Individual Differences in the Radiosensitivity of Hematopoietic Progenitor Cells Detected in Steady-State Human Peripheral Blood

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Hematopoietic progenitor cells/Radiosensitivity/Individual differences/Antioxidants.

The aim of this study is to evaluate the individual differences in radiosensitivity of lineage-committed myeloid hematopoietic progenitors, colony-forming cells (CFC), detected in steady-state human peripheral blood (PB). Mononuclear cells were prepared from the buffy-coat of 30 individuals PB, and were assayed for CFC by semi-solid culture supplemented with cytokines. X irradiation was performed in the range of 0.5 – 4 Gy at a dose rate of about 80 cGy/min. The mean number of hematopoietic progenitor cells is 5866 ± 3408 in 1 ml of buffy-coat, suggesting that the erythroid progenitor cells are the major population. The total CFC radiosensitivity parameter $D_0$ and $n$ value are 1.18 ± 0.24 and 1.89 ± 0.98, respectively. Using a linear regression analysis, a statistically significant correlation is observed between the $D_0$ value and the surviving fraction at 4 Gy ($r = 0.611$ p < 0.001). Furthermore, we evaluate the relationship between individual radiosensitivity and the level of antioxidants, plasma uric acid, plasma bilirubin, and intracellular glutathione. No statistically significant correlations are observed, however, between the $D_0$ parameter and the level of antioxidants, plasma uric acid, plasma bilirubin, and intracellular glutathione. The present study demonstrates that there are large individual differences in the radiosensitivity of hematopoietic progenitor cells as detected in steady-state human PB. These differences demonstrate almost no correlation with plasma or intracellular antioxidants. The prediction of individual differences in radiosensitivity of CFC can only be measured by 4 Gy irradiation.

INTRODUCTION

The biological characteristics of human hematopoietic stem/progenitor cells subjected to ionizing radiation have been described in many clinical studies. These studies provide important information on predicting the tolerance of the hematopoietic system to radiation exposure, and on clinically applying recombinant cytokines after irradiation.1–5 In steady-state hematopoiesis, a small fraction of stem cells circulate in the peripheral blood (PB), and play a decisive role in the homeostasis of blood cell production.5,6 Hematopoietic stem/progenitor cells contained in steady-state PB are more mature when compared to equivalent cells in bone marrow (BM) and placental/umbilical cord blood (CB).7,8 In addition, various antioxidative molecules/radical scavengers can be detected in the PB of steady-state normal individuals.9–11 When considering the radiation exposure of steady-state normal individuals, the radiation damage to hematopoietic stem/progenitor cells circulating in the PB may tell a different story from BM and CB due to the differences in their biological characteristics and in the presence of various components. Therefore, the heterogeneity of hematopoietic stem/progenitor cells and their antioxidative capacities may result in a differential sensitivity to radiation. Although many in vitro studies have been reported to quantify individual radiosensitivity,12–18 there have so far been few studies on the relationship among individual differences in of circulating hematopoietic stem/progenitor cells in steady-state PB, their radiosensitivity and antioxidative molecules contained in PB.

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In this study, the individual differences in the radiosensitivity of lineage-committed myeloid hematopoietic progenitor cells, colony-forming cells (CFC), contained in each individual mononuclear cells (MNC) prepared from the buffy-coat of steady-state human PB were evaluated. The CFC is composed of colony-forming unit granulocyte-macrophage (CFU-GM) which is a progenitor for granulocytes and macrophages, burst-forming unit erythroid (BFU-E) which differentiate into erythroblasts and erythrocytes, and colony-forming unit granulocyte-erythroid-megakaryocyte-macrophage (CFU-Mix) which is a pluripotent hemopoietic progenitor, thus giving rise to erythroid, granulocyte-macrophage and megakaryocytic cells. We focused our attention on determining the primary effector(s) on the individual differences in radiosensitivity of hematopoietic progenitor cells.

MATERIALS AND METHODS

Growth factors and reagents
Recombinant human stem cell factor (SCF) and interleukin-3 (IL-3) were provided by Kirin Brewery Co. Ltd. (Tokyo, Japan). Recombinant human granulocyte colony-stimulating factor (G-CSF) and erythropoietin (Epo) were purchased from Sankyo Co. Ltd. (Tokyo, Japan), and the recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech Inc. (London, UK). The amounts of these factors added per ml of medium were: SCF, 100 ng; IL-3, 50 ng; G-CSF, 10 ng; Epo, 4 units and GM-CSF, 10 ng.

