The Influence of Gender- and Age-related Differences in the Radiosensitivity of Hematopoietic Progenitor Cells Detected in Steady-state Human Peripheral Blood

Kengo KATO1, Mikinori KUWABARA2 and Ikuo KASHIWAKURA1*

Hematopoietic progenitor cells/Peripheral blood/Radiosensitivity/Gender/Age.

To investigate the importance of gender and aging on the individual radiosensitivity of lineage-committed myeloid hematopoietic stem/progenitor cells (HSPCs) detected in mononuclear cells (MNCs) of steady-state human peripheral blood (PB), the clonogenic survival of HPCs, including colony-forming unit-granulocyte macrophage; burst-forming unit-erythroid; colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte cells derived from MNCs exposed to 0.5 Gy and 2 Gy X-irradiation were estimated. MNCs were prepared from the buffy-coats of 59 healthy individual blood donors. The results showed that large individual differences exist in the number of HSPCs, as well as in the surviving fraction of cells. Furthermore, the number of progenitor cells strongly correlated with their surviving fraction, suggesting that the radiosensitivity of hematopoietic progenitor cells decreases with the number of cells in the 10^5 cells population. A statistically significant negative correlation was observed between the surviving fraction observed at a dose of 0.5 Gy and the age of an individual, however, none of these correlations were observed after 2 Gy irradiation. No statistically significant difference was observed in individual radiosensitivity between males and females at either radiation dose. The present results indicated a correlation between the individual responsiveness of HSPCs to ionizing irradiation, especially to low dose irradiation, and aging.

INTRODUCTION

Hematopoietic stem cells (HSCs) can self-renew and differentiate into all hematopoietic lineages throughout the lifetime of an organism. Due to their high proliferative potential, HSCs are sensitive to extracellular oxidative stresses such as radiation and chemotherapeutic agents.1–6) The peripheral blood (PB) of steady-state healthy individuals contains a small fraction of hematopoietic stem/progenitor cells (HSPCs) which play a decisive role in the homeostasis of blood cell production.7,8) In the case of radiation exposure, HSPCs circulating in PB also suffer radiation damage, but this may differ from that of bone marrow (BM) and placental/umbilical cord blood (CB) due to the differences in their biological characteristics and the various components present in plasma. That is, HSPCs in steady-state PB are more mature compared to equivalent cells in BM and CB.9,10) Therefore, HSPCs and their antioxidative capacities may result in a differential sensitivity to radiation. Müller et al. suggested that individuals show marked differences in radiation sensitivity, which has considerable consequences in the fields of both radiation protection and radiation therapy.11) Our previous study also demonstrated that there are large individual differences in the radiosensitivity of lineage-committed myeloid hematopoietic progenitor cells detected in steady-state human PB.12) These differences were not observed to correlate with either the plasma or intracellular antioxidants. Although many in vitro studies have been reported to quantify individual radiosensitivity,13–17) there have so far been few studies on the relationship between individual differences in circulating HSPCs in steady-state PB, their radiosensitivity, and gender and aging. An understanding of this relationship can help predict hematopoietic recovery from radiation exposure as well as the extent of radiation damage in hematopoiesis.

In this study, individual differences in the radiosensitivity of lineage-committed myeloid HSPCs, colony-forming cells (CFCs), including colony-forming unit-granulocyte mac-
rophere (CFU-GM); burst-forming unit-erythroid (BFU-E); and colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFU-Mix) cells, detected in mononuclear cells (MNCs) derived from steady-state human PB were evaluated. In addition, we focused on the relationship between their clonogenic survival and the gender and age of the subject.

