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Ex vivo Expansion and Clonal Maintenance of CD34+ Selected Cells from Cord Blood and Peripheral Blood

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Purpose : Because of the unavailability of marrow transplantation, umbilical cord blood (CB) is increasingly being used. We evaluated the potential of ex vivo expansion and clonality in CD34+ cells separated from cord blood source and mobilized peripheral blood (PB) in a serum-free media.
Methods : The CD34+ cells, selected from CB and mobilized PB, were expanded with hematopoietic growth factors. They were then cultured for burst-forming units of erythrocytes (BFU-E), colony-forming units of granulocytes and monocytes (CFU-GM) and colony-forming units of megakaryocytes (CFU-Mk) at culture days 0, day 4, day 7, and day 14 with various growth factors.
Results : The CB-selected CD34+ cells showed significantly higher total cell expansion than those from the PB at day 7 (2 fold increase than PB). The CB-selected CD34+ cells produced more BFU-E colonies than did the PB on culture at days 7 and at day 14. Also, the CB-selected CD34+ cells produced more CFU-Mk colonies than did the PB on culture at day 4 and at day 7.
Conclusion : The ex vivo expansion of the CB cells may be promising in producing total cellular expansion, CFU-Mk and BFU-E compared with PB for 7 to 14 days. The growth factors combination including megakaryocyte growth and development, flt3-ligand and interleukin-3 showed more expansion in the view of total cells and clonal maintenance compared with less combination. **(Korean J Pediatr 2005;48:894-900)**

Key Words : CD34+ cells, Colony-forming unit of megakaryocyte, Cord blood, Mobilized peripheral blood, Ex vivo expansion

Introduction

The umbilical cord blood (CB) is receiving increasing acceptance for allogeneic transplantation as an alternative source of stem cells instead of bone marrow or mobilized peripheral blood[1-7]. For patients whom no suitable related donor is available, cord blood transplantation offers substantial advantages : relatively easy access, prolonged storage, speed of donor search, viral safety and relatively low rate of graft-versus-host-disease. However, the insufficient numbers of hematopoietic stem cells (HSCs) in CB is potentially a major limitation to their widespread use.

Megakaryocyte growth and development (MGDF) is known to be a cytokine for promoting all phases of megakaryocytopiesis including hematopoietic stem cell proliferation[8-11]. Various combinations of cytokines including recombinant human MGDF, stem cell factor, flt3-ligand and interleukin-3 have been used to expand hematopoietic cells [8, 9, 12-15]. To overcome the small amount of hematopoietic cells in CB, cytokine-mediated ex vivo expansion has been proposed and practiced as a means of increasing the number of cord blood HSCs for transplantation[16-20].

This study was performed to compare the total cellular expansion, and erythropoietic and megakaryocytopoietic capacities by each combination of growth factors using

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CD34+ cells selected from CB and PB.

Materials and Methods

1. CB

CB collections were performed at the end of full-term deliveries following vaginal delivery of infants, from the maternal end of the severed cord, while the placenta was still in utero. After collection, the samples were processed within 24 hrs at room temperature. The cells were subjected to a Ficoll-Paque density gradient (Pharmacia Biotech, Uppsala, Sweden), followed by positive selection of CD34+ cells using a magnetic cell sorting and separation method with a Miltenyi MiniMACS column[21](Miltenyi Biotec GmbH), purified to 90% CD34+ content, and cryopreserved in liquid nitrogen.

2. Mobilized PB

The G-CSF (Filgrastim, 10 μg/kg/day)-primed CD34+ cells were harvested by leukapheresis from healthy donors or from patients with breast cancer without bone marrow metastasis, purified to 95% CD34+ content by magnetic cell sorting, and cryopreserved in liquid nitrogen. No difference has been observed in colony count of colony-forming unit of granulocyte/monocyte (CFU-GM), burst-forming unit of erythrocyte (BFU-E), and colony-forming unit of megakaryocyte (CFU-Mk) between normal donor PB and patients with breast cancer (unpublished data).

