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Initial CD34$^+$ Cell-Enrichment of Cord Blood Determines Hematopoietic Stem/Progenitor Cell Yield Upon Ex Vivo Expansion

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ABSTRACT

Since umbilical cord blood (UCB), contains a limited hematopoietic stem/progenitor cells (HSC) number, successful expansion protocols are needed to overcome the hurdles associated with inadequate numbers of HSC collected for transplantation. UCB cultures were performed using a human stromal-based serum-free culture system to evaluate the effect of different initial CD34$^+$ cell enrichments (*Low*: 24 ± 1.8%, *Medium*: 46 ± 2.6%, and *High*: 91 ± 1.5%) on the culture dynamics and outcome of HSC expansion. By combining PKH tracking dye with CD34$^+$ and CD34$^+$CD90$^+$ expression, we have identified early activation of CD34 expression on CD34$^-$ cells in *Low* and *Medium* conditions, prior to cell division (35 ± 4.7% and 55 ± 4.1% CD34$^+$ cells at day 1, respectively), affecting proliferation/cell cycle status and ultimately determining CD34$^+$/CD34$^+$CD90$^+$ cell yield (*High*: 14 ± 1.0/3.5 ± 1.4-fold; *Medium*:22 ± 2.0/3.4 ± 1,0-fold; *Low*:31 ± 3.0/4.4 ± 1.5-fold) after a 7-day expansion. Considering the potential benefits of using expanded UCB HSC in transplantation, here we quantified in single UCB units, the impact of using one/two immunomagnetic sorting cycles (corresponding to *Medium* and *High* initial progenitor content), and the average CD34$^+$ cell recovery for each strategy, on overall CD34$^+$ cell expansion. The higher cell recovery upon one sorting cycle lead to higher CD34$^+$ cell numbers after 7 days of expansion (30 ± 2.0 vs. 13 ± 1.0 × 10^6 cells). In particular, a high (>90%) initial progenitor content was not mandatory to successfully expand HSC, since cell populations with moderate levels of enrichment readily increased CD34 expression ex-vivo, generating higher stem/progenitor cell yields. Overall, our findings stress the importance of establishing a balance between the cell proliferative potential and cell recovery upon purification, towards the efficient and cost-effective expansion of HSC for cellular therapy. J. Cell. Biochem. 112: 1822–1831, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: CD34 ENRICHMENT; UMBILICAL CORD BLOOD; EX-VIVO EXPANSION; HEMATOPOIETIC STEM/PROGENITOR CELLS

Ex-vivo expansion of hematopoietic stem/progenitor cells (HSC) aims to generate clinically relevant cell numbers from the limited amount of cells in available samples for multiple settings including bone marrow (BM) transplantation, somatic gene therapy, or production of specific blood products for transfusion [Traycoff et al., 1995; Koller et al., 1996; Douay, 2001]. In particular for umbilical cord blood (UCB), the low cell number available per unit makes it unsuitable for transplantation in adults [Kelly et al., 2009]. To overcome this limitation, clinical trials have been designed featuring double cord transplantation [Barker et al., 2001], intra-marrow injection [Francesco et al., 2010], or ex-vivo expansion of HSC [Jaroscak et al., 2003; de Lima et al., 2008], with promising results. However, the major focus in HSC expansion is still the rational delineation of optimal culture conditions to provide an amplification of the primitive HSC pool [Cabrita et al., 2003] namely: cytokine cocktails used [Andrade et al., 2010], initial stem/progenitor cell enrichments, the presence/absence of stroma [da Silva et al., 2003, 2005, 2010], or culture duration [Douay, 2001],

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which have a critical impact on the final quality of the expanded graft and on the cost effectiveness of the expansion process [Kirouac and Zandstra, 2008].

Most studies to date have been directed towards the expansion of $CD34^+$ cells since a fraction of the HSC pool is characterized by the expression of the CD34 surface antigen [Berenson et al., 1988; Koller et al., 1995b]. Indeed, $CD34^+$ cells have been shown to reconstitute hematopoiesis in humans after high-dose chemotherapy [Shpall et al., 1994; Steiner et al., 2009]. The delineation of experimental conditions for expanding the number of long-term engrafting cells within this population would be clinically valuable [da Silva et al., 2005; Hofmeister et al., 2007].

However, some studies revealed the uncertainty as to whether or not all HSC express CD34 [Steiner et al., 2009] and that CD34 expression by human stem cells is a reversible process [Zanjani et al., 1998; Zanjani et al., 2003]. In fact, it has been hypothesized that the BM $CD34^-$ stem cells exist in an extremely quiescent state [Osawa et al., 1996], which are capable of long-term multilineage engraftment in vivo [Zanjani et al., 2003], and up-regulation of CD34 expression by these cells is required to more readily be able to engraft a mouse recipient [Sato et al., 1999]. This population was shown to act as a reservoir for generation of $CD34^+$ cells, showing reversibility of CD34 expression on HSC that retain the capacity for secondary in vivo reconstitution [Dao et al., 2007]. In addition, Hess et al. [2003] indicate that lineage-depleted (Lin-) $CD34^+$, and $CD34^-$ cells from UCB, possess repopulating capacity and are functionally related components of the human stem cell compartment, suggesting an emerging functional relationship between $CD34^-$ and $CD34^+$ stem cells, with both populations contributing towards hematopoietic reconstitution [Osawa et al., 1996]. We have recently shown that inherent modulation of CD34 and CD38 during ex-vivo expansion of both human BM and UCB may lead to erroneous quantification of the stem cell content of the expanded graft [da Silva et al., 2009] suggesting that the $CD34^{+/-}$ dichotomy might influence the efficiency of the HSC expansion process. Furthermore, other authors suggest that the expression of CD34 in peripheral blood (PB) is tightly linked to the proliferation status in vitro [Dooley et al., 2004].

