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Ex vivo expansions and transplantations of mouse bone marrow-derived hematopoietic stem/progenitor cells*

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Abstract: To examine the effects of co-culture with bone marrow mesenchymal stem cells on expansion of hematopoietic stem/progenitor cells and the capacities of rapid neutrophil engraftment and hematopoietic reconstitution of the expanded cells, we expanded mononuclear cells (MNCs) and $CD34^+$/c-kit^+ cells from mouse bone marrow and transplanted the expanded cells into the irradiated mice. MNCs were isolated from mouse bone marrow and $CD34^+$/c-kit^+ cells were selected from MNCs by using MoFlo Cell Sorter. MNCs and $CD34^+$/c-kit^+ cells were co-cultured with mouse bone marrow-derived mesenchymal stem cells (MSCs) under a two-step expansion. The expanded cells were then transplanted into sublethally irradiated BDF1 mice. Results showed that the co-culture with MSCs resulted in expansions of median total nucleated cells, $CD34^+$ cells, GM-CFC and HPP-CFC respectively by 10.8-, 4.8-, 65.9- and 38.8-fold for the mononuclear cell culture, and respectively by 76.1-, 2.9-, 71.7- and 51.8-fold for the $CD34^+$/c-kit^+ cell culture. The expanded cells could rapidly engraft in the sublethally irradiated mice and reconstitute their hematopoiesis. Co-cultures with MSCs in conjunction with two-step expansion increased expansions of total nucleated cells, GM-CFC and HPP-CFC, which led us to conclude MSCs may create favorable environment for expansions of hematopoietic stem/progenitor cells. The availability of increased numbers of expanded cells by the co-culture with MSCs may result in more rapid engraftment of neutrophils following infusion to transplant recipients.

Key words: Hematopoietic stem cells, Mesenchymal stem cells, Expansion, Transplantation
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INTRODUCTION

High dose chemotherapy followed by stem cell transplantation (SCT) is routinely used for treatment of patient with hematological malignancies and selected solid tumors. Patients receiving SCT have periods of neutropenia and thrombocytopenia and prolonged periods of depressed immune function, particularly recipients of cord blood (CB) and bone marrow (BM) grafts. The use of peripheral blood progenitor cells (PBPCs) rather than CB and BM as a source of hematopoietic reconstitution has been shown to significantly reduce the duration of pancytopenia (To *et al*., 1992; Faucher *et al*., 1994; Schmitz *et al*., 1996; Hartmann *et al*., 1997). The time of neutrophil and platelet engraftment varies for different sources of hematopoietic cells. PBPCs engraft neutrophils and platelets at around days 9 and 14 respectively while bone marrow cells engraft on days 14 and 28 respectively (Peters *et al*., 1988; Sheridan *et al*., 1992). The time of CB to neutrophil (20 to 30 days) and platelet (50 to 200 days) en-

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graftment are significantly delayed compared to BM and PBPC (Gluckman *et al*., 1997). Although there is a clear dose-response relationship between the number of $CD34^+$ cells infused and the time to neutrophil recovery, a plateau is reached at approximately 5×10^6 $CD34^+$ cells/kg, above which dose there is little incremental improvement in recovery time (Glaspy *et al*., 1997). These are most likely due to the time required for the $CD34^+$ cells to differentiate into mature neutrophils following their infusion and the number of matured progenitor cells committed to nuetrophil and platelet recovery.

Ex vivo expansion of hematopoietic grafts could provide more rapid engraftment and decrease graft failure in CB and BM. Clinical studies of ex vivo expanded PBPCs demonstrated a correlation of rapid neutrophil engraftment with the total nucleated cells (TNC) per kilogram, whereas no significantly correlation was obtained with the number of $CD34^+$ cells/kg (McNiece *et al*., 1999). Also, the ex vivo expanded PBPC products contained more mature neutrophil cells compared to the expanded CB cells (McNiece *et al*., 1999; Shpall *et al*., 1999). With a two-step culture, we improved culture conditions for the ex vivo expansion of CB and increased expansion of both committed and primitive progenitor cells (McNiece *et al*., 2000b). In the present studies, we developed a modified expansion methodology using mouse bone marrow mesenchymal stem cells (MSCs) as an adherent cell feeder layer in the two-step culture system to support the ex vivo expansion of hematopoietic stem cell from mouse bone marrow. Effects of hematopoietic cells expanded with this modified expansion method on hematopoietic recovery following high-dose irradiated mouse and expanded hematopoietic cell transplantation were analyzed.

