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Proliferation, Apoptosis, and Telomerase Activity in Human Cord Blood CD34+ Cells Cultured with Combinations of Various Cytokines

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Abstract Umbilical cord blood (UCB), a rich source of hematopoietic stem/progenitor cells, has been proposed as an alternative to bone marrow and peripheral blood for transplantation treatment. *Ex vivo* expansion of cord blood stem cells could make the use of cord blood transplant feasible even for adult patients. However, the optimal cytokine cocktail for expansion of stem cells is yet to be established. This study compares proliferation, apoptosis, and telomerase activities in human cord blood stem cells cultured *ex vivo* with FLT3 ligand (FL)/thrombopoietin (TPO) or FL/TPO/ stem cell factor (SCF), with a view to determine optimal combination of cytokines. CD34+ cells were cultured in DMEM containing either FL (50 ng/ml) and TPO (10 ng/ml) (FT group) or FL (50 ng/ml), TPO (10 ng/ml) and SCF (50 ng/ml) (FTS group). The cell proliferation rate was ten times higher in the FTS group. Although cells cultured with the two different combinations of cytokines were maintained for a long term (up to 8 weeks), a large number of cells underwent differentiation during this period. Cells cultured in FTS displayed lower levels of apoptosis compared to those of the FT group during the initial 7 days of culture. The CD34+ fraction in both groups was markedly decreased to 21–30%, and only 5–6% was detected at 14 days of culture. Telomerase activity detected in human CD34+ cord blood at low levels was upregulated during the early phase of culture and decreased to baseline levels in the later phase. The telomerase activity of cord blood cultured in FT was lower than that of the FTS group. Our results suggest that, on adding stem cell factors to the FT cytokines, cultured CD34+ cord blood cells display a greater degree of cell proliferation and decreased apoptosis. However, during CD34+ cord blood cell culture, a large number of cells undergo differentiation, indicating that more potent novel cytokines or new culture conditioning methods should be developed to maintain their ability to engraft and sustain long-term hematopoiesis.

Key words: Cord blood stem cells, *ex vivo* expansion, CD34, apoptosis, telomerase activity, cytokines

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Umbilical cord blood contains a significantly higher number of early and committed progenitor cells than adult peripheral blood [3]. Moreover, the number of colony forming unit-granulocyte-macrophages (CFU-GM) is much greater in the umbilical cord blood than peripheral blood [2, 8]. Therefore, umbilical cord blood is proposed as an ideal alternative to bone marrow and peripheral blood for hematopoietic stem cell transplantation. Hematopoietic reconstitution in a patient with severe Fanconi's anemia, who received cryopreserved umbilical cord blood nucleated cells from a sister, was initially reported by Gluckman *et al.* [10]. This finding suggests that cord blood-derived hematopoietic progenitor cells may be used for an effective stem cell transplantation process in a clinical setting. However, a major problem associated with the use of umbilical cord blood is that it is a one-off collection and may not contain enough stem cells to successfully transplant to an adult, since 5×10^8 mononuclear cells/ml ($2-5\times10^6$ CD34+ cells/ kg) are necessary for a full recovery of neutrophil and platelet count after transplantation. Generally, the numbers of progenitors obtained from cord blood samples are only useful for patients with a body weight of <40 kg. Consequently, cord blood transplantation is mainly restricted to children and small-sized adults.

Ex vivo expansion of cord blood stem cells may make transplantation feasible for adult patients [5, 13, 21, 22, 24]. A number of investigators have focused on the development of a novel method of *ex vivo* expansion of cord blood stem cells with high cell proliferation rates and

sustained hematopoiesis. The growth of cord blood stem/progenitor cells may be sustained and expanded by using a combination of hematopoietic growth factors. FLT3 ligand, thrombopoietin, and stem cell factors play a key role in maintaining functional repopulation of hematopoietic stem cells. However, the optimal cytokine cocktail for stem cell expansion remains to be established. This investigation was performed to compare proliferation, apoptosis, and telomerase activity in human cord blood stem cells cultured *ex vivo* with FLT3 ligand (FL)/thrombopoietin (TPO) or FL/TPO/stem cell factor (SCF) to determine the optimal combination of cytokines.

