Interleukin-6 precludes the differentiation induced by interleukin-3 on expansion of CD34+ cells from cord blood

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Background and Objectives. *Ex vivo* expansion of hematopoietic progenitor cells (HPC) from umbilical cord blood (UCB) is an interesting strategy to obtain a sufficient number of transplantable cells for adults. To define the optimal culture conditions allowing the generation of HPC that retain their proliferative capacity without loss of long-term culture-initiating cells (LTC-IC), the effect of different cytokine combinations on the expansion of CD34+ cells from UCB was assessed.

Design and Methods. CD34+ cells were cultured in serum-free culture medium with four cytokine combinations: stem cell factor plus thrombopoietin plus flk2/flt3 ligand (STF), STF plus interleukin-3 (IL-3), STF plus interleukin-6 (IL-6) and STF plus IL-6 plus IL-3. After a 1-week culture, the number of CD34+ and CD133+ cells, colony forming units (CFU), LTC-IC and telomerase activity were determined.

Results. The addition of IL-6 or IL-3 to the combination of STF significantly enhanced the expansion of CD34+, CD133+ cells and CFU. All cytokine combinations tested induced a slight increase in LTC-IC number except that composed by STF plus IL-3. The greatest induction of telomerase activity was observed with the combination of STF plus IL-3 or plus IL-3 plus IL-6. Decay of the activity along time was observed when the combination of STF plus IL-3 was used, and this effect was reverted by the addition of IL-6.

Interpretation and Conclusions. Our results demonstrate that the inclusion of IL-6 in a serum-free short-term culture has a beneficial effect on HPC expansion from UCB, and precludes the negative effects induced by IL-3 on LTC-IC expansion and telomerase activity.

Key words: cord blood, CD34+ and CD133+ expansion, CFU, LTC-IC, telomerase activity.

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Umbilical cord blood (UCB) has become an alternative source of hematopoietic progenitor cells (HPC) for transplantation. However, the small number of HPC in a single cord blood unit limits their use in adults. The *ex vivo* expansion of HPC could be a useful procedure to achieve a sufficient number of cells for transplantation in adults or *ex vivo* genetic manipulation. Antibodies to CD34 are commonly being used as a marker to select HPC, but this antigen is expressed on a phenotypically and functionally heterogeneous cell fraction that contains early and more committed hematopoietic progenitors. Recently a novel antigen, CD133 (formerly named AC133), present in early HPC has been described.[1] Moreover, it has been reported that the $CD34^{bright}CD133^{+}$ cell subset contains early high proliferating stem/progenitor cells and early committed progenitors[2] and long-term culture-initiating cells (LTC-IC) and NOD/SCID repopulating cells.[3] These findings suggest that CD133+ cell selection for *ex vivo* expansion of progenitor cells could be a better option than selection based on the most widely used CD34 expression.

Many investigators have used various combinations of cytokines that act on primitive hematopoietic cells in order to optimize culture conditions for HPC expansion. In particular, stem cell factor (SCF) and flk2/flt3 ligand (FL) are essential for the maintenance and expansion of HPC.[4-6] In addition to these cytokines, thrombopoietin (TPO), a regulator factor for megakaryopoiesis, has also been shown to stimulate HPC expansion.[7,8] Moreover, these three factors together recruit the more quiescent HPC into cell cycling.[9] On the other hand, interleukin-6 (IL-6) synergizes with c-kit and flk2/flt3 signal to expand HPC.[10-12] In contrast, the role of interleukin-3 (IL-3) is controversial. Data on increased, maintained as well as loss of stem cell potential,[13-16] determined as LTC-IC or engraftment ability have been reported, in both murine and human systems.

It has been reported that primitive HPC have a low level of telomerase activity.[17] Telomerase is essential, but not sufficient to maintain a stable telomere length, implying that telomeres will shorten with each cell replication.[18] However, it has been shown that telomerase expression is associated with self-renewal potential[19] and is upregulated when cells enter the cell cycle,[20] suggesting that telomerase expression must be correlated with the cells' proliferative capacity as well as the stage of differentiation.