Collection of MNC from PB
This study was approved by the Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan). After obtaining informed consent from normal human blood donors, PB was collected by Aomori Red Cross Blood Center (Aomori, Japan). The buffy-coat (approximately 30 ml) was prepared from whole-blood (400 ml) by the Red Cross and was supplied to our research team. Light-density MNC were separated from the buffy-coat by centrifugation for 30 min at 300×g on a cushion of Lymphosepar I (1.077 g/ml; Immuno-Biological Laboratories Co. Ltd, Takasaki, Japan). After centrifugation, the supernatant plasma was kept at 4°C for 30 min at 300×g. The plasma was transferred onto 24-well cell culture plates (Falcon, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) at 0.3 ml/well, and then incubated at 37°C for 14 days in a humidified atmosphere containing 5% CO2. The colonies consisting of more than 50 cells were counted using an inversion microscope.

X-ray exposure, the dose intensity was evaluated by the probe with ionization chamber.

Methylcellulose cultures
Colony-forming cells (CFC), including BFU-E, CFU-GM, and CFU-Mix, were assayed using methylcellulose culture, as described previously, with minor modifications. Non-irradiated and X-irradiated MNC (1 × 10^6 – 1 × 10^7) were suspended in 1 ml methylcellulose medium (Methocult H4230, Stem Cell Technologies INC, Vancouver, BC, Canada) supplemented with SCF, IL-3, G-CSF, GM-CSF, and Epo. This mixture was transferred onto 24-well cell culture plates (Falcon, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) at 0.3 ml/well, and then incubated at 37°C for 14 days in a humidified atmosphere containing 5% CO2. The colonies consisting of more than 50 cells were counted using an inversion microscope.

Quantitative analysis of total bilirubin and uric acid in plasma
A quantitative analysis of total bilirubin and uric acid in individual plasma was analyzed using an autoanalyzer (TBA-200FR, Toshiba Medical Systems Co., Otawara, Japan). A Nescote LV T-BIL kit (enzymatic method, Alfresa Pharma Corporation, Osaka, Japan) and Uricolor-liquid S (enzymatic method, Ono Pharmaceutical Group, Osaka, Japan), were applied to determine the total uric acid and bilirubin, respectively.

Quantitative analysis of intracellular total glutathione
The total intracellular glutathione was analyzed using a total glutathione quantification kit (Dojindo Molecular Technologies, Inc, Gaithersburg, MD, USA) in accordance with the manufacturer’s instructions. MNC prepared from each individual were applied to the kit.

Statistical analysis
The significance of differences between the control and experimental groups were determined by a Student’s t test. Data from multiple groups were analyzed using a one-way layout analysis of variance (ANOVA) and a Fisher’s least significant difference test. The values for D0, which is the radiation dose that reduces survival to e^-1 (i.e., 0.37) of its previous value on the exponential portion of the survival curve, and the extrapolation number, n which is the point on the survival scale to which the straight part of the curve back-extrapolates, were determined using a single-hit multitarget equation programmed into a computer. Dose-survival curves were fitted by the method of Levenberg-Marquardt. The correlation coefficient (r) between the two parameters was estimated by least square linear regression analysis. To estimate, each data point was inputed to the program Origin (OriginLab, Northampton, MA, USA) and the calculation was performed on a Windows PC. The normal distribution

RESULTS

The determination of BFU-E, CFU-GM, and CFU-Mix in each individual PB

The number of CFC prepared from steady-state PB of 30 individuals was measured by methylcellulose culture supplemented with a combination of SCF, IL-3, G-CSF, GM-CSF, and Epo (n = 30). This combination supports maximum colony formation, and each concentration is a saturated amount. The number of progenitor cells, CFU-GM, BFU-E, CFU-Mix and CFC, detected in 1 × 10⁵ MNC and 1 ml of buffy-coat is summarized in Table 1. The distributions of each progenitor cell are shown in Fig. 1. The mean data of CFC detected in 1 × 10⁵ MNC and 1 ml of buffy-coat were roughly 77.6 ± 47.2 and 5866 ± 3408, respectively. As determined by the calibration of these results, both were a normal distribution (data not shown). The data shows that BFU-E is a major population (70%) in the buffy-coat of steady-state PB.

