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RESEARCH ARTICLE

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Experimental study on ex vivo expanded hematopoietic stem/progenitor in the two step culture from human umbilical cord blood transplanted into NOD/SCID mice

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Abstract The effects of hematopoietic stem/progenitor cells (HSPCs) expanded in the two step coculture with human bone marrow mesenchymal stem cells (hMSCs) on the hematopoietic reconstruction of irradiated NOD/SCID mice were studied. Mononuclear cells (MNCs) were isolated from human umbilical cord blood (UCB) and cultured in the non-coculture scheme of rhSCF + rhG–CSF + rhMDGF combination and the coculture scheme of rhSCF + rhG–CSF + rhMDGF + hMSCs. Sublethally-irradiated NOD/SCID mice were transplanted with ex vivo expanded HSPCs with the dose of 8.5×10^6 cells per mouse. After transplantation, the dynamics of WBC in the transplanted mice was measured periodically, and the Alu sequence fragment special for human in the transplanted mice was inspected by PCR. Results showed that the coculture scheme increased proliferation of UCB-derived HSPCs. After transplantation with expanded HSPCs, the population of WBC in the transplanted mice increased in 12 d and reached the first peak in 25 d, then showed the second increasing of WBC in 45~55 d. Expanded cells from the coculture scheme appeared to be favorable for the second increasing of WBC in the transplanted mice. After 85 d, the Alu sequence fragment was detected in the probability of 87.5% (7/8) for the non-coculture scheme and 88.9% (8/9) for the coculture scheme.

Keywords human umbilical cord blood, hematopoietic stem/progenitor cells, ex vivo expansion; NOD/SCID mice, transplantation

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1 Introduction

Human umbilical cord blood (CB) is an attractive alternative to bone marrow or peripheral blood mobilized with growth factors as a source of hematopoietic stem cells. CB contains a high rate of primitive stem/progenitor cells and possesses lower risk of graft-versus-host disease (GVHD) in transplantation. In addition, CB has a rich resource and is convenient to collect. However, a major disadvantage of CB is the low cell dose, which results in slower time to engraftment and higher rates of engraftment failure than bone marrow transplantation. Therefore, ex vivo expansion of hematopoietic stem/progenitor cells (HSPCs) has been proposed as a method to increase the number of cells available for transplantation. Ex vivo expansion of hematopoietic grafts could provide more mature neutrophil cells and more rapid engraftment (Bridclell et al., 1997; Shpall et al., 1999). With a two-step culture, we have previously established a new co-culture system with human bone marrow mesenchymal stem cells (hMSCs) for the ex vivo expansion of HSPCs and increased expansion of both committed and primitive progenitor cells (Wang et al., 2003). In this paper, we study the transplantation model with the nonobese diabetic/severe combined immunodeficiency disease (NOD/SCID) mice to analyze the engraftment and differentiation of expanded HSPCs in NOD/SCID mice after transplantation. Meanwhile, the effects of expanded cells on hematopoietic recovery of high-dose irradiated mice are studied and the clinical availability of HSPCs expanded in the two step culture is discussed.

2 Materials and methods

2.1 Umbilical cord blood (CB) products and animals

After obtaining informed consent, frozen cord blood samples were provided by Blood Center of Zhejiang Province, Hangzhou. Five samples, each with a volume of 75~85 mL, were used in this experiment. Frozen CB was diluted with 4 times volume of phosphate-buffered saline (PBS, Gibco BRL, Grand Island, NY) after it thawed completely in 37 ℃ water bath. Cells were collected by centrifugation at 1500 r / min at 4 ℃. Cells from the pellet were lysed with red blood lysing buffer (Gibco BRL) and then digested with DNase (Sigma, St. Louis, MO). Mononuclear cells (MNCs) were isolated using Ficoll-Hypaque (1.077±0.001g/mL, Sigma) per manufacturer's instructions. MNCs were washed and resuspended in PBS supplemented with 1% human serum albumin (HSA, New Century Pharmaceuticals, Huntsville, AL). $CD34^+$ cell proportion was determined by flow cytometric analysis.

Eight-week-old male NOD/SCID mice, weighting 19~23 g, were obtained from the Central Institute for Experimental Animals in Zhejiang Academy of Medical Sciences, and maintained in the defined flora animal facility. All animals were handled under sterile conditions.

2.2 HSPCs expansion and detection

Granulocyte-macrophage colony forming cells (GM-CFC) and high proliferative potential colony-forming cells (HPP-CFC) in MNCs were evaluated as progenitor assays before expansion (Wang et al., 2003).

