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I mmunophenotype of hematopoietic stem cells from placental/umbilical cord blood after culture

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Abstract

Identification and enumeration of human hematopoietic stem cells remain problematic, since *in vitro* and *in vivo* stem cell assays have different outcomes. We determined if the altered expression of adhesion molecules during stem cell expansion could be a reason for the discrepancy. $CD34^+CD38^-$ and $CD34^+CD38^+$ cells from umbilical cord blood were analyzed before and after culture with thrombopoietin (TPO), FLT-3 ligand (FL) and kit ligand (KL; or stem cell factor) in different combinations: TPO + FL + KL, TPO + FL and TPO, at concentrations of 50 ng/mL each. Cells were immunophenotyped by four-color fluorescence using antibodies against CD11c, CD31, CD49e, CD61, CD62L, CD117, and HLA-DR. Low-density cord blood contained 1.4 ± 0.9% $CD34^+$ cells, 2.6 ± 2.1% of which were CD38-negative. $CD34^+$ cells were isolated using immuno-magnetic beads and cultured for up to 7 days. The TPO + FL + KL combination presented the best condition for maintenance of stem cells. The total cell number increased 4.3 ± 1.8-fold, but the number of viable $CD34^+$ cells decreased by 46 ± 25%. On the other hand, the fraction of $CD34^+CD38^-$ cells became 52.0 ± 29% of all $CD34^+$ cells. The absolute number of $CD34^+CD38^-$ cells was expanded on average 15 ± 12-fold when $CD34^+$ cells were cultured with TPO + FL + KL for 7 days. The expression of CD62L, HLA-DR and CD117 was modulated after culture, particularly with TPO + FL + KL, explaining differences between the adhesion and engraftment of primary and cultured candidate stem cells. We conclude that culture of $CD34^+$ cells with TPO + FL + KL results in a significant increase in the number of candidate stem cells with the $CD34^+CD38^-$ phenotype.

Key words
- Hematopoietic stem cells
- $CD34^+CD38^-$ cells
- Human umbilical cord blood
- *Ex vivo* expansion
- Adhesion molecules

Introduction

A small population of primitive hematopoietic stem cells (HSCs) is present in the bone marrow. These cells are defined by their ability to self-renew as well as to differentiate into committed progenitors of the different myeloid and lymphoid compartments generating all of the blood cell lineages (1). The complexity of this system is enormous, since as many as 10^{10} erythrocytes and 10^8-10^{10} white blood cells are produced each hour each day during the lifetime of the individual. Over the past 15 years,

human umbilical cord blood (HUCB) has been clinically investigated as an alternative source of HSCs for allogeneic transplantation of patients lacking a human leukocyte antigen-matched marrow donor. However, the number of HSCs in HUCB samples is limited. Identification of conditions that support the self-renewal and expansion of human HSCs remains a major goal of experimental and clinical hematology. Expansion of human stem cells in *ex vivo* culture will likely have important applications in transplantation, stem cell marking, and gene therapy (2). The $CD34^+$ protein is a surface glycoprotein expressed on HSCs and progenitor cells in early developmental stages in HUCB and bone marrow, as well as on endothelial cells. The $CD34^+CD38^-$ immunophenotype defines a primitive subpopulation of progenitor cells in fetal liver and fetal or adult bone marrow (3-5). About 1% of bone marrow cells express CD34, and generally less than 1% of these cells are CD38-negative. Hence, the frequency of the $CD34^+CD38^-$ population is about 1 in 10,000, or even lower. Phenotypic analyses of several cell surface markers reveal that even this rare population is heterogeneous (6). *Ex vivo* culture is a crucial component of several clinical applications of stem/progenitor cells. A single stem cell has been proposed to be capable of more than 50 cell divisions or doublings *in vivo* and as such has the capacity to generate up to 10^{15} cells, or sufficient cells for up to 60 years. The proliferation and differentiation of cells is controlled by a group of hematopoietic growth factors. Replication of this enormous cell amplification with hematopoietic growth factors *in vitro* would allow the generation of large numbers of cells that could be used for a variety of clinical applications (7).

Several culture systems have been developed to expand HSCs (5). Piacibello et al. (1) reported the differential ability of FLT-3 ligand (FL), thrombopoietin (TPO), kit ligand (KL), and interleukin-3 (IL-3), alone or combined, to support different stages of hematopoiesis in long-term stroma-free suspension cultures of HUCB $CD34^+$ cells. Several studies have described the effects of TPO alone in culture, which can stimulate early proliferation, survival or differentiation of progenitor cells in cord blood or bone marrow (8).

The proliferation and differentiation of HSCs are controlled not only by soluble growth factors, but also by adhesion to stromal cells and matrix molecules. The expression of adhesion molecules has attracted special attention, as their expression on HSCs and on endothelial and stromal cells plays a pivotal role in the process (9). These molecules permit the interaction with various regulatory elements present in the microenvironment, which includes stromal cells, extracellular matrix molecules and soluble regulatory factors such as cytokines and growth/differentiation factors (10).

Adhesion molecules include integrins, selectins and molecules from the immunoglobulin superfamily.

The objective of the present study was to investigate the behavior of umbilical cord blood $CD34^+CD38^+$ and $CD34^+CD38^-$ cells cultured with different combinations of growth factors, with respect to their viability, immunophenotype and self-renewal and differentiation capacities. Adhesion molecules representing the integrins (CD11c or integrin α-chain, CD49e or α-5 chain and CD61 or ß-3 chain), selectins (CD62L or LECAM-1) and the immunoglobulin superfamily (CD31 or PECAM-1) were analyzed. The expression of HLA-DR and CD117 (c-kit or stem cell factor receptor), which represent differentiation markers for $CD34^+$ cells, was also investigated.

Material and Methods

Human umbilical cord blood cells

A total of 27 cord blood samples were used in this study. Umbilical cord blood

samples obtained after deliveries (≥29 weeks) were collected in sterile bags containing citrate-phosphate-dextrose. Samples were obtained at the Umbilical Cord Blood Bank of the New York Blood Center (New York, NY, USA). Blood was collected according to an Institutional Review Board-approved protocol. Units that are not used in the Placental Blood Program are destined to research. Since collection is performed on delivered placentas, the blood is considered discarded tissue. Blood not used for clinical transplants is not identified and is used without informed consent.