In this study, we investigated the effect of adding IL-6 and/or IL-3 to the growth factor combination composed of SCF, TPO and FL on the expansion of HPC from UCB in serum and stroma-free cultures. Their progenitor cell characteristics were compared using clonogenic cell and LTC-IC assays. Moreover, telomerase activity was measured using a very sensitive polymerase chain reaction (PCR)-based telomeric repeat amplification protocol and compared with the cell proliferation and CD34+ cell expansion.

Design and Methods

Isolation of cells

After informed consent from the mother, UCB samples were collected from normal full-term deliveries, by standard procedures. Erythrocyte depletion was achieved by centrifugation in the presence of HES. Mononuclear cells (MNC) were obtained by Ficoll-Hypaque (Amersham Pharmacia, Upsala, Sweden) density gradient centrifugation. CD34+ cells were isolated by immunomagnetic cell separation, using two columns (MACS system, Miltenyi Biotec, Bergisch Gladbach, Germany), in accordance with the manufacturer's recommendations.

Expansion cell culture

Isolated CD34+ cells were cultured in 24-well plates (4×10^4/mL) in serum-free medium composed of X-VIVO 15 (BioWhittaker, Walkersville, MD, USA) supplemented with 1% bovine serum albumin, 200 µg/mL transferrin, 40 µg/mL low density lipoproteins (Sigma, St Louis, MO, USA), 10 µg/mL insulin, 0.1 mM 2-mercaptoethanol, 50 µg/mL gentamicin and 2.5 µg/mL fungizone (GibcoBRL, Life Technologies, Paisley, UK). CD34+ cells from each UCB unit were incubated in the presence of four different cytokine combinations: SCF plus TPO plus FL (STF), STF plus IL-6 (STF6), STF plus IL-3 (STF3), and STF plus IL-6 plus IL-3 (STF63). Human recombinant cytokines (R&D Systems Inc, Minneapolis, MN, USA) were used at final concentrations of 50 ng/mL (SCF and FL), 10 ng/mL (TPO) and 20 ng/mL (IL-6 and IL-3). The cultures were incubated at 37°C in 5% CO_2 and 95% air in a fully humidified atmosphere for seven days and cytokines replaced every 3 days.

Flow cytometry

Flow cytometry analysis was performed on a Facs-Calibur equipped with two lasers (Becton Dickinson, San José, CA, USA) using the Cell Quest Software (Becton Dickinson). The cells were labeled according to standard protocols. The monoclonal antibodies used were anti-CD45-fluorescein isothiocyanate (FITC), anti-CD34-allophycocyanin (APC) (Becton-Dickinson Immunocytometry Systems, Heidelberg, Germany [BDIS]) and anti-CD133/2-phycoerythrin (PE) (Miltenyi Biotec). 7-amino-actinomycin D (Sigma) was used for discarding non-viable cells in the analysis. Matched labeled isotypes were used as controls.

Colony-forming unit (CFU) assay

CFU were evaluated in isolated and cultured CD34+ cells by clonogenic assay using the methylcellulose-based medium Methocult™ GF H4434 (Stem Cell Technologies, Vancouver, Canada), containing SCF, granulocyte/monocyte colony-stimulating factor, IL-3 and erythropoietin. The density of the culture was 1000 cells per 3 mL for the STF and STF6 combinations and 2000 cells per 3 mL for the STF3 and STF63 cocktails. The cultures were made in duplicate in 35-mm Petri dishes and incubated at 37°C in 5% CO_2 and 95% air in a fully humidified atmosphere. On day 14, colonies were scored using an inverted microscope for myeloid (CFU-GM), erythroid (BFU-E) and mixed colonies (CFU-GEMM) defined by morphologic criteria.

