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Short-term, serum-free, static culture of cord blood-derived CD34+ cells: effects of FLT3-L and MIP-1α on *in vitro* expansion of hematopoietic progenitor cells

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ABSTRACT

Background and Objective. **The use of *ex vivo* expanded cells has been suggested as a possible means to accelerate the speed of engraftment in cord blood (CB) transplantation. The aim of this study was to fix the optimal condition for the generation of committed progenitors without affecting the stem cell compartment.**

Design and Methods. **Analysis of the effects of FLT3-L and MIP-1α when combined with SCF, IL-3 and IL-6, in short-term (6 days), serum-free expansion cultures of CB-selected CD34+ cells.**

Results. **An important expansion was obtained that ranged between 8-15 times for CFU-GM, 21-51 times for the BFU-E / CFU-Mix population and 11 to 30 times for CD34+ cells assessed by flow cytometry. From the combinations tested, those in which FLT3-L was present had a significant increase in the expansion of committed progenitors, while the presence of MIP-1α had a detrimental effect on the generation of more differentiated cells. However, stem cell candidates assessed by week 5 CAFC assay could be maintained in culture when both MIP-1α and FLT3-L were present (up to 91% recovery). This culture system was also able to expand megakaryocytic precursors as determined by the co-expression of CD34 and CD61 antigens (45-70 times), in spite of the use of cytokines non-specific for the megakaryocytic lineage.**

Interpretation and Conclusions. **The results obtained point to the combination of SCF, IL-3, IL-6, FLT3-L and MIP-1α as the best suited for a pre-clinical short-term serum-free static *ex vivo* expansion protocol of CB CD34+ cells, since it can generate large numbers of committed progenitor cells as well as maintaining week 5 CAFC.**

Key words: cord blood, CD34+ cells, FLT3-L, MIP-1α, expansion

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The transplantation of cord-blood (CB) derived hematopoietic progenitors has become an alternative to other hematopoietic progenitor sources (bone marrow and mobilized peripheral blood) in those cases in which no suitable donor is available.[1-5] In two reports that analyzed engraftment following CB transplantation, median time to neutrophil recovery and platelet transfusion independence was 23 and 75 days respectively.[3,4] This delay, particularly in platelet engraftment, has conditioned a high rate of transplant related mortality. In order to overcome this problem, the use of ex vivo expanded cells has been suggested as a possible means of accelerating the speed of engraftment.[6-8]

Previous reports have shown that it is possible to generate considerable numbers of committed hematopoietic progenitors *in vitro* using cytokine combinations that include early acting (SCF, IL-6) and proliferative cytokines (IL-3).[7,9-12] However, the *ex vivo* expansion of more immature progenitors in stroma-free cultures has yielded more controversial results.[7,8,13,14] It has recently been suggested that fetal liver tyrosine kinase ligand (FLT3-L) and macrophage inhibitory protein 1α (MIP-1α) play roles in the maintenance of stem cells in stroma-free cultures. FLT3 (also known as FLK2 and CD135) is expressed in 88% to 95% of CB-derived CD34+ cells,[15] and its ligand (FLT3-L) has been found to induce proliferation of CD34+ cells, in synergy with other cytokines such as IL-3, IL-6, G-CSF and GM-CSF.[7-9,16,17] MIP-1α is a chemokine that inhibits the proliferation of immature subsets of progenitors in clonogenic cultures, while it stimulates the proliferation of more mature progenitors such as CFU-E, CFU-G and CFU-M.[18,19] The use of MIP-1α in liquid cultures has shown that its effects are not only dependent on the phenotype of the cultured cells, but also on the cytokines present in the culture.[20] Taken together, the results obtained have led to the suggestion that MIP-1α might be involved in preventing the terminal differentiation of immature progenitors.[21]

In view of these initial reports and in order to define a clinically focused procedure, we sought to test the effects of FLT3-L and an MIP-1α analog (BB10010) on the short term *ex vivo* expansion of CB-derived

CD34+ cells. These cytokines were added to SCF, IL-3 and IL-6, which in our hands[22] have been able to expand CB-derived CD34+ cells efficiently in a serum-dependent medium. The first part of the report describes the results of the *ex vivo* expansion of CD34+ cells in the presence of a serum-free medium (StemPro 34; Life Technologies). In the second part of the study we compare several combinations of cytokines containing *early acting* cytokines, such as SCF and FLT3-L, and an inhibitory cytokine MIP-1α, and analyze the effect that the presence of these cytokines during short *ex vivo* expansion cultures has on immature and committed progenitors, including megakaryocytic progenitors, as assessed by flow cytometry.