Isolation of CD34+ cells

Low-density mononuclear cells (MNCs) were isolated using density gradient centrifugation on Ficoll-Paque 1.077 g/cm^2 (Amersham Pharmacia, Piscataway, NJ, USA), modified by the addition of 1 M phosphate buffer, pH 7.6, to the Dulbecco's phosphate-buffered saline (GibcoBRL, Gaithersburg, MD, USA) used to dilute the blood. This modification improved the isolation of the mononuclear fraction, since the harvested cell population contained 50% less reticulocytes and less than 50 to 60% erythrocytes. CD34+ cells were harvested from the MNCs using automated magnetic cell sorting (MACS) High Gradient Magnetic Separation Columns for positive selection (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany). The magnetically labeled cells were enriched by passing them twice through positive selection columns.

Antibodies

For the analysis of CD34+ cells, monoclonal antibodies (PharMingen/Becton Dickinson, San Jose, CA, USA) specific for the following human antigens were used: CD34/FITC (clone 34374X lot MO46959), CD38/APC (clone HIT2), CD11c/PE (clone B-IY6), CD31/PE (clone WM59), CD49e/PE (clone IIA1), CD61/PE (clone VI-PL2), CD62L/PE (clone Dreg 56), CD117/PE (clone YB5.B8), and HLA-DR/PE (clone G46-6), as well as isotype control antibodies (clones MOPC-21): mouse IgG1,k/FITC, IgG1,k/PE, and IgG1,k/APC.

Flow cytometry analyses

Processing for four-color fluorescence flow cytometry was done within 36 h of collection using at least 10,000 CD34+ cells, before culture and after 4 and 7 days of culture. Cells were incubated with anti-CD34/FITC and anti-CD38/APC antibodies combined with PE-conjugated antibodies specific for CD11c, CD31, CD49e, CD61, CD62L, CD117, or HLA-DR. All incubations were done for 30 min at 4ºC, and cells were washed with phosphate-bufered saline. 7-Aminoactinomycin D (Molecular Probes, Inc., Eugene, OR, USA) at a final concentration of 1 µg/mL was used to identify dead cells. Flow cytometry was performed on a FACScalibur instrument (Becton Dickinson) equipped with an argon-ion laser tuned at 488 nm. The CELLQuest software (Becton Dickinson) was used for data analysis. Between 5,000 and 50,000 events were collected for each analysis. The gating strategy used can be summarized as follows. First, viable cells were gated, followed by a gating of the cell cluster in forward and side scatter, and using the FITC channel of CD34+ cells. Among the CD34+ cells, CD38-negative and CD38-positive cells were gated and the frequency of cells positive for the third antibody was analyzed.

Analysis of the cell frequency among the different populations was done using the Hendrikx and Pranke Plot program (data not shown) (11), a novel method to facilitate visualization of complex flow cytometry data sets across four dimensions in just a few graphs. In short, samples are divided into clusters, and the mean fluorescence of the cluster versus the frequency of the cluster is

plotted per cluster. In addition, data and cell suspension are shown as third and fourth dimensions, identified as symbol shape and symbol color.

Ex-vivo expansion cultures

MACS-isolated CD34+ cells were cultured in 24-well plates (Multiwell™ Tissue Culture Plate, Becton Dickinson) in 2 mL Iscove's modified Dulbecco's medium with L-glutamine and 25 mM HEPES buffer (GibcoBRL), supplemented with hydrocortisone (10^{-5} M, Sigma, St. Louis, MO, USA), 2-mercaptoethanol (5.5 x 10^{-5} M, GibcoBRL), penicillin G (100 units/mL, GibcoBRL), streptomycin (0.1 mg/mL, GibcoBRL) and 1% bovine serum albumin (Sigma). Cell concentrations varied between 2.5 and 5.5 x 10^5/well. Human growth factors used were: TPO (Kirin Brewery, Kirin Brewery, Chuo-ku, Tokyo, Japan), FL (Amgen Inc., Thousand Oaks, CA, USA) and KL (Amgen Inc.), also called steel factor or stem cell factor, at concentrations of 50 ng/mL each. The combination of hematopoietic growth factors used were: TPO + FL + KL; TPO + FL and TPO. The cultures were maintained at 37ºC and 5% CO_2 in a humidified atmosphere and analyzed on days 4 and 7.

Table 1. Volume of the umbilical cord blood samples and frequency of mononuclear and CD34+ cells obtained after the isolation on Ficoll-Paque and automated magnetic cell sorting columns, respectively.

	Mean ± SD	Range
Blood volume (mL)	45.2 ± 5.6	32-57
MNC (x 10^7)	10.4 ± 5.7	2.5-23.0
MNC (/mm^3 blood)	2287 ± 1132	532-4510
CD34+ cells (x 10^6)	1.3 ± 0.8	0.3-4.0
CD34+ cells (/mm^3 blood)	28.0 ± 16.7	6.4-85.0
% CD34+ cells among MNCs	1.4 ± 0.9	0.4-4.9

Data are reported as mean ± SD for N = 27. MNC = mononuclear cells.

Results

CD34+ cells were isolated from a total of 27 cord blood samples, and the number of MNCs as well as CD34+ cells was analyzed in a Neubauer chamber. As shown in Table 1, the concentration of CD34+ cells was 28 cells/mm^3 of cord blood (yield after Ficoll and MACS procedure). The purity of this cell fraction was 93.2 ± 3.6% (87-99%; N = 10). Since 22.2 ± 11.3% (12.5-50.6%) of the CD34+ cells were dead, the average frequency of viable CD34+ among total cells was 71.0 ± 13.1% (39.4-86.5%).