Long-term culture-initiating cell (LTC-IC) assay

Isolated and culture-generated CD34+ cells were plated in limiting dilutions for determination of LTC-IC frequency, using as stroma the cell line AFT024, a generous gift from Dr. F. Prosper, with the consent of Dr I. Lemischka, (Princeton University, Princeton, NJ, USA).[21] Ten dilutions (from 2 to 500 for isolated CD34+ cells and from 25 to 2500 for culture-generated CD34+ cells) and six replicates per dilution were seeded for each sample on pre-irradiated AFT024-coated 96-well plates. The medium used was Iscove's modified Dulbecco's medium (IMDM) supplemented with 12.5% FCS, 12.5% horse serum, 1% L-glutamine, 2.5 µg/mL fungizone, 50 µg/mL gentamicin, 50 µM 2-mercaptoethanol (GibcoBRL) and 1 µM hydrocortisone (SIGMA). Cultures were maintained at 37°C in 5% CO_2 and 95% air in a fully humidified atmosphere with a half-medium change weekly. After 5 weeks, the cultures were scored under an inverted microscope considering positive for LTC-IC those wells that had at least one cobblestone area-forming cell. The frequency of LTC-IC was calculated by a Poisson statistic as described elsewhere.[22]

To determine the number of CFU per LTC-IC, cells present in some positive wells were harvested and replated for clonogenic assay, as described above.

Photometric enzyme immunoassay-based telomeric repeat amplification protocol (TRAP)

Telomerase activity was assessed using the *T*elo TAGGG telomerase PCR ELISA^PLUS kit, according to the manufacturer's instructions (Roche Molecular Biochemicals, Germany). The test principle of this

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protocol is a double-step procedure. In a first step, telomerase adds repeats (TTAGGG) to the 3'-end of the biotin-labeled synthetic P1-TS primer. The elongation products as well as the internal standard (IS) included in the same reaction cocktail are amplified by PCR using the primers P1-TS and the anchor primer P2. The IS generates a 216 bp PCR product. In the second step, the PCR products are divided into two aliquots, denatured and hybridized separately to digoxigenin-(DIG)-labeled detection probes, specific for the telomeric repeats and for IS. The resulting products are detected by ELISA. Quantification of telomerase activity was carried out using 1-3 μL of cell extract, corresponding to 25-100 cell equivalents. The relative telomerase activity (RTA) in the samples was determined by comparing the signal from the sample to the signal obtained using a control template provided in the kit, according to the kit's protocol.

Statistical analysis

Results are expressed as the mean $\pm$ standard error. The significance of differences between mean values was determined using the Student's t-test for paired samples. The statistical analysis was performed with Excel Software, Microsoft, USA.

Results

Expansion of total, CD34$^+$ and CD133$^+$ cells

Immunomagnetic isolated cells showed a purity in CD34$^+$ cells of 94.89$\pm$2.86%, in CD133$^+$ cells of 92.31$\pm$4.37%, and 94.18$\pm$5.01% of CD34$^+$ cell population co-expressed the antigen CD133. As shown in Table 1, the total cell number was 9-fold higher in the presence of SCF, TPO and FL (STF). Addition of IL-6 or IL-3 to the combination of STF significantly stimulated cell proliferation, the highest proliferative effect being observed in the presence of IL-3.

When the combinations of STF and STF plus IL-6 (STF6) were used, about 36% of expanded cells were CD34$^+$. However, the addition of IL-3 to STF (STF3) or IL-6 plus IL-3 to STF (STF63) reduced the percentage of CD34$^+$ cells by one half (Table 1). Thus, addition of IL-6 to the STF cocktail enhanced the expansion of CD34$^+$ cells by about 143%, similarly to that observed for nucleated cells. No significant differences were observed with the rest of the cytokine combinations assayed.

A similar pattern was observed when the CD133$^+$ cells generated in culture were analyzed, although the absolute numbers of cells obtained were lower than those of CD34$^+$ cells, according to published data.[23] As shown in Table 2, the percentage of CD133$^+$ cells was about double in those cultures carried out in the absence of IL-3 than in its presence. However, in this case, the addition of IL-6 to the combination of STF3 induced a slight but significant increase ($p < 0.001$) in the percentage of positive cells. Absolute expansion of CD133$^+$ cells with the STF cocktail was 2.38 fold and was further significantly increased by the addition of IL-6, IL-3 or both together, the increase being more notable with the combination of STF63 ($p < 0.013$ and 0.034 vs. STF6 and STF3, respectively).

