

Regular Article

Proliferation and Differentiation of Human Hematopoietic Stem/Progenitor Cells Exposed to Low-dose X-irradiation

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Hematopoietic stem/progenitor cells (HSPCs) can self-renew and differentiate into all hematopoietic lineages, and are extremely sensitive to oxidative stresses. Exposure of HSPCs to ionizing radiation causes a marked suppression of mature blood cell production in a dose-dependent manner. However, little information about the long-term effects of low-dose X-irradiation on the stemness of human HSPCs has been reported. The present study investigated the biological characteristics of the differentiation and proliferation of low-dose X-irradiated human CD34⁺ HSPCs. Highly purified CD34⁺ cells exposed to low-dose X-rays were cultured in liquid and semisolid media supplemented with an optimal cytokine combination. The liquid medium was cultured for 14 days; no significant differences in total cell number were observed between non-irradiated and X-irradiated cultures. The expression levels of cell surface antigens such as CD34, CD38, and CD45 on cells harvested from X-irradiated culture were similar to those of cells from non-irradiated culture. However, a significant reduction was observed in the number of burst-forming unit erythroid (BFU-E) cells between non-irradiated and 0.5 Gy X-irradiated cells in the myeloid progenitor assay on day 14; moreover, concurrently, radiation-induced γ -H2AX foci were significantly greater on day 14 than on day 0. These results suggest that low-dose X-irradiation is associated with clonal growth suppression of BFU-E cells.

Key words: hematopoietic stem/progenitor cells, low-dose X-irradiation, radiosensitivity, burst-forming unit erythroid cells

1. Introduction

Ionizing radiation with low-linear energy transfer such as X-rays generates reactive oxygen species (ROS) by indirect action of energy on water molecules and simultaneously generating double-strand breaks (DSBs), which are known to be lethal, by direct action on DNA

itself, with sequence apoptosis or stress-related responses. The biological effects of low-dose irradiation have been investigated, and the cumulative evidence suggests that the cellular response induced by low-dose irradiation may differ from that induced by higher doses.¹⁻⁴⁾ Radiation-induced bystander effects, radioadaptive response, and activation of the defense system or immune response represent phenomena that have an important impact on novel biological responses induced by low-dose irradiation.

Hematopoietic stem/progenitor cells (HSPCs) can differentiate into all hematopoietic lineages throughout the lifetime of an organism and self-renew to maintain

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their undifferentiated phenotypes,⁵⁻⁸⁾ which are abundant in not only bone marrow but also placental/umbilical cord blood. Because of their high proliferative potential, HSPCs are extremely sensitive to oxidative stresses such as ionizing radiation, chemotherapeutic agents, and extensive ROS accumulation.⁹⁻¹⁵⁾ In previous reports, damage to the hematopoietic system caused by ionizing radiation markedly suppressed the production of mature blood cells in a dose-dependent manner,^{6, 7, 9, 10, 16)} because of loss of stemness caused by oxidative DNA damage.^{17, 18)} The cellular response induced by lower doses of irradiation may differ from that induced by higher doses; however, the long-term effects of low-dose X-irradiation on the proliferation and differentiation of human HSPCs have not been studied extensively. In the present study, the biological characteristics of the myeloid differentiation and proliferation of low-dose X-irradiated CD34⁺ HSPCs derived from human placental/umbilical cord blood were investigated.

2. Materials and Methods

2.1. Growth factors and fluorescent antibodies

Recombinant human interleukin-3 (IL-3) and human stem cell factor (SCF) were purchased from BioSource (Tokyo, Japan). Recombinant human erythropoietin (EPO) and granulocyte colony-stimulating factor (G-CSF) were purchased from Sankyo Co. Ltd. (Tokyo, Japan). Recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). These growth factors were administered at the following concentrations into the medium: IL-3, 100 ng/ml; SCF, 100 ng/ml; EPO, 4 U/ml; G-CSF, 10 ng/ml; GM-CSF, 10 ng/ml. The following fluorescence-labeled monoclonal antibodies (mAbs) were purchased from Beckman Coulter Immunotech (Marseille, France): fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (CD34-FITC), phycoerythrin (PE)-conjugated anti-human CD38 (CD38-PE), PE-cyanin-5-forochrome tandem (PC5)-conjugated anti-human CD45 (CD45-PC5), and mouse IgG₁-FITC, IgG₁-PE, and IgG₁-PC5, which were used as isotype controls. Anti-phospho-Histone H2AX monoclonal antibodies (JBW301) were purchased from Upstate Biotechnology (Lake Placid, NY, USA), and Alexa Fluor 488[®]-conjugated anti-mouse IgG secondary antibodies were purchased from Molecular Probes (CA, USA).