**MATERIALS AND METHODS**

**Growth factors**

Recombinant human interleukin-3 (IL-3) and recombinant human stem cell factor (SCF) were purchased from Bio-source (Tokyo, Japan). The recombinant human granulocyte-colony stimulating factor (G-CSF) and erthropoietin (EPO) were purchased from Sankyo Co. Ltd. (Tokyo, Japan). Recombinant human granulocyte-macrophage-colony stimulating factor (GM-CSF) and thrombopoietin (TPO) were purchased from Pepro Tech Inc (Rocky Hill, New Jersey, USA). These factors were administered at the following concentrations: IL-3 and SCF, 100 ng/ml; TPO, 50 ng/ml; G-CSF and GM-CSF, 10 ng/ml; and EPO, 4 U/ml medium.

**Collection of MNCs from PB**

This study was approved by the Committee of Medical Ethics of Hirosaki University Graduate School of Medicine (Hirosaki, Japan). After obtaining informed consent from 59 healthy individual blood donors (41 males and 18 females), PB was collected by the Aomori Red Cross Blood Center (Aomori, Japan). The mean donor age was 42.7 ± 11.6 years, with a range of 22–69 years. The buffy-coat was prepared from whole-blood by the Red Cross and was supplied to our laboratory. Light-density MNCs were separated from the buffy-coat by centrifugation for 30 min at 300 × g on Lymphosepar I (1.077 g/ml; Immuno-Biological Laboratories, Gunma, Japan). The light-density MNCs were then washed twice with phosphate-buffered saline (Sigma-Aldrich, Stockholm, Sweden) containing 5 mM ethylenediamine -N, N, N’, N’-tetraacetic acid (Wako, Tokyo, Japan).

**In vitro irradiation**

The X-ray irradiation (150 kVp, 20 mA, 0.5 mm Al and 0.3 mm Cu filters) was performed using an X-ray generator (MBR-1520R; Hitachi Medical Co., Tokyo, Japan) with a distance of 45 cm between the focus and the target at a dose rate of approximately 80 cGy/min. During X-ray exposure, the dose intensity was evaluated using an ionization chamber.

**Methylcellulose culture**

The colony-forming cells (CFCs) including colony-forming unit-granulocyte-macrophage (CFU-GM), burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte (CFU-Mix) cells were assayed by methylcellulose culturing using MethoCult (StemCell Technologies Inc, Vancouver, Canada). Non-irradiated (1 × 10^5 cells/ml) and X-irradiated cells (0.5 Gy, 1.5 × 10^5 cells/ml; 2 Gy, 5 × 10^5 cells/ml) were plated onto each well of a 24-well plate (Falcon, Becton Dickinson Biosciences, New Jersey, USA) at 0.3 ml/well with culture medium containing IL-3 (100 ng/ml), SCF (100 ng/ml), G-CSF (10 ng/ml), EPO (4 U/ml), GM-CSF (10 ng/ml), penicillin (100 U/ml) and streptomycin (100 U/ml). Each plate was incubated at 37°C in a humidified atmosphere containing 95% air/5% CO2 for 14 days. The colonies containing more than 50 cells were counted using an inverted microscope (×40, Olympus, Tokyo, Japan). The surviving fraction was obtained by the following calculation; (colonies

![Fig. 1. The numbers of each type of hematopoietic progenitor cell per 1 × 10^5 MNCs from the 59 samples. MNCs were cultured in a methylcellulose medium supplemented with a combination of five cytokine (IL-3, SCF, G-CSF, EPO and GM-CSF), and were assayed for the number of CFU-GM, BFU-E, CFU-Mix and CFCs. The Tukey-Kramer test was performed to analyze the results.](image_url)

<p>| Table 1. The number of progenitor cells of each type |
|---------------------------------|-----------------|------------------|</p>
<table>
<thead>
<tr>
<th>Gender</th>
<th>Progenitor</th>
<th>Number of progenitors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Male</td>
<td>CFU-GM</td>
<td>10.9 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>47.7 ± 35.3</td>
</tr>
<tr>
<td></td>
<td>CFU-Mix</td>
<td>1.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>CFC</td>
<td>59.9 ± 41.8</td>
</tr>
<tr>
<td>Female</td>
<td>CFU-GM</td>
<td>7.9 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>36.1 ± 36.7</td>
</tr>
<tr>
<td></td>
<td>CFU-Mix</td>
<td>0.8 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>CFC</td>
<td>44.8 ± 34.8</td>
</tr>
</tbody>
</table>

There were 41 PB samples from males and 18 from females. The mean individual age was 41.6 ± 11.6 years in males, with a range of 36–69 years and 43.2 ± 11.7 years in females, with a range of 22–35 years.
formed per $1 \times 10^5$ X-irradiated cells) / (colonies formed per $1 \times 10^5$ non-irradiated cells).