MATERIALS AND METHODS

Isolation of Umbilical Cord Blood CD34+ Cells

Normal umbilical cord blood scheduled for discarding after delivery was obtained with a maternal consent. Low-density mononuclear cells were isolated on Ficoll-Paque (1.077 g/ml) (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), washed twice in phosphate buffered saline (PBS) containing 2 mM EDTA, and centrifuged for 10 min at 200 ×g at 20°C. The pellet was resuspended in a final volume of 300 µl buffer/10^8 total cells. CD34+ cells were isolated with a monoclonal anti-human CD34+ antibody (QBEND/10), followed by a positive selection with immunomagnetic beads (Miltenyl Biotech, Auburn, CA, U.S.A.) and mini-MACS according to the manufacturer's recommendations. The purity of CD34+ cells routinely exceeded 95%, as determined by fluorescence-activated cell sorting (FACS) analyses (Becton Dickinson, San Jose, CA, U.S.A.).

Cell Culture

CD34+ cells were seeded at 1×10^5/mL DMEM with 10% heat-inactivated fetal bovine serum (FBS) containing either FLT3 ligand (FL: 50 ng/ml) and thrombopoietin (TPO: 10 ng/ml) or FL (50 ng/ml), TPO (10 ng/ml), and stem cell factor (SCF: 50 ng/ml) in 35-mm dishes at 37°C in a humidified atmosphere of 5% CO_2 in air. Every 3–4 days, all the wells were demidepopulated by removing half of the culture volume and replacing them with fresh medium and growth factors. At various intervals (days 0, 4, 7, 10, and 14), cells were harvested. Viability was assessed by using the trypan blue exclusion assay, and 1×10^5 cells were cytospinned and stained with Wright-Giemsa stain.

FACS Analysis for the CD34+ Fraction

For each cytokine cocktail, freshly isolated or incubated cord blood stem cells were stained at various time intervals with CD34-fluorescein isothiocyanate (FITC) or isotype control-FITC for 15 min and fixed with 2% paraformaldehyde solution. Fixed cells were suspended in PBS and analyzed by FACScalibur flow cytometry (Becton Dickinson, San Jose, CA, U.S.A.). Data for 10,000 events were analyzed by using a CellQuest version 3.11 software (Becton Dickinson, San Jose, CA, U.S.A.).

Determination of Apoptosis

To evaluate apoptosis during the cell culture with different cytokines, harvested cells were incubated with Annexin V-FITC (R&D systems, McKinley, NE, U.S.A.) and propidium iodide (PI; Sigma, St. Louis, MO, U.S.A.) at various time intervals and then analyzed by FACS according to the manufacturer's instructions [17]. Briefly, 1×10^6 cells were resuspended in 1 ml of PBS with 9 ml of ice-cold 70% ethanol and incubated for 15 min. Cells were centrifuged, washed with 5 ml of PBS, followed by 0.5 ml of the DNA extraction buffer (192 ml of 0.2 M Na_2HPO_4, 8 ml of 0.1 M citric acid; pH 7.8), and incubated at room temperature for 5 min. Next, the cells were re-centrifuged, suspended in 1 ml of DNA staining solution (500 µg PI dissolved in 10 ml of PBS with 2 mg of DNase-free RNase A) and incubated for 30 min at room temperature. Data for 10,000 events were obtained by a FACScalibur flow cytometer with CellQuest version 3.11 software.

Analysis of Telomerase Activity

Telomerase activity in cord blood stem cells was analyzed, as reported previously [11, 27], by using a telomerase polymerase chain reaction (PCR) enzyme-linked immunosorbent assay (ELISA) method, which is an extension of the original telomeric repeat amplification protocol described by Kim *et al.* [15]. Briefly, cord blood stem cells were lysed in ice-cold lysis buffer for 30 min. Initially, a volume of cell extract containing 6 µg of the total protein was incubated with a biotin-labeled synthetic telomerase-specific primer. Under established conditions, telomerase in cellular extracts adds telomeric repeats (TTAGGG) to the 3' end of the primer. Next, these elongation products were amplified by PCR by using specific primers. An aliquot of the PCR products was denatured, hybridized to a digoxigenin-labeled telomeric repeat-specific probe, and bound to a streptavidin-coated microtiter plate. Immobilized PCR products were detected with an antibody against digoxigenin which was conjugated to peroxidase. Finally, the probe was visualized by using a peroxidase-metabolizing agent to form a colored reaction product and semiquantified photometrically (450 nm). An extract of the telomerase-positive 293 embryonic kidney cell line was used as a positive control. The procedure was sufficiently sensitive to detect telomerase activity in an extract containing 10 telomerase-positive cells. In each case, negative controls were prepared by treating cell extracts by heat.