In vitro expansion studies, using perfusion cultures of BM mononucleated cells (MNC) have demonstrated progenitor expansion values that exceed total cell expansion, resulting in an enrichment of the immature cell populations [Koller et al., 1993]. This suggests that complete $CD34^+$ selection might not be strictly necessary to obtain net cell expansion, which also results in significant cell losses [Berenson et al., 1988; Koller et al., 1995a]: the waste fraction from separation columns and/or fluorescence-activated cell sorting (FACS) should contain important cells subsets [Koller et al., 1995a], either in the form of lost $CD34^+$ cells, as primitive hematopoietic cells lacking the CD34 surface antigen and/or accessory cells [Lobato da Silva, 2006].

In the present studies, UCB cultures were performed using a human stromal-based serum-free culture system using BM derived mesenchymal stem cells (MSC) as feeder layers [da Silva et al., 2005; Goncalves et al., 2006] to evaluate the effect of the degree of initial $CD34^+$ cell enrichment on the outcome and culture dynamics of hematopoietic stem/progenitor cell expansion. The presented results suggest the existence of highly dynamic culture events regarding CD34 modulation in HSC-MSC co-cultures, prior to cell division, affecting cell cycle and proliferation status in culture and ultimately the final hematopoietic cell yield, pointing to the need to establish a balance between the cell recovery upon purification and the stem/progenitor cell proliferative potential of cultured cells.

MATERIALS AND METHODS

HUMAN DONOR CELL PREPARATION

The UCB samples were kindly provided by Crioestaminal – Saúde e Tecnologia, SA, and were collected after maternal donor consent. Low density UCB mononuclear cells (MNC) were separated by a Ficoll density gradient (1.077 g/ml; GE Healthcare) and washed twice in Iscove's Modified Dulbecco's Medium (IMDM; GibcoBRL). UCB MNC were kept cryopreserved in liquid nitrogen and kept frozen until further use.

HUMAN BM MSC CULTURES

BM aspirates were harvested from healthy adult donors, after informed consent. BM MSC were isolated, kept in liquid nitrogen and then thawed as previously described [Dos Santos et al., 2010]. Upon thawing, BM MSC were expanded for 3–5 passages (3,000 cells/cm^2), using Dulbecco's Modified Essential Medium (DMEM) containing 10% fetal bovine serum (FBS) MSC-qualified (GibcoBRL), supplemented with streptomycin (0.025 μg/ml) and penicillin (0.025 U/ml; GibcoBRL), at 37°C and 5% CO_2 in a humidified atmosphere. Medium was changed twice a week. Near cell confluence (80–90%), cells were washed with phosphate buffered saline (PBS, GibcoBRL) and harvested from the flask by adding Accutase (Sigma), for 7 min at 37°C. In order to be used as stromal feeder layers, cells were seeded on 24-well plates (3,000 cells/cm^2) and grown until confluency. Before starting co-cultures with UCB HSC, BM MSC were treated with Mitomycin C (Sigma) (0.5 μg/ml solution prepared in IMDM + 10% FBS) to prevent stromal overgrowth as previously described [da Silva et al., 2010].

EX-VIVO EXPANSION OF HUMAN $CD34^+$-ENRICHED CELLS

Upon thawing, UCB MNC were $CD34^+$-enriched using magnetic cell sorting (MACS, Miltenyi Biotec). Two magnetic cell-sorting cycles were performed for each UCB sample in order to obtain high initial $CD34^+$ enrichments (*High*, >90%). *Medium* (≈50%) and *Low* (≈25%) $CD34^+$ enrichments were attained by proper dilution of the *High* fraction with MNC in the flowthrough of the column (CD34-depleted cells) (1:1 and 1:3 (v/v), respectively). A control with non-selected MNC was also performed. Cells were then seeded on 24-well plates (3×10^4 total cells/ml) (on top of confluent mytomicin-treated BM MSC-derived stromal cell layers).

Cells were cultured in QBSF-60 serum-free medium (Quality Biological, Inc) with the cytokine cocktail previously optimized by our group focusing UCB stem/progenitor cell expansion [Andrade et al., 2010] (SCF-60 ng/ml, Flt-3-55 ng/ml, TPO-50 ng/ml, bFGF-5 ng/ml – all from Peprotech) at 37°C and 5% CO_2 humidified air, for 7 days, without medium change.

PROLIFERATIVE AND PHENOTYPIC ANALYSIS

Hematopoietic cell numbers were determined using the Trypan Blue (GibcoBRL) exclusion method for each different culture condition. Fold increase (FI) in total nucleated cells (TNC) was calculated by dividing the number of cells at each day by the number of cells at day 0. Cells were also analyzed by flow cytometry (FACSCalibur equipment, Becton Dickinson) using anti-CD34 (FITC-conjugated) and anti-CD90 (Thy-1; PE-conjugated) monoclonal antibodies for stem/progenitor cells (Becton Dickinson Immunocytometry Systems). Isotype controls were also prepared for every experiment. A minimum of 10,000 gated events was collected for each sample.

CELL DIVISION KINETIC STUDIES

CD-34-enriched cell populations (both for *High, Medium*, and *Low* enrichment conditions) were labeled using PKH26 Red Fluorescent Cell Linker Kit (Sigma) prior to cell culture according to manufacturer's instructions. This stable membrane dye allows the tracking of cell division, by the decrease in fluorescence: each generation of cells is half as fluorescent as the previous one [da Silva et al., 2009]. Flow cytometry data was then analyzed using the Proliferation Wizard module of the ModFit software (Becton Dickinson).