3. Hematopoietic growth factors

The cytokines and growth factors used in the cultures were as follows : recombinant methionyl human G-CSF (Amgen Inc., Thousand Oaks, CA), recombinant methionyl stem cell factor (SCF, Kirin Brewery Company Limited, Tokyo, Japan), and pegylated recombinant human MGDF (Kirin Brewery Company Limited), recombinant human flt3-ligand (FL, StemCell Technologies Inc, Vancouver, British Columbia), interleukin-3 (IL-3, Kirin Brewery Company Limited) and interleukin-11 (IL-11). Each cytokine was added to the purified CD34+ cells obtained from CB and PB in a 24-well plate. The purified CD34+ cells were cultured in quadruplicate flat-bottomed 24-well plates in 1 mL of Iscove's serum-free media (IMDM) supplemented with 10% fetal calf serum, and the following combination of cytokines.

MGDF (100 ng/mL)+FL-3 (50 ng/mL), designated as MF group
MGDF (100 ng/mL)+FL-3 (50 ng/mL)+IL-11 (10 ng/mL) : MFI_{11} group
MGDF (100 ng/mL)+FL-3 (50 ng/mL)+SCF (100 ng/mL) : MFS group
MGDF (100 ng/mL)+FL-3 (50 ng/mL)+SCF (100 ng/mL)+IL-11 : $MFSI_{11}$ group

All the wells were demi-depopulated by removal of one half of the culture volume, which was replaced with fresh medium and growth factors twice a week. Cell count was counted by a Coulter counter. Cell culture and assays for BFU-E, CFU-GM and CFU-Mk cultures of purified CD34+ cells were performed. Cells were suspended at 10^6 per mL in IMDM, supplemented with 10% FBS, which also included : 2 mM glutamine, 100 U/mL penicillin and 100 (μg/mL streptomycin, and were transferred to 24-well plates at a starting volume of 1mL per well. About $1-2\times10^5$ cells were implanted in each well. Recombinant hematopoietic growth factors were added as described above. The wells were grown at 37℃ in a 5% CO_2 fully humidified atmosphere. The cell count for CFU-Mk, BFU-E and CFU-GM was as explained in the previous article[20]. The purified CB CD34+ cells were cultured in a stroma-free liquid culture system with a combination of growth factors for up to 3 weeks. Demi-depopulation was carried out twice a week.

In vitro CFU assays were performed to detect primitive progenitor cells at ex vivo expansion days 0, 4, 7, 14 and 17. For the CFU-GM and BUF-E, we used MethoCult HCC-4434, which included a methylcellulose medium (StemCell Technologies Inc). For the CFU-Mk, we used a MegeCultTM Kit (StemCell Technologies Inc), which included a collagen solution. CFU colonies of the CFU-GM and BFU-E were enumerated after 14 days under an inverted microscope.

After culturing for 12 days, slides of the CFU-Mk were prepared, dried and fixed with a methanol/acetone (1 : 3) mixture, and immunostained with anti-GPIIb/IIIa; avidin-alkaline phosphatase conjugate; secondary biotin-conjugated goat anti-mouse IgG antibody; alkaline phosphatase substrate and buffer tablets (StemCell Technologies Inc.). The CFU-Mk colonies were scored under the inverted microscope.

All statistical analyses were made using the SAS system for Windows (version 8.0). Mean, standard deviation, and Student's t-test were used in the statistical analysis. Differences in proportions were tested for statistical signifi-

Table 1. Total Cell Expansion (Mean Fold±SD) according to the Combination of Cytokines

Cytokines	Cell sources	Day 4	Day 7	Day 11	Day 14	Day 17
MF	CB (n=7)	2.5±0.6	10.5±4.9	24.3±17.2	56.5±41.7	108.4±42.4
	PB (n=3)	1.8±0.5	4.8±3.1	8.7±2.7	30.8±33.0	21.7±26.0
MFI_{11}	CB (n=5)	2.7±0.3	14.0±4.6*	37.2±11.6	73.6±28.3*	126.8±69.6
	PB (n=3)	3.1±1.8	5.9±0.1	15.9±14.1	20.1±11.5	35.0±30.4
MFS	CB (n=5)	4.7±2.2	36.8±10.4*	75.6±22.0	138.6±77.8	239.3±105.0
	PB (n=3)	4.1±1.3	18.8±5.5	59.0±34.9	84.3±55.7	115.6±82.8
$MFSI_{11}$	CB (n=5)	5.7±0.9	45.3±17.5*	90.3±44.4	151.6±52.5	278.9±131.5
	PB (n=3)	5.0±3.3	18.3±3.1	47.2±37.3	95.5±62.3	113.2±79.5
Total	CB (n=22)	3.9±1.8	26.5±18.2*	56.2±37.7	104.3±65.2	186.8±109.7*
	PB (n=12)	3.5±2.1	12.5±7.6	36.8±32.8	64.2±53.2	65.9±63.9