2.2. Collection and purification of HSCs

This study was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine (Hirosaki, Japan). Informed consent was obtained from the mothers, who had delivered full-term infants, after written and verbal explanation was provided.

Upon delivery, placental/umbilical cord blood samples were collected into sterile collection bags (CBC-20; Nipro, Osaka, Japan) containing the anticoagulant citrate phosphate dextrose. The samples were isolated and maintained separately before each experiment. Within 24 h of cord blood collection, light-density mononuclear cells were separated by centrifugation on a Lymphosepar I (1.077 g/ml; Immuno-Biological Laboratories, Takasaki, Japan) for 30 min at 300 g and were washed three times with calcium- and magnesium-free phosphate-buffered saline [PBS (-); Sigma-Aldrich, Stockholm, Sweden] containing 5 mM ethylenediamine-N,N,N',N'-tetraacetic acid (Wako, Tokyo, Japan). The cells were then processed for CD34⁺ enrichment according to the manufacturer's instructions. An Automacs[™] Pro Separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used for the sorting of CD34⁺ cells. The CD34⁺-enriched cell population is referred to as HSPCs in this study.

2.3. In vitro irradiation

CD34⁺ cells were exposed to X-irradiation in a serum-free medium within 24 h of isolation. Radiation (0.1, 0.3, or 0.5 Gy; 150 kVp; 20 mA; 0.5 mm aluminum and 0.3 mm copper filters) was administered using an X-ray generator (MBR-1520R; Hitachi Medical Co., Tokyo, Japan) at a distance of 45 cm between the focus and the target. The dosage was monitored with a thimble ionization chamber placed next to the sample during irradiation. The dose rate was approximately 1 Gy/min.

2.4. Methylcellulose culture

Lineage-committed myeloid hematopoietic progenitor cells included colony-forming unit-granulocyte macrophages (CFU-GM), burst-forming unit-erythroid (BFU-E) cells, and colony-forming unit-granulocyte erythroid, macrophage, megakaryocyte (CFU-Mix) cells. The CD34⁺ cells were assayed in methylcellulose culture by suspending them in 1 ml of methylcellulose medium (MethoCult H4230, StemCell Technologies Inc.) supplemented with a cytokine mixture that included five growth factors (GFs; IL-3, SCF, EPO, G-CSF, and GM-CSF). This mixture was transferred into 24-well cell culture plates (Falcon, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) at 0.3 ml/well and was subsequently incubated for 14 days at 37°C in a humidified atmosphere with 5% CO₂. Colonies consisting of more than 50 cells were counted using an inversion microscope (Olympus, Tokyo, Japan).

2.5. Liquid culture

The CD34⁺ cells resuspended in serum-free Iscove's Modified Dulbecco's Medium (Gibco Invitrogen, Grand Island, NY, USA) supplemented with BIT 9500 serum substitute (StemCell Technologies, Vancouver, Canada)

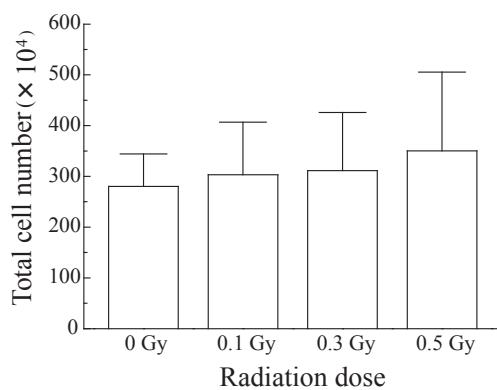


Fig. 1. The total number of cells generated in liquid culture. CD34⁺ cells were exposed to 0, 0.1, 0.3, or 0.5 Gy X-irradiation and cultured in serum-free medium supplemented with a combination of G-CSF, GM-CSF, IL3, SCF, and EPO. The cells were harvested from the culture on day 14. The total number of viable cells was counted using trypan blue dye exclusion. Values represent the means \pm SD of four separate experiments.