The analysis of the co-expression of antigens CD34 and CD133 showed a 1.62 fold-expansion for this cell subset with the combination of STF (Table 2), which was significantly increased with the other cytokine combinations, especially STF6 and STF63 ($p < 0.048$ and 0.003 vs. STF3, respectively). As can be observed in Figure 1, cultured cells exhibited a gradual expression of CD34 antigen, not so clearly observed for CD133. Moreover, most CD133$^+$ cells present in the culture had a high expression of CD34 antigen.

Table 1. Fold expansion of total and CD34$^+$ cells and CD34$^+$ cell percentage after 7 days of *ex vivo* expansion of UCB-isolated CD34$^+$ cells in the presence of 4 cytokine combinations. Values are the mean ± standard errors of 11 to 16 independent experiments. Each experiment corresponding to a different UCB sample was conducted in duplicate.

Cytokine Combination	*Total cell expansion*	*CD34$^+$ cell expansion*	*CD34$^+$ cell (%)*
STF	9.17±2.65	35.63±9.97	4.13±1.20
STF6	13.58±4.37	36.78±10.92	5.89±1.98
STF3	29.67±5.04	16.40±7.98	6.05±2.64
STF63	30.52±4.93	18.25±8.56	6.56±2.17

S: SCF; T: TPO; F: FL; 6: IL-6 and 3: IL-3.

Table 2. Fold expansion and percentage of CD133$^+$ cells and CD34$^+$CD133$^+$ cells, after 7 days of *ex vivo* expansion of UCB-isolated CD34$^+$ cells in the presence of 4 cytokine combinations. Values are the mean ± standard errors of 11 to 13 experiments. Each experiment corresponding to a different UCB sample was conducted in duplicate.

Cytokine Combination	*CD133$^+$ cells (%)*	*CD133$^+$ cell expansion*	*CD34$^+$ CD133$^+$ cells (%)*	*CD34$^+$ CD133$^+$ cell expansion*
STF	22.69±5.90	2.38±0.82	14.50±5.76	1.62±0.46
STF6	22.83±5.99	3.59±1.34	15.79±5.41	2.78±0.89
STF3	11.34±3.35	3.60±1.26	6.77±2.33	2.33±0.73
STF63	13.04±2.84	4.11±1.15	8.43±2.25	2.94±0.73

S: SCF; T: TPO; F: FL; 6: IL-6 and 3: IL-3.

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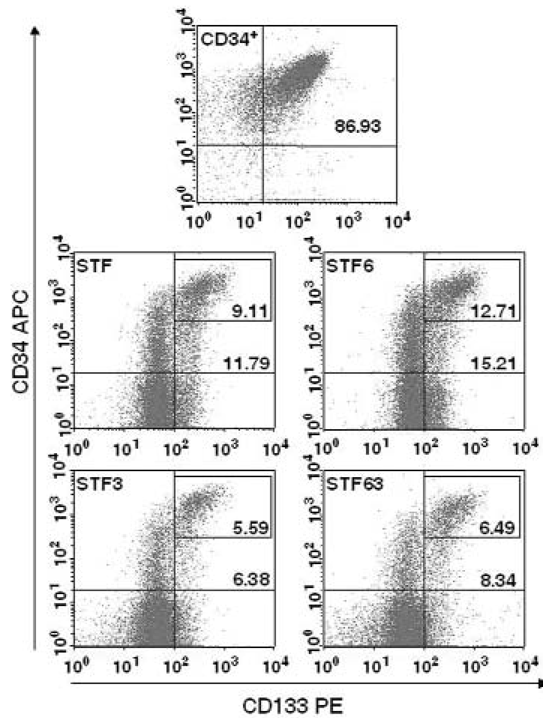

Figure 1. An example of FACS analysis of CD34+CD133+ cells in fresh CD34+ cells (upper figure) and after 7 days of *ex vivo* expansion of UCB-isolated CD34+ cells in the presence of 4 cytokine combinations as indicated. Cultured cells had higher autofluorescence for PE, thus markers according to the matched isotypes should be displaced to the right. Numbers correspond to percentages of CD34+CD133+ cells (upper right quadrant) and CD34brightCD133+ (drawn region) of viable cells. S: SCF; T: TPO; F: FL; 6: IL-6 and 3: IL-3.