Radiosensitivity of hematopoietic progenitor cells

The reaction of each hematopoietic progenitor cell to X irradiation was determined by a clonal assay. After preparing MNC from the buffy-coat, each MNC was irradiated with X-rays ranging in dosage from 0.5 to 4 Gy. The survival curve of each individual sample was plotted by using a single-hit multi-target equation. The typical radiation survival curves are shown in Fig. 2[A], one is from relative radioresistant individuals (open circle) and the other is from radiosensitive individuals (closed circle). The parameters D₀ and n, which characterize the radiosensitivity of each progenitor cell obtained from the radiation survival curves, are shown in Fig. 2[B, C].

The D₀ of each hematopoietic progenitor cell, CFU-GM, BFU-E, CFU-Mix, and the total CFC, was 1.20 ± 0.53, 1.09 ± 0.26, 1.07 ± 0.36 and 1.18 ± 0.24, respectively (Fig. 2[B, C]). There were no significant differences among the D₀ values of each hematopoietic progenitor cell. The n value corresponding to the above indicates, was 2.26 ± 2.14, 2.75 ± 2.78, 1.66 ± 1.01 and 1.89 ± 0.98, respectively. A statistically significant difference (p < 0.05) was only observed

Table 1. Steady-state peripheral blood hematopoietic progenitor cells.

<table>
<thead>
<tr>
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<th>Mean ± SD</th>
<th>Range</th>
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<tbody>
<tr>
<td>CFCs per 1 × 10⁵ MNC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-GM</td>
<td>15.9 ± 15.3</td>
<td>3 – 68</td>
</tr>
<tr>
<td>BFU-E</td>
<td>53.9 ± 30.2</td>
<td>12 – 127</td>
</tr>
<tr>
<td>CFU-Mix</td>
<td>7.8 ± 5.9</td>
<td>1 – 28</td>
</tr>
<tr>
<td>Total CFC</td>
<td>77.6 ± 47.2</td>
<td>19 – 205</td>
</tr>
<tr>
<td>CFCs per 1 ml of buffy-coat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU-GM</td>
<td>1213 ± 979</td>
<td>138 – 3825</td>
</tr>
<tr>
<td>BFU-E</td>
<td>4087 ± 2349</td>
<td>727 – 10680</td>
</tr>
<tr>
<td>CFU-Mix</td>
<td>566 ± 384</td>
<td>68 – 1575</td>
</tr>
<tr>
<td>Total CFC</td>
<td>5866 ± 3408</td>
<td>1071 – 13920</td>
</tr>
</tbody>
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Freshly prepared PB MNC was cultured in a methylcellulose medium supplemented with five cytokine combinations, IL-3 + SCF + G-CSF + GM-CSF + EPO. Values are the mean ± SD of each performed in three wells (n = 30).

Fig. 1. A histogram of total hematopoietic progenitor cells. Freshly prepared PB MNC was cultured in a methylcellulose medium supplemented with five cytokine combinations, IL-3 + SCF + G-CSF + GM-CSF + EPO. [A] the mean value is 77.6 ± 47.2 per 1 × 10⁵ MNC and [B] 5866 ± 3408 per 1 ml buffy-coat, each performed in three wells (n = 30).

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between the BFU-E and CFU-Mix.

Using these results, we analyze the relationship between the $D_0$ values and the CFC number. Linear regression analysis demonstrated no statistical correlation between the $D_0$ and the CFC measurements contained in $1 \times 10^5$ cells ($r = -0.234$, $p = 0.213$) (Fig. 3-[A]), or CFC number contained in 1 ml of buffy-coat derived from the steady-state PB.

The relationship between $D_0$ of each progenitor cell and surviving fraction

To see the relationship between the $D_0$ value and the surviving fraction of hematopoietic progenitor cells in each dose of X irradiation, a correlation coefficient was estimated. Statistically significant correlations were found between the $D_0$ of CFU-GM and the surviving fraction obtained from 1.5 Gy ($r = 0.576$, $p < 0.005$) to 4 Gy ($r = 0.768$, $p < 0.0001$). The relationship obtained by 4 Gy exposures is shown in Fig. 4-[A]. In the case of BFU-E, the major population in the total CFC, the $D_0$ value was significantly correlated with the surviving fraction at only 4 Gy ($r = 0.695$, $p < 0.0001$) (Fig. 4-[B]).
The CFU-Mix also showed statistically significant correlations between the $D_0$ value and the surviving fraction ranging from 1.0 Gy ($r = 0.485, p < 0.001$) to 3 Gy ($r = 0.639, p < 0.0001$) (Fig. 4-[C]). In the total CFC however, no correlation was observed from 0.5 Gy to 3 Gy, but instead, statistically significant correlations were found between the $D_0$ value and surviving fraction at 4 Gy ($r = 0.611, p < 0.001$) (Fig. 4-[D]). Therefore, these results suggest that the individual radiosensitivity is only predictable in surviving fractions at 4 Gy X irradiations.