The two-step culture method was adopted in the expansion (Wang et al., 2003). Briefly, cells were seeded into 100 mL Teflon culture bags (American Fluoroceal, Gaithersburg, MD) in 50 mL Iscove's modified Dulbecco's medium (IMDM, Gibco). The cultures were incubated for 7 days in 100% humidified, 5% CO_2 in air at 37 ℃. After 7 days' culture, the contents of the 100-mL bags were harvested and transferred into 1 L Teflon culture bags (American Fluoroceal,) in 1 L IMDM. The cultures were incubated for another 7 days in 100% humidified, 5% CO_2 in air at 37 ℃. Cells were incubated for total 14 days and then were harvested for transplantation. For the non-coculture scheme, the MNCs were cultured in IMDM supplemented with 100 ng / mL each of rhSCF, rhGCSF and rhMGDF (Amgen, Thousand Oaks, CA). For the coculture scheme, the MNCs were cultured in IMDM with three growth factors mentioned above and with hMSCs as feeder layers. hMSCs were from the cell line constructed in our lab (hMSC-ZJ0311).

After incubation as above, cells were harvested, washed, and counted for total nucleated cells (TNC) and evaluated for phenotype by flow cytometry. Also, expanded cells were plated as progenitor assays for GM-CFC and HPP-CFC.

2.3 Transplantation into NOD/SCID mice

In 24 h before transplantation, male NOD/SCID mice at 8 weeks of age were irradiated with a $^{60}Co\gamma$ in a split dose with a 4 h interval between doses. Each dose consists of 25 min at 24.1 r / min.

The expanded HSPCs were transplanted by tail-vein injection into sublethally irradiated NOD/SCID mice. Group A of 10 irradiated mice was used for transplantations of cells expanded in non-coculture scheme, group B of 10 irradiated mice for transplantation of cells expanded in coculture scheme, and group C of 10 irradiated mice as control by being injected with the same volume (200 μL) of PBS/1% HSA. Each group was performed in triplicate. The number of transplanted cells for each irradiated mouse was 8.5×10^6 cells in 200 μL suspension.

2.4 Analysis of Hematopoietic reconstruction of NOD/SCID mice

Since the second day after transplantation, 100 μL peripheral blood was collected by retro orbital sampling from each mouse at regular intervals. Peripheral blood was diluted up to 250 μL with PBS / 1% HSA, and then analyzed for hematopoietic recovery using Advia 120 (Bayer, Leverkusen, Germany).

Polymerase chain reaction (PCR) amplification of human Alu repetitive sequence gene was employed for testing the presence of human cells in the NOD/SCID mice that had received transplants. Mice were killed in 12 weeks after transplantation, and the bone marrow (from the femurs and tibiae) was harvested. Genetic DNA was isolated from bone marrow cells. Genetic DNA isolated from the human peripheral blood leucocytes was used as positive control, and genetic DNA isolated from the untreated NOD/SCID mice bone marrow cells as negative control. PCR amplification was performed as previously described by Wu et al. (2002) with slight modifications. Briefly, the sequences of primers were 5'-CTG GGC GA C AGA ACG AGA TTC TAT-3' and 5'-CTC ACT ACT TGG TGA CAG GTT CA-3'. Reaction system (50 μL): 5 μL 10 × amplification buffer, 3 μL 25 mmol / L $MgCl_2$, 2.5 U Taq DNA polymerase, mmol / L dNTP 0.2, 0.25 μmol / L each primer, and 0.2 μg template DNA. The amplification conditions were as follows: pre-denaturation at 95 ℃ / 5 min, denaturation at 95 ℃ / 30 s, annealing at 54 ℃ / 45 s and extension at 72 ℃ / 45 s during 30 cycles, finally extended at 72 ℃ for 5 min. The amplification product was visualized as a 224-bp band on 2% agarose gel electrophoresis and ethidium bromide staining.

2.5 Statistics

Numbers of TNC, $CD34^+$ cells in expansion experiment and results of GM-CFC, HPP-CFC assays are expressed as

mean ± SD. The significance of differences was assessed by the Student' s *t*-test.

3 Results

3.1 Expansion of HSPCs

Following MNCs separation from thawed frozen CB, the mean MNCs of each sample was 7.4×10^7 cells (5.4×10^7~9.1×10^7, $n = 5$). The mean percentage of $CD34^+$ cells was 2.2% (1.5%~3.2%, n=5).

After expansion of two-step culture for 14 days, the expanded fold of total nucleated cells (TNCs) in coculture scheme was significantly higher than that in the non-coculture scheme. TNCs increased by 312-fold for coculture scheme, and 139-fold for non-coculture scheme (Table 1).