and low density lipoprotein (Calbiochem®, US and Canada). The CD34⁺ cells were treated with a cytokine combination of GFs in 24-well cell culture plates at a concentration of 2.5×10^2 cells/0.5 ml/well and were incubated at 37 °C in a humidified atmosphere with 5% CO₂. The number of viable cells was counted on day 14 using the trypan blue dye exclusion method (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA).

2.6. Flow cytometry analysis

The expression of specific cell surface antigens was analyzed by direct immunofluorescence flow cytometry (FC500, Beckman Coulter Inc., Fullerton, CA, USA) using triple staining combinations of mAbs. In brief, the cells were incubated with saturated concentrations of the relevant mAbs for 20 min at room temperature. The cells were washed and subjected to flow cytometry. For each experiment, the isotype-matched control mAb was used as a negative control.

2.7. Immunofluorescence detection of γ-H2AX

Following treatment with cytokines and X-irradiation for the indicated time periods, CD34⁺ cells (2×10^5 cells/sample) were harvested, washed with PBS (-), and fixed with ice-cold 75% ethanol for 10 min at room temperature. Fixed cells were washed with PBS (-), permeabilized in 0.5% Triton X-100 (Wako, Osaka, Japan) on ice for 5 min, and then washed twice with PBS (-). Cells were incubated with anti-phospho-Histone H2AX monoclonal antibody diluted 1:300 with 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 0.1% Tween-20 (TBST) containing 5% skim milk at 37 °C for 120 min. Thereafter, cells were washed with PBS (-) and were incubated with Alexa Fluor 488 conjugated anti-mouse IgG secondary antibody diluted

1:400-fold with TBST containing 5% skim milk at 37 °C for 60 min. Following a second wash with PBS (-), cells were adhered to microscope glass slides (Matsunami Glass Ind., Osaka, Japan) using a StatSpin® CytoFuge 2 (Iris Sample Processing, Inc., MA, USA), and mounted with Vectashield® Mounting Medium with DAPI (Vector Laboratories, Inc., CA, USA). Images of the cells were captured using an LSM 710 laser scanning microscope (Carl Zeiss Microscopy Co., Ltd., Tokyo, Japan), and the expressions of γ-H2AX foci per cell counting were performed until at least 50 cells in every sample under blinded condition.

2.8. Statistical analysis

Statistical analysis was performed using the Origin software package (OriginLab® Pro v8.1; Northampton, MA, USA) for Windows operating system. Data were obtained from four independent experiments and were compared between control and experimental groups using the paired *t*-test and Tukey-Kramer test. A *P*-value of < 0.05 was considered to be statistically significant. Data were analyzed using the two-sided Student's *t*-test and Mann-Whitney *U*-test. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. Proliferation and differentiation of CD34⁺ cells exposed to low-dose X-irradiation

Freshly prepared CD34⁺ cells X-irradiated at doses ranging from 0.1 to 0.5 Gy were cultured in serum-free medium supplemented with an optimal cytokine combination and then harvested on day 14. As displayed in Figure 1, the control culture containing 2.5×10^2 non-irradiated CD34⁺ cells increased to 2.8×10^6 cells on day 14. A similar increase was observed in the culture of cells exposed to X-irradiation, thereby indicating that cell growth of CD34⁺ cells was not affected by low-dose X-irradiation ranging from 0.1 to 0.5 Gy. Among the harvested cells described above, the expression of early-stage hematopoiesis-related cell surface antigens, including CD34, CD38, and CD45, were analyzed by flow cytometry. The CD34 antigen is a novel marker of human HSPCs, the CD45 antigen is a common leukocyte antigen, and CD38 antigen is a novel multifunctional ectoenzyme that is widely expressed in cells and tissues and most notably in leukocytes¹⁹. In particular, CD38⁻CD45⁺ cells are more mature cells than CD38⁺CD45⁺ cells, and CD34⁺CD38⁻ cells are more primitive cells than CD34⁺CD38⁺ cells. As displayed in Fig. 2, the positive proportions of CD34⁺CD45⁺, CD38⁺CD45⁺, and CD34⁺CD38⁺ cells detected in the non-irradiated culture were reduced dramatically to approximately one twenty-sixth, one third, and one twenty-sixth, respectively, on