CELL CYCLE ANALYSIS

Cell cycle analysis was performed at days 0, 2, and 7. Cells were fixed in 2% Paraformaldehyde (PFA, Sigma) for 20 min, washed with 1% normal goat serum (NGS, Sigma), resuspended in 3% NGS and permeabilized with a 0.5% saponin solution (Sigma). Cells were then incubated with a staining solution consisting of 5 μg/ml propidium iodide (BD Biosciences), 25 μg/ml RNAse A (Easyspin) and 3% NGS in PBS for 30 min at 37°C. To distinguish cells in cell cycle from G0 cells, the anti-Ki-67-FITC antibody (dilution 1:38, Invitrogen) was used. Mouse IgG1 antibody, FITC-conjugated (dilution 1:30, BD Biosciences), was used as control for each sample in all conditions. Cells were also analyzed by flow cytometry. A minimum of 40,000 gated events was collected for each sample.

CLONOGENIC ASSAYS (CFUs) AND COBBLESTONE AREA-FORMING CELLS (CAFCs)

UCB cells at day 0 (fresh) and expanded after 7 days in culture were characterized in terms of clonogenic potential. The clonogenic assays were performed in triplicate in MethoCult GF H4434 (Stem Cell Technologies, Inc.) as previously described [da Silva et al., 2005]. After 14 days, colonies were counted and evaluated as colony-forming unit-granulocyte, macrophage (CFU-GM), colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-Mix) and burst-forming unit-erythroid (BFU-E) progenitors. For the assessment of CAFCs, expanded cells (after 7 days) (2×10^3 cells/ml) were cultured on top of a confluent mytomycin-treated monolayer of MS-5 murine fibroblasts in long-term BM culture medium (Myelocult™, StemCell Technologies) as previously described by us [Andrade et al., 2010]. Briefly, cultures were half-fed once a week and cobblestone areas of more than five tightly packed cells were scored 2 weeks after seeding [Andrade et al., 2010]. For both CFUs and CAFCs, expansion results were calculated as FI relatively to day 0.

CLINICAL-SCALE EXPANSION – SIMULATIONS

A clinical-scale expansion of a single UCB unit was simulated for three different strategies: non-enriched cells (MNC) and CD34-enriched cells upon one or two immunomagnetic isolation cycles. For each time point (days 2, 3, 4, and 7), we calculated the number of $CD34^+$ cells by multiplying the mean FI in the number of $CD34^+$ cells corresponding to each strategy by the number of $CD34^+$ cells available at day 0. In order to determine the number of cells at day 0, we considered an average number of 1×10^9 MNC and 5×10^6 $CD34^+$ cells [Gunetti et al., 2008]. In addition, we took into consideration the cell losses associated to the cell processing, namely cryopreservation/thawing and magnetic sorting. In the absence of literature data, we used the collected data from our laboratory. For these studies, we assumed a UCB unit cryopreserved into two cohorts (60 and 40%) [Shpall et al., 2002]. Considering cell losses of 40% (estimated), the two cohorts available were assumed to have 3.6×10^8 and 2.4×10^8 MNC, respectively. We simulated the expansion of the 40% cohort after 7 days in culture, whereas the 60% would, in theory, be infused without manipulation (2.4×10^6 $CD34^+$ cells).

STATISTICAL ANALYSIS

Values are presented as mean ± standard error of mean (SEM). Comparisons between experimental results were determined by a Paired Sampled Wilcoxon Signed Rank Test, when appropriate. A P-value <0.05 was considered statistically significant.

RESULTS

EFFECT OF INITIAL CD34 ENRICHMENT ON STEM/PROGENITOR CELL YIELD AND IMMUNOPHENOTYPE

The effect of different levels of initial $CD34^+$ cell enrichment on the expansion of UCB HSC co-cultured with BM MSC was studied. *High* (91 ± 1.5%; n = 7), *Medium* (46 ± 2.6% n = 7), and *Low* (24 ± 1.8%; n = 7) initial $CD34^+$ cell enrichments were obtained by proper dilutions with CD34-depleted MNC and cultured for 7 days using the cytokine-supplemented serum-free medium, as previously described [Andrade et al., 2010]. Unselected MNC were also included in this panel. We limited the culture duration to 7 days in order to minimize potential cellular defects occurring during ex-vivo expansion, as well as culture costs with medium renewal, while attaining meaningful cell numbers in a relatively short period [Hofmeister et al., 2007].

Despite the significantly different initial $CD34^+$ enrichment (91 ± 1.5; 46 ± 2.6; 24 ± 1.8%) (n = 7; $P < 0.05$), there was a significant increase in the percentage of $CD34^+$ cells for both *Medium* and *Low* conditions from day 0 to day 1 and from day 1 to day 2 ($P < 0.05$). For *High* condition, the $CD34^+$ content was roughly maintained until day 4 (Fig. 1A). Interestingly, at day 4, all conditions presented similar % $CD34^+$ cells (95 ± 1.6%, 90 ± 1.8%, 83 ± 3.1%, respectively; n = 6; $P > 0.05$), with the highest FI in terms of $CD34^+$ cell expansion achieved in *Low* (31 ± 3.0) when