Table 1. Time-course analysis of expansion of total nucleated cells cultured with combinations of either FL/TPO or FL/TPO/SCF.

Days	Total nucleated cells		*P*-value
	FL/TPO/SCF (Mean±SE)	FL/TPO (Mean±SE)	
Day 0	1×10^5	1×10^5	-
Day 2	$0.95\pm0.07\times10^5$	$0.65\pm0.05\times10^5$	0.257
Day 6	$1.33\pm0.27\times10^7$	$2.7\pm0.40\times10^6$	0.051
Day 10	$1.99\pm0.01\times10^8$	$2.20\pm0.36\times10^7$	<0.001
Day 13	$7.83\pm0.67\times10^8$	$8.96\pm0.27\times10^7$	<0.001
Day 15	$1.13\pm0.15\times10^9$	$1.52\pm0.48\times10^8$	<0.001

Purified CD34+ cord blood cells were cultured in stroma-free liquid with FL/TPO or FL/TPO/SCF. Cells were cultured with FLT3 ligand (FL: 50 ng/ml) and thrombopoietin (TPO: 10 ng/ml) or FL (50 ng/ml), TPO (10 ng/ml) and stem cell factor (SCF: 50 ng/ml) in 35-mm dishes at 37°C, 5% CO_2 in air. Viability was assessed by using the trypan blue exclusion assay.

Statistical Analysis

Cell proliferation results are represented by mean±SE. The statistical significance between two groups was analyzed by one-way ANOVA (SPSS11.0; SPSS Inc. Chicago, IL, U.S.A.).

RESULTS

Cell Proliferation

Purified CD34+ cord blood cells were cultured in a stroma-free liquid with FL/TPO (FT group) or FL/TPO/SCF (FTS group) for more than 8 weeks. In both cultures, a progressively rapid increase in the cell production was noted (Table 1). In the FT group, the cell output after 14 days was 1,000-fold greater than the input number. In contrast, for the FTS group, the cell output after 10 days was 1,000-fold greater than the input number (Fig. 1), and 10,000-fold greater after 14 days. The cells cultured in FL/TPO/SCF proliferated almost ten times more rapidly than those of the FT group. Although cultures with FT and FTS lasted for 8 and 10 weeks, respectively, the former group displayed a rapid decrease in cell number after 5 weeks of culture. The cells cultured with FL/TPO/SCF exhibited viability rate higher than the FL/TPO group any given day of the culture, determined by the trypan blue exclusion assay (data not shown).

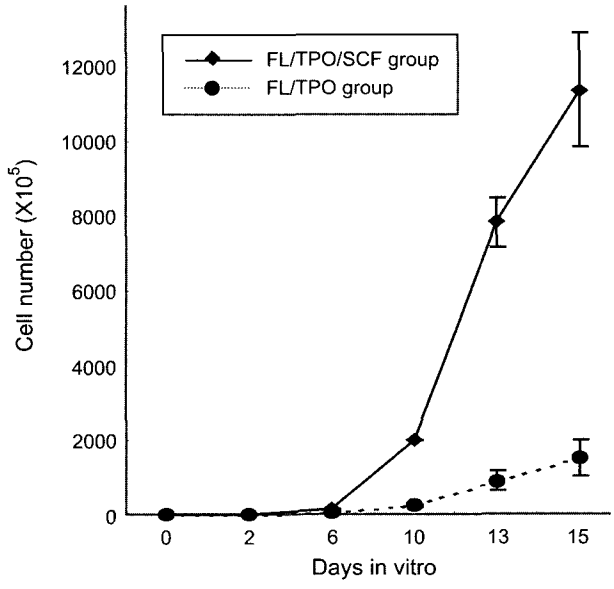

Fig. 1. Cell proliferation from *ex vivo* expansion of CD34+ cord blood cells in the presence of FL/TPO or FL/TPO/SCF.
CD34+ cord blood cells (1×10^5) were seeded and every 3–4 days, all the wells were demidepopulated by removal of half the culture volume and replacing with fresh medium and growth factors. At each time interval, cells were harvested and counted by trypan blue exclusion.