CD34+ cells were cultured with three

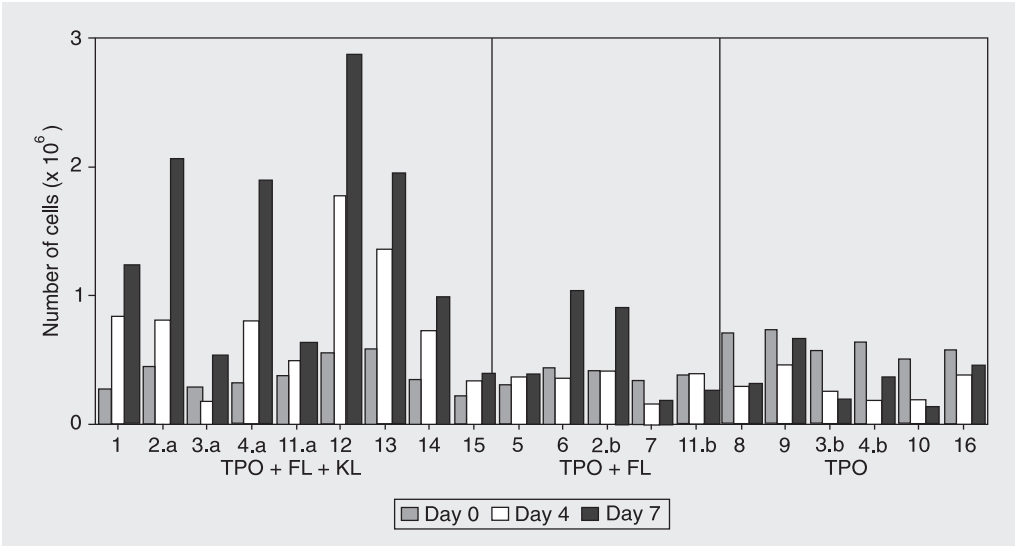

Figure 1. Effect of different combinations of growth factors on cell proliferation after 4 and 7 days in culture. Sixteen different units of cord blood were used, and samples 2, 3, 4 and 11 were split into two different cultures (a and b), using 2 combinations of growth factors. TPO = thrombopoietin; FL = FLT-3 ligand; KL = kit ligand.

combinations of growth factors (Figure 1). With TPO + FL + KL, the average increase in cell number in 9 samples studied was 3.55 ± 1.6-fold after 7 days of culture. Only 1 sample (3.a) showed a 0.56-fold decrease in cell number on day 4, but by day 7 the number of the cells in this sample had increased. The observation of individual cultures (Figure 1) showed some heterogeneity among samples, particularly when TPO + FL was used. With this combination of growth factors, the total number of cells changed very little until day 4. From day 4 until day 7 of culture, however, in one sample the cell number decreased 0.65-fold, in 2 samples it showed no major variation, and in the remaining 2 a considerable increase (2.57 ± 0.5-fold) in total cell number was observed. Considering the total culture period, in 2 samples the cell number decreased 0.59 ± 0.11-fold and in 3 of them it increased 1.92 ± 0.56-fold. In all 6 samples cultured with TPO, the total cell number decreased during the first 4 days. After that, in 4 of them there was an increase, not enough, however, to reach the original number. Taken as a whole, cell numbers decreased 0.55 ± 0.26-fold with TPO only.

Further analyses were done in 10 of the samples. Three of them were separated into two cultures submitted to different treatments. The analysis of cell viability during the period of culture (Table 2) showed that, for cultures in the presence of TPO + FL + KL, the absolute number of viable cells increased 4.27 ± 1.82-fold in the 4 samples studied. In one sample (3.a) the number of viable cells decreased 0.5-fold from day 0 to day 4, but increased 3.4-fold from the 4th to the 7th day. In cultures with TPO + FL, the number of viable cells increased 1.94 ± 0.56-fold in 3 samples, and decreased 0.76-fold in one sample. In the 5 samples cultured with TPO, the number of viable cells decreased 0.35 ± 0.28-fold.

The viability of CD34+ cells was increased after culture with TPO + FL + KL and with TPO + FL, and maintained in the presence of TPO, but the frequency of viable CD34+ cells among total viable cells decreased (Table 2). From day 0 to day 7 of culture with TPO + FL + KL, the number of viable CD34+ cells decreased 0.46 ± 0.25-fold in the 4 samples studied, whereas with TPO + FL and TPO alone this decrease was 0.30 ± 0.12- and 0.13 ± 0.08-fold, respectively.

Of particular interest are the results regarding the frequency of CD34+CD38- cells during the culture period. As shown in Table 2, the number of viable CD34+CD38- cells increased in some of the culture conditions. With TPO + FL + KL, this increase was

Table 2. Total and CD34+ cell viability and frequency of CD34+ and CD34+CD38- cells on day 0 and after culture with three different combinations of growth factors.

		TPO + FL + KL (N = 4)		TPO + FL (N = 4)		TPO (N = 5)	
	Day 0	Day 4	Day 7	Day 4	Day 7	Day 4	Day 7
Total cell viability	76.1±13.0 (N = 10)	81.7±6.0	78.6±5.8	68.9±17.6	72.2±8.1	54.2±14.9	52.9±13.0
CD34+ cell viability	76.0±12.7 (N = 10)	90.2±4.0	92.3±5.9	83.2±7.5	87.7±7.0	78.6±11.0	79.8±14.0
Viable CD34+ cells among viable cells	93.3±2.1 (N = 10)	35.0±23.3	10.9±5.0	63.9±16.9	23.9±22.0	80.7±7.0	41.8±21.1
Viable CD38- cells among CD34+ cells	2.6±2.1 (N = 8)	16.0±19.9	52.0±28.8	1.6±0.9	9.1±8.6	1.8±1.6	2.5±2.4

Data are reported as % ± SD. TPO = thrombopoietin; FL = FLT-3 ligand; KL = kit ligand.

14.59 ± 11.81-fold in 3 samples. In one sample, cell numbers were not determined on day 0, but from day 4 to day 7 the number of viable $CD34^+CD38^-$ cells increased 4.27-fold. In one sample (3.a), the number of $CD34^+CD38^-$ cells decreased 0.23-fold from day 0 to day 4, but increased 23-fold until day 7. For cultures with TPO + FL, these cells increased 2.79 ± 2.29-fold in the 3 samples studied from day 0 to day 7 and in one sample for which the analysis was not done on day 0, they increased 1.22-fold from day 4 to day 7. In the presence of TPO, however, the number of viable $CD34^+CD38^-$ cells decreased 0.26 ± 0.31-fold in 5 samples.

