Effects of a 2-step Culture with Cytokine Combinations on Megakaryocytopoiesis and Thrombopoiesis from Carbon-ion Beam-irradiated Human Hematopoietic Stem/progenitor Cells

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Hematopoietic stem/progenitor cells/Cytokine combination/Megakaryocytopoiesis and thrombopoiesis.

To evaluate whether the continuous treatment of two cytokine combinations is effective in megakaryocytopoiesis and thrombopoiesis in hematopoietic stem/progenitor cells exposed to heavy ion beams, the effects of a 2-step culture by a combination of recombinant human interleukin-3 (IL-3) + stem cell factor (SCF) + thrombopoietin (TPO), which just slightly protected against carbon-ion beam-induced damages, and a combination of IL-3 + TPO, which selectively stimulated the differentiation of the hematopoietic stem/progenitor cells to megakaryocytes and platelets, were examined. CD34+-hematopoietic stem/progenitor cells isolated from the human placental and umbilical cord blood were exposed to carbon-ion beams (LET = 50 keV/μm) at 2 Gy. These cells were cultured under three cytokine conditions. The number of megakaryocytes, platelets and hematopoietic progenitors were assessed using a flow cytometer and a clonogenic assay at 14 and 21 days after irradiation, respectively. However, the efficacy of each 2-step culture was equal or lower than that of using the IL-3 + SCF + TPO combination alone and the 2-step culture could not induce megakaryocytes and platelets from hematopoietic stem/progenitor cells exposed to high LET-radiation such as carbon-ion beams. Therefore, additional cytokines and/or hematopoietic promoting compounds might be required to overcome damage to hematopoietic cells by high LET radiation.

INTRODUCTION

The high radio-sensitivity of hematopoietic stem/progenitor cells is a severe factor in radiation damage.1,2) Although hematopoietic stem cell transplantation has been utilized to treat hematopoietic syndrome following high dose exposure in irradiated victims, allogeneic cell transplantation is incomplete from an immunological point of view.2) Therefore, other strategies to recover radiation-damaged hematopoietic cells have been studied. Cytokines, powerful growth factors, have been used to treat irradiated experimental animals.3-6) In fact, the partial recovery of granulocytes after about 10 Gy or more acute total body exposure in a radiation accident victim treated with supportive measures, transfusions, and cytokines (granulocyte-macrophage colony-stimulating factor [GM-CSF] and interleukin [IL]-3) was observed although no recovery of platelet number was observed.7)

Platelets, which play an important role in hemostasis, are released from mature megakaryocytes. A previous study demonstrated that megakaryocytes exhibit especially high radiation-sensitive among hematopoietic cells.8) Moreover, specific thrombopoietic cytokines, such as thrombopoietin (TPO) and IL-11, have not yet been clinically approved for the treatment of thrombocytopenia.9,10) Therefore, the ex vivo expansion with many cytokines is anticipated as a method for amplifying autologous platelets and megakaryocytes.11-13) A recent study showed that cytokine combinations of IL-3 and TPO effectively provided protection and proliferation of X-irradiated immature megakaryocytes (colony-forming unit-megakaryocyte, [CFU-Meg]).14)

On the other hand, the recovery from damage from high
LET radiation has been investigated since the cruel accident in Tokai-mura in 1999.\textsuperscript{15,16} Protons, neutrons, $\alpha$ particles and heavy ion particles, which are known to be high sources of LET, have created a higher relative biological effectiveness (RBE).\textsuperscript{17–19} Other studies have also shown that in vitro carbon-ion beam-irradiated CD34$^+$ CFU-Meg were narrowly protected by treatment with the combination of IL-3 + stem cell factor (SCF) + TPO. However, the specific expansion of megakaryocytes and platelets was insufficient with this combination alone.\textsuperscript{20}

To expand the population specific megakaryocytes and platelets as much as possible, protocols using several cytokine combinations have been tried.\textsuperscript{21–23} Sun and co-workers demonstrated a two-step culture with IL-3 + SCF + TPO and TPO alone to induce a sufficient number of megakaryocytes from cord blood-derived CD34$^+$ cells.\textsuperscript{23} Therefore, in this study, to clarify whether a 2-step culture, including the radio-protection of IL-3 + SCF + TPO (3ST) combination in the ex vivo expansion of hematopoietic stem/progenitor cells exposed to high LET-radiation, we investigated the recovery of megakaryocytopoiesis and thrombopoiesis from the carbon-ion beam-induced damage of CD34$^+$ cells derived from the human placental and umbilical cord blood.