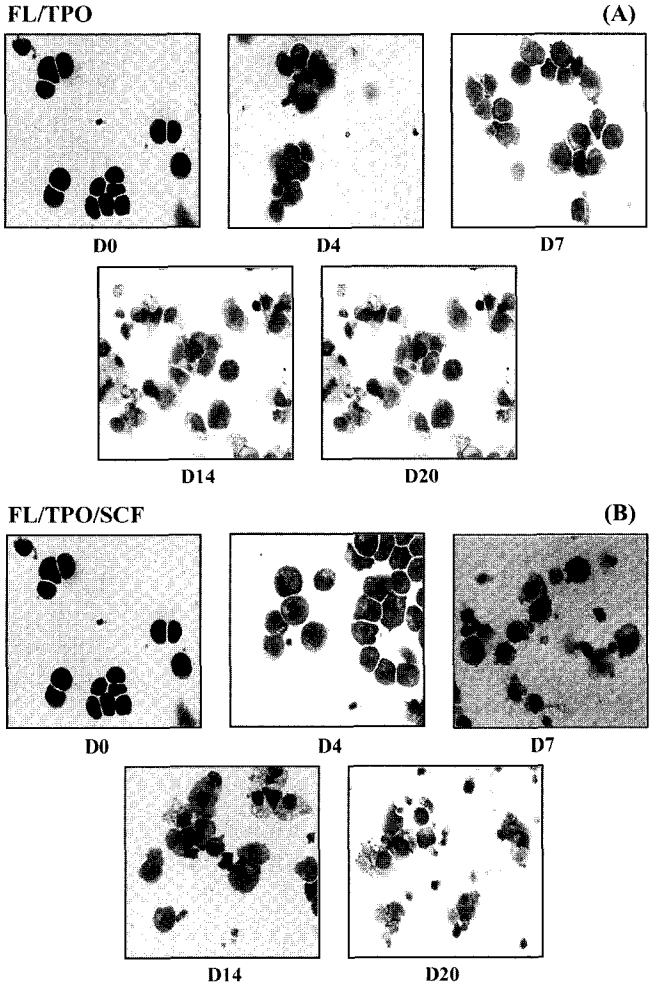

Fig. 2. Morphological changes of CD34+ cord blood cells during short-term culture in the presence of FL/TPO (A) or FL/TPO/SCF (B).
Wright-Giemsa staining was performed to evaluate the morphology of cells at each time-point. Cells displayed condensed nuclei and decreased nuclear/cytoplasm ratio, even at 4 days after culture, suggesting differentiation into committed cells.

Morphological Changes

Since the FT group displayed slower proliferation than the FTS group, Wright-Giemsa staining was performed to compare the morphological changes between the cells cultured in the two cytokine groups at the same time intervals. The cells from both groups displayed condensed nuclei, morphological heterogeneity, and decreased nuclear/cytoplasm ratio even at 4 days after the culture, suggesting differentiation into committed cells (Fig. 2). However, no significant differences between the two groups were observed regarding morphological changes.

CD34+ Cell Proportion

To compare CD34+ cell counts during a short-term culture in the two different cytokine combinations, cells were counted by using a flow cytometry at different time-points. During the cell culture, the proportion of CD34+ cells in both groups decreased rapidly, even at a very early phase of the culture. After seven days, the CD34+ fraction was markedly decreased to 21–30% and was only 5–6% at 14 days in both groups. However, the cells cultured in FT contained more CD34+ cells when compared to those of the FTS group, until day 10 (Fig. 3).