Colony-forming units

Expansion of committed HPC, determined as CFU, is presented in Table 3. As shown, CFU number was doubled by the combination of STF, and significantly enhanced when IL-6 was added ($p < 0.015$). Further increases were obtained with the STF3 and STF63 cocktails ($p < 0.018$ and 0.041 vs. STF6, respectively). The cloning efficiency of CD34+ cells generated after 7 days of culture with cytokine combinations containing IL-3 was significantly higher than that observed in the presence of the STF and STF6 cocktails and similar to that presented by freshly isolated CD34+ cells (Table 3). When CFU were expressed per 100 CD133+ cells, a higher cloning efficiency, with respect to that of the freshly isolated CD34+ cells, was observed in cultured cells with all the combinations. The only significant difference was observed between the STF3 and STF63 groups ($p < 0.001$). Analysis of the different subtypes of CFU (Table 3) showed that, independently of the cytokine combination employed, expanded cells generated a higher number of BFU-E and a lower number of CFU-GM than isolated CD34+ cells ($p < 0.05$ in all cases). In the presence of STF and STF6 cocktails, CD34+ cells produced more CFU-GEMM than initial CD34+ cells and even more than with the other two combinations, although differences were not statistically significant.

Long-term culture-initiating cells

As shown in Table 4 the number of LTC-IC present in freshly isolated CD34+ cells was similar when expressed per CD34+ or CD133+ cells. After 7 days of expansion culture, the LTC-IC frequency decreased with all the cytokine combinations tested, although it was higher in the CD133+ cell subset than in the CD34+ cell one in the expanded cells. The highest frequency in both cellular subsets was obtained in

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Table 3. Fold expansion of CFU, cloning capacity of CD34+ and CD133+ cells, and CFU type generated after 7 days of *ex vivo* expansion of UCB-isolated CD34+ cells in the presence of 4 cytokine combinations. Values are the mean ± standard errors of 6 to 11 experiments. Each experiment corresponding to a different UCB sample was conducted in duplicate.

Cytokine combination	*CFU expansion*	*CFU/100 CD34+ cells*	*CFU/100 CD133+ cells*	*BFU-E/100 CFU*	*CFU-GM/100 CFU*	*CFU-GEMM/100 CFU*
Initial CD34+ cells		41.61±10.98	41.31±12.63	38.13±6.82	57.87±6.53	4.00±1.74
STF	1.98±0.69	28.52±5.75	69.58±28.16	51.32±9.85	42.66±8.82	6.03±1.53
STF6	3.48±1.31	28.37±11.86	68.85±34.34	54.74±10.57	39.76±10.66	5.50±2.54
STF3	5.46±1.04	48.86±14.05	80.41±19.85	53.43±10.73	41.85±10.52	4.72±1.66
STF63	5.36±1.95	43.07±14.57	59.98±21.22	50.52±12.37	45.54±11.29	3.94±2.27

S: SCF; T: TPO; F: FL; 6: IL-6 and 3: IL-3.

Table 4. Frequency, fold expansion, and cloning capacity of LTC-IC obtained after 7 days of *ex vivo* expansion of UCB-isolated CD34⁺ cells in the presence of 4 cytokine combinations. The frequency of LTC-IC is expressed as number of LTC-IC, determined by limiting dilution assays, per 100 CD34⁺ cells and per 100 CD133⁺ cells. Values are the mean ± standard errors of 5 experiments. Each experiment corresponding to a different UCB sample was conducted in duplicate.