Abbreviations : M, megakaryocyte growth and development (MGDF), F, flt-3 ligand; S, stem cell factor; I_3, interleukin-3; I_{11}, interleukin-11; CB, cord blood; PB, mobilized peripheral blood
*Significantly different between groups ($P<0.05$)

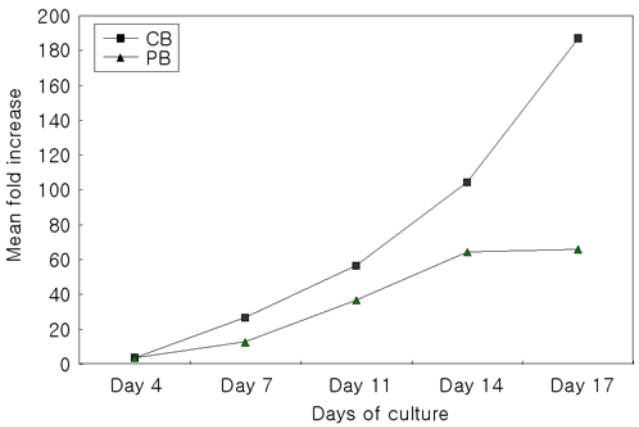

Fig. 1. Mean fold increase of total white blood cells after culture of cord blood- and peripheral blood- derived CD34+ cells.

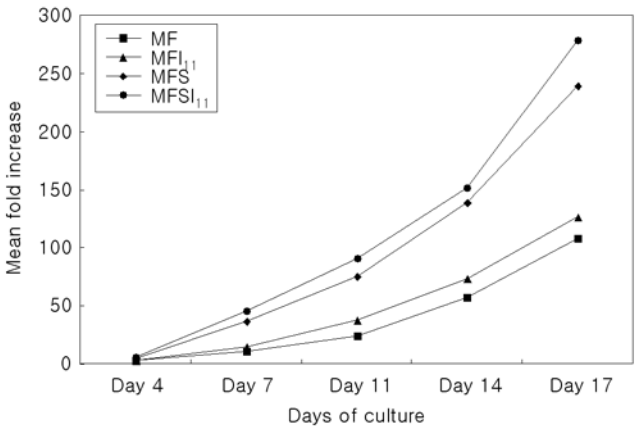

Fig. 2. Mean fold increase of white blood cells after culture of cord blood derived CD34+ cells with the combination of cytokines.

cance by a chi-square test.

Results

As shown in Table 1, total white blood cells (WBC) were calculated using a Coulter counter at days 4, 7, 11, 14 and 17, and were expressed by their fold increase, compared with initial cell counts (day 0). The CB-derived CD34+ cells had a higher total WBC counts than those derived from PB (Fig. 1). Ex vivo expansion in serum-free media revealed that the total cell quantities on day 7 were increased 26-fold in the CB-derived cells, compared with 12-fold increase in the PB-derived cells.

The WBC counts on day 14 were increased 104-fold (SD 65.2) in CB-derived cells, 56-fold (SD 35.5) in the PB, compared to the initial numbers. Ex vivo expansion from the CB-derived CD34+ cells at day 7 showed significantly higher total cell counts than PB-derived CD34+ cells.

Table 2. The Number of BFU-E Colonies and CFU-Mk/BFU-Mk Colonies at Day 0 per CD34+ Cell

Cell sources	BFU-E colonies per CD34+ cell	CFU-Mk/BFU-Mk colonies per CD34+ cell
CB (n=6)	0.14±0.02	0.047±0.011
PB (n=6)	0.12±0.01	0.036±0.006

In CB-derived CD34+ cells, the MFS and MFSI11 groups were significantly more expanded on day 11 and day 14 than those from MF and MFI_{11} groups (Fig. 2). In PB, total WBC in the MFS and $MFSI_{11}$ groups were also higher than those in MF and MFI_{11} groups on day 4, day 7, day 11 and day 14 (Fig. 3).