Statistical analysis
The differences between the two groups were analyzed by the paired t-test and Wilcoxon signed-ranks test. A univariate analysis was performed using Pearson’s correlation coefficient. Data from multiple groups were also analyzed using a Tukey-Kramer test. The correlation analysis was performed using the software program Origin (OriginLab® Co. Northampton, Massachusetts, USA) for Windows. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Determination of CFU-GM, BFU-E, and CFU-Mix in individuals
The number of CFCs prepared from the steady-state PB of 59 individuals was measured by methylcellulose culture supplemented with a combination of IL-3, SCF, G-CSF,

Fig. 2. The relationship between the age of the blood donors and the number of progenitor cells of each type per $1 \times 10^5$ MNCs. [A] CFU-GM, [B] BFU-E and [C] total number of hematopoietic progenitor cells (CFCs). The Pearson’s correlation coefficient was calculated using the Origin software program.

Fig. 3. The distribution of the surviving fractions of hematopoietic progenitor cells by type. [A] 0.5 Gy-surviving fractions, [B] 2 Gy-surviving fractions. The Tukey-Kramer test was performed.

Fig. 4. The relationship between the number of hematopoietic progenitor cells per $1 \times 10^5$ MNCs and the surviving fraction. [A], [B] and [C] 0.5 Gy-surviving fractions, [D], [E] and [F] 2 Gy-surviving fractions. The Pearson’s correlation coefficient was calculated using the Origin software program.
EPO and GM-CSF. This combination supports maximum colony formation, and each concentration represents the saturated amount. The number of progenitor cells, CFU-GM, BFU-E, CFU-Mix and CFCs, detected in $1 \times 10^5$ MNCs are shown in Fig. 1. The mean number of CFCs detected in $1 \times 10^5$ MNCs was $55.3 \pm 40.1$, thus indicating that BFU-E is the major population (80%) in CFCs (data not shown). The number of BFU-E varied by approximately 20-fold among samples, the average number being significantly higher than the numbers of CFU-GM and CFU-Mix. Next, the relationship between the number of progenitor cells and the gender of each individual was analyzed. Although the average number in males was higher than in females, no significant intergender difference was observed (Table 1). In addition, no significant correlation was observed between the number of progenitor cells and the age of the individuals (Fig. 2).

### Radiosensitivity of HSPCs

HSPCs in MNCs prepared from steady-state PB were exposed to 0.5 Gy and 2 Gy X irradiation, and their survivals was determined by colony formation assay. As a result, the surviving fraction of each CFU-GM, BFU-E, CFU-Mix and CFCs detected in the 0.5 Gy-irradiated MNCs was $0.64 \pm 0.39$, $0.74 \pm 0.23$, $0.30 \pm 0.39$ and $0.70 \pm 0.21$, respectively.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Gender</th>
<th>Progenitor</th>
<th>Survival Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>BFU-E</td>
<td>$0.77 \pm 0.24$</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>CFU-GM</td>
<td>$0.60 \pm 0.45$</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>BFU-E</td>
<td>$0.67 \pm 0.18$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CFU-GM</td>
<td>$0.30 \pm 0.14$</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>BFU-E</td>
<td>$0.33 \pm 0.11$</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>BFU-E</td>
<td>$0.31 \pm 0.016$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CFU-GM</td>
<td>$0.23 \pm 0.24$</td>
</tr>
</tbody>
</table>

There were 41 PB samples from males and 18 from female samples in the experiment using 0.5 Gy irradiation and 21 male and 11 female PB samples used in the experiment with 2 Gy irradiation.