MATERIAL AND METHODS

BM products

Mouse hematopoietic stem/progenitor cells (HS/PCs) were obtained from ♂BDF1 mouse. The mouse was infused with 5 Fu (8.5 ml NaCl (0.9%) +1.5 ml 5-fluorouracil) at dose of 20 μl/g in 8 days before bone marrow was collected by rushing back legs with PBS/1%FBS. Low-density mononuclear cells (MNCs) were isolated from the heparinized bone marrow samples using Ficoll-Hypaque (Sigma Chemical Company, St. Louis, MO) per manufacturer's instructions. The low-density MNCs were washed and resuspended in phosphate-buffered saline (PBS) and 1% human serum albumin (HAS) (Baxter Healthcare Corp.). Half of each MNCs unit was selected for $CD34^+$/c-kit^+ cells using MoFlo cell selection system (MoFlo Cell Sorter, Fort Collins). $CD34^+$/c-kit^+ cell purity was determined by flow cytometric analysis using anti-CD34 and c-kit antibodies.

Stroma conditions

Confluent layers of mouse bone marrow mesenchymal stem cell lines UC-OZ-17 were grown in 100 ml Teflon culture bags (American Fluoroceal Inc., Gaithersburg, MD). The cells were cultured in 50 ml of minimum essential medium alpha medium (Alpha; GIBCO, Grand Island, NY) supplemented with 20% fetal calf serum (FCS; Summit Fort Collins, CO). When the layers were confluent, the medium was removed and rinsed once with Hanks balanced salt solution (HBSS; GIBCO, Grand Island, NY). Then MNCs or $CD34^+$/c-kit^+ cells were seeded on the stromal layers.

HS/PC expansion

MNCs or $CD34^+$/c-kit^+ cells were seeded into the above culture bags in 50 ml of modified ex-vivo expansion medium (DM; Amgen Inc.) containing 100 ng/ml each of rrSCF, rhG-CSF and rhMGDF (Amgen Inc.) per experiment. The cultures were incubated for 7 days in 100% humidified 5% CO_2 in air at 37 ºC. After 7 days of culture, the contents of the bags were refed with another 50 ml of modified ex-vivo expansion medium (DM; Amgen Inc.) containing 100 ng/ml each of rrSCF, rhG-CSF and rhMGDF (Amgen Inc.). The cultures were incubated for another 7 days in 100% humidified 5% CO_2 in air at 37 ºC. After incubation, the cultures were harvested. Expanded cells from the supernant were collected from each bag. Then each bag was scraped with a cell scraper to collect all adherent cells. The expanded cells and adherent cells were pooled to-

gether, sieved through a 100-μm nylon filter, and after washing the cells in PBS/1%HSA were re-suspended in PBS/1%HSA. The cell suspension was evaluated for white blood cell (WBC) content using Advia 120 (Bayer Corp.). Phenotype was determined by flow cytometry. The cells were plated in progenitor assays for committed myeloid progenitor cells (GM-CFC) and primitive progenitor cells (HPP-CFC).

Advia 120

Complete blood counts (CBC) with white blood cell differential (Diff) were performed using Advia 120 hematology system (Bayer Corp, Norwood, MA). CBC/Diff were taken on the initial cells seeding day as a baseline. At various time points during the culture, 250 μl of supernant was collected and analyzed by Advia 120. Upon harvesting the cells, a final CBC/Diff was analyzed for each bag's total cells. After transplantation of expanded cells, the peripheral blood was collected from each transplanted or non-transplanted mouse at various time points and analyzed for hematopoietic recovery using Advia 120.