1. Relative frequency of ex vivo expanded BFU-E and CFU-Mk

At initial culture, the colony counts for the BFU-E per

Table 3. Fold Change of Burst-Forming Unit of Erythrocyte (BFU-E) Colonies in Culture according to the Combination of Cytokines (Mean Fold±SD)

Cytokines	Cell sources	Day 0 fold	Culture at day 4	Culture at day 7	Culture at day 14	Culture at day 21
BFU-E	CB (n=6)	1.0	2.8±0.8	8.5±2.1[a]	3.9±1.8[a]	3.0±2.2
	PB (n=6)	1.0	1.3±0.3	2.7±0.6[b]	0.9±0.7[b]	0.1±0.2
CFU-MK	CB (n=8)	1.0	3.6±0.7[a]	4.9±1.9[a]	5.0±6.9	1.9±0.48
	PB (n=6)	1.0	1.7±0.5[b]	0.89±0.66[b]	1.6±2.0	1.5±1.06

a & b : significant at the 0.05 level

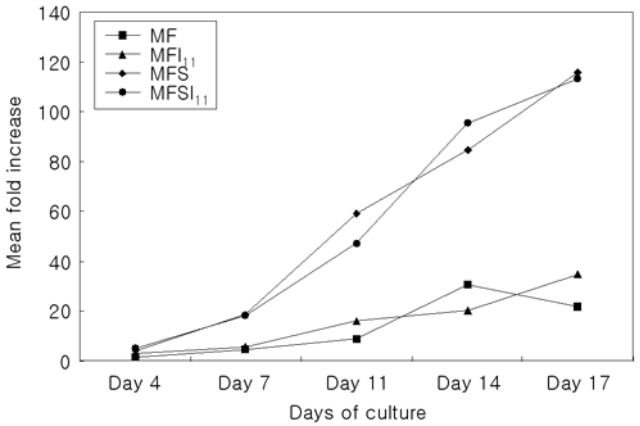

Fig. 3. Mean fold increase of white blood cells after culture of peripheral blood derived CD34+ cells with cytokines.

CD34+ cells was 0.14±0.02 in CB-derived CD34+ cells, and 0.12±0.01 in PB-derived CD34+ cells (Table 2). Initially the colony counts for the CFU-Mk per CD34+ cell in the initial culture was 0.047±0.011 for the CB- derived CD34+ cells, and 0.036±0.006 for the PB-derived CD34+ cells.

As shown in Table 3, the CB-derived CD34+ cells produced significantly more BFU-E colonies than the PB-selected cells at day 7 and 14, compared with the initial cultures. The erythroid progenitors from the PB-derived cells increased a little at day 4 and at day 7, but were decreased at day 14 culture, compared to that in the initial day 0 culture. In contrast, the BFU-E colonies from the CB-selected cells increased markedly on days 4 and 7.

CB-derived CD34+ cells produced more CFU-Mk colonies than did those selected from the PB cells at day 4 and at day 7. The number of CFU-Mk colonies from the CB cells on days 7 and 14 were about 3- or 5-fold greater than those from the PB-selected cells. The megakaryocytic progenitors from the PB-derived cells increased a little on day 4, but decreased below that of the initial culture by day 7. Thus, the cells separated from the CB-derived cultures yielded more erythroid, and megakaryocyte, progenitors than the PB-derived cells cultures at day 7. On culturing the expanded cells, the colony-size for the BFU-E and CFU-Mk gradually decreased as long as the expansion duration was prolonged for these two hematopoietic sources. Compared to the PB-derived cells, however, the sizes of the BFU-E and CFU-Mk colonies were larger after culture of CB-derived cells.

Discussion

As the numbers of CD34+ cells in stem cell transplantations are critical in determining the engraftment, the small cord blood volume is a major limitation to the use of CB. The median time to recovery is known to be delayed in CB transplantations compared to that with BM or PB[3, 23]. To overcome the limited amount of hematopoietic stem and progenitor cells, ex vivo expansion has been attempted using thrombopoietin and other hematopoietic growth factors. Infusion of ex vivo expanded megakaryocytic progenitor cells is a strategy for shortening the duration of thrombocytopenia following hematopoietic stem cell transplantation. Expanded PB progenitor cells can shorten the time to engraftment of neutrophils compared with historical controls and that the rate of engraftment is related to the dose of expanded cells transplanted[24, 25].