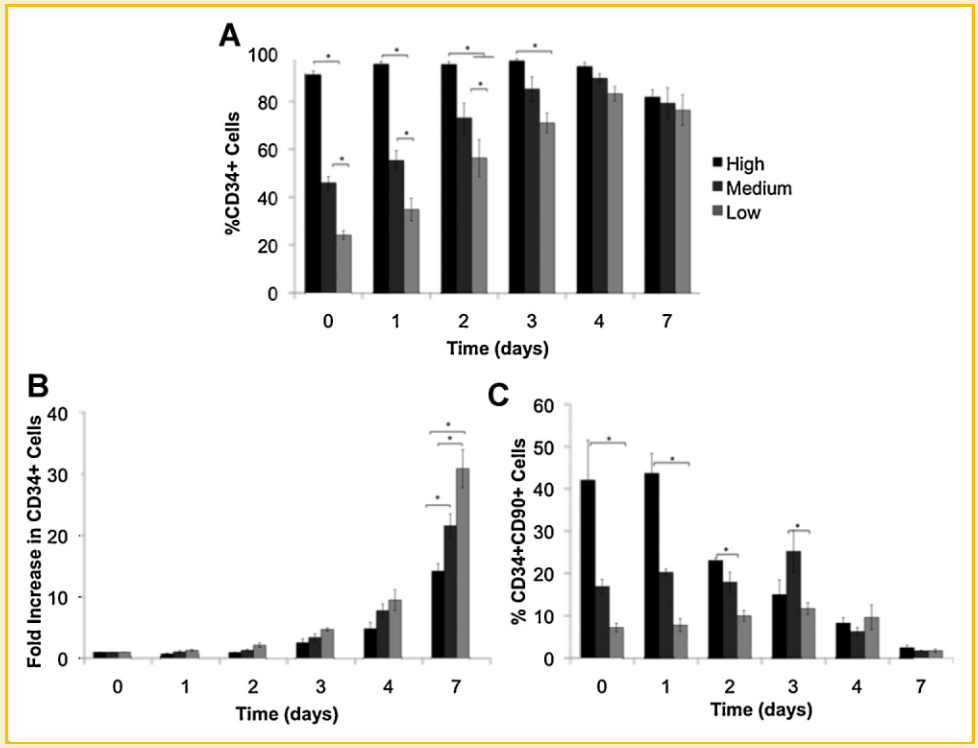

Fig. 1. Influence of initial $CD34^+$ cell enrichment (High ≈ 100%-black bars; Medium ≈ 50%-dark grey; Low ≈ 25%-light grey and MNC-white) on the FI in CD34+ cells (A) (n = 6) and the corresponding expression levels (%) of CD34+ cells (n = 6) (B) and CD34+CD90+ cells (n = 4) (C). Results are presented as mean ± SEM (*$P < 0.05$).

compared to *High* (14 ± 1.0), and *Medium* (22 ± 2.0) conditions (n = 6; $P < 0.05$; Fig. 1A,B). As expected, higher initial $CD34^+$ cell enrichments provided higher values of the HSC phenotype $CD34^+CD90^+$ [Danet et al., 2001] in early stages of the co-culture (Fig. 1C; n = 4; $P < 0.05$); the expression of this phenotype in this condition consistently decreased throughout time in culture, reaching 2.5 ± 0.6% at day 7. Furthermore, upon culture initiation, there was an increase of the expression of $CD34^+CD90^+$ phenotype for *Medium* and *Low* conditions. Moreover, while in culture, *Medium* and *Low* conditions reached the highest expression levels of $CD34^+CD90^+$ cells at day 3 (25 ± 5.0%) and 4 (12 ± 1%), respectively (Fig. 1C).

The clonogenic potential (CFU-MIX and CFU-GM) and the ability to form cobblestone areas in vitro (CAFCs) of the expanded hematopoietic cells was determined, for the different initial $CD34^+$ cell enrichments tested (Table I). At day 7, the FI in the number of CFU-MIX and CFU-GM progenitors, as well as CAFCs are in agreement with the levels of expansion of the $CD34^+$ cells, with higher values obtained by the lowest initial $CD34^+$ cell enrichments (80 ± 12 vs. 28 ± 4.4 for CAFCs ($P < 0.05$), 50 ± 9.5 vs. 15 ± 1.4 for CFU-MIX ($P < 0.05$) and 20 ± 3.3 vs. 11 ± 1.9 for CFU-GM, for *Low* and *High* conditions, respectively; n = 6). In addition, non-enriched populations (MNC) showed negligible expansion of CFU and CAFCs (1.1 ± 0.1, 3.2 ± 1.7, and 1.7 ± 1.0 for CAFCs, CFU-MIX and CFU-GM, respectively). At day 7, the differentiative potential of the expanded cells was evaluated, with similar expression patterns of the early lymphoid (CD7) and common myeloid (CD15, CD33, and CD14) markers [Andrade et al., 2010].

EFFECT OF INITIAL $CD34^+$ ENRICHMENT ON CELL DIVISIONAL HISTORY AND CELL CYCLE STATUS OF EXPANDED CELLS

In order to further depict the influence of initial $CD34^+$ cell enrichment in cell proliferation, we performed cell divisional history studies. Cells with *High*, *Medium*, and *Low* initial $CD34^+$ enrichments were labeled with PKH26, processed daily by flow cytometry and respective data were analyzed using the Proliferation Wizard of the ModFit Software, where the percentage of cells for each generation was determined (Fig. 2).