The expansion and proliferation of $CD34^+CD38^-$ cells were significantly increased in the presence of TPO + FL + KL as compared to the other two combinations of growth factors. An interesting correlation can be seen between this effect and total increase in cell numbers, which is directly proportional to the increase or decrease in $CD34^+CD38^-$ cells (Figures 1 and 2). When TPO + FL + KL were used, in 4 samples, total cell numbers as well as $CD34^+CD38^-$ cells were gradually increased during culture. In Figure 1, it can be seen that total cell numbers increased from the first to the fourth day in 8 of 9 samples cultured in the presence of TPO + FL + KL. Only one sample (3.a) showed a 0.6-fold decrease in cell number until the fourth day in culture, but this number increased around 3.4-fold from day 4 to day 7. The same pattern was observed for $CD34^+CD38^-$ cells (Figure 2), as well as in cultures where TPO + FL or TPO alone were used as growth factors. On the other hand, no correlation was observed between the initial number of $CD34^+CD38^-$ cells and total cell growth or with the expansion of the $CD34^+CD38^-$ cells. Similarly, no correlation was detected between the initial number of viable $CD34^+$ cells and cell growth or number of $CD34^+CD38^-$ cells (results not shown).

The immunophenotypic profile of freshly isolated $CD34^+CD38^+$ and $CD34^+CD38^-$ cells was also investigated. In 7 of 8 samples analyzed, the number of $CD34^+CD38^+$ cells positive for CD11c was low (less than 20%) and the fluorescence was dim or, alternatively, all cells were negative. Only one sample showed around 45% of positive cells with dim fluorescence. The $CD34^+CD38^-$ population could be analyzed in only 5 samples due to low event numbers. The

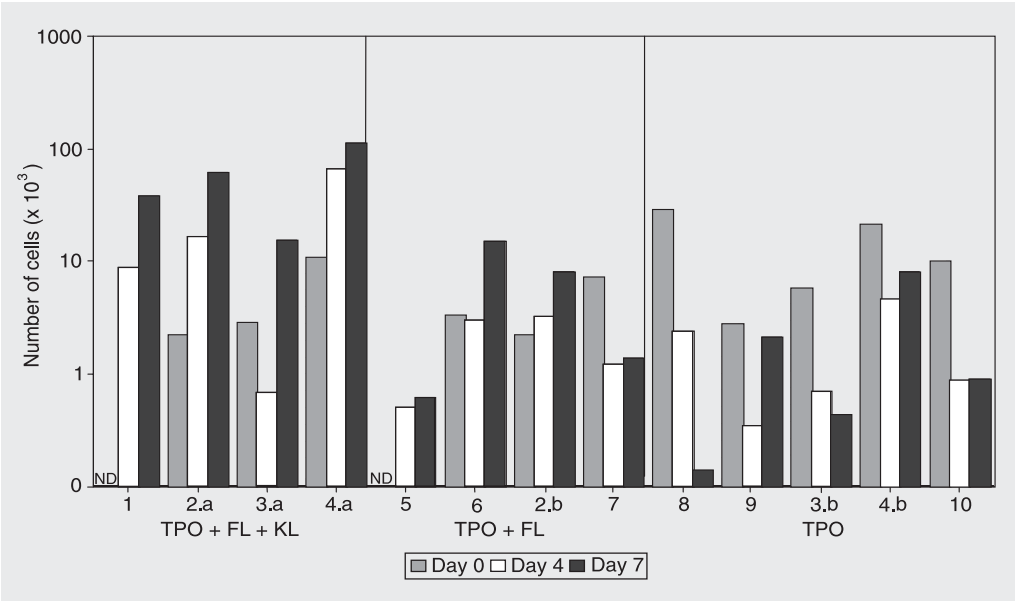

Figure 2. Number of viable $CD38^-$ cells among viable $CD34^+$ cells in culture. Note the logarithmic scale on the ordinate. ND = not determined; TPO = thrombopoietin; FL = FLT-3 ligand; KL = kit ligand.

samples presented the same predominant pattern as observed for $CD34^+CD38^+$ cells.

All 8 samples showed 100% of the cells positive for CD31, with a bright fluorescence, and about 70-100% of the cells with regular fluorescence for CD49e. For CD61, however, among $CD34^+CD38^+$ cells either the number of positive cells was low (less than 15%), with regular fluorescence, or 100% of the cells were negative. In one of the samples, the positive population contained a small (around 3%) subpopulation of bright cells. Because the number of the events was low in $CD34^+CD38^-$ cells, we could analyze only 4 of 8 samples.

Cells were heterogeneous for CD62L expression. About 43 ± 17 and 27 ± 17% of $CD34^+CD38^+$ and $CD34^+CD38^-$ cells, respectively, were positive with a regular mean fluorescence intensity. In one sample, a second population of around 20%, among $CD34^+CD38^+$ cells, presented bright fluorescence for CD62L. In 2 samples the number of events among $CD34^+CD38^-$ cells was too low to be analyzed.

The pattern for HLA-DR was very heterogeneous among samples. This marker was present in about 54 ± 28 and 34 ± 31% of $CD34^+CD38^+$ and $CD34^+CD38^-$ cells, respectively, with a regular mean fluorescence intensity. In one of the samples, a small population of the bright cells was also seen among the $CD34^+CD38^+$ population, whereas in another two populations, very few positive cells with dim and bright cells were observed.

The fluorescence pattern observed for CD117 was complex. Among $CD34^+CD38^+$ cells, two clusters could be observed, one with a high frequency of cells (80 ± 10%, range: 59-91%) with regular fluorescence and another composed of few cells (6 ± 5%) with bright or very bright fluorescence. Among the $CD34^+CD38^-$ cells, 56 ± 24% presented regular mean fluorescence intensity, with no bright cells observed.

The immunophenotypic profile of umbilical cord blood $CD34^+$ cells was analyzed after 4 and 7 days of culture with TPO + FL + KL, TPO + FL and TPO. In some of the samples, particularly among $CD34^+CD38^-$ cells, the analysis was not possible due to very small numbers of events. Culture with TPO + FL or TPO alone was also a factor which decreased the cell number to a level below meaningful analysis in some cases.

The patterns for CD11c, CD31, CD49e, and CD61 between $CD34^+CD38^+$ and $CD34^+CD38^-$ cells were not modified after the culture in all combinations of growth factors (data not shown). For CD62L, however, the number of positive cells and the fluorescence intensity increased from day 0 to day 4 and again from day 4 to day 7 in all culture conditions (Figure 3). In only one sample, cultured with TPO alone, did the number of positive cells increase from day 0 to day 4 but it decreased a little from day 4 to day 7 (data not shown).