Flow cytometric analysis

Initial and cultured cells were analyzed for phenotypic expression of surface proteins specific for subpopulations of hematopoietic cells. Cells were stained with monoclonal antibodies (mAbs) conjugated to either phycoerythrin (PE) or fluorescein isothiocynate (FITC). The mAbs included anti-CD34-PE, anti-CD3-FITC, anti-CD14- PE, and anti-CD15-FITC (Becton Dickinson, San Jose, CA). Approximately 0.5×10^6 cells were removed from the culture and washed once in PBS/1%HSA. Conjugated antibody was added, and then the cells were incubated at 4 ºC in the dark for minutes. Following one wash with PBS/1%HSA, cells were fixed with 1% paraformaldehyde and analyzed by flow cytometry within 24 hours. Flow cytometry analysis was performed using FACSComp Flow Cytometer (Coulter Electronic Inc.). Nonspecific binding was determined by staining an aliquot of cultured cells with an isotype control for both PE and FITC fluorescence. A minimum of 50 000 cells were collected in a list mode file format. List mode data were analyzed using CellQuest software (Coulter Electronic Inc.).

Progenitor cell assays

Initial and cultured cells were analyzed for committed (granulocyte-macrophage colony-forming) progenitor cells (GM-CFC) and primitive (high proliferative potential colony-forming) progenitor cells (HPP-CFC). Initial cells and post expansion cells were plated in 1ml complete methylcellulose without erythropoietin (Gencyte, Amherst, NY) based assays at 10 000 to 100 000 cells per culture. Cultures were incubated for 14 days and GM-CFC colonies identified as colonies of ≥50 translucent cells using a dissecting microscope at 20×magnification. After that, the cultures were incubated for another 14 days and scored for HPP-CFC, defined as compact large colonies (diameter>0.5 mm) that contained>50 000 cells per colony.

Transplantation

At one day before transplantation of expanded cells, ♀BDF1 mice at 8 week age were irradiated with ^{60}Co γ-ray for two times in a 4 hour interval, each time for 25 min at dose of 24.1 R/min. Five irradiated mice were used for transplantations of cells expanded respectively from MNCs and $CD34^+$/c-kit^+ cells, and 5 irradiated mice were used as control. The number of transplanted cells for each irradiated mouse was 8.7×10^6 cells expanded from MNCs and 4.7×10^5 cells expanded from $CD34^+$/c-kit^+ cells. After transplantation, 100 μl of peripheral blood was collected from the orbit of each mouse. Peripheral blood was diluted up to 250 μl with PBS/1%HSA, and then analyzed for hematopoietic recovery using Advia 120.

RESULTS

Expansion of TNCs

Following MNCs separation of the bone marrow, the mean number of MNCs from 10 mice was 15.2×10^6 cells, half of which was selected $CD34^+$/c-kit^+ cells. The mean number of $CD34^+$/c-kit^+ cells after selection was 7×10^4 cells (with the mean recovery of 2.1%). MNCs or $CD34^+$/c-kit^+ cells were

expanded in the bag layered with and without UC-OZ-17. After expansion of two-step culture for 14 days in the culture with UC-OZ-17, the total nucleated cells (TNCs) from MNC culture increased 10.8-fold, and TNCs from $CD34^+$/c-kit^+ cell culture increased 76.1-fold. In the culture without UC-OZ-17, TNCs from MNC culture and $CD34^+$/c-kit^+ cell culture increased only 6.3- and 58.7-fold respectively.

During expansion, TNCs in MNC culture decreased gradually from the second day to the fifth day after seeding, then increased gradually until TNCs were harvested at the 14th day (Fig.1). TNCs in $CD34^+$/c-kit^+ cell culture also increased from the fifth day after seeding; there was no the decreasing trend as in the MNC culture.

Expansion of GM-CFC and HPP-CFC

As shown in Fig.2, the modified expansion conditions resulted in increased numbers of GM-CFC and HPP-CFC. The mean number of GM-CFC and HPP-CFC in the starting MNC fraction was 7.6×10^4 and 3.2×10^4, respectively. Following expansion, there were 501×10^4 GM-CFC and 124×10^4 HPP-CFC (65.9- and 38.8-fold expansion, respectively). The mean number of GM-CFC and HPP-CFC in the starting $CD34^+$/c-kit^+ cell fraction was 0.23×10^3 and 0.11×10^3, respectively. Following expansion, there were 16.5×10^3 GM-CFC and 5.7×10^3 HPP-CFC (71.7- and 51.8-fold expansion, respectively).