**MATERIALS AND METHODS**

**Growth Factors and Reagents**

TPO, IL-3 and SCF were purchased from Biosource (Tokyo, Japan). Recombinant human granulocyte-colony stimulating factor (G-CSF), GM-CSF and erythropoietin (Epo) were purchased from Sankyo Co. Ltd. (Tokyo, Japan), PeproTech (Rocky Hill, NJ) and Kirin Pharma Co. Ltd. (Tokyo, Japan). These factors were administered at the following concentrations: TPO, 50 ng/ml; SCF and IL-3, 100 ng/ml; G-CSF and GM-CSF, 10 ng/ml and Epo, 4 U/ml medium. Human AB plasma was provided from the Aomori Red Cross Blood Center. The fluorescence-labeled monoclonal antibodies (MAbs) fluorescein isothiocyanate (FITC)-conjugated anti-human CD34 (CD34), FITC-conjugated anti-human CD41 (gpIIbIIIa, CD41), FITC-conjugated anti-human CD42a (gpIX, CD42a) and phycoerythrin (PE)-conjugated anti-human CD41 (gpIX, CD42a) were purchased from Beckman Coulter Immunotech (Marseille, France). Mouse IgG$_1$-FITC, mouse IgG$_1$-PE and mouse IgG$_2a$-FITC (Becton Dickinson Biosciences, San Jose, CA) were used as isotype controls.

**Collection and Purification of cord blood CD34$^+$ Cells**

This study was approved by The Committee of Medical Ethics of Hirosaki University School of Medicine (Hirosaki, Japan). After obtaining informed consent from the mothers, cord blood (7 samples) for use in this study was collected at the end of full-term deliveries using a sterile collection bag containing the anticoagulant, citrate-phosphate dextrose, according to the guidelines of the Tokyo Cord Blood Bank (Tokyo, Japan). These samples were separately isolated and used for each experiment. Within 48 hr after the collection of cord blood, light-density mononuclear cord blood cells were separated by centrifugation on Lymphosep I (1.077 g/ml, Immuno-Biological Laboratories, Takasaki, Japan) for 30 min at 300 g and washed three times with phosphate-buffered saline (PBS) containing 5 mM ethylenediaminetetraacetic acid (EDTA). These cells were then processed for CD34$^+$ cell enrichment, according to the manufacturer’s instructions. EasySep$^\text{TM}$ human CD34 selection kit (StemCell Technologies, Vancouver, Canada) was used for the positive selection of CD34$^+$ cells. At the end of the procedure, the purity was measured using a fluorescence cell analyzer (EPICS$^\text{TM}$ XL, Beckman-Coulter, Fullerton, CA) (90.0–97.0%).

**In Vitro Irradiation of cord blood CD34$^+$ Cells**

Monoenergetic carbon-ion beams (290 MeV/nucleon, LET = 50 keV/$\mu$m, about 2 Gy/min) were generated by an accelerator (Heavy Ion Medical Accelerator in Chiba) at the National Institute of Radiological Sciences (Chiba, Japan).\textsuperscript{18,19} In brief, a combination of wobbler magnets and a scatterer was used to obtain a uniform irradiation field. The range shifter made of Lucite disks was inserted just upstream of the samples to decrease the energy and to modulate the LET at the sample position. In the acrylic irradiation chambers (1 mm thickness), the LET ranged from 60 to 72 when the energy was adjusted to give 50 keV/$\mu$m at the sample center. The dose and the dose rate were monitored during irradiation with a parallel-plate ionization chamber placed upstream of the range shifter. Before the irradiation of the cell samples, this monitoring ionization chamber was calibrated with a standard ionization chamber placed at the sample position. The standard ionization chamber was calibrated to the national standard. The LETs of the beams were calculated by fitting the measured residual range to the theoretical depth vs. LET relation.