As shown in the previous section, *Medium* and *Low* cultures presented increases in the percentage of $CD34^+$ cells (Fig. 1C) upon culture initiation. This increase was clear even after 24 h ($P < 0.05$), when no significant cell division had occurred for any of the culture conditions tested (Fig. 2), suggesting that $CD34^-$ cells upmodulated CD34 expression prior to cell division. In addition, the primitive $CD34^+CD90^+$ phenotype was also increased upon this culture period. The cell divisional history revealed that proliferation started after day 1 for all culture conditions, since at day 2 all settings displayed a significant decrease in the percentage of undivided cells (Generation 0) compared to day 1 (Fig. 2). In addition, a significantly lower percentage of undivided cells was found at day 2 for *High* $CD34^+$ cell enrichment (30 ± 3.0% in Generation 0, compared to *Medium* (72 ± 2.0%; $P < 0.05$) and *Low* (62 ± 2.0%; $P < 0.05$)

TABLE I. Influence of Initial % CD34$^+$ Cells (*High*, *Medium*, *Low*, and *MNC*) on the Proliferation of CAFCs and the Clonogenic Progenitors Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte (CFU-MIX), and CFU-GM After 7 days in Culture (n = 6). For Both CFUs and CAFCs, Expansion Results Were Calculated as FI Relatively to day 0.

	CAFCs*	CFU-MIX*	CFU-GM
High	28 ± 4.4	15 ± 1.4	11 ± 1.9**
Medium	57 ± 8.3	30 ± 5.2	19 ± 4.2
Low	80 ± 12.1	50 ± 9.5	20 ± 3.3**
MNC	1.1 ± 0.1	3.2 ± 1.7	1.7 ± 1.0***

Results are expressed as mean ± SEM.
*$P < 0.05$ for all conditions.
**$P < 0.05$ between the highlighted conditions.
***$P < 0.05$ when compared to the remaining conditions.

conditions (Fig. 2)), with a consequent significant increase in the fractions of cells at Generation 1 and 2. However, from day 4 on, the cell division profile was similar regardless the initial CD34$^+$ enrichment tested ($P > 0.05$) and at day 7 of culture, hematopoietic cells were distributed mostly in Generations 5, 6, and 7, in agreement with our previously reported results [da Silva et al., 2009].

Cell cycle analyses were performed at different stages of culture to confirm the results previously obtained upon evaluation of the Ki67 antigen [Gothot et al., 1997]. Propidium iodide was used to distinguish between G1 and G2/S/M phases, and cells were analyzed by flow cytometry.

At day 0, a lower percentage of quiescent cells (G0) was detected for *High* conditions (72 ± 0.9%), compared to *Medium* (77 ± 2.8%) and *Low* (85 ± 1.1%) initial CD34$^+$ cell enrichments (Fig. 3). Upon culture initiation, cells readily responded to the culture stimuli, entering into cell cycle; as a result, the fraction of quiescent cells (G0) decreased significantly for all culture conditions and at day 2 they represented 10 ± 7.9%, 38 ± 2.7%, and 64 ± 3.2% of *High*, *Medium*, and *Low* cell populations, respectively. Concomitantly, from day 0 to day 2, the percentages of cells at G1 and G2/S/M phases increased significantly, for all conditions tested (Fig. 3; n = 4, $P < 0.05$). These results are in agreement with the cell division kinetics previously shown (Fig. 2). Of notice, more than 90% of MNC were at G0 throughout time in culture (data not shown).

In agreement with CD34$^+$ cell expression and cell division kinetics, later in culture (at day 7), the cell cycle status was similar for all conditions tested, with low numbers of cells remaining quiescent (1 ± 1%, 2 ± 1% and 1 ± 1% cells in G0, for *High*, *Medium*, and *Low* enrichments, respectively) and most of the cells in culture at G1 (Fig. 3; $P > 0.05$).

CD34$^+$ CELL ENRICHMENT: IMPACT ON CLINICAL-SCALE EXPANSION

In the previous sections, we presented the intrinsic phenomena associated to the culture dynamics under different initial CD34$^+$ cell

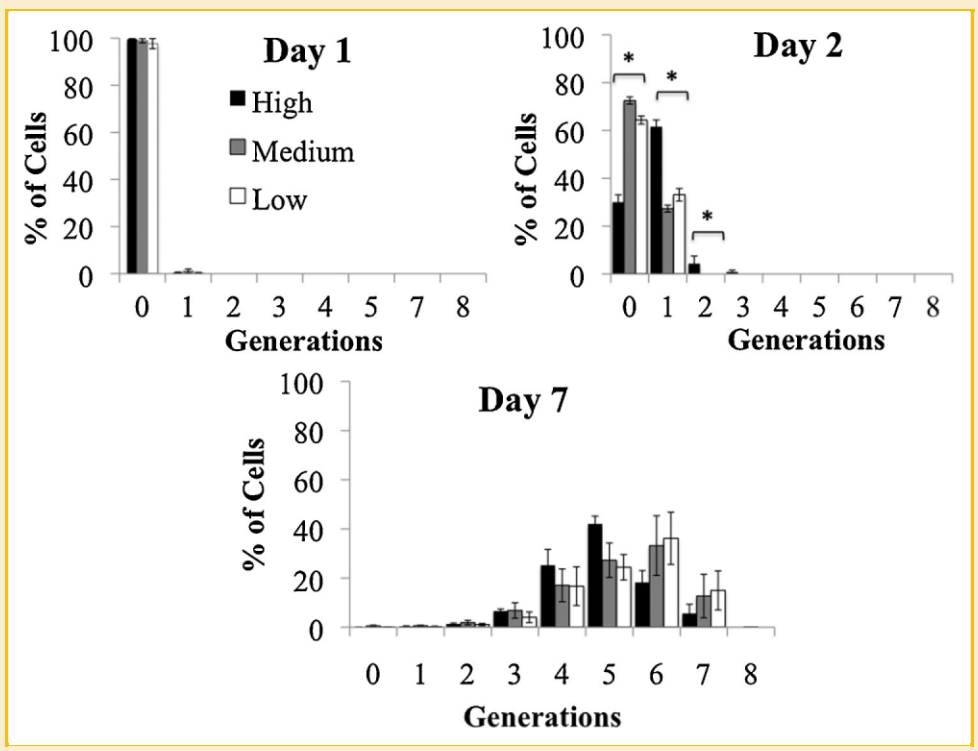

Fig. 2. Influence of initial CD34+ cell enrichment on cell divisional history of expanded cells. Cell divisional history of UCB HSC in co-culture with BM-MSC under different initial CD34$^+$ cell enrichment (High ≈ 100%-black bars; Medium ≈ 50%-dark grey; Low ≈ 25%-white). Data was acquired by flow cytometry and analyzed using the Proliferation Wizard of ModFit software. Each bar represents the percentage of cells in each doubling generation. The mean percentages are presented as mean ± SEM (n = 5; *$P < 0.05$).