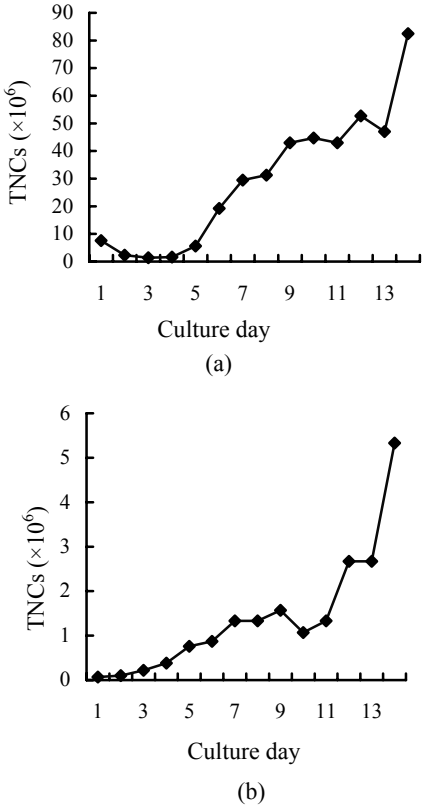

Fig.1 Expansion trends of MNCs and $CD34^+$/c-kit^+ cells
(a) for MNC culture; (b) for $CD34^+$/c-kit^+ cell culture

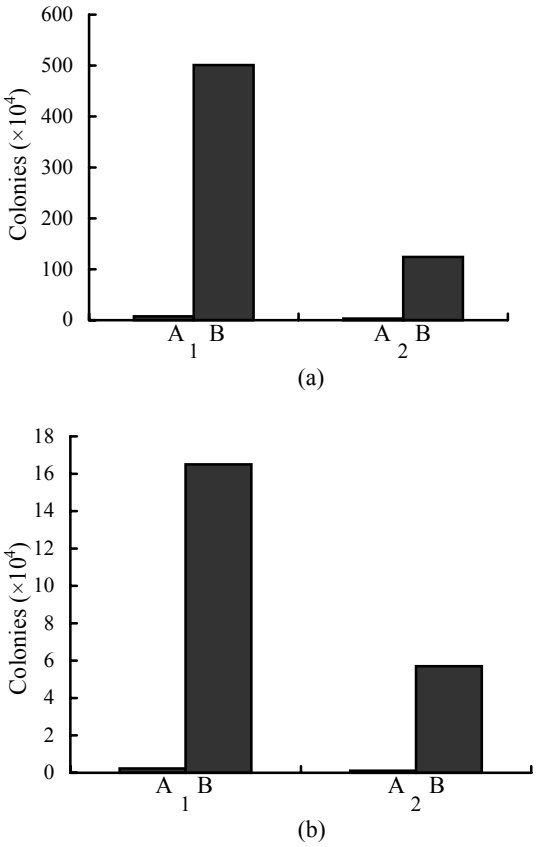

Fig.2 Expansion of MG-CFC and HPP-CFC (A is the starting number and B is the expanded number; 1 is the expansion for MNCs and 2 is the expansion for $CD34^+$/c-kit^+ cells)
(a) GM-CFC; (b) HPP-CFC

Expanded cell immunophenotype

The cell products harvested after 14 days had significantly higher percentages of $CD15^+$ and $CD14^+$ mature myeloid cells (Table 1). The percentages of $CD15^+$ and $CD14^+$ cells were 49% and 13% respectively for the MNC culture, and 41% and

Table 1 Percentage of expanded cells expressing specific phenotypes (%)

	CD15	CD14	CD34	Fold expansion of $CD34^+$ cells
MNC expansion	49	13	0.41	4.8
$CD34^+$/c-kit^+ cell expansion	41	17	3.80	2.9

Results are given as the median of 3 separate experiments

17% respectively for $CD34^+$/c-kit^+ cell culture.