Cytokine combination	*LTC-IC frequency in CD34⁺ cells*	*LTC-IC frequency in CD133⁺ cells*	*LTC-IC expansion*	*CFU/ LTC-IC*
Initial CD34⁺ cells	2.16±1.19	2.06±1.24	–	14.58±2.86
STF	0.67±0.54	1.35±1.14	1.20±0.87	11.79±3.41
STF6	0.41±0.17	1.08±0.73	1.25±0.52	23.68±4.91
STF3	0.38±0.22	1.07±0.58	0.90±0.48	14.20±6.08
STF63	0.43±0.10	1.14±0.74	1.55±0.93	23.15±4.06

S: SCF; T: TPO; F: FL 6: IL-6 and 3: IL-3.

the presence of STF. A slight LTC-IC expansion was observed with the STF, STF6 and STF63 combinations, although without statistically significant differences. The STF3 combination failed to increase the number of LTC-IC, but did maintain it. Nevertheless the addition of IL-6 to the STF3 combination reverted the effect induced by IL-3 and induced the highest expansion in LTC-IC.

The proliferative capacity of LTC-IC was determined by measuring the number of CFU in clonogenic assays (Table 4). After culture expansion, a higher capacity was obtained for LTC-IC generated with combinations of STF6 and STF63, whilst STF and STF3 combinations maintained the LTC-IC capacity.

Telomerase activity

We measured the telomerase activity of CD34⁺ isolated cells and expanded cells after 3 and 7 days of culture. This activity was expressed as relative telomerase activity (RTA), as described in the kit protocol, per CD34⁺ cell. As shown in Figure 2, after 3 days of culture, telomerase activity of cord blood-derived CD34⁺ cells was increased 8-, 11-, 27-, and 35-fold with STF, STF6, STF3, and STF63 combinations, respectively. After 7 days of culture, telomerase activity of the cells treated with the STF and STF6 combinations was further increased and reached a similar level. However, a slight decline in activity was observed in the cells treated with the STF3 combination: this decline was reversed and even overcome when IL-6 was also present.

As telomerase activity can be considered a parameter that reflects the replicative potential of cells, a correlation between values of the enzyme activity along the culture time and fold expansion of total and CD34⁺ cells was studied. As can be observed in Figure 3A, an increase in cell proliferation paralleled telomerase activity, but a better correlation, witnessed by the r^2 value was obtained when the activity was plotted against CD34⁺ cell expansion: this correlation reached a $r^2 = 0.91$ with the STF6 combination (Figure 3B).

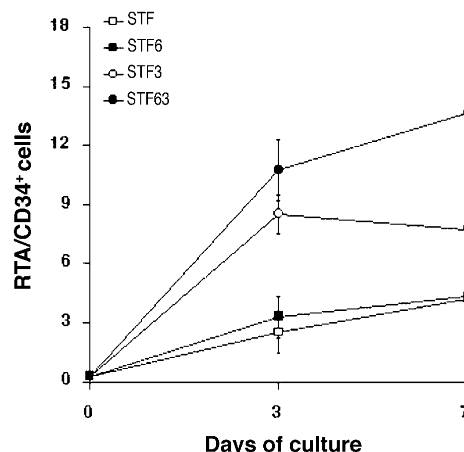

Figure 2. Telomerase activity (RTA/CD34⁺ cell) of cells obtained at days 0, 3 and 7 of expansion in the presence of the 4 cytokine combinations. Each point is the mean value of 3-5 independent experiments. Each experiment corresponding to a different UCB sample was conducted in triplicate. Vertical bars correspond to standard error. The RTA/CD34⁺ isolated cell was 0.29±0.06. S: SCF; T: TPO; F: FL; 6: IL-6 and 3: IL-3.

Discussion

The aim of this work was to evaluate the effect of IL-3 and IL-6 on *ex vivo* expansion of HPC from human UCB. It is accepted that early cytokines, SCF, TPO, and FL, are particularly effective in expanding primitive HPC, maintaining the viability of these cells in culture.[9,12,24] Nevertheless, in our laboratory the effect of these cytokines, alone or in combination, on CD34⁺ cell expansion was tested and a quite low total cell proliferation was found, together with a high mortality (*data not shown*). However, the combination of these three cytokines was considered the basal cocktail and was included in all cytokine combinations used.