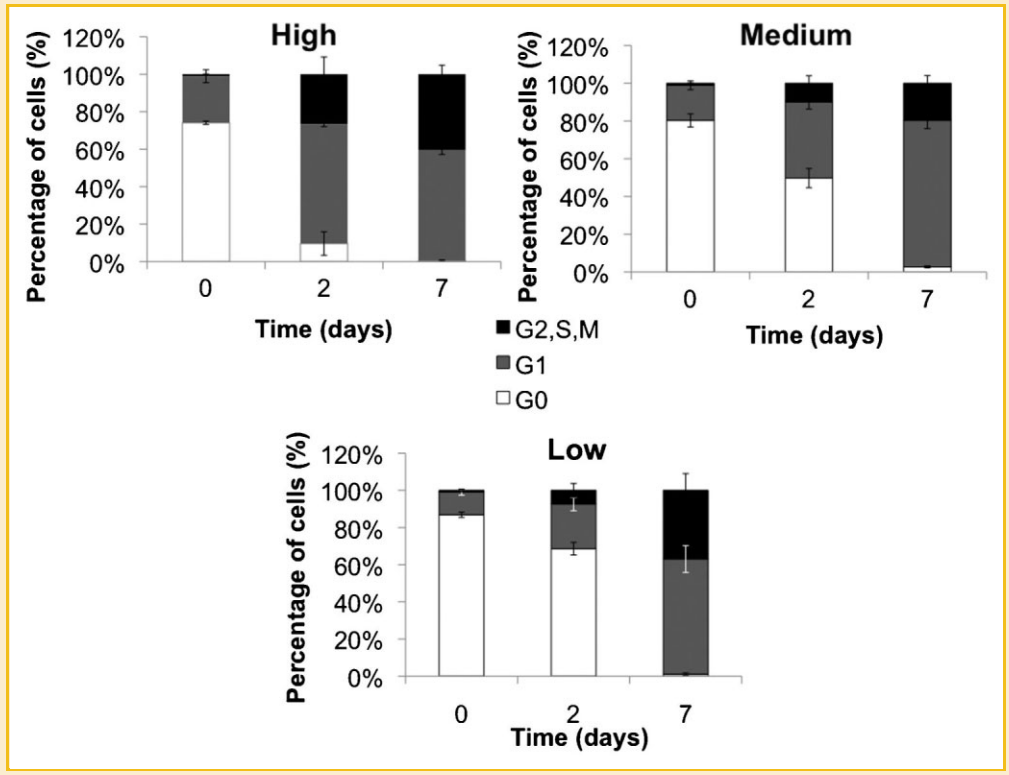

Fig. 3. Cell cycle status of CD34$^+$ enriched cells. Cells were periodically stained with anti-Ki67-FITC and Propidium iodide. Data was acquired by flow cytometry and the percentages of cells in G0-white, G1-dark grey and G2, S, M-black bars were determined for days 0, 2, and 7 of co-culture. The mean percentages are presented as mean ± SEM (n = 4).

enrichments. In this section, we anticipated the impact of those phenomena on the final CD34$^+$ cell output in a putative clinical scale expansion.

According to Materials and Methods Section, and considering the expansion of a 40% cohort of a typical UCB unit, cells can be CD34$^+$ enriched using clinical grade magnetic cell sorting (e.g., CliniMACS®), before cultivation with a specific cocktail of cytokines. We foresee three strategies (Table II):

A – The cell sample is enriched for CD34$^+$ expression using one immunomagnetic isolation cycle, leading to a starting population of 69 ± 5.2% CD34$^+$ cells with 78 ± 14% of cell recovery;

B – The same cell sample is enriched using two cycles of immunomagnetic sorting, leading to a starting population of 93 ± 1.0% CD34$^+$ cells, with a 52 ± 8.5% cell recovery;

C – All the UCB MNC in the cell sample are used, without any CD34$^+$ cell enrichment.

After 7 days of culture, the simulated final CD34$^+$ cell yield of situation A (1 immunomagnetic cycle) was $30 \pm 2.0 \times 10^6$ cells, which is twice the amount of cells needed for a putative 80 kg-patient transplant, whereas situation B (two CD34$^+$ cell enrichment cycles) originates $13 \pm 1.0 \times 10^6$ cells CD34$^+$ cells and C (MNC) does not provide CD34$^+$ cell expansion in our conditions ($1.0 \pm 0.5 \times 10^6$; Fig. 4). The number of CD34$^+$ cells throughout

TABLE II. Immunomagnetic Sorting of CD34$^+$ Cells From UCB. Summary of UCB Characteristics Including Percentage of CD34$^+$ Expression Before Sorting and CD34$^+$ Purity and Cell Recovery After One or Two Immunomagnetic Isolation Cycles, for a Total of 29 Thawed Samples (Data From Stem Cell Bioengineering Laboratory, Institute for Biotechnology and Bioengineering)