The percentages of $CD34^+$ cells decreased after 14 days of expansion (0.41% and 3.8% respectively for MNC culture and $CD34^+$/c-kit^+ cell culture) compared to the starting percentages (2.1% and 100% respectively for MNC fraction and $CD34^+$/c-kit^+ cell fraction). The number of $CD34^+$ cells in harvested products increased 4.8- and 2.9-fold after expansion by calculating the product of the percentage of $CD34^+$ cells in TNCs and the number of TNCs.

Transplantation with expanded cells

As shown in Fig.3, all irradiated mice without transplantation of expanded cells were dead before 20 days after irradiation. The mortality of the irradiated mice with transplantation of expanded cells were 16.7% and 20% for the transplantations of expanded cells respectively from MNC culture and $CD34^+$/c-kit^+ cell culture at the 20th day after irradiation.

The number of each blood cell lineage in the

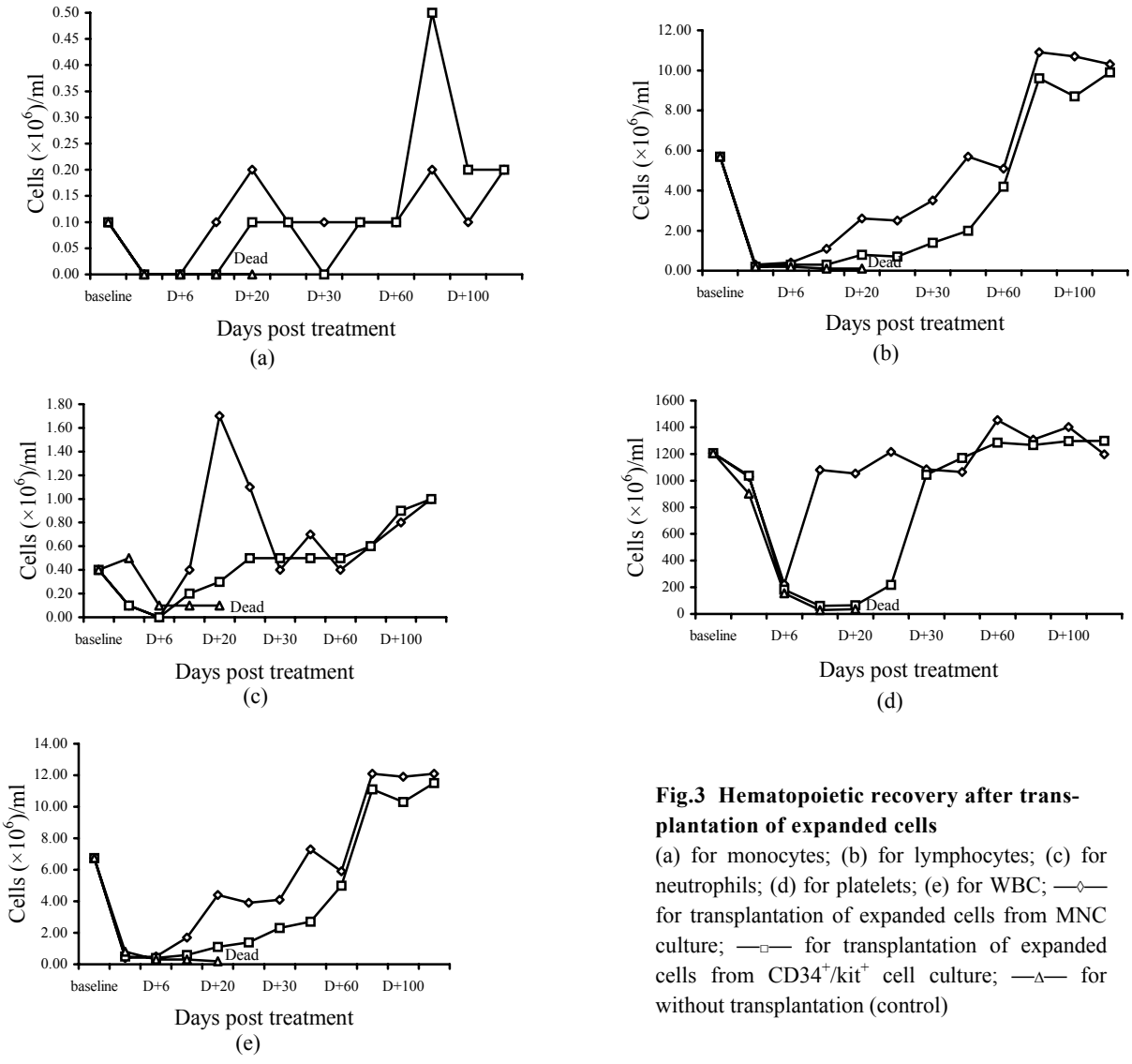

Fig.3 Hematopoietic recovery after transplantation of expanded cells

(a) for monocytes; (b) for lymphocytes; (c) for neutrophils; (d) for platelets; (e) for WBC; —◊— for transplantation of expanded cells from MNC culture; —□— for transplantation of expanded cells from $CD34^+$/kit^+ cell culture; —△— for without transplantation (control)

transplanted mice decreased in 3–6 days after transplantation, but increased from then on. In the transplantation of expanded cells from MNC culture, each blood cell lineage reached the first peaks in 20–25 days after transplantation, and then decreased, especially for monocytes and neutrophils. At the 40–60th day after transplantation, the number of each blood cell lineage in mice transplanted with cells expanded from MNC culture or from $CD34^+$/c-kit^+ cell culture increased significantly until the number reached and even went beyond the baseline.

DISCUSSION

Some studies showed that culture duration beyond 10 days would increase the yield of expanded cells and their maturity, thus permitting further reductions in the duration of neutropenia (McNiece *et al.*, 2000b; Paquette *et al.*, 2002; Wang *et al.*, 2003). We used mesenchymal stem cells (MSCs) as stromal layers in the two-step culture to expand hematopoietic stem/progenitor cells (HS/PCs) from human cord blood (CB) (Wang *et al.