Design and Methods

Progenitor cells

Cord blood samples (n=16) were obtained from term deliveries, informed consent having previously been obtained from the mothers. Briefly, immediately after delivery and while the placenta was still *in utero*, the umbilical cord was clamped and sectioned and the umbilical vein was punctured. Blood was allowed to drain by gravity into a blood collection bag containing CPD-A as anticoagulant, and was processed within 24 hours of collection.

The mononuclear cell fraction (MNC) present in CB was isolated by density gradient separation (Ficoll-Hypaque, density 1.077 g/mL; Pharmacia, Uppsala, Sweden). CD34+ cells were purified from the MNC by positive selection of CD34+ cells, using an immunomagnetic system for peripheral blood (Isolex-50, Baxter, Deerfield, IL, USA)[23] as previously described . The CD34-enriched cell fraction was washed and re-suspended in serum-free culture medium (StemPro-34, Life Technologies, Grand Island, NY, USA).

Cytokines

Recombinant human IL-3 and IL-6 were obtained from Sandoz (Basel, Switzerland); recombinant human SCF and FLT3-L were a kind gift from Amgen (Thousand Oaks, California, USA); the MIP-1α analog BB-10010 was a kind gift from British Biotech (Oxford, UK).

SCF, IL-3 and IL-6 were used at a concentration of 50 ng/mL each; FLT3-L was used at 100 ng/mL and the MIP-1α analog was used at 20 ng/mL. To supplement the cultures, 4 different cytokine combinations were used: to a basic combination containing SCF, IL-3 and IL-6 (s,3,6), either FLT3-L (s,3,6,f), MIP-1α (s,3,6,m) or both (s,3,6,f,m) were added.

Expansion cultures

A static, short term, stroma-free culture system was used to expand CD34+ cells. Fifty thousand cells of the enriched fraction were seeded in 1 mL of culture medium in 4-well dishes (Nunc, Roskilde, Denmark), and cultured at 37ºC and 5% CO_2 for 6 days. Cytokines were added to the culture on days 0 and 3. Two different culture media were used; a serum-dependent medium consisting of IMDM (Life Technologies) supplemented with 25% fetal calf serum (FCS; Life Technologies); and a serum free medium (StemPro-34, Life Technologies; SP34) supplemented with 2% human albumin (Instituto Grifols, Parets del Vallès, Spain). Nine initial experiments were done to evaluate the performance of a serum-free media compared to a serum-dependent condition using SCF, IL-3 and IL-6 for expansion. Following this, seven experiments were performed in order to evaluate the different cytokine combinations proposed using a serum-free media.

Expansion cultures were monitored on days 0 and 6. At both these times, CFU-Mix assay, flow cytometry and cobblestone-area-forming cell (CAFC) assays were performed.

CFU assay

Cells for the assay were plated in methylcellulose containing 30% fetal bovine serum, SCF (50 ng/mL), recombinant human GM-colony-stimulating factor (rh-GM-CSF; 10 ng/mL), rhIL-3 (10 ng/mL) and rh-erythropoietin (3 U/mL; Methocult GF H4434; Stem Cell Technologies, Vancouver, Canada). Cells of the enriched fraction were plated at 750 cells/mL and cells obtained after 6 days of *ex vivo* expansion were plated at a concentration of 1,200 cells/mL. Cultures were kept at 37ºC and 5% CO_2 for 2 weeks, and were then scored for the presence of CFU-GM, BFU-E and CFU-Mix. For analysis BFU-E and CFU-Mix were considered together. Only immature erythroid colonies (BFU-E) were scored; CFU-E were not included in the BFU-E/CFU-Mix group. Colonies that contained cells from erythroid and myeloid lineages were scored as CFU-Mix. When used, colony-forming cells (CFC) were related to the total amount of colonies including all the lineages scored.