Table 1 Expansion of total nucleated cells from CB mononuclear cells

Scheme	Sample	Starting cells / $\times 10^7$ ± SD	Expanded cells / $\times 10^9$ ± SD	Expanded fold ± SD
Coculture	5	1.48 ± 0.21	4.62 ± 0.21	312.2 ± 21.5
Non-coculture	5	1.48 ± 0.21	2.07 ± 0.29	139.9 ± 27.7

Expanded cells from the two schemes were labeled by monoclonal antibodies, then analyzed using flow cytometry. The results were shown in Table 2.

Table 2 Percentages (%) of expanded cells expressing specific phenotype

Scheme	Sample	CD33	CD15	CD14	CD34
Coculture	5	84 ± 2.6	69 ± 4.2	37 ± 3.5	0.35 ± 0.04
Non-coculture	5	72 ± 4.8	61 ± 5.3	42 ± 5.1	0.17 ± 0.09

The expanded cells harvested from both of the schemes had significantly higher percentage of $CD33^+$ and $CD15^+$ cells, while the percentage in the coculture scheme was higher than that in the non-coculture scheme ($P < 0.05$). It indicated that the coculture scheme was also propitious for the maturation of neutrophilic granulocyte. The percentage of $CD34^+$ cells decreased distinctly after expansion, and was 0.35% for cocultur scheme and 0.17% for non-coculture scheme. The total number of $CD34^+$ cells calculated according to the percentage of $CD34^+$ cells in expanded cells showed that $CD34^+$ cells increased 49.7-fold for coculture scheme, and 10.8-fold for non-coculture scheme, which suggested that the coculture scheme was also significantly propitious for the self-renewal of $CD34^+$ cells ($P < 0.05$).

The mean GM-CFC and HPP-CFC in the starting MNC fraction were 21×10^4 and 13×10^4, respectively. Following 14 days of expansion, there were 1.72×10^7 GM-CFC and 0.62×10^7 HPP-CFC (81.9- and 47.7-fold expansion, respectively) for coculture scheme. And there were 0.92×10^7 GM-CFC and 0.49×10^7 HPP-CFC (43.8- and 37.7-fold expansion,respectively)for non-coculture scheme. Therefore, the expansion proportions of GM-CFC and HPP-CFC increased by 1.87-fold and 1.27-fold respectively in coculture scheme compared with non-coculture scheme.

3.2 Transplantation of expanded cells

All irradiated mice without transplantation of expanded cells (group C) were dead within 15 days after irradiation. For the irradiated mice transplanted with expanded cells in non-coculture scheme (group B), two mice died respectively at the 13th and 17th day after transplantation. For the irradiated mice transplanted with expanded cells in coculture scheme (group A), one mouse died at 25th day after transplantation. No death was observed in the following time for these two transplantation groups.

The number of white blood cells (WBC) in groups transplanted with expanded cells began to increase at the 15th day after transplantation. The leucocytes number reached baseline value at the 25th day after transplantation, and then decreased distinctly. The number returned to the baseline value again till the 45~55th days after transplantation. Moreover, it increased significantly faster for coculture scheme compared with non-coculture scheme in this process (Fig. 1).

Polymerase chain reaction (PCR) amplification of human Alu repetitive sequence gene was employed for testing the presence of human cells in the NOD/SCID mice that had received transplants (Fig. 2). Human Alu repetitive sequence gene was detected in 8out of 9 NOD/SCID mice that survived after transplantation with expanded cells in coculture scheme. For non-coculture scheme, human Alu repetitive sequence gene was detected in 7 out of 8 NOD/SCID mice survived after transplantation. The positive percentage was 88.9% for coculture scheme and 87.5% for non-coculture scheme. The Alu repetitive sequence was not detected in NOD/SICD mice without transplantation.

4 Discussion

It has been widely demonstrated that HSPCs have significant impact on hematopoietic recovery following high-dose actinotheraphy or chemotheraphy. In previous