The relationship between the individual differences in radiosensitivity of hematopoietic progenitor cells and the level of antioxidants

In order to determine the primary effector(s) of individual differences in radiosensitivity of hematopoietic progenitor cells, the total amount of antioxidant molecules/radical scavengers existing in non-irradiated plasma, uric acid and bilirubin, and intracellular glutathione (GSH), were analyzed. During the estimation of the effects of total bilirubin and uric acid, X irradiation was carried out with whole blood before MNC preparation. X irradiation was carried out with 3 Gy exposures. The total uric acid and bilirubin, ranged from 1.5 to 3.9 mg/dl and from 0.26 to 0.68 mg/dl, respectively ($n = 20$, data not shown). The total amount of intracellular GSH ranged from 8.8 to 130 μmol/l ($n = 20$, data not shown). At this time, no significant difference in the content of each molecule was observed between the findings obtained before and after irradiation (data not shown).

The correlation coefficient between the total amount of plasma antioxidant, bilirubin and uric acid, and surviving fraction of total progenitor cells obtained by 3 Gy exposures, is 0.087 ($p = 0.716$, Fig. 5-[A]) and 0.170 ($p = 0.474$, Fig. 5-[B]), respectively, indicating no statistically significant correlations. Similarly, no significant correlations were observed between the total amount of intracellular GSH and the surviving fraction of total progenitor cells ($r = 0.224, p = 0.356$, Fig. 5-[C]). However, the $D_0$ value of CFU-Mix was

![Images of graphs showing survival fraction vs. $D_0$ values for different progenitor cells.](http://jrr.jstage.jst.go.jp)
significantly correlated with the total amount of bilirubin ($r = 0.705$, $p < 0.001$, Fig. 5-[D]). With the exception of this case, no statistically significant correlations were also found between the amount of each antioxidant and the surviving fraction of each progenitor cell.

### DISCUSSION

In the present study, we evaluated the individual differences in radiosensitivity of lineage-committed myeloid hematopoietic progenitor cells detected in MNC prepared from steady-state human PBuffy-coat by using methylcellulose cultures supplemented with a combination of recombinant human cytokines to give maximal colony formation. Under our culture conditions, the mean data of total CFC detected in $1 \times 10^5$ MNC and 1 ml of buffy-coat were about $77.6 \pm 47.2$ and $5866 \pm 3408$, respectively (Table 1). There were large differences, approximately 10 and 13-times, respectively, from the smallest number to the largest number.

We used a PBuffy-coat instead of whole blood for the evaluation; our findings are consistent with those studies to some extent. For example, BFU-E was a major population and the total number of CFC was similar.\(^{19,20}\) The in vitro radiosensitivity of human hematopoietic progenitor cells, CFU-GM, BFU-E, and CFU-Mix, has been investigated in previous studies.\(^{21-23}\) Grilli et al. demonstrated that the $D_0$ for PB-derived CFU-GM is $1.46 \pm 0.13$ Gy, and BFU-E is $0.93 \pm 0.06$ Gy.\(^{11}\) The results we obtained cannot be compared directly with the data obtained by those previous studies because of the differences in cytokine combinations, culture systems, and the purity of cells. However, the $D_0$ values obtained in this study (1.20, 1.09, and 1.07 Gy, for CFU-GM, BFU-E, and CFU-Mix, respectively) were almost the same as in the previous reports (Fig. 2-[B]), demonstrating the validity of our analysis. The $n$ value ranged from 1.66 to 2.75 (Fig. 2-[C]), with a statistically significant difference only between the BFU-E and CFU-Mix. However, the reason for this difference remains unclear based on the