Flow cytometry analysis

The following monoclonal antibodies were used for flow cytometry: phycoerythrin-labeled anti CD34, clone 8G12 (HPCA-2, Becton-Dickinson, San José, CA, USA); tri-color labeled anti-CD45, clone HI30 (Caltag Laboratories, Burlingame, CA, USA); fluorescein-labeled anti-CD61, clone RUU-PL 7F12 (Becton-Dickinson). Isotypic controls (phycoerythrin-IgG1 and fluorescein-IgG1) were obtained from Becton-Dickinson and Coulter (Miami, Florida). Samples were processed as described elsewhere,[23] and analyzed using an Epics XL-MCL flow cytometer and Epics-XL software (version 1.5, Izasa-Coulter, Barcelona, Spain). CD34+ cells that showed a level of fluorescence superior to that of the isotypic control were termed CD34+, or total CD34+ cells. This population includes cells with low intensity of fluorescence for CD34 antigen that could not be clonogenic. Cells, that after culture showed a level of fluorescence equivalent to that shown by the cells of the enriched fraction on day 0 were termed $CD34^{bright}$ cells. In Fig-

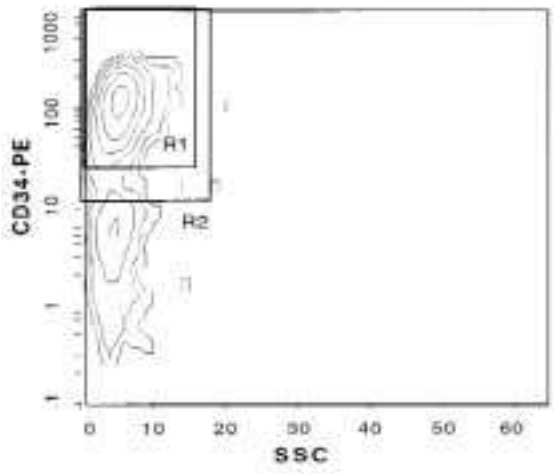

Figure 1. Strategies for defining the CD34bright and CD34+ regions. The histogram shows the definition of the region for analysis after positive selection. Two regions were created using the same instrument settings at day 0 and after 6 days of expansion. R1 is a fixed region created after selection (day 0) and shows the cluster population positive for CD34 antigen. The events in this region assessed after 6-day expansion were termed CD34bright. R2 is the region that includes the events with a level of fluorescence superior to that of the isotypic control. These cells were termed overall CD34+ cells in each point of analysis.

ure 1, the gating strategies used to monitor the follow-up of this population are presented. In order that the analyses at day 0 and day 6 were comparable, the same antibody concentration, labeling time and instrument settings were used. Previous reports[24,25] have shown an enrichment of immature progenitors in CB-CD34+ cells with a high intensity fluoresence level (CD34+++).

CAFC assay

Cobblestone-area-forming cell (CAFC) assay was performed as described previously,[26] except that irradiated human stroma was used as a feeder layer instead of a stromal cell line. Briefly, 20×10^6 MNC obtained from human bone marrow were seeded in 25 cm^2 flasks and cultured at 33ºC and 5% CO_2 in long-term culture medium. This medium consists of McCoy's medium (Life Technologies) supplemented with 12.5% fetal calf serum (Life Technologies) and 12.5% horse serum (Biological Industries, Kibbutz Beit Haemek, Israel), 1 µM hydrocortisone (Biological Industries), 30 mM HEPES (Biological Industries), 2 mM L-glutamine (Biological Industries) and vitamins and amino acids (Biological Industries) at appropriate concentrations. When the cultures had reached 75% confluence (3-4 weeks), cells were trypsinized, irradiated with 15 Gy and seeded onto 96 well plates at a concentration of 30,000 cells/well. Plates thus prepared were overlaid in a limiting dilution set-up with cells from the CD34+ cell-enriched fraction or with cells obtained after 6 days of *ex vivo* expansion; 4 dilutions (range between 5000 and 500 cells per well) and 15 replicates per dilution were seeded for each sample. Cells were cultured for 5 weeks at 33ºC in long-term culture medium, with weekly medium feedings. Plates were scored for the presence of cobblestone areas (CA) in week 5 of culture. The frequency of CAFC was calculated using Poisson statistics as described elsewhere.[27] Stroma from the same donor were used for all the CAFC assays, in order to ensure that the results obtained were comparable.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, release 6.1). The level of significance was set at $p<0.05$. The Student's t-test was used. Unless otherwise stated, data are expressed as mean and standard deviation.