% CD34$^+$ cells in MNC fraction	% CD34$^+$ Purity after magnetic immuno-isolation		% Cell recovery after CD34$^+$ magnetic immuno-isolation	
	1st cycle	2nd cycle	1st cycle	2nd cycle
0.8 ± 0.2	69 ± 5.2	93 ± 1.0	77.5 ± 14.4	52 ± 8.5

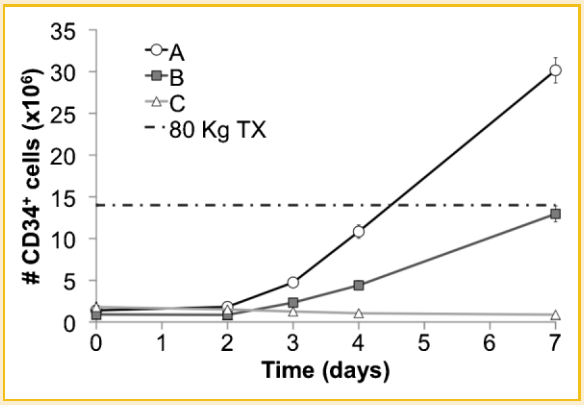

Fig. 4. Simulation of a clinical-scale expansion of UCB CD34+ cells under three different initial CD34+ cell enrichments: (A) using one immunomagnetic sorting cycle (n = 10) – black line; (B) using two cycles of immunomagnetic sorting, (n = 19) – dark grey line and (C) without any CD34+ cell enrichment (n = 7) – light grey line. The dashed line indicates the minimum CD34+ cell dose requirement for HSC transplantation of a 80 kg patient. Results are presented as mean ± SEM.

time in culture was simulated using the FI data for each condition obtained in the present studies (Fig. 1), under the same initial cell density (3×10^4 cells/ml), and is presented in Figure 4.

DISCUSSION

Most of the studies regarding hematopoietic stem/progenitor cell expansion and/or differentiation have focused on the culture of CD34-selected cells, and it had been previously stated that hematopoietic cells must be enriched before culture in order to be successfully expanded ex-vivo [Douay, 2001]. However, presently, it is still not clear whether protocols for enrichment of immature cells are mandatory prior to culture in order to achieve a higher degree of expansion. Furthermore, studies using diverse culture conditions such as cytokine combinations, blood sources, feeder cell layers, culture media and feeding protocols lead to different conclusions [Koller et al., 1995a; Douay, 2001; Balducci et al., 2003]. Indeed, it can be argued that the approach of cell enrichment is inconsistent with in vivo hematopoiesis, in which the more primitive cells proliferate in close association with supportive stromal cells including $CD34^-$ cells of hematopoietic origin, such as macrophages, known to produce cytokines [Mazini et al., 1998]. However, the question regarding the proper initial stem/progenitor cell enrichment remains highly pertinent [Hai-Jiang et al., 2008], with crucial implications as to HSC yield, quality of the expanded graft and the total cost of the expansion process.

In this work, we evaluated the influence of different initial $CD34^+$ cell enrichment on the culture dynamics namely cell divisional history, cell cycle status and clonogenic potential, and ultimately on HSC yield.

In order to eliminate the donor-to-donor variability, each of the UCB unit studied was processed to test the different $CD34^+$ enrichments (*High*-90%, *Medium*-50%, and *Low*-25%). Interestingly, *Low* and *Medium* enriched populations readily upmodulated their expression of the CD34 antigen upon day 1, reaching $CD34^+$ expression levels similar to the *High* condition by day 4. Cell divisional history and cell cycle studies showed that for all $CD34^+$ enrichments studied, proliferation started only after 24 h, between days 1–2 (in agreement with [da Silva et al., 2009]), with significantly lower percentages of undivided cells for *High* $CD34^+$ cell enrichment at day 2. Thus, CD34 activation in primitive $CD34^-$ cells must have occurred prior to cell division in *Medium* and *Low* conditions. CD34 modulation in hematopoietic cultures has been described by us [da Silva et al., 2009] and others [Hess et al., 2003; Dooley et al., 2004] for UCB, BM, and PB. However, this work constitutes, to our best knowledge, the first report where this phenomenon is precisely identified in time, correlated with the proliferative status of the culture, and intrinsically occurring within the same population of UCB cells, for high, moderate or low levels of initial $CD34^+$ cell enrichment. Within the same biological sample, UCB cells cultivated at moderate (*Medium* and *Low*) initial $CD34^+$ cell enrichments readily modulated CD34 expression ex-vivo; the modulation of CD34 expression observed preceded an intense cellular proliferation particularly evident upon days 4 and 7. Interestingly, the levels of expansion of $CD34^+$ cells are higher in less $CD34^+$-enriched cultures, suggesting that a potential exhaustion of the proliferative potential in highly enriched cultures might have occurred earlier in culture. We hypothesized that along with the cytokine cocktail, BM MSC-derived feeder layer might play an important role on the intricate regulation of this process, as previously described by Bhatia and colleagues, where BM cells in $CD34^-Lin^-$, or $CD34^+Lin^-$ fraction can become SCID repopulating cells only when accessory and/or feeder cells are present [Bhatia et al., 1998].

Of notice, non-enriched populations (MNC) resulted in non-significant cell proliferation, which hindered further analysis, suggesting that the initial amount of $CD34^+$ cells in culture was insufficient for the CD34 modulation phenomenon to occur and subsequent proliferation, in our culture conditions. In addition, a high concentration of mature cells in non-selected cultures may have a strong suppressive effect of hematopoietic cell growth [Douay, 2001].