Our results show that the addition of IL-3 or IL-6 to the basal cytokine combination has a positive effect on expansion of total cells, CD34⁺ cells and CFU. While a significant difference in fold expansion of CFU and total cells was observed in response to the addition of these two interleukins, the number of CD34⁺ cells generated in culture was similar. Consequently, CD34⁺ cells derived from cul-

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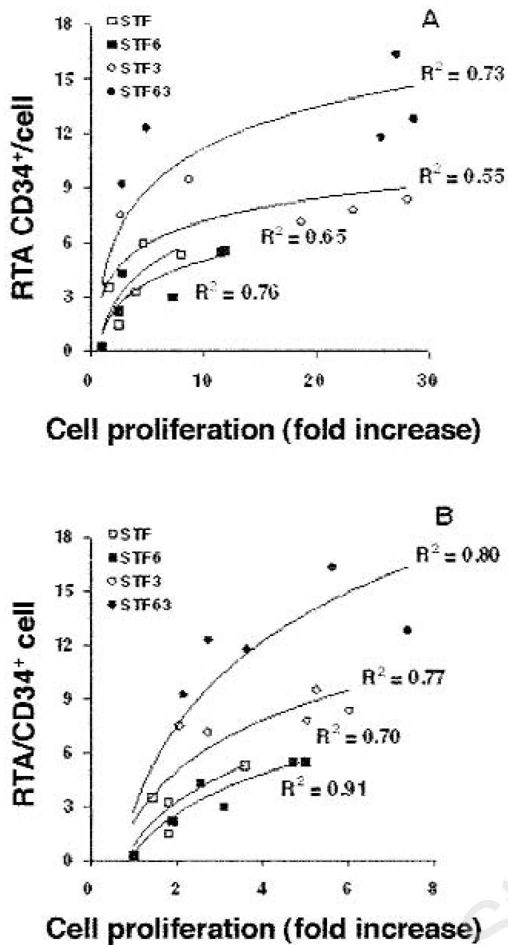

Figure 3. Correlation between (A) total cell proliferation and (B) $CD34^+$ cell proliferation and telomerase activity (RTA/$CD34^+$ cell) of generated cells in culture in the presence of the 4 cytokine combinations. Each point is the mean value of one sample tested in duplicate. n: 4–6 experiments. S: SCF; T: TPO; F: FL 6: IL-6 and 3: IL-3.

tures with the STF3 combination have a greater cloning efficiency than those generated in the presence of the STF6 cocktail. These results are consistent with other observations regarding the effect of differentiation induced by IL-3 on hematopoietic progenitors in culture[7] and corroborate the accelerated CFU development induced by IL-3.[15]

Long-term cultures allow determination of more immature progenitor cells *in vitro*. A slight increase in the number of LTC-IC after 7 days was found with the basal combination of cytokines and this was not increased by the addition of IL-6. The addition of IL-3 failed to promote LTC-IC expansion.

High concentrations of early-acting cytokines are required to induce division of more primitive cells[25] and to amplify the LTC-IC content,[26] so it is possible that the moderate increase in LTC-IC observed with STF could be due to the lower concentrations of cytokines employed. Moreover, the cultures were performed with $CD34^+$ cells without cell subset enrichment of more primitive hematopoietic progenitors, such as $CD34^+$c-kit^-,[27] $CD34^+$CD38-[28] or $CD34^+$$Thy^-$$1^+$.[9] Furthermore, different cytokines are required to expand LTC-IC, depending on the source of the $CD34^+CD38^-$ cells (bone marrow or UCB), so while IL-3 had a beneficial effect on bone marrow-derived cells, it was detrimental for UCB.[29] Our results support the negative effect of IL-3 on LTC-IC expansion.