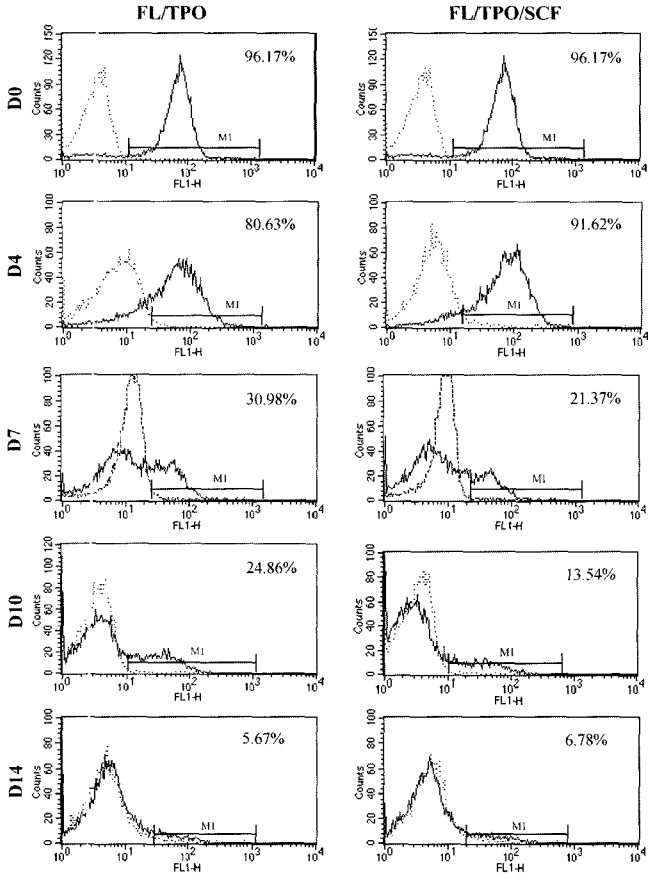

Fig. 3. Expression of CD34 on *ex vivo*-expanded cord blood cells stimulated by either FL/TPO or FL/TPO/SCF. FACS plots of CD34 expression from a representative experiment are shown. CD34+ cells were counted by flow cytometry at different time intervals.

Apoptosis

Since a large number of cells proliferated and underwent differentiation during the culture, apoptosis was compared between the FT and FTS groups. Increased apoptosis was observed at each time-point in both groups. After 14 days of culture, about 13–17% cord blood cells underwent apoptosis. However, the cells cultured in FTS displayed a low apoptosis compared to the FT group until day 7 (Fig. 4).

Telomerase Activity

Telomerase activity was quantitatively measured with ELISA. CD34+ cord blood stem cells displayed a very low activity, compared to the 293 cell line. During the culture

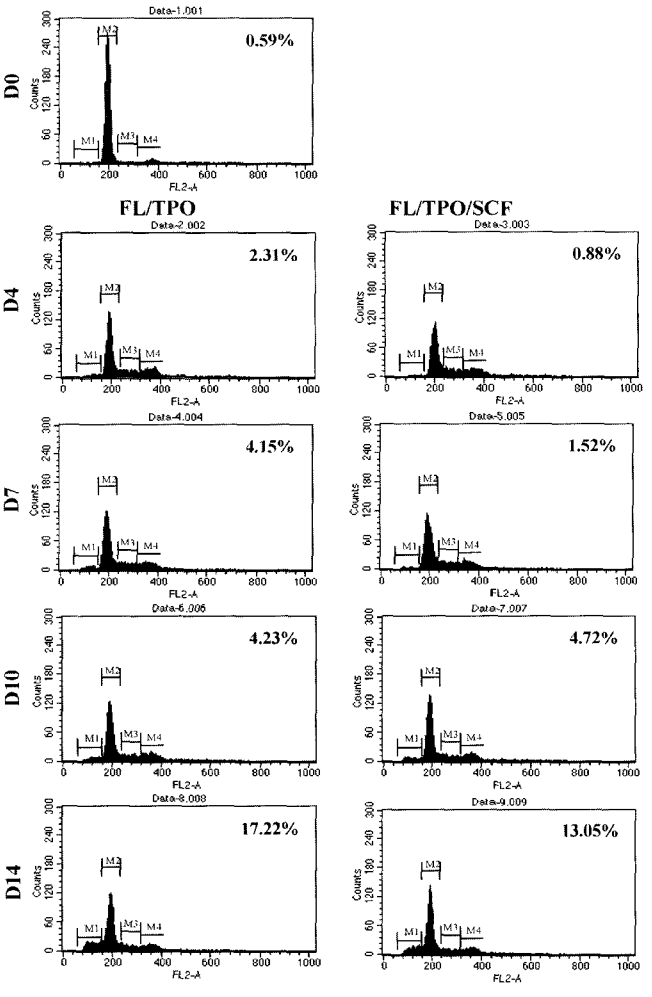

Fig. 4. The proportion of apoptosis of *ex vivo*-expanded cord blood cells in the presence of either FL/TPO or FL/TPO/SCF. At each time-point of culture, harvested cells were incubated with Annexin V-FITC and propidium iodide, and subsequently analyzed by FACS. FACS plots of apoptosis from a representative experiment are depicted.