Results

Positive selection

A total of 16 cord blood units were used in these experiments. The median percentage of CD34+ cells present in whole cord blood was 0.31% (0.13-0.48). After Ficoll separation, the median percentage of CD34+ cells was 0.74% (0.38-1.76), with a median recovery of CD34+ cells of 83% (52-117). In the enriched fraction, the median purity of CD34+ cells was 86% (72-93), with a median yield of 44% (38-57) of the CD34+ cells present before selection (after density gradient separation).

Performance of a serum-free medium vs. a serum-dependent medium

In order to evaluate the effect of a serum-free medium on the *ex vivo* expansion of cord-blood derived hematopoietic progenitors, cells from the enriched fraction (n=9) were expanded in both serum-free and serum-dependent media, in the presence of SCF, IL-3, and IL-6. The results are summarized in Table 1. No significant differences were observed between the

Table 1. Fold expansion of nucleated cells (NC), CD34+ cells, CD34bright cells and colony-forming cells (CFC) in the presence of serum (IMDM + FCS) or in its absence (SP34), after 6 days of *ex vivo* expansion of CB-derived CD34+ cells in the presence of SCF, IL-3 and IL-6 (50 ng/mL each). Mean and standard deviation of 9 experiments.

Culture medium	*Fold expansion*			
	NC	*Total CD34+ cells*	*CD34bright cells*	*CFC*
IMDM+ FCS	18.19 (± 10.05)	9.16 (± 7.06)	4.26 (± 3.94)	12.50 (± 14.51)
SP34	16.71 (± 7.23)	10.97 (± 5.86)	3.53 (± 2.11)	13.60 (± 8.77)

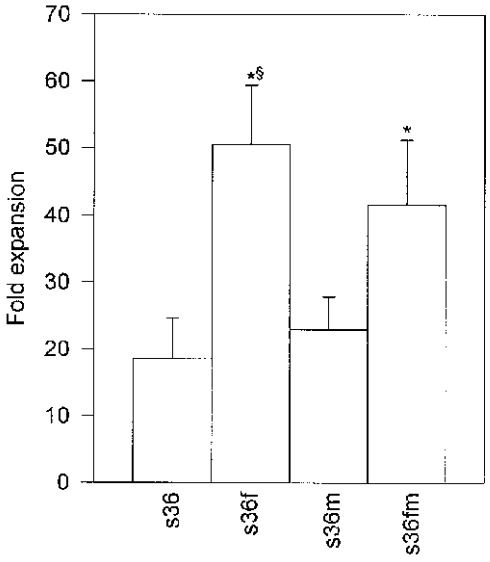

Figure 2. Expansion of nucleated cells. Fold expansion of nucleated cells in the presence of 4 cytokine combinations after 6 days of *ex vivo* expansion culture of CB-derived CD34+ cells. Data shown are the mean and standard deviation of 7 experiments. Abbreviations: s = SCF (50 ng/mL); 3 = IL-3 (50 ng/mL); 6 = IL-6 (50 ng/mL); f = FLT3-L (100 ng/mL); m = MIP-1α (20 ng/mL); *= fold expansion in groups containing FLT3-L was significantly higher than in groups without FLT3-L ($p \leq 0.05$); § = the rate of expansion in the s,3,6,f group was significantly higher than in the s,3,6,f,m ($p \leq 0.05$).