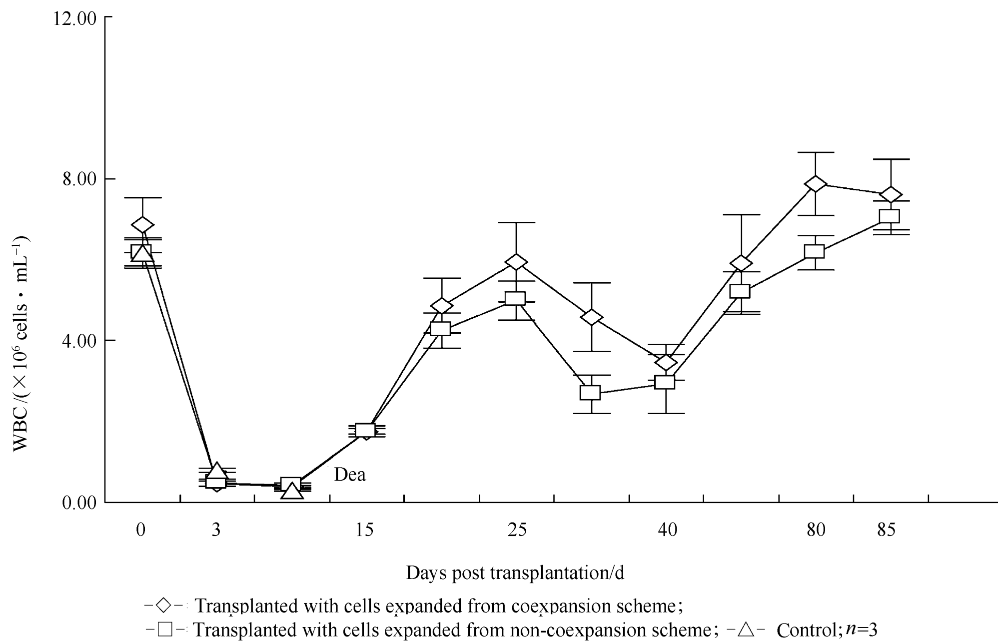
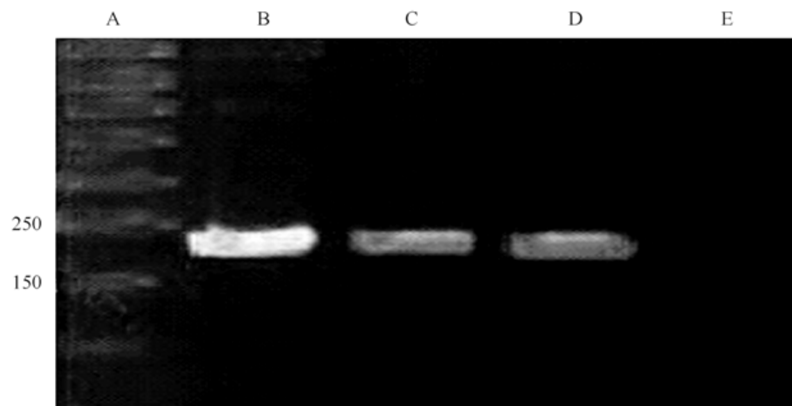


Fig. 1 Recovery tenders of WBC population in irradiated NOD/SCID mice transplanted with or without expanded hematopoietic stem/progenitor cells



A: Marker; B: human WBC genome; C: BM genome of mice transplanted with HSPCs from the coexpansion scheme; D: BM genome of mice transplanted with HSPCs from the no-coexpansion scheme; E: control

Fig. 2 PCR detection of Alu sequence fragment special for human in NOD/SCID mice BM

clinical studies, the use of peripheral blood progenitor cells (PBPCs) and BM as a source of hematopoietic reconstitution has shown that they can generate matured neutrophils in vivo within a short period of time after transplantation. Recently, CB cells are used increasingly as a source of HSPCs for hematopoietic recovery especially for allogeneic transplantation of patients lacking a human leukocyte antigen-matched marrow donor. However, the CB transplantation is associated with higher rates of delayed and failed engraftment than the bone marrow transplantation, particularly for adult patients. *Ex vivo* expansion of CB cells has been proved to generate increased mature cells and progenitors that are capable of more rapid engraftment of neutrophils following infusion to transplanted recipients. At present, there are more and more studies on hematopoietic reconstruction in irradiatied mouse transplanted with expanded HSPCs from CB (Cao et al., 1997; Liu et al., 1999; Su et al., 2002; Liu et al., 2003). In the current study, effects of *ex vivo* expanded HSPCs with modified two-step culture method on hematopoietic recovery of high-dose irradiated mouse and expanded hematopoietic cell engraftment were analyzed. Results demonstrated that the expanded cells could rapidly engraft in the sublethally irradiated mice and reconstitute their hematopoiesis (Wang and Wu 2004)

In the present paper, we firstly expanded HSPCs using

two-step culture method with / without mesenchymal stem cells (MSCs) as feeder layers, and then transplanted these expanded cells into the sublethally irradiated NOD/SCID mice to assess the effects of expanded cells on hematopoietic recovery. It is showed that the coculture scheme increased ex vivo expansion of CB-derived HSPCs more effectively than non-coculture scheme, and MSCs are propitious to expansion of HSPCs as feeder layers.

As for the transplantation analysis, WBC numbers in mice transplanted with expanded cells in both coculture scheme and non-coculture scheme reached the baseline value rapidly (began to increase at 15th day and reached the peak at 25th day after transplantation). However, expanded cells from the coculture scheme appeared to be more favorable for the second increasing of WBCs and reconstructed hematopoiesis more rapidly in the transplanted mice. This conclusion is also confirmed in the detection of human Alu repetitive sequence fragment in BM of NOD/SCID mice transplanted with expanded cells at the 12th week after transplantation.

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