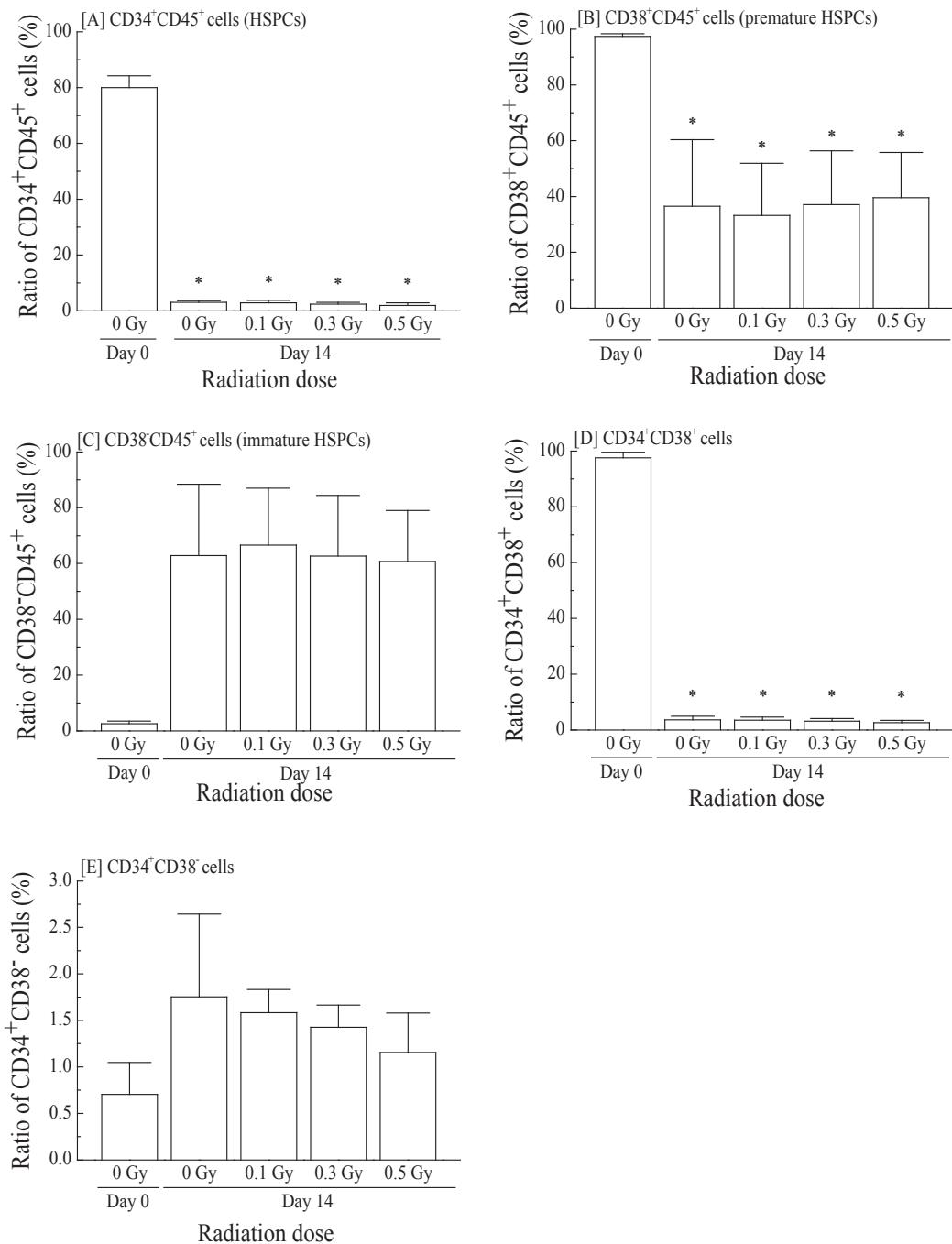


Fig. 2. The expression of early-stage hematopoiesis-related cell surface antigens. The positive proportions of CD34⁺CD45⁺ (panel A), CD38⁺CD45⁺ (panel B), CD38⁻CD45⁺ (panel C), CD34⁺CD38⁺ (panel D), and CD34⁺CD38⁻ (panel E) cells were determined by flow cytometry on day 0 and 14 postirradiation. Values represent the means \pm SD of four separate experiments. * $P < 0.05$ vs. non-irradiated cells on day 0.