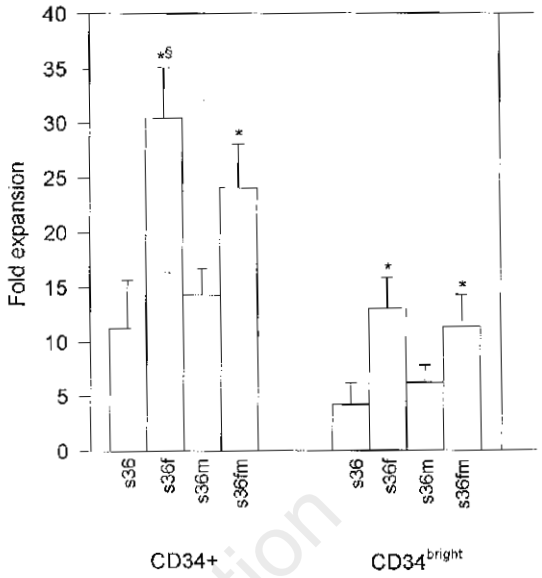

Figure 3. Expansion of CD34+ and CD34bright cells. Fold expansion of CD34+ and CD34bright cells in the presence of several cytokine combinations after 6 days of *ex vivo* expansion culture of CB-derived CD34+ cells. Data are expressed as mean and standard deviation (n=7). Abbreviations: s = SCF (50 ng/mL); 3 = IL-3 (50 ng/mL); 6 = IL-6 (50 ng/mL); f = FLT3-L (100 ng/mL); m = MIP-1α (20 ng/mL). *=fold expansion in groups containing FLT3-L was significantly higher than in groups without FLT3-L ($p<0.05$); § = the rate of expansion in the s,3,6,f group was significantly higher than in the s,3,6,f,m group ($p<0.05$).

fold-increase of nucleated cells (NC), total CD34+ cells, CD34bright cells and clonogenic precursors (Table 1). Moreover, serum-free expansion cultures showed a lower variability between samples than when the expansion was carried out in the presence of serum. The variation coefficients between experiments were 42% vs 62% for nucleated cells, 51% vs 96% for CD34+ cells, 57% vs 115% for CD34bright cells and 58% vs 91% for colony-forming cells, in serum-free and fetal calf serum-containing medium, respectively. These results suggest that the use of a serum-free media would allow greater reproducibility of results between samples, as well as permitting a better analysis of the effect of cytokines by eliminating possible interactions with unknown factors present in serum.

Ex vivo expansion of CD34+ cord-blood derived cells in a serum-free medium

In a second series of experiments (n=7), the effects of the above described 4 different cytokine combinations (s,3,6; s,3,6,f; s,3,6,m; s,3,6,f,m) in *ex vivo* expansion cultures carried out in SP34 were tested.

Nucleated cell expansion

The results of the expansion of NC are summarized in Figure 2. The greatest increase in nucleated cells was obtained with the s,3,6,f combination (50 fold ± 9), while the cytokine combinations least efficient at achieving NC expansion were those that did not contain FLT3-L (s,3,6: 18 fold ± 6 and s,3,6,m: 23 fold ± 5). Analysis of the fold-increase obtained with each cytokine combination showed that the groups containing FLT3-L caused significantly greater increase than those groups in which this cytokine was not present. Furthermore, the fold-increase achieved in the s,3,6,f group was significantly higher than in the other group containing FLT3-L (s,3,6,f,m: 42 fold ± 10), suggesting that the presence of MIP-1α has an inhibitory effect on the total number of cells generated when FLT3-ligand is also present.

Expansion of CD34+ cells

Analysis of the generation of CD34+ and CD34bright cells revealed a similar pattern to that obtained for NC (Figure 3). Thus, the greatest expansion of CD34+ and CD34bright cells was achieved with the s,3,6,f combination (30 fold ± 5 and 13 fold ± 3, respectively). In the case of CD34+ cells, as with nucleated cells, the expansion was significantly higher in the 2 groups containing FLT3-L (s,3,6,f: 30 fold ± 5; s,3,6,f,m: 24 fold ± 4) than in the 2 groups in which this cytokine was not present (s,3,6: 11 fold ± 4; s,3,6,m: 14 fold ± 2). The fold expansion in the group supplemented with s,3,6,f was also significantly higher than the expansion with the s,3,6,f,m combination.