*, 2003). This resulted in increased expansion compared to the culture without MSC layers. In the present research, the modified ex vivo expansion method resulted in increases of 10.8-fold expansion of TNCs in the MNS culture and of 76.1-fold expansion of TNCs in the $CD34^+$/c-kit^+ cell culture. Most of the expanded cells were the matured hematopoietic progenitor cells ($CD15^+$ and $CD14^+$ cells) that would contribute to the rapid neutrophil recovery in the irradiated mice.

Recently, our and other researches showed that the ex vivo administration of expanded peripheral blood progenitor cells (PBPCs) can have significant impact on hematopoietic recovery following high-dose chemotherapy (Paquette *et al.*, 2000; Reiffers *et al.*, 1999; McNiece *et al.*, 2000a). The ideal ex vivo expanded cell product should contain cells capable of additional proliferation after they are transplanted, to minimize the cell dose required, and cells differentiated enough to generate matured neutrophils in vivo within a short period of time after transplantation. For hematopoietic recovery in irradiated mice transplanted with the expanded cells from MNC culture, it is inferred that the large percentage of matured progenitor cells result in a first peak of hematopoietic recovery. This rapid recovery creates not only the opportunity for additional proliferation and differentiation of primitive hematopoietic stem cells (HSCs) transplanted but also the opportunity for endogenous recovery. The second increase of each blood cell lineage after 40−60 days should be resulted from additional proliferation and differentiation of infused primitive HSCs and/or from endogenous recovery. The infused dose of cells expanded from the $CD34^+$/c-kit^+ cell culture was less than that from the MNC culture, but there was a higher percentage of primitive hematopoietic stem cells, which resulted in the smooth increase of each blood lineage after transplantation.

On the basis of the above results, we suggest that MNC culture and $CD34^+$ cell culture should be conducted together in the preclinical expansion of HS/PCs; and that both expanded products should be infused together into the patient followed by high-dose chemotherapy, which may result in not only rapid neutrophil recovery but also intermediate and long term engraftments.

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