In cultures with TPO + FL + KL, the number of cells positive for HLA-DR and the intensity of fluorescence increased from the day 0 to day 4, but decreased a little from day 4 to day 7 between $CD34^+CD38^+$ and $CD34^+CD38^-$ cells (Figure 3). In cultures with TPO + FL, the reactivity pattern did not change among $CD34^+CD38^+$ cells, except for one sample in which the number of positive cells increased from day 0 to day 4 but remained unaltered until the end of the culture. Similar patterns were observed in $CD34^+CD38^-$ cells, but the number of events was very low. The results were heterogeneous in cells cultured with TPO alone (data not shown). Among $CD34^+CD38^+$ cells, the number of positive cells increased significantly in 3 samples, whereas in the remaining 2 no changes were observed. Two of the 4 samples analyzed for $CD34^+CD38^-$ cells remained the same and in 2 the number of HLA-positive cells increased.

Culture with TPO + FL or TPO alone reduced the number of cells brightly positive for CD117 among $CD34^+CD38^+$ cells in

comparison with day 0. The presence of TPO + FL + KL, however, induced a decrease in CD117-positive cells (Figure 3). Among $CD34^+CD38^-$ cells, the results were more heterogeneous. After culture with TPO + FL + KL, a cluster of few cells with dim or regular fluorescence could be observed, but no bright cells. The number of positive cells increased after culture with TPO + FL but no bright cells were seen. When TPO alone was used, two clusters were observed: a high number of cells with mean fluorescence around 100 and another one with few cells of mean fluorescence around 1000.

In some samples, after culture with TPO + FL (samples 5 and 6) or TPO alone (samples 9 and 3.b) the number of $CD34^+CD38^-$ cells was too low to analyze for CD117 reactivity. On day 0, the average frequency of CD117-negative cells was 44.2 ± 26.3% (N = 5). After culture with TPO + FL + KL, 84.5 ± 11.8% of the cells were negative for CD117 on day 4 (N = 3) and 96.0 ± 2.7% (N = 4) on day 7. After culture with TPO + FL, 11.3 ± 5.1 and 7.5 ± 9.2% of the $CD34^+CD38^-$ cells were CD117-negative on day 4 (N = 3) and day 7 (N = 2) respectively, whereas culture with TPO resulted in 7.3 ± 2.1 and 7.5 ± 6.4% CD117-negative cells on days 4 (N = 3) and 7 (N = 2), respectively.

Discussion

Lack of CD38, HLA-DR and lineage-committed antigens, as well as the co-expression of Thy-1 (CDw90) and c-kit receptor (CD117), have been shown to identify the candidate hemopoietic stem cells (12). However, a better knowledge and standardization of the phenotype of umbilical cord blood $CD34^+$ cells is critical since HUCB volume is limited (13). The present study aimed to contribute to the characterization of $CD34^+$ cells from umbilical cord blood, analyzing their phenotype and behavior before and after culture with different combinations of growth factors.

The frequency of $CD34^+$ cells among

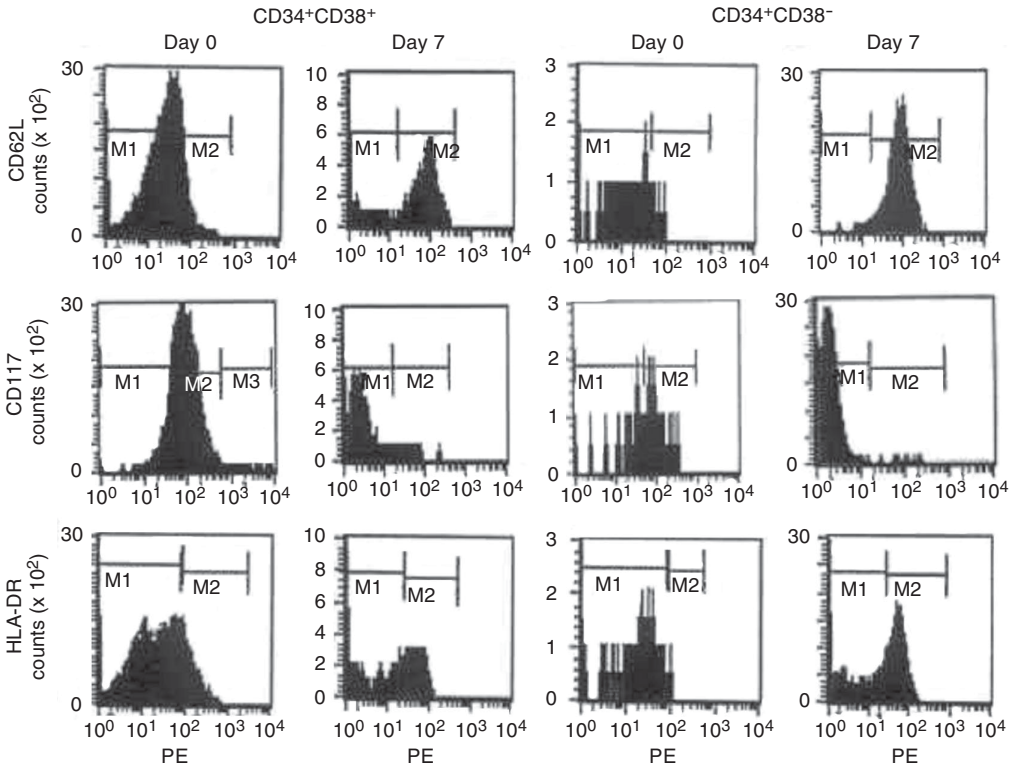

Figure 3. Frequency of cells positive for CD62L/PE, CD117/PE and HLA-DR/PE among $CD34^+CD38^+$ and $CD34^+CD38^-$ cells on day 0 and after 7 days of culture with TPO + FL + KL. TPO = thrombopoietin; FL = FLT-3 ligand; KL = kit ligand. PE = fluorochrome phycoerythrin. M1 = isotype control; M2 = cells with regular fluorescence; M3 = cells with bright fluorescence.