**Liquid Culture**

Cord blood CD34$^+$ cells were plated on 24-well plates (Falcon, Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) and cultured in serum-free Iscove’s modified Dulbecco’s medium (IMDM, Gibco$^\text{TM}$ Invitrogen, Carlsbad, CA) supplemented with BtT9500 (a serum substitute for serum-free culture, StemCell Technologies, INC) and low density lipoproteins (CALBIOCHEM, Darmstadt Germany). As shown in Fig. 1, the schedules and cytokine combinations for step culture were performed. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO$_2$. On
day 14 and 21, the cells were harvested and the number of viable cells determined by trypan blue exclusion. The total number of megakaryocytes was calculated by the total cell number harvested from the culture and the proportion of CD41+ in the harvested cells. The platelet proportion was analyzed as CD42a+ particles according to a previous description. Each hematopoietic progenitor (burst-colony forming unit-erythroid [BFU-E], colony-forming unit-granulocyte and monocyte [CFU-GM], colony-forming unit-granulocyte, erythroid, monocyte and megakaryocyte [CFU-Mix] and CFU-Meg) included in the harvested cells was assayed using methylcellulose culture and plasma clot culture supplemented with suitable cytokines. The total number of these hematopoietic progenitors was calculated from the total number of cells harvested and the number of colonies per well.

**Methylcellulose culture**

BFU-E, CFU-GM, and CFU-Mix were assayed using a methylcellulose culture as described previously with minor modifications. The harvested cells from each liquid culture (1 × 10^6) were suspended in 1 ml methylcellulose medium (Methocult H4230, Stem Cell Technologies INC) supplemented with SCF, IL-3, G-CSF, GM-CSF, and Epo. This mixture was transferred onto 24-well cell culture plates 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.

**Plasma Clot Culture**

CFU-Megs were assayed by the plasma clot technique using platelet-poor human plasma. 10% human platelet-poor AB plasma, TPO, SCF, penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), MEM non-essential amino acids (1%), MEM vitamins (1%), MEM non-essential amino acids (1%) (GIBCO® Invitrogen), thioglycerol (1 × 10^-5 M) (Sigma, St. Louis, MO), L-asparagine (2 μg/ml), CaCl₂ (74 μg/ml) (Wako Pure Chemicals, Tokyo, Japan), and 0.2% bovine serum albumin (BSA, Boehringer Mannheim GmbH, Germany) were added to the culture medium based on IMDM. On day 14 and 21, harvested cells (2 × 10^5 cells/ml) from each liquid culture were added to the culture medium. This baseline culture was plated onto 24-well cell culture plates (BD Falcon, Franklin Lakes, NJ) at 0.3 ml per well, and then was incubated at 37°C for 14 days in a humidified atmosphere containing 5% CO₂.

**Identification of Megakaryocyte Colonies by Immunofluorescence**

The plasma clot cell cultures were fixed by two 15-min incubations in acetone: methanol (2:1). The plates were dried in airflow overnight and stored at –20°C. Prior to staining, the plates were returned to room temperature and then PBS containing 0.5% BSA (PBS-B) was added to soften the clot. The plates were incubated at room temperature with FITC-CD41 MAAb diluted 1:100 in PBS-B for 1 hr, followed by a single wash with PBS-B. The nuclei were then counterstained with propidium iodine (PI, 0.3 ng/ml, Sigma).
After a final wash, the colonies were counted using a fluorescence microscope at 100X magnification (Olympus, Tokyo, Japan). The megakaryocyte colonies were classified by size: large colonies containing more than 50 cells (from immature CFU-Meg), and small colonies containing 3 to 50 cells (from mature CFU-Meg). The total number of CFU-Meg (total CFU-Meg; i.e., progenitor cells) among the original CD34+ cells was calculated by the summation of the total number of colonies derived from CFU-Meg.

Flow Cytometry Analysis

The expression of specific cell surface antigens was analyzed by direct immunofluorescence flow cytometry using double staining combinations of MAbs, including PE-CD41 and FITC-CD42a. Briefly, the cells were incubated with saturated concentrations of the relevant MAbs for 20 min at room temperature, washed, and analyzed by flow cytometry. For each experiment, an isotype-matched irrelevant control MAb was used as a negative control. The CD42a-positive particles detected in the fraction of low forward scatter were determined to be platelet-like particles using flow cytometry as described previously. The production rate of platelet-like particles was determined by the percentage of total events recorded by flow cytometry.