![Fig. 5. The relationship between the age of the blood donors and the surviving fraction of total hematopoietic progenitor CFCs. [A], [B] and [C] 0.5 Gy-surviving fractions, [D], [E] and [F] 2 Gy-surviving fractions. The Pearson’s correlation coefficient was calculated using the Origin software program.](image-url)
significant correlations were observed between the number of progenitor cells in steady-state human PB (Fig. 1). In addition, no CFU-Mix-derived colony was detected after 2 Gy irradiation, thus suggesting the high radiosensitivity of CFU-Mix.

The correlation between the number of progenitor cells and their surviving fraction was analyzed, as shown in Fig. 4. The number of progenitor cells derived from MNCs exposed to 0.5 Gy irradiation correlated with the surviving fraction of CFU-GM and CFCs (Fig. 4-[A]). With regard to 2 Gy irradiation, the number of progenitor cells strongly correlated with the surviving fraction of that cell population (Fig. 4-[B]), thus suggesting that the radiosensitivity of hematopoietic progenitor cells decreases with the number of cells in the $10^5$ cells population.

**The relationship between radiosensitivity and gender- and age-related differences**

To investigate the relationship between the radiosensitivity of HSPCs and gender, surviving fractions in males and females was compared, as shown in Table 2. No significant difference was observed between the surviving fraction, including CFU-GM, BFU-E and CFCs from males and females after 0.5 Gy and 2 Gy-irradiation.

The relationship between the radiosensitivity of HSPCs and age was also analyzed. A significant correlation was observed between the surviving fraction of CFCs obtained after 0.5 Gy irradiation and the age of the blood donors (Figs. 5-[A]–[C]). In particular, this correlation was more evident in males (Fig. 5-[B]), with a correlation coefficient of -0.318 ($p = 0.043$). In contrast, no significant correlation was observed between the surviving fraction of CFCs derived from 2 Gy-irradiated HSPCs and age (Figs. 5-[D]–[F]).

**DISCUSSION**

To investigate the importance of gender and aging on the individual radiosensitivity of lineage-committed myeloid hematopoietic progenitor cells detected in MNCs of steady-state human PB, the clonogenic potential of each type of progenitor cell (CFU-GM, BFU-E and CFU-Mix) was determined. The present study demonstrated that there are large individual differences in the number of hematopoietic progenitor cells in steady-state human PB (Fig. 1). In addition, there were only limited correlations with gender or age in the present study (Table 1, Fig. 2), although statistically significant correlations were observed between the number of progenitors (CFCs) and the surviving fraction (Fig. 4). In particular, the number of progenitor cells strongly correlated with surviving fractions in the case of 2 Gy irradiation (Fig. 4-[B]). These findings suggest that the number of progenitor cells may predict the radiosensitivity of an individual.

In the present study, the surviving fraction obtained after 0.5 Gy irradiation decreased with age (Fig. 5), showing a statistically significant correlation. This correlation was more clearly observed in males (Fig. 5-[B]). At this time, approximately 17% of the CFU-GM surviving fraction was greater than 1.0, while 12% and 9% of BFU-E and CFU-Mix in the surviving fraction were greater than 1.0, respectively (Fig. 3-[A]). Although, these values contained random errors of the survival fraction individually obtained by the calculation based on a small number of colonies due to the fact that the number of progenitors widely varied, hematopoiesis was augmented by 0.5 Gy irradiation in some individuals. In particular, these phenomena were frequently observed in the CFU-GM-derived colony formation, thereby suggesting a leukopoietic response. Although no precise mechanism can explain the present results, numerous positive responses caused by low-dose irradiation are well known. The nature of these responses, known as the adaptive response, caused by priming low-dose irradiation have been examined by a few investigators. For example, Yonezawa et al. reported a radioadaptive survival response where 0.45 Gy irradiation 12 days prior to 6.75 Gy challenging irradiation resulted in significant survival in a mouse model. They speculated that preirradiation increased the stem cell numbers, allowing the production of cytokines such as IL-4 and IL-11. Nevertheless, in order to elucidate the precise mechanisms of 0.5 Gy X-irradiation enhanced hematopoiesis in healthy individuals, further studies using a larger number of PB samples will be required.