HUCB MNCs in our study was 1.4% (0.4-4.9%), in agreement with other studies. This frequency is similar to that in harvested pelvic bone marrow (1.0 ± 0.3 *vs* 0.8 ± 0.4%) (14). About 1% of bone marrow cells express CD34, and generally less than 1% of these cells are CD38-negative (6,11,15). In other studies, Campagnoli et al. (16) showed that the concentration of CD34+ cells in whole blood samples in term fetal blood was 0.4 ± 0.03% of total CD45+ cells, and Hao et al. (3) showed that the frequency of CD34+ cells among total MNCs in cord blood was 0.36 ± 0.33% with a large variation among samples (range 0.02 to 1.43%; N = 30).

In the present study, we found a large variation in the frequency of CD34+ cells among HUCB MNCs, from 0.4 to 4.9%. Although correlation between total nucleated cell and CD34+ cells in HUCB has been reported, within groups of samples with similar total nucleated cell counts a high degree of variation (at times exceeding 10-fold) in CD34+ cells is observed. CD34 counts in HUCB can be as low as 0.1% of total nucleated cell as reported by Yap et al. (17), and D'Arena et al. (18) observed 0.01-1.71% CD34+ cells among HUCB cells. Different explanations have been given for the variability found in the frequency of CD34+ cells in HUCB. There is evidence that, although the CD34 population frequency is a reliable indicator of the progenitor potential of HUCB, it is nevertheless heterogeneous in nature. On the other hand, these heterogeneous results can reflect differences in the sensitivity of the methods employed by the different groups. CD34+ HSCs have also been shown to vary with gestational age, mode of delivery and positioning of the delivered neonate after delivery. Yap et al. (17) found that CD34+ cells accounted for 5.1 ± 1.0% of CD45+ cells in first trimester blood, significantly more than in term cord blood (0.4 ± 0.03%).

Controversial results have been published regarding the frequency of CD38- cells among cord blood CD34+ cells. We found that 2.6 ± 2.1% (range 0.55-5.57) of the CD34+ cells were CD38-negative on day 0, which agrees with reports showing that most CD34+ cells present the CD38 antigen in HUCB (4) and in mobilized peripheral blood cells (19). Campagnoli et al. (16) reported that the percentage of CD34+ cells which are CD38- was 3.9 ± 0.9% (N = 5), whereas CD34+CD38- cells have been reported to comprise 0.05 ± 0.08% of the MNCs present in cord blood (3). Timeus et al. (9) observed that the number of CD34+CD38- cells was significantly higher in cord blood than in bone marrow (16 ± 8.8 and 4.7 ± 3% of total CD34+ cells, respectively). However, the number of CD38- cells among HUCB CD34+ cells was reported as 11% (18), or 34.9 ± 3.4% (20).

Studies comparing the three different compartments have shown that the proportion of CD34+CD38- cells is greater in HUCB as compared to peripheral blood (9,21,22). This might explain the successful clinical use of HUCB even when a small number of cells is used, making the presence of these antigens candidate predictive parameters for clinical use of HUCB samples (13).

The frequency of viable CD34+ cells after isolation was lower than that found, for instance, in samples processed in cord blood centers. It is possible that the extensive manipulation involved in the immunomagnetic procedure, not performed in cord blood centers, decreases cell viability. It is known that different factors involved in the processing of hematopoietic cells, such as a 48-h delay in their analysis or freezing and thawing have a negative impact on their biology (23).

Although cell numbers were higher after culture, particularly in the presence of TPO + FL + KL, and cell viability was increased or did not show a difference, the number of CD34+ cells showed a marked decrease. These results indicate that, during culture, a proportion of stem cells differentiate and lose the CD34 surface marker. In our study

we used three different combination of hematopoietic growth factors. TPO is a primary regulator of megakaryocyte and platelet production and might also play an important role in early hematopoiesis (24). It is an important cytokine in the early proliferation of human primitive as well as committed hematopoietic progenitors, and in the *ex vivo* manipulation of human hematopoietic progenitors (9). TPO has also been observed to suppress apoptosis of $CD34^+CD38^-$ cells in culture, showing a potential role in maintaining quiescent primitive human progenitor cells viable (25). Liu et al. (26) used a combination of growth factors with and without TPO and showed a significant expansion of $CD34^+$ cells from HUCB and neonatal blood to early and committed progenitors, in the presence of this factor. This potential role of TPO in the early hematopoietic differentiation was explored in the present study, in which TPO was used in all combinations of growth factors.

FL, a class III tyrosine kinase receptor expressed on primitive human and murine hematopoietic progenitor cells, is able to induce proliferation of $CD34^+CD38^-$ cells that are non-responsive to other early acting cytokines and to improve the maintenance of progenitors *in vitro* (5). The expansion of nonadherent cells from umbilical cord blood, for instance, has been shown to be greater with TPO + FL + KL than TPO + FL, and greater in this combination than with TPO alone. Similarly, the expansion of $CD34^+$ $CD38^-$ was greater with TPO + FL than with TPO alone, and the percentage of $CD34^+$ cells was greater with TPO + FL than with TPO + FL + KL (1). Our data agree with these results, since the absolute number of $CD34^+CD38^-$ cells increased considerably when TPO, FL and KL were used as growth factors. The number of those cells increased in a few samples when we used TPO and FL and decreased when we used just TPO. It has already been shown that, although TPO alone can stimulate limited clonal growth, it synergizes with the KL, FL, or IL-3 to potently enhance clonogenic growth. Ramsfjell et al. (24) showed that whereas KL and FL in combination stimulate the clonal growth of only 3% of $CD34^+CD38^-$ cells, 40% of those cells are recruited by TPO + FL + KL, demonstrating that TPO promotes the growth of a large fraction of $CD34^+CD38^-$ progenitor cells.

An interesting correlation can be made between the number of $CD34^+CD38^-$ cells and total increase in cell numbers, which was directly proportional to the increase or decrease in $CD34^+CD38^-$ cells. When TPO + FL + KL were used total cell numbers as well as $CD34^+CD38^-$ cells presented a gradual increase during culture. These results indicate that self-renewal and differentiation of $CD34^+CD38^-$ cells were significantly increased in the presence of TPO + FL + KL as compared to the other two combinations of growth factors.