For CD34bright cells, the expansion achieved in the 2 groups containing FLT3-L (s,3,6,f: 13 fold ± 3; s,3,6,f,m: 11 fold ± 3) was again significantly higher than in the 2 groups that did not contain it (s,3,6: 4 ± 2; s,3,6,m: 6 ± 2); however, no significant differences resulted from the addition of MIP-1α in the presence of FLT3-L. This suggests that MIP-1α affects mainly the generation of mature cells rather than a more immature population (represented by the CD34bright cells).

Expansion of CD34+ CD61+ cells

The mean percentage of cells co-expressing CD34 and CD61 antigens in the enriched fractions used for this study was 1.43% (± 1.00). All the combinations of cytokines analyzed were capable of expanding this subpopulation of cells after 6 days of *in vitro* culture, although a great deal of variation between samples was observed. Fold-increases obtained were 45±18, 70±35, 57±32, and 61±36 for the combinations s,3,6; s,3,6,f; s,3,6,m; and s,3,6,f,m respectively. The increase observed was as high as the myeloid/erythroid expansion as assessed by CFU-Mix assay, suggesting a non-lineage restricted (multilineage) expansion using these combinations of cytokines, all of which include early-acting cytokines. No significant differences exist between the different groups analyzed.

Expansion of clonogenic precursors (CFC)

The results of the expansion of progenitor cells (CFU-GM and BFU-E/CFU-Mix) are illustrated in Figure 4. As is the case with CD34bright cells, the fold-increases of CFU-GM and BFU-E/CFU-Mix were significantly higher in the groups supplemented with FLT3-L than in the groups that did not include FLT3-L. No differences were observed between the s,3,6,f group and the group containing FLT3-L and MIP-1α. The MIP-1α analog did not affect the generation of CFC, in contrast with the results obtained for NC and CD34+ cells. In all the combinations tested the expansion of BFU-E/CFU-Mix population was higher than the CFU-GM. This was mainly due to a considerable generation of mature erythroid colonies with burst-forming unit characteristics. Cloning efficiency (CE) of CD34+ cells was lower after six days of expansion, suggesting a generation of CD34+ cells, expressing low antigen intensity, that is not clonogenic. After positive selection CE was 42±30% (including CFU-GM, and BFU-E/CFU-Mix group). In contrast, the mean CE ranged from 25-37% after six days of expansion culture, depending on the combination used.

Evolution of CAFC during ex vivo *expansion cultures*

Week 5 CAFC were assessed in order to evaluate the functional behavior of the stem cell compartment in the short-term cultures (n=4). In the conditions used, week 5 CAFC failed to expand, even though input levels could be maintained when FLT3-L or MIP-1α were added to the cultures. Thus, while the combination s,3,6 only maintained 63% (± 26) of input CAFCs after 6 days of *ex vivo* expansion, addition of FLT3-L or MIP-1α alone increased the percentage to 79% (±57) and 84% (±44) respectively, and addition of both FLT3-L and MIP-1α allowed the maintenance of 91% (±49) of the input week 5 CAFC. Figure 5 shows the mean recovery after 6-day expansion of 5th week CAFC depending on the combination of cytokines used for proliferation.