The present results suggest that the leukopoietic response observed after low-dose (0.5 Gy) irradiation decreases with aging, although a promoting response occurs in some individuals; no similar correlations were observed in the case of 2 Gy irradiation (Fig. 5). Previous experiments using mouse models showed that the level of a specific product of endogenous oxidative damage to DNA, 8-hydroxy-2'-deoxyguanosine, which is a proposed indicator of oxidative damage in DNA in vitro and in vivo, increased with age in various organs. In addition, when comparing older and younger erythrocytes, decreased concentrations of total free thiol, reduced glutathione, and decreased glutathione reductase activity were observed. In humans, the age-associated decrease in the repair of UV-induced DNA damage at least partially results from decreased levels of proteins that participate in the repair process. Takahashi et al. described that the reduced post-UV DNA repair capacity due to aging results from impairment in the latter step of nucleotide excision repair by a decrease in the expression of factors required for repair. In contrast, Marcon et al. reported that no age- or gender-related differences in mutagen sensitivity
or DNA repair capacity after 2 Gy γ-irradiation were observed in freshly prepared human peripheral lymphocytes.\textsuperscript{29} An age-related decrease in the DNA repair capacity in the general population was reported by some authors,\textsuperscript{30,31} but not by others.\textsuperscript{32,33} However, recent data suggests that age dependent and acquired loss of DNA repair may contribute to physiologic aging and could contribute to the increase in cancer incidence with age.\textsuperscript{34} In addition, recent studies proposed that unrepaired double-strand breaks in KLS (c-Kit\textsuperscript{+}, Sca1\textsuperscript{+}, lineage\textsuperscript{−}) cells and their progenitors lead to decreased proliferative potential, increased turnover of the hematopoietic stem cell population, decreased self-renewal and hence age-dependent decline in multipotent cells within the KLS population, which becomes manifested as loss of bone marrow cellularity and erythropoiesis, features characteristic of normal ageing.\textsuperscript{35,36} In the present study, although no significant differences were observed between males and females with regard to the number of hematopoietic progenitor cells (Table 1) and surviving fractions of cells (Table 2), the mean values of surviving fractions observed in males were higher than those in females. Sari-Minodier \textit{et al.}\textsuperscript{37} assessed occupationally induced chromosomal damage in a large population of hospital workers exposed to low doses of ionizing radiation by the cytokinesis-block micronucleus assay.\textsuperscript{37} They showed that the average binucleated micronucleated cell rate was significantly higher in females than males. In contrast, Koturbash \textit{et al.}\textsuperscript{38} reported that the ionizing radiation-induced increase in the level of DNA strand breaks in male mice was significantly higher than that in females.\textsuperscript{38} They suggested that male mice are more susceptible to the damaging effects of radiation than female mice. Nevertheless, because the individual sample numbers of females analyzed in this study was half of that in males, and gender and age are the most important demographic variables affecting the formation of micronuclei,\textsuperscript{37} further investigation under similar conditions is required to accurately assess the effect of gender on the radiosensitivity of human HSPCs using a larger number of individual samples.

In conclusion, although the findings of the present study are still limited with regard to explaining the mechanism of individual radiosensitivity, a correlation between the individual responsiveness of hematopoietic progenitor cells to ionizing irradiation, particularly to a low-dose of irradiation (such as 0.5 Gy), and aging has been established. We speculate that the individual DNA repair capacity may play an important role in individual differences in the radiosensitivity of human HSPCs, and that this is likely more important than age or gender.

\section*{Acknowledgments}

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