It was observed that most $CD133^+$ cells generated in cultures showed the brightest intensity for CD34 expression, and that the addition of IL-6 to the STF3 cocktail induced a significant increase in $CD133^+$ and $CD34^+CD133^+$ cell numbers. These data, together with a higher frequency of LTC-IC in the $CD133^+$ cell subset than in the $CD34^+$ one within the expanded cells, explain the observed increase in LTC-IC number when cultures were supplemented with STF3 plus IL-6. Moreover, the findings support the idea that $CD34^+CD133^+$ cells are the most highly proliferative cells,[2] and that they are more enriched in clonogenic cells and in LTC-IC.[3]

IL-6 is a potent co-factor for the survival and proliferation of primitive progenitor cells[30] and acts on cells through a receptor system comprising IL-6 receptor (IL-6R, gp 80) and gp 130.[31] It was reported that progenitor expansion and level of engraftment in NOD/SCID mice were enhanced when a recombinant molecule of soluble IL-6 receptor fused to IL-6 was added to SCF and FL.[32] More recently, Ueda *et al.*[12] demonstrated that the addition of IL-6/sIL-6R to the combination of SCF, FL and TPO increased total number of cells, CFU, and also stimulated the expansion of HSC capable of repopulating in NOD/SCID mice, and that this effect was abrogated by IL-3. Here we demonstrated that the addition of IL-6 to the basal combination of cytokines had a positive effect on $CD34^+$ cell expansion, but failed to increase the number of LTC-IC, probably because of the lack of effect on the more primitive hematopoietic cells, $CD34^+$IL-$6R^-$.[33] However, in our study IL-6 precluded the inhibitory effect of IL-3 on LTC-IC expansion, and enhanced the proliferative capacity of LTC-IC, thus demonstrating and extending the beneficial effects of this interleukin.

The question remained whether the $CD34^+$ cells generated in culture retained capacity for self-renewal and/or proliferative potential. It was reported that telomerase activity was consistently low in $CD34^+CD38^-$ and $CD34^+$c-kit^- cells from cord

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blood cell subsets enriched in LTC-IC.[27] Recently Minamiguchi *et al*.[34] demonstrated that the $CD34^+IL\text{-}6R^-$ cell fraction, enriched in primitive hematopoietic cells as determined by LTC-IC and ELTC-IC, had a lower level of telomerase activity, a higher upregulation of telomerase activity and a higher replicative potential than its counterpart, $CD34^+IL\text{-}6R^+$. The telomerase activity over culture time was investigated. A good correlation between the level of this activity and cell proliferation was found, indicating that telomerase activity can be an indicator of proliferative capacity of $CD34^+$ cells in culture. Given that telomerase activity was essentially confined to $CD34^+$ cells, as we assessed by selecting $CD34^+$ and $CD34^-$ cells after expansion (*data not shown*), there was a better correlation between telomerase activity and $CD34^+$ cell expansion than with total cell proliferation.

Interestingly, after 7 days of culture with the STF3 and STF63 cytokines, the number of $CD34^+$ cells was similar but from the third day, telomerase activity decayed with the STF3 cocktail while it increased with the STF63 one, thus indicating that IL-6 was able to prevent the loss of proliferative potential of $CD34^+$ cells generated in the presence of IL-3.

In summary, IL-3 was beneficial in the expansion of total, $CD34^+$, $CD133^+$ cells and CFU, important for quicker neutrophil and platelet recovery after transplantation. However, IL-3 was detrimental to the increase of telomerase activity and to the number of LTC-IC, which are the more immature hematopoietic precursors. These detrimental effects of IL-3 could be reversed by addition of IL-6. These results lead us to suggest that SCF, FL, TPO, IL-6 and IL-3 was the best cytokine combination, of all those tested in this work, to expand cord blood $CD34^+$ cells for clinical use.

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Pre-Publication Report & Outcomes of Peer Review

Contributions

AE, EM and MDM performed cellular isolation and cell culture. AE and FC-U performed the flow cytometry analysis. EM and MDM performed PCR. AE, EM, FC-U and MDM analyzed and discussed the results. AE and MDM wrote the manuscript. The order of authorship was a joint decision agreed by all co-authors.

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Disclosures

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Manuscript processing

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In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

Expansion of hematopoietic cells may, overall, result in differentiation and loss of engraftment potential. In particular, this has been a common problem in studies on expansion of cord blood hematopoietic cells.

What this study adds

In vitro IL-6 prevented the loss of proliferative potential of expanded CD34-positive cells from cord blood

Caveats

There is no evidence that this approach may be useful in cord blood transplantation.

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