Finally, it is interesting to observe that, although an increase in total cell counts and in $CD34^+CD38^-$ cell number was induced by the growth factors, particularly in the TPO + FL + KL combination, this increase was smaller than reported in other studies (1). In those studies, as well as in several other ones, however, cells were cultured with fetal calf serum or pooled human serum, whereas we employed serum-free media. We believe that serum-free medium allows a better control of the role that individual cytokines and their combination have on cell growth and differentiation. Furthermore, many of these studies analyze the behavior of the cells in long-term culture, whereas in the present study cells were cultured for only one week. Our aim was to expand $CD34^+CD38^-$ cells within a short period of time to use the expanded population for transplants.

We investigated the expression of several cell adhesion molecules and other proteins among $CD34^+CD38^+$ and $CD34^+CD38^-$ cells in umbilical cord blood, before and after culture with TPO + FL + KL, TPO + FL

or TPO. Adhesion molecules play a role in the migration of hematopoietic progenitor cells and in the regulation of hematopoiesis. Cell adhesion molecules are highly expressed in both HUCB and bone marrow $CD34^+$ $CD38^-$ cells. It has been shown that molecules such as CD11a and CD62L are more expressed in HUCB than in the bone marrow $CD34^+CD38^-$ subset, suggesting a possible advantage in homing and engraftment of more undifferentiated HUCB as opposed to bone marrow HSCs (27).

The expression of CD11c on HUCB $CD34^+$ cells in fresh samples was rare, as already reported for bone marrow (28) and HUCB (6) $CD34^+$ cells. This adhesion molecule has a role in the linkage to receptors on stimulated endothelium (Nancy Hogg, www.ncbi.nlm.nih.gov/prow). PECAM-1 expression was observed on all $CD34^+$ cells, with high fluorescence, in all samples. Other reports have also shown high expression of CD31 on bone marrow (28) and cord blood (6) $CD34^+$ cells. This molecule is involved in the adhesion between cells such as endothelial cells and leukocytes (Muller WA, www.ncbi.nlm.nih.gov/prow), as well as in the interaction between hematopoietic cells and extracellular matrix components in bone marrow. CD11c and CD31 were homogeneously expressed, presenting the same pattern among $CD34^+$ $CD38^+$ or $CD34^+CD38^-$ before and after culture.

We found a large number of $CD34^+$ $CD38^+$ and $CD34^+CD38^-$ cells positive for CD49e in all samples both before and after culture. This molecule corresponds to the α-chain of the VLA-5 integrin, and is strongly involved in the binding of bone marrow progenitor cells to extracellular matrix components (29). It is interesting, however, that different studies report conflicting results. Asosingh et al. (28) showed that all $CD34^+$ cells in normal bone marrow expressed CD49e, while cord blood and mobilized $CD34^+$ cells had a lower expression of this molecule than bone marrow $CD34^+$ cells. On the other hand, Timeus et al. (9) showed greater expression of this molecule on $CD34^+$ of cord blood than bone marrow. In other studies, cord blood $CD34^+$ cells have been reported to express VLA-5 in a pattern remarkably similar to that of bone marrow $CD34^+$ cells. Denning-Kendall et al. (30) showed that the expression of VLA-5 on $CD34^+$ and $CD34^+CD38^-$ cells increased after 7 days of culture with KL, FL, TPO, and G-CSF.

CD61 has been observed in small levels on HUCB $CD34^+$ cells: less than 20% (13) or $20.2 \pm 16.1\%$ (4). In our study, the expression of this antigen on HUCB $CD34^+$ cells was also rare in $CD34^+CD38^+$ or $CD34^+$ $CD38^-$ cells before and after culture. L-selectin is involved in the homing of $CD34^+$ cells after peripheral blood MNC transplantation. The majority of the $CD34^+$ cells had CD62L on the membrane surface. HUCB and mobilized blood $CD34^+$ have been shown to present a higher expression of CD62L than bone marrow $CD34^+$ cells (28). CD62L was also more frequently expressed in the HUCB than in the bone marrow $CD34^+CD38^-$ subset, suggesting a possible advantage in homing and engraftment (9,27). In the present study, CD62L expression was heterogeneous, and the $CD34^+CD38^+$ population presented a slightly higher frequency of positive cells. Surbek et al. (31) showed that CD62L on $CD34^+$ stem and progenitor cells in umbilical cord blood change during gestation. This could explain the great variability in the frequency of CD62L-positive cells observed in different samples, since in the present study the gestational age presented a range from 29 weeks to term.

An interesting effect was observed for the expression of CD62L after culture. The number of CD62L-positive $CD34^+CD38^-$ cells and the CD62L expression on these cells increased during culture in all conditions. Denning-Kendall et al. (30) have also found increased expression of L-selectin (or CD62L) on $CD34^+$ and $CD34^+CD38^-$ cells

after 7 days of culture with KL, FL, TPO, and G-CSF.

Timeus et al. (9) have shown that a short exposure to cytokines increases L-selectin expression in the more differentiated hematopoietic progenitors, $CD34^+CD38^+$ cells, which could improve their homing in a transplant setting. After transplantation of HSCs, adhesion molecules play a major role in the multistep process of engraftment in which L-selectin is suggested to be of relevance. Gigant et al. (32) showed a higher frequency of CD62L-positive cells in peripheral blood than in bone marrow or cord blood, and Young et al. (33) reported increased expression of CD62L expression on mobilized peripheral blood $CD34^+$ cells cultured with TPO + FL + KL. The present study showed a significant increase of L-selectin-positive cells, suggesting improved homing ability in HUCB cultured with growth factors in all combinations.

There is evidence that cord blood, bone marrow and peripheral blood-derived HSCs are highly heterogeneous for a number of antigens useful for HSC enumeration by flow cytometry (6). HLA-DR is expressed in the majority of HUCB (4,13) and peripheral blood (13,19) $CD34^+$ cells. While De Bruyn et al. (21) showed that the co-expression of CD34 with HLA-DR was not significantly different in HUCB and bone marrow (86.3 ± 2.7 and 92.7 ± 5.1%, respectively), Cho et al. (22) showed that $CD34^+HLA\text{-}DR^+$ cell frequencies did not differ significantly between those two compartments and MNCs. Very heterogeneous results were found for HLA-DR in the present study which, due to the small number of cells in some of the experimental conditions, made their interpretation difficult. The great heterogeneity of positive cells in fresh samples, as well as small differences after culture, could be explained by different gestational ages. Fetal liver cells, for instance, have been shown to present lower proportions of $CD34^+HLA\text{-}DR^+$ than HUCB, showing that the composition of fetal leukocytes changes during development and with gestational age (34). The frequency of HLA-DR-positive cells was a little higher among $CD34^+CD38^+$ than $CD34^+CD38^-$ cells. This result supports the conclusion that these molecules are more expressed in more differentiated cells.