Importantly, we have also tracked a primitive hematopoietic subset, the $CD34^+CD90^+$ cells for all conditions tested, since this phenotype has been described as the most reliable predictor for SCID repopulating potential [Danet et al., 2001]. During time in culture, the expression of this phenotype decreased in culture for *High* condition. However, for *Medium* and *Low* conditions, an increase of $CD34^+CD90^+$ expression was observed after 24 h, in the absence of cell division. Cells at *Medium* and *Low* conditions attained their maximum $CD34^+CD90^+$ expression at days 3 and 4, respectively. Despite the decrease of $CD34^+CD90^+$ expression occurring later in culture for these two culture conditions, and also for *High* cultures (readily upon cultivation), there was an efficient expansion of this primitive cell-subset in terms of cell numbers generated. Concomitantly with $CD34^+$ and $CD34^+CD90^+$ modulation in culture, expansion of CAFCs and CFUs after 7 days in culture was higher in lower initial cell enrichments, due to similar frequencies by the end of the culture, despite their initial lower number. Therefore, our

results indicate that, within the range of different initial CD34 enrichments studied for UCB cells, the highest initial $CD34^+$ cell content might not be advantageous to produce a higher expansion in terms of total cells or more primitive stem/progenitor cells, in agreement with other published results [Koller et al., 1995a]. In fact, high levels of enrichment may be associated to important cell losses which may also account for primitive $CD34^-$ cells with SCID repopulating activity [Bhatia et al., 1998] or some HSC that may not express enough CD34 on their surface to be selected [Chou et al., 2010].

The impact of the findings presented herein has to be considered not only in terms of the intrinsic culture phenomena they present, but also on the overall cell yield after ex-vivo expansion towards the generation of a clinical significant hematopoietic stem/progenitor cell graft [Kirouac and Zandstra, 2008]. In fact, although $CD34^+$ selection prior expansion is of major importance (e.g., to obtain lower processing volumes or facilitate eventual gene therapy approaches, as well as tumor cell purging), significant stem/progenitor cell losses after immunomagnetic sorting have been reported [Firat et al., 1998].

Strategies combining ex-vivo expanded and unmanipulated UCB are being explored to optimize the initial engraftment kinetics, as well as the long-term durability upon HSC transplantation. A fraction of the UCB unit to be used (typically 40–60%) may be expanded ex-vivo prior to transplantation [Shpall et al., 2002; Kelly et al., 2009] while the other is infused without manipulation.

Since the number of HSC in the graft is the essential parameter in the clinical setting, any isolation/purification process that results in extensive HSC loss should clearly be avoided in order to maximize the number of primitive cells transplanted into the patient [Chou et al., 2010]. Due to the limited data available in the literature on cell losses associated to cryopreservation, concentration of the nucleated cell component and magnetic sorting, we used the collected data from our laboratory to study the influence of the number of immunomagnetic sorting cycles, the $CD34^+$ purity and cell recovery after each cycle on the stem/progenitor cell expansion. There may also exist potential cell losses due to stress and eventual cell death during the various processing steps [Chou et al., 2010]. In this case, we used the traditional protocol of a Ficoll density gradient to isolate UCB MNC followed by cryopreservation prior to immunomagnetic cell sorting. It should be noticed that presently there are fully automated and integrated systems for the concentration of the MNC component (e.g., Sepax® system) with better performances in terms of MNC and $CD34^+$ cell recoveries, which are not commonly available in most laboratories on stem cell research.

Here, we decided to perform a side-by-side comparison of three different expansion strategies in terms of cell yield towards the generation of a clinical significant hematopoietic stem/progenitor cell expanded graft. The higher cell recovery upon one immunomagnetic sorting cycle (corresponding to *Medium* initial $CD34^+$ cell enrichment) leads to higher $CD34^+$ cell numbers after 7 days of expansion, compared to the extremely purified initial CD34+ cell population (2 cycles of immunoisolation), representing twice the minimum recommended UCB freshly isolated $CD34^+$ cell dose for transplantation of an average 80 kg patient, 1.75×10^5 $CD34^+$ cells/kg [Wagner et al., 2002]. Cell loss in magnetic sorting may be in part to failure to capture the target cells, given that positive selection always leads to cell loss due to inefficient binding to the beads. We assumed that the values of $CD34^+$ cell purity and recovery obtained after one magnetic sorting cycle are comparable to the ones achieved using clinical-grade magnetic sorting [Shpall et al., 2002]. The third strategy explored – expansion of non-enriched MNC- was clearly inefficient.

Of notice, we used as reference the minimum recommended dose of freshly isolated UCB $CD34^+$ cells [Wagner et al., 2002] for transplantation of a 80 kg patient, which refers to non-expanded cells, due to the lack of data on cell doses for expanded cells to be infused. Importantly, the expansion results presented in our study (TNC: $>$13-fold; $CD34^+$: $>$ 14-fold; $CD34^+CD90^+$: $>$3-fold) are superior to the ones reported in the literature for clinical trials using ex-vivo expanded UCB HSC for transplantation [Kelly et al., 2009], and achieved in a shorter period (7 days) without culture manipulation (e.g., feeding).

The results presented herein suggest the existence of highly dynamic culture events regarding CD34 modulation in HSC-MSC co-cultures affecting cell cycle, proliferation status and clonogenic potential throughout time in culture. In particular, our studies indicate that a highly enriched $CD34^+$ starting population is not mandatory in order to successfully expand hematopoietic stem/progenitor cells, since cell populations with moderate levels of initial $CD34^+$ enrichment readily increase their CD34 expression ex-vivo, presenting a high stem/progenitor cell yield.

Overall, our findings highlight the need to establish a balance between the expansion potential of the selected cell subsets, the number of cells lost during purification and culture volumes to be processed towards the efficient and cost-effective expansion of HSC for cellular therapy.

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