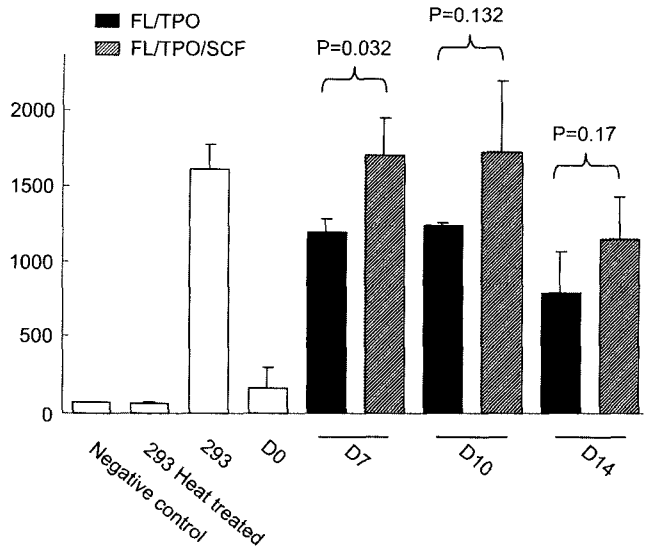

Fig. 5. Telomerase activity of CD34+ cord blood cells in liquid culture. Telomerase activity was quantitatively measured by PCR ELISA.

In purified CD34+ cord blood cells, telomerase activity was very low, significantly upregulated at day 7, peaked at day 10, and declined thereafter. Telomerase activity in the FL/TPO/SCF group was higher than that of FL/TPO. 293 and heat-treated cells were employed as positive and negative controls, respectively.

period, telomerase activity increased in both groups, showing a peak at 10 days and decreasing after 14 days. The telomerase activity of cord blood cultured in FTS was higher than that cultured in FT (Fig. 5).

DISCUSSION

Allogeneic stem cell transplantation is successfully used to treat patients with hematologic malignancies, bone marrow failure syndromes, and metabolic disorders. The major limitation of stem cell transplantation is the availability of a suitable donor. Umbilical cord blood has been clinically investigated as an alternative source of hematopoietic tissue for allowing allogeneic transplantation in patients who lack an HLA-matched donor. Compared to hematopoietic stem cells from adults, those in cord blood have distinctive proliferative advantages, including a capacity to form more colonies in a culture, rapid cell-cycle, autocrine production of growth factors, and longer telomeres [21, 30]. Moreover, the relative immaturity of lymphocytes in a cord blood may reduce the risk and severity of GVHD, which in turn permits more HLA mismatching between donor and recipient than it is usually acceptable with transplants of blood or marrow hematopoietic stem cells from adults. Since the number of stem cells in cord blood is limited, its use in adult patients would benefit from *ex vivo* expansion. Considerable efforts are currently being made to define culture conditions that facilitate optimal expansion of hematopoietic cells for clinical applications. The ultimate goal of *ex vivo* expansion is to promote cell division and proliferation of stem cells, while maintaining their ability to engraft and sustain long-term hematopoiesis.

FL, TPO, and SCF play a key role in maintaining functional repopulation of hematopoietic stem cells [6, 18]. FLT3 belongs to the type III receptor tyrosine kinase family that includes Kit, FMS, and platelet-derived growth factor receptors [25]. FL functions are similar to the Kit ligand. Both proteins stimulate the proliferation of primitive hematopoietic progenitors [20, 26]. TPO, a recently isolated ligand of the MPL receptor, independently stimulates megakaryocyte progenitor division and maturation. MPL receptor expression has been detected by polymerase chain reaction in human hematopoietic cells throughout the megakaryocyte lineage and in primitive CD34+CD38- cells [7]. SCF, also known as Kit ligand, mast cell growth factor, or steel factor, is a hematopoietic cytokine that triggers biologic effects by binding to its receptor, c-Kit. Earlier *in vitro* experiments revealed that it is a potent growth factor for primitive hematopoietic cells and multiple differentiating lineage, acting in synergy with other cytokines [1, 19]. In terms of hematopoietic progenitor cell expansion, the best results are obtained when cytokines, such as early-acting growth factors (FL, TPO, and SCF), are used in combination to sustain and augment the number of primitive progenitor cells *in vivo* and *in vitro* [16, 18].