Statistical Analysis

In all points, the differences among three culture conditions were analyzed by Student’s t-test or Welch’s t-test.

RESULTS

Effects of the 2-step culture on total cells and megakaryocytes expansion from carbon-ion beam-irradiated cord blood CD34+ cells

In non-irradiated cord blood CD34+ cells, the expansion rate of total cells reached to a 100 – 1000 fold-increase on day 14. However, the total cell number on day 21 just increased in comparison to day 14. In carbon-ion beam-irradiated cord blood CD34+ cells, the rate of expansion was about 1/10 of non-irradiated controls. The culture schedule of Day 7 3ST-3ST showed most effective hematopoiesis in both of 0 and 2 Gy-irradiated cord blood CD34+ cells (Fig. 2-A). On the other hand, the megakaryocytic population indicated the same pattern in non- and carbon-ion beams irradiation, respectively (Fig. 2-B). In Day 7 3ST-3ST treatment, the megakaryocyte purity was low although the total megakaryocyte number was highest of the 3 groups (Fig. 2-B and C).

Effects of the 2-step culture on the expansion of hematopoietic progenitor cells from carbon-ion beam-irradiated cord blood CD34+ cells

The step culture of Day 7 3ST-3ST showed a strong ability to expand hematopoietic progenitor cells in comparison to the other two types of culture in non-irradiated cord blood.
Effects of the 2-step culture on the platelet production from mature megakaryocytes in carbon-ion beam-irradiated cord blood CD34+ cells

The platelets released from differentiated mature megakaryocytes in non- and carbon-ion beam-irradiated cord blood CD34+ cells were assayed in the particle population by a flow cytometer.14) The long period of IL-3 + SCF + TPO treatment decreased the platelet-like CD42a+-population (Fig. 3-A). However, the total platelet number were equal among the three conditions in non- and carbon-ion beam-irradiated cord blood CD34+ cells, respectively (Fig. 3-B).

DISCUSSION

High LET-radiation induced severe hematopoietic damage in radiation accident victims at Tokai-mura in 1999.15,16) Ex vivo expansion is a promising method to recover autologous damaged hematopoietic cells because few cytokines have been given clinical approval. A previous study demonstrated the combination of IL-3 + SCF + TPO to comparatively protect radio-sensitive CD34+ CFU-Meg from carbon-ion beams at 2 Gy and that it is possible to expand hematopoietic cells by the cytokine combination from hematopoietic stem/progenitor cells exposed to high LET-radiation in an ex vivo culture. However, this combination could not effectively induce megakaryocytopenia.20) Recently, the use of a few cytokine combinations have been reported to induce selective and large-scale proliferation of hematopoietic cells in ex vivo.21–23) Therefore, the ability of cord blood CD34+ cells protected by the combination of IL-3 + SCF + TPO (a first

Table 1: Hematopoietic progenitor cells expanded by a two-step culture from non- and carbon-ion beam-irradiated cord blood CD34+ cells.

<table>
<thead>
<tr>
<th>Day 14</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>CFU-Mix</th>
<th>CFU-Meg</th>
<th>CFU-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 3ST-3T</td>
<td>0 Gy</td>
<td>27 ± 22</td>
<td>378 ± 673</td>
<td>5 ± 7</td>
<td>159 ± 200</td>
</tr>
<tr>
<td></td>
<td>2 Gy</td>
<td>0.5 ± 0.7</td>
<td>3 ± 3</td>
<td>N. D.</td>
<td>6 ± 8</td>
</tr>
<tr>
<td>Day 7 3ST-3T</td>
<td>0 Gy</td>
<td>15 ± 27</td>
<td>413 ± 650</td>
<td>N. D.</td>
<td>169 ± 139</td>
</tr>
<tr>
<td></td>
<td>2 Gy</td>
<td>1 ± 1</td>
<td>9 ± 11</td>
<td>0.1 ± 0.2</td>
<td>6.5 ± 6.2</td>
</tr>
<tr>
<td>Day 7 3ST-3ST</td>
<td>0 Gy</td>
<td>170 ± 222</td>
<td>3538 ± 6393</td>
<td>34 ± 50</td>
<td>729 ± 597</td>
</tr>
<tr>
<td></td>
<td>2 Gy</td>
<td>6 ± 8</td>
<td>62 ± 105</td>
<td>1 ± 2</td>
<td>26 ± 24</td>
</tr>
</tbody>
</table>

N. D.: not detected
The values are the means ± SD of three separate experiments.
step) from heavy ion beams to be effectively differentiated into megakaryocytopoiesis and thrombopoiesis by the combination of IL-3 + TPO (a second step) was investigated.