The expression of c-kit (CD117) on $CD34^+CD38^+$ cells separated the cells into two populations in all samples, with more than 60% of the cells showing regular fluorescence, and a small population of bright or very bright cells. In this way, three fractions were described: negative cells, cells with regular fluorescence, and cells with high fluorescence. Among $CD34^+CD38^-$ cells, however, the frequency of c-kit-positive cells was slightly lower and bright cells were not observed. Although the number of samples in the present study was relatively small, the results show that, after culture with TPO + FL + KL, the number of $CD34^+CD38^-CD117^-$ cells increased. This number decreased when we used TPO + FL or TPO alone. Culture of $CD34^+$ cells with TPO + FL + KL thus significantly increases the number of candidate stem cells with the $CD34^+CD38^-$ ($c\text{-}kit^-$) phenotype. On the other hand, the down-regulation of c-kit may be due to the presence of KL in the growth factor combination, since this factor was essential to expand $CD34^+$ $CD38^-$ cells.

The most primitive human hematopoietic progenitor cells have demonstrated co-expression of c-kit, FLT-3 and Thy-1, being negative for HLA-DR, CD38 and lineage markers (12,24). CD117 expression has thus been reported to characterize true stem cells (35). The c-kit receptor, a member of the Ig superfamily of adhesion molecules, is involved in the interactions of $CD34^+$ cells with other cells and stroma in bone marrow, mobilized peripheral blood and HUCB. The c-kit receptor was detected on the majority of $CD34^+$ HSCs, particularly on HUCB in the studies of D'Arena et al. (12). On the other hand, Sakabe et al. (19) showed that

the expression of the c-kit receptor on mobilized peripheral blood CD34+ cells was approximately 20% of that on bone marrow- or HUCB-derived CD34+ cells which express high levels of c-kit receptor.

Studies about the expression of c-kit on mobilized peripheral blood CD34+ cells showed three fractions, namely CD34+c-kit^{high}, CD34+c-kit^{low} and CD34+c-kit^{-} cells (19). Different levels of CD117 antigen were also shown in HUCB. While Gunji et al. (36) demonstrated that myeloid progenitors are enriched in CD34+c-kit^{high} cells and erythroid progenitors are more enriched in CD34+ c-kit^{low} cells, Sakabe et al. (19) showed that erythroid progenitors are highly enriched in mobilized peripheral blood CD34+c-kit^{high} cells, and that CFU-GM is enriched in mobilized peripheral blood CD34+c-kit^{-} cells. Primitive progenitors with self-renewal potential may be present in the mobilized peripheral blood CD34+c-kit^{-} or CD34+c-kit^{low} cell population. Laver et al. (37) reported that the HUCB-derived CD34+c-kit^{low} cell population contains the majority of quiescent progenitors and blast cell colony forming cells. Thus, the CD34+CD38^{-} or CD34+c-kit^{-} or low immunophenotype defines primitive progenitor cells in fetal liver, fetal bone marrow, adult bone marrow, mobilized peripheral blood, and HUCB (19). The expression of c-kit may therefore be useful in identifying HUCB progenitors with long-term engraftment capability (37).

HUCB has recently been explored as an alternative HSC source for allogeneic transplantation in both adults and pediatric patients with hematological malignancies and marrow failure syndromes. HUCB transplantation is particularly important in patients who lack HLA-matched marrow donors, permitting the use of HLA-mismatched grafts at 1-2 loci (or antigens) without higher risk for severe graft-versus-host disease relative to bone marrow transplantation from a full-matched unrelated donor (38). Since the first case reported in 1988, more than 3700 patients have received HUCB transplants for a variety of malignant and non-malignant diseases. Due to the relatively low number of stem cells in HUCB, limited by the blood volume which can be collected, the vast majority of recipients (2/3) were children with an average weight of 20 kg (www.netcord.org). The *ex vivo* expansion of HSCs thus represents an attractive approach to overcoming the current limitations regarding adult HUCB transplantation.

CD34 selection and *ex vivo* expansion of HUCB prior to transplantation are feasible. Serum-free media, in some cases with complementation of growth factors such as TPO, KL and FL (39,40), have been shown to allow the expansion and transplantation of HSCs. The *ex vivo* expansion of HUCB hematopoietic stem and progenitor cells has been shown, for instance, to increase cell dose and reduce the severity and duration of neutropenia and thrombocytopenia after transplantation. Additional accrual, however, will be required to assess the clinical efficacy of expanded HUCB progenitors. Some studies have shown that the *ex vivo* expansion of cord blood CD34+ cells results in the generation of increased mature cells and progenitors that are capable of more rapid engraftment in fetal sheep compared to unexpanded HUCB CD34+ cells (39). Enumeration of CD34+CD38^{-} cells is correlated with the number of committed progenitors and the capacity of generating CD34+ cells. This is an important parameter if expansion protocols are to be used in clinical transplantation, since CD34+CD38^{-} cells are a good predictive marker of cloning ability and expansion potential of CD34+ cord blood cells (40).

In conclusion, in this study the results indicating that culture of HUCB CD34+ cells with the combination TPO + FL + KL induced an increase in total cell counts as well as in CD34+CD38^{-} cell number suggest that this growth factor combination induces an expansion of very primitive stem cells. The

use of allogeneic cord blood products as a source of cellular support for patients receiving high-dose chemotherapy has been limited primarily by the low numbers of cells in a HUCB unit. The results of the present study and others, however, show that the true stem cell can be expanded *in vitro*. Furthermore, our data show that the discrepancy between current *in vitro* and *in vivo* read-out systems to assess candidate stem cells may be affected by changes in adhesion molecules. Further studies should determine what culture conditions and cell populations are needed for a range of clinical applications, improving the use of cord blood for transplantation in adults.

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