This study demonstrated that CD34+ cord blood stem cells successfully proliferated in our culture system with different combinations of cytokines. Cells cultured with a combination of FL, TPO, and SCF cytokines proliferated more rapidly and retained ten times higher proliferation capacity, compared to those cultured in FL and TPO. Piacibello *et al.* [24] reported that growth of cord blood CD34+ cells could be sustained and greatly expanded for more than 6 months by using a simple combination of two growth factors, FL and TPO. However, in our experiments, cord blood cells cultured with FL/TPO were maintained for 8 weeks and the proportion of viable cells rapidly decreased thereafter. This discrepancy may be explained by the differences in the culture systems. Although cord blood cells in the presence of FL, TPO, and SCF proliferated for 8 weeks and displayed more sustained long-term culture than the FT group, a large number of cells exhibited condensed nuclei and decreased nuclear/cytoplasm ratio on a Wright-Giemsa stain, indicating continuous differentiation to committed cells in this culture system.

Cells cultured in FTS displayed a lower induction of apoptosis than those in FT at an early phase of the culture (7 days), due to high proliferation potential in the FTS group. Although CD34+ cord blood cells lasted for 8 weeks in the culture with FL/TPO/SCF, more than 13% of the cells displayed apoptosis after 14 days of culture. This

result is consistent with an earlier study demonstrating that proliferation-induced decline of primitive hematopoietic cellular activity correlated with an increase in the proportion of apoptotic CD34+ cells [28]. The findings collectively confirm the detrimental effect of cell proliferation on the maintenance of self-renewal activity of primitive hematopoietic progenitor cells.

During the cell culture, the percentage of CD34+ cells in both groups rapidly decreased, even at a very early phase. After 7 days, the CD34+ fraction in both groups decreased tremendously to 21-30% and was only 5-6% at 14 days. Although cells cultured with FT displayed slightly higher CD34+ fractions until 10 days of culture, compared to the FTS group, the absolute number of CD34+ cells in the FTS group was higher than that in the FL group due to ten times more rapid proliferation capacity.

Low levels of telomerase activity have recently been reported in human primitive hematopoietic stem and progenitor cells [4, 14]. Telomerase is a ribonucleoprotein, RNA-dependent DNA polymerase and acts as a reverse transcriptase-like enzyme that maintains telomere length by adding telomeric repeat units of TTAGGG to the telomere end [12]. Telomerase activity was detected in CD34+ cells of hematopoietic stem cells from all sources and increased significantly after the *ex vivo* expansion. In this study, initial CD34+ cord blood cells showed very low levels of telomerase activity, compared to the 293 cell line. Proliferation of *ex vivo* expanded CD34+ cells, and cell cycle activation in response to cytokine support was closely linked with telomerase upregulation, suggesting that telomerase is activated as cells progressed from G_0 into S phase. Moreover, compared to the FT group, FTS cultures displayed higher telomerase activity. However, after 2 weeks in an *ex vivo* expansion culture, telomerase activity declined, indicating that the levels of the enzyme may decrease with a reduction in cell renewal and expansion potential.

Engelhardt *et al.* [9] reported that within 48 to 72 h of *in vitro* culture of CD34+ cells in the presence of cytokines, telomerase acitivity was upregulated, peaked after one week of culture, and decreased to the baseline or below detection levels after 3 to 4 weeks. The cytokines which were used included stem cell factor, interleukin-3 (IL-3), IL-6, erythropoietin, and granulocyte colony-stimulating factor. This discrepancy may be attributed to the different cytokines and culture systems employed. Otsuka *et al.* [23, 29] additionally demonstrated that human telomeric protein, TRF1, and hTERT were downregulated, consistent with telomerase downregulation during the course of myeloid differentiation in CD34+ cord blood cells cultured *in vitro* with growth factors.

In conclusion, a combination of three cytokines, specifically, FL, TPO, and SCF, induced higher proliferation rates, lower levels of apoptosis, and decreased downregulation of telomerase activity during *ex vivo* culture of CD34+ cord blood stem cells, compared to a combination of FL and TPO. These results suggest that FL, TPO, and SCF constitute the best cytokine combination for the *ex vivo* expansion of cord blood cells. Although progress has been made in the expansion of cord blood hematopoietic stem and progenitor cells, further advancements in *ex vivo* expansion technology are required for making a clinical application to induce cell proliferation of stem cells, while maintaining their ability to engraft and sustain a long-term hematopoiesis.

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