SCF was discovered as c-kit ligand at 1990^24,25^ and was shown to play a role as a radioprotector in vitro and in vivo. Therefore, it did not have clinical marketing approval. During hematopoietic ex vivo expansion, SCF has been shown to synergize with other growth factors, increasing the number of mature cells, mature progenitor cells or primitive progenitor cells. Certainly, the continuous treatment of hematopoietic stem/progenitor cells with SCF (Day 7 3ST-3ST) increased the total cell number (Fig. 2-A). The treatment with Day 7 3ST-3ST also effectively induced megakaryocyte expansion and protected hematopoietic stem/progenitor cells against high LET-radiation-induced damage (Fig. 2-C). However, the specific induction of megakaryocytopoiesis was low (Fig. 2-B). The treatment with SCF + TPO decreased apoptosis of cord blood CD34+ CFU-Meg at 24 hrs after X-irradiation by half in comparison to TPO alone. Therefore, the protection against carbon-ion beam-induced apoptosis might occur due to SCF-treatment for 24 hr under the Day 1 3ST-3T conditions and this result was an improvement over that observed with the regimen of IL-3 + TPO alone (data not shown). However, the proliferative effects of SCF were immediately lost after the removal of SCF although the treatment on Day 1 3ST-3T exclusively induced megakaryocytopoiesis (Fig. 2, Table 1). The number of megakaryocytes by Day 7 3ST-3T did not exceed that of Day 7 3ST-3ST (Fig. 2). The hematopoietic progenitors, including CFU-Meg, were increased by Day 7 3ST-3ST although no significant differences was observed (Table 1). Therefore, the continuous treatment with SCF seemed to be effective for the protection from carbon-ion beams in hematopoietic stem/progenitor cells.

Carbon-ion beams considerably decreased the number of platelets in the CD34+ cells and the number of platelets were closely similar among the three cytokine conditions (Fig. 3-B) although the specificities of thrombopoiesis were different among them (Fig. 3-A). It seemed that the treatment with Day 7 3ST-3ST inhibited the ability of platelet release from mature megakaryocytes although this treatment effectively increased the number of CFU-Meg and megakaryocytes. Therefore, other cytokines may be necessary to directly stimulate the maturation of megakaryocytes and the production of platelets. Cortin and co-workers demonstrated that the combination of TPO, SCF, IL-6 and IL-9 efficiently induced the maturation of megakaryocytes and the production of platelets. Moreover, they showed that increasing SCF concentrations from 1 to 150 ng/ml led to a decrease in megakaryocytic purity but to an increased number of mature megakaryocytes and platelets. The concentration of SCF (50 ng/ml) used by Sun and co-workers might be insufficient according to the results reported by Cortin and co-workers. Nevertheless, this concentration was thought to have resulted in the success of the 2-step culture. Certainly, the expansion rate of the total cells in present study was higher than that at day 7 (data not shown). Each concentration of IL-3 + SCF + TPO might be optimal as a 1-step culture. Therefore, in this study, 2-step culture both IL-3 + SCF + TPO and IL-3 + TPO were unable to dramatically improve the megakaryocytopoiesis and thrombopoiesis.

Recently, novel thrombopoietic agents, such as non-peptide compounds and TPO peptide mimetics have been developed in many laboratories due to the fact that TPO is no longer prescribed for such patients. We also discovered that liquid crystal-related compounds enhanced the platelet production in ex vivo expansion from peripheral blood CD34+ cells with TPO (unpublished data). Therefore, these stimuli for platelet production, such as cytokines may stimulate effective megakaryocytopoiesis and thrombopoiesis with IL-3 + SCF + TPO in heavy ion beam-irradiated hematopoietic stem/progenitor cells.

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