![Fig. 5. The relationship between $D_0$ of total CFC or CFU-Mix, and antioxidants. (A) $D_0$ of total CFC and the concentration of uric acid in each individual plasma ($n = 20$). (B) $D_0$ of total CFC and the concentration of total bilirubin ($n = 20$). (C) $D_0$ of total CFC and the concentration of intracellular total glutathione ($n = 19$). (D) $D_0$ of CFU-Mix and the concentration of total bilirubin ($n = 21$).](image-url)
findings of the present experiment. We confirmed the existence of large differences among the individual radiosensitivity of hematopoietic progenitor cells. In addition to the individual differences in radiosensitivity of hematopoietic progenitors, large differences were found among the progenitors detected in each individual. In particular, there were large differences in the D0 value obtained in CFU-GM which is a progenitor of granulocyte, monocyte and macrophage. This is also very important from the viewpoint of the response after radiation exposure. Based on the findings of an analysis between D0 and the CFC number or the surviving fraction, the results suggest that the individual radiosensitivity is only predictable in surviving fractions at 4 Gy X irradiations (Fig. 4). The evaluation of the radiosensitivity of hematopoietic progenitor cells is usually complicated in some extent, such as the optimal cytokine combinations, the cell preparation required for a colony assay and the skill required to accurately performing colony scoring. Therefore, it is very meaningful that the individual radiosensitivity of hematopoietic progenitors can be evaluated only by 4Gy irradiation.

The levels of antioxidants are thought to influence radiosensitivity or antioxidative capacity, and thereby, potentially effecting radiosensitivity indirectly.24,25 In order to know whether the antioxidant level can be used as a predictor(s) of the radiosensitivity of progenitors, the relationship between individual differences in radiosensitivity of hematopoietic progenitor cells and the level of antioxidants was analyzed. In human blood plasma, free radicals occurring in an aqueous phase are scavenged by the various antioxidants such as uric acid, ascorbate, bilirubin, and sulfhydryl compounds.9–11 However, the level of uric acid in human plasma is several times higher than ascorbate.26,27 Benzie et al. reported that the most important component in fresh plasma is uric acid, which constitutes about 60% of the total antioxidant capacity.24 However, the present results showed no significant correlations between D0 and the total amount of uric acid, bilirubin, and intracellular GSH (Fig. 5). A statistically significant correlation (r = 0.705) was only observed between the D0 of CFU-Mix and total plasma bilirubin (Fig. 5-[Dj]). Neuzil et al. have reported that bilirubin protects the protein by the oxidative damage derived from oxygen radicals, including hydroxyl and hydroperoxyl radicals and superoxide generated by radiation.28 As one possibility, bilirubin may play as a part of the defense system, because bilirubin can protect tissues against oxidative damage caused by free radicals and other reactive oxygen species.29 Severin et al. investigated blood assays for the prediction of the individual radiosensitivity in leukemia patients.30 They focused an association with oral mucositis, which they pose hypothetically as an early symptom of enhanced radiosensitivity. They concluded that three blood assays are characteristic for increased radiosensitivity in leukemia patients: low leukocyte count after 4 Gy TBI, damaged lymphocyte score after 4 Gy in vitro exposure, and reduced antioxidative capacity following 8 Gy TBI. This current study shows that the level of antioxidants is not a predictor for individual radiosensitivity of hematopoietic progenitor cells. On the other hand, as shown in a previous report,31–34 radiation induces the change in the antioxidant level such as the case of the criticality accidents which demonstrated elevation in the serum uric acid level in the lethally irradiated victims. Although little is known regarding the precise mechanisms, antioxidants are nevertheless considered to play an important role in the radiation response. When hematopoietic stem cells are exposed to radiation in vivo, we should consider the degree of proliferative capacity on each progenitor, cell cycle status within the in vivo, p53 status, the repair capacity for DNA damage response and signal transductions by cytokines.35,36 We should also consider the environment surrounding hematopoietic stem cells. In particular, the microenvironments of these cells, known as stem cell niches, which are critical for maintaining stem cell properties, including the self-renewal capability and the ability for differentiation into single and multiple lineages.77 The stem cell niches consist of osteoblasts. Therefore, more precise investigations will be necessary regarding the relationship between the radiation effects on hematopoietic stem/progenitor cells and the effects of co-existing molecule and the environment surrounding the cells.

In conclusion, the present data demonstrates that there are large individual differences in the radiosensitivity of lineage-committed myeloid hematopoietic progenitor cells detected in steady-state human PB. The antioxidant level cannot use a predictor against the radiosensitivity of progenitors; however, the individual differences of hematopoietic progenitor cells can be predicted at a 4 Gy irradiation. We feel that the individual differences of radiosensitivity may depend on the cytokine responsiveness of heterogeneous hematopoietic progenitor cells, the cellular repair and antioxidative capacities after X irradiation.

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