorporation of BrdUrd in the group without IR was higher than that with IR. (c) The plateau level, i.e., the cumulative cycling-cell fraction was calculated from the plateau phase curve. The vertical axis indicates the difference in plateau level ratio between the age-matched groups with and without irradiation with standard deviations. Again, only CFU-S13 in old mice showed the opposite trend, i.e., the plateau level ratio of the group with IR was significantly higher than that without IR. (* $p < 0.05$ between age-matched groups with and without IR). BrdUrd: bromodeoxyuridine; CFU-S9: colony-forming unit in the spleen on day9; CFU-S13: colony-forming unit in the spleen on day 13; IR: irradiation

difference in the cell cycle of CFU-GM or CFU-S9 between the nonirradiated young and old groups, the irradiated young and old groups showed marked differences in the cell-cycle parameters of HPCs. Unlike the young mice that showed significant decreases in generation doubling time and increases in the percent incorporation of BrdUrd at time zero, such tendencies in change with the differentiation of HPCs are unclear for old groups (Figure 5a and b). Interestingly, whereas the good recovery of peripheral blood parameters can be seen 4 weeks after 2-Gy whole-body irradiation even in old mice (Figure 2), the accelerated cell cycle seen in immature CFU-S13 of old groups was associated with a shorter generation doubling time and a larger cumulative cycling-cell fraction (Figures 4 and 5).

In this study, although we did not focus on HSCs, the upregulation of beta-catenin, cyclin D1, and PiK3r1 in the steady state was reported in the lineage-marker-negative, c-kit-positive, and stem-cell-antigen-1-positive (LKS) cell population in 21-month-old mice,² which is abundant among cells with the hematopoietic reconstitution ability among bone marrow cells.²⁸ In addition, single-dose (2-Gy) whole-body irradiation at 6 weeks or 6 months of age induced further upregulation of cyclin D1 and PiK3r1 in the LKS cells in 21-month-old mice.² On the basis of these previous observations, it can be hypothesized that, in the steady-state hematopoiesis in old mice, cells in the LKS population proliferate to compensate for their functional impairments within downstream progenitorial daughters. Simultaneously, it may be considered that ineffective hematopoiesis also progresses without any increase in the number of differentiated HPCs with aging. Moreover, these changes induced by aging may be enhanced by irradiation.

Finally, no significant difference was observed in the acute recovery of peripheral blood cells following exposure to xenobiotic stimuli between the old and young groups, as observed following the single-dose whole-body irradiation in this study. However, regardless of this absence of

significant difference, hematopoietic cells of old mice with selective genetic and/or epigenetic alterations^{29–31} may easily further alter their capacity to recover from hematological stresses such as repeated exposures to xenobiotic stimuli.³² Accordingly, more mature HPCs, such as CFU-GM and CFU-S9, in young mice can participate in hematopoiesis not only in the steady state but also as they recover from damage caused by xenobiotic stimuli such as 2-Gy whole-body irradiation. However, these more mature HPCs in old mice are solely responsible for the steady-state hematopoiesis, but they lose the ability to act as a buffer and proliferate as they recover from damage caused by xenobiotic stimuli. On the other hand, CFU-S13 in old mice were found to show an accelerated response as they recover from damage caused by the 2-Gy whole-body irradiation in this study. Thus, primitive HPCs such as CFU-S13, whose cell cycle decelerates in the steady state during aging, have the potential to overcome damage caused by whole-body irradiation, and the mature peripheral blood cell population can be restored to its stable level.

Author contributions: YH designed and performed the experiments, analyzed the data, and wrote the manuscript; IT performed experiments, analyzed the data, and edited the manuscript; KK provided aged mice and edited the manuscript; YK analyzed the data and edited the manuscript; and TI designed and supervised the research, and analyzed the data. All the authors have read and approved the final manuscript except TI, who passed away on 6 October 2014 during the follow-up study.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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