day 14. A similar decrease was observed in the culture of cells exposed to X-irradiation. In contrast, the positive proportions of CD38⁻CD45⁺ and CD34⁺CD38⁻ cells detected in the non-irradiated culture were increased, but not statistically significant. A similar increase was observed in the culture of cells exposed to X-irradiation, thereby indicating that proliferation and differentiation of

CD34⁺ cells was not affected by low-dose X-irradiation.

3.2. Low-dose effects on the clonal growth of CD34⁺ HSPCs

X-irradiated CD34⁺ cells were plated in methylcellulose semisolid culture supplemented with an optimal cytokine combination on day 0. In addition, the generated cells

Table 1. Total number of myeloid hematopoietic progenitors generated in liquid culture

	Dose (Gy)	Day 0	Day 14
0 Gy	CFU-GM	66 ± 11	88603 ± 44748
	BFU-E	71 ± 18	50490 ± 46525
	CFU-Mix	6 ± 3	3490 ± 6980
	CFC	143 ± 30	142583 ± 93686
0.1 Gy	CFU-GM	67 ± 18	80666 ± 18110
	BFU-E	77 ± 27	18633 ± 4648
	CFU-Mix	4 ± 3	4530 ± 5313
	CFC	147 ± 45	103830 ± 9530
0.3 Gy	CFU-GM	60 ± 21	81426 ± 74001
	BFU-E	68 ± 18	26896 ± 13078
	CFU-Mix	3 ± 1	9613 ± 11138
	CFC	131 ± 31	117936 ± 75072
0.5 Gy	CFU-GM	50 ± 14	101913 ± 55745
	BFU-E	69 ± 25	2356 ± 4713*
	CFU-Mix	6 ± 3	3430 ± 6860
	CFC	125 ± 38	107700 ± 59569

Values are means of four separate experiments.

*P < 0.05 compared with BFU-E in the non-irradiated cells on Day 14.

in the liquid culture on day 14 were plated in the same manner. As summarized in Table 1, the total number of lineage-committed myeloid hematopoietic progenitor cells, including CFU-GM, BFU-E, and CFU-Mix cells, detected in the non-irradiated culture was 143 ± 30 on day 0; moreover, similar clonal growth of each of the progenitors exposed to X-irradiation was observed as well. After the liquid medium was cultured for 14 days, no statistically significant difference was observed between the total number of hematopoietic progenitor cells in the non-irradiated and X-irradiated cells. However, a significant difference was observed in the number of BFU-E between non-irradiated cells and 0.5 Gy X-irradiated cells, whose proportion was dramatically reduced to approximately one twenty-fifth. These results indicated that clonal growth of BFU-E cells, but not of total colony-forming cells, was suppressed by low-dose X-irradiation.

3.3. γ-H2AX expression

To monitor the repair of DNA DSBs induced by ionizing radiation, the expression of γ-H2AX, a marker of DNA damage response, was measured in cells harvested from liquid culture. The expression of radiation-induced γ-H2AX was not observed immediately following administration of 0.5 Gy X-irradiation. In contrast, a significant increase in γ-H2AX expression was observed in the cells harvested from liquid culture on day 14 (Fig. 3).