In our study, the potential for clinical application of the ex vivo expansion of CB progenitor cells is manifest due to the greater expansion of the BFU-E and CFU-Mk in the CD34+ cells derived from CB, compared with those from PB. That is, the number of BFU-E colonies from the CB-derived cells expanded 3-fold and 4-fold than those from the PB-derived cells, on days 7 and 14, respectively. The number of CFU-Mk colonies from the CB-derived cells expanded approximately 5-fold and 3-fold, respectively, on both days 7 and 14, compared to those from the PB-derived cells. Therefore, the optimal duration for the ex vivo expansion from a cord blood origin may be between 7

and 14 days, when conventional culture methods are used under serum-free media.

Although we also assayed CFU-GM colonies by using a methylcellulose media, we could not objectively quantify the colonies counts because of the overlap of white colonies. Therefore, standardized quantification methods should have been applied in measuring the number of CFU-GM colonies, i.e., by inoculating a smaller amount of CD34+ cells, or by shortening the culture duration. Several authors have shown the number of CFU-GM per mononuclear cell from CB-derived cells to be 2-fold greater than those from PB-derived cells[26, 27].

Various combinations of cytokine, in a previous study, have shown more cellular expansion, compared with MGDF alone in liquid cultures, and a decreasing total cellular expansion[22, 28]. FL-3 has been shown to exhibit striking structural homology with SCF and M-CSF, and synergy with a range of other human growth factors, including IL-3, IL-6, IL-7, IL-11, G-CSF and GM-CSF[29, 30]. FL-3 was effective in the ex vivo expansion of CB CD34+ cells, by itself, and in combination with MGDF and/or G-CSF. The SCF, also known as steel factor or c-Kit ligand, has been known to enhance colony formation by primitive cell populations, and displays synergistic activity with other growth factors on erythroid, myeloid and megakaryocytic progenitors[31]. In our study, the combination of MGDF, FL-3 and SCF showed more expansion regardless of IL-11. And the CD34+ cells separated from CB yielded more total cells, and were more prominent with the combination of MGDF, FL-3 and SCF.

A reason for the CB cells being more proliferative than PB-derived cells might be that the CB cells have a higher frequency of the highly primitive progenitors, or a more immature compartment[32, 33]. More CFU-Mk colonies were observed in CB-derived CD34+ cell cultures than in those derived from PB, and these colonies were much larger in cell number and size. Therefore, the clinical applications of the ex vivo expansion from CB cells seems to be promising, although longer culturing of hematopoietic stem cells results in the loss of multilineage potential[34].

In conclusion, ex vivo expansion of CB-derived CD34+ cells, rather than with PB-derived cells, may be very promising, as the former were the more productive hematopoietic source on a per volume basis. The growth factors combination including MGDF, FL and IL-3 showed more expansion in the view of total cells and clonal maintenance compared with less combination.

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한글 요약

제대혈 및 말포혈로부터 분리한 CD34 양성 세포의 체외 증폭 및 클론 유지

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목 적 : 조직적합 항원의 불일치로 인하여 골수이식을 할 수 없는 경우에 점점 더 제대혈이 사용되고 있다. 그러나 제대혈의 조혈모세포의 수가 적기 때문에 이를 증가시킬 대책이 필요한 바, 여러 성장인자를 조합하여 체외증폭하여 말초혈의 체외증폭과 비교하였다.

방 법 : 저자들은 제대혈 및 말초혈로부터 분리한 CD34+ 세포를 혈청이 아닌 배양체에서 체외 증폭하여 비교하였다. Miltenyi 방법으로 분리한 CD34+는 조혈성장인자들과 함께 체외증폭 시켰다. 증폭 당일, 4일 후, 7일 후 및 14일에 증폭된 세포를 가지고 burst-forming units of erythrocytes (BFU-E), colony-forming units of granulocytes and monocytes (CFU-GM) 및 colony-forming units of megakaryocytes (CFU-Mk)의 생성 능력을 알아보았다.

결 과 : 말초혈에 비하여 제대혈로부터 분리한 CD34+ 세포의 증폭 능력이 2배로 컸다. 체외에서7일 및 14일 동안 증폭된 제대혈이 더 많은 BFU-E를 생성하였고, 4일 및 7일 동안 증폭된 제대혈이 더 많은 CFU-Mk를 생성하였다.

결 론 : MGDF, FL 및 IL-3를 포함한 성장인자의 자극 하에서 제대혈의 체외 증폭이 더 많은 BFU-E 및 CFU-Mk를 생성하였으므로, 이를 이용한 체외 증폭을 시도하는 것의 가능성을 시사하고 있다.

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