4. Discussion

In the present study, the biological characteristics of the

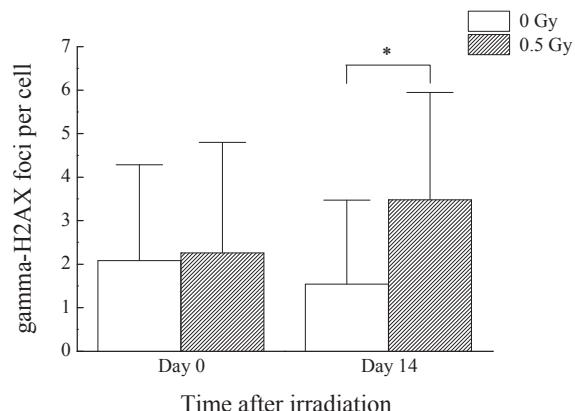


Fig. 3. Effects of X-irradiation on DNA damage in CD34⁺ HSPCs. CD34⁺ cells exposed to 0 or 0.5 Gy X-irradiation were cultured in liquid medium supplemented with a combination of G-CSF, GM-CSF, IL3, SCF, and EPO. Cells were harvested on day 14, and γ-H2AX expression was analyzed (as described in the Materials and Methods section). Nuclear and γ-H2AX foci were stained using DAPI and Alexa Fluor 488, respectively. *P < 0.05 vs. non-irradiated cells.

differentiation and proliferation of low-dose X-irradiated CD34⁺ HSPCs derived from human placental/umbilical cord blood were investigated. In the analysis of liquid culture, the total number of cells generated in the culture of CD34⁺ HSPCs exposed to 0.1, 0.3, and 0.5 Gy X-rays was similar to the number of cells in the non-irradiated culture (Fig. 1). In addition, the positive proportions of CD34⁺CD45⁺, CD38⁺CD45⁺, and CD34⁺CD38⁺ cells detected in the non-irradiated culture were reduced dramatically on day 14 (Fig. 2). A similar decrease was observed in the cultures of cells exposed to X-rays. In contrast, the positive proportions of CD38⁻CD45⁺ and CD34⁺CD38⁻ cells detected in the non-irradiated culture were increased, but were not statistically significant. A similar increase was observed in the culture of cells exposed to X-rays, indicating that proliferation and differentiation of CD34⁺ cells are not affected by low-dose X-irradiation.

In the myeloid progenitor assay, the generation of hematopoietic progenitor CFCs from primitive hematopoietic stem cells, which can produce HSPCs, was not decreased by radiation in the liquid culture on day 0 (Table 1); however, a significant difference in the number of BFU-E between non-irradiated and 0.5 Gy X-irradiated cells was observed in the liquid culture on day 14. At that time, DNA damage was assessed by measuring γ-H2AX expression in CD34⁺ HSPCs (Fig. 3), but the expression was not observed immediately following administration of 0.5 Gy X-rays. In contrast, a significant increase in γ-H2AX expression was observed in the cells harvested from liquid culture on day 14. In general, the main target in biological damage by ionizing radiation is DNA, and ROS generated by ionizing radiation induces DNA DSBs directly and exerts various cytotoxic effects.²⁰⁻²³

Furthermore, it has been reported that irradiation induced a persistent increase in ROS production in HSPCs, and the induction of chronic oxidative stress was associated with sustained increase in oxidative DNA damage, inhibition of clonogenic function, and induction of cell senescence but not apoptosis.^{17,18)} In this study, it was confirmed that low-dose X-irradiation did not affect cell proliferation or differentiation but led to the accumulation of persistent DNA damage in the CD34⁺ HSPCs (Figs. 1, 2, 3). However, significant suppression of clonal growth was observed only in the BFU-E and not in the total colony-forming cells by low-dose X-irradiation in liquid culture on day 14 (Table 1). Although no definitive mechanism can be proposed on the basis of the present results alone, radiation-induced accumulation of intracellular ROS and subsequent DNA damage of hematopoietic progenitors may be keys to the induction of erythrocyte senescence, considering that regulation of oxidative stress particularly affects early erythropoiesis.²⁴⁾ It is possible that low-dose X-irradiation stimuli direct common myeloid progenitor (CMP) differentiation because erythropoiesis and granulopoiesis are derived from CMP.

In conclusion, the present results suggest that erythropoiesis derived from CD34⁺ HSPCs is suppressed by low-dose X-irradiation. Further studies will be necessary to determine whether the expressions of early-stage erythropoiesis-related cell surface antigens, such as CD36 or CD235a, on γ-H2AX-positive, low-dose X-irradiated cells are higher or lower than those on non-irradiated cells in liquid culture on day 14. Additional studies are being performed in our laboratory to examine this mechanism in detail using a larger number of cord blood samples.

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