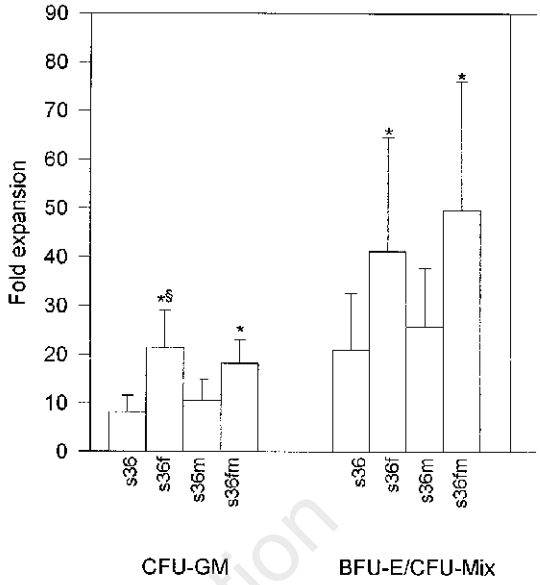

Figure 4. Expansion of clonogenic precursors. Fold expansion of CFU-GM and BFU-E / CFU-Mix in the presence of several cytokine combinations after 6 days of *ex vivo* expansion culture of CB-derived CD34+ cells. Data are expressed as mean and standard deviation of 7 different experiments. Abbreviations: s = SCF (50 ng / mL); 3 = IL-3 (50 ng / mL); 6 = IL-6 (50 ng / mL); f = FLT3-L (100 ng / mL); m = MIP-1α (20 ng / ml). *= fold expansion in groups containing FLT3-L was significantly higher than in groups without FLT3-L (*p*<0.05); § = the rate of expansion in the s,3,6,f group was significantly higher than in the s,3,6,f,m group (*p*<0.05).

Discussion

This report describes the results obtained after *ex vivo* expansion of CB-derived CD34+ cells in a short-term, stroma-free, static culture which, once scaled up, could be used in a clinical setting. In the first part of the study, the performance of a serum-free medium (StemPro-34) in the *ex vivo* expansion of cord-blood derived CD34+ cells was analyzed; a serum-free medium would avoid the risk of immunologic reactions and transmissible diseases linked to the use of FCS, thus making it better suited for clinical applications. In addition, a serum-free medium allows better assessment of the effects of individual cytokines on the culture. The results obtained using 2 different combinations of cytokines (s,3,6 and s,3,6,f) show

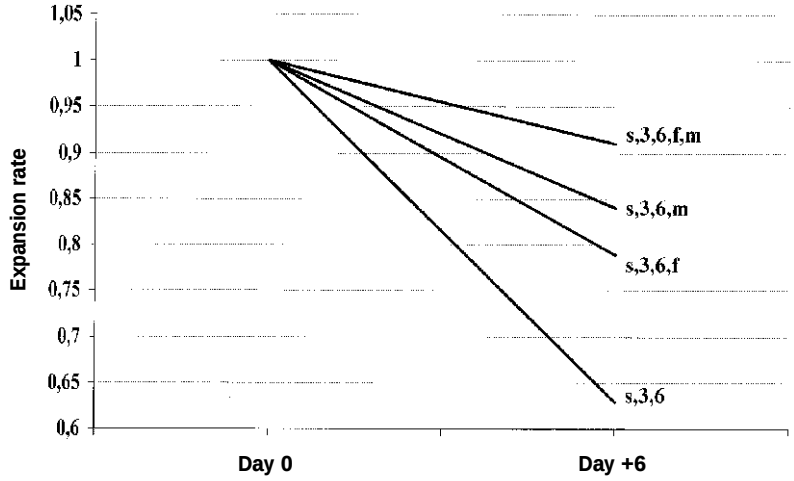

Figure 5. Recovery of week 5 CAFC in 6-day cultures. Evolution of week 5 CAFC after 6 days of *ex vivo* expansion cultures in the presence of 5 different cytokine combinations. The data shown are the mean of 4 independent experiments.
Abbreviations: s = SCF (50 ng/mL); 3 = IL-3 (50 ng/mL); 6 = IL-6 (50 ng/mL); f = FLT3-L (100 ng/mL); m = MIP-1α (20 ng/mL).

that the presence of serum is not necessary for efficient expansion of progenitor cells, and suggest that no additional factors contained in FCS appear to be necessary for an optimal expansion in culture. Furthermore, better reproducibility was observed using serum-free media than when serum was present in the cultures; this is probably due to the lack of effects from factors contained in the serum.[28]

The second part of the study was aimed at determining the effect of several cytokine combinations containing the early acting cytokine FLT3-L and the inhibitory chemokine MIP-1α on the expansion of CB-derived $CD34^+$ cells. To evaluate progenitor expansion, the fold increases of NC, CFC, $CD34^+$ cells were assessed, while in order to determine the expansion of the immature compartment, the week 5 CAFC assay was used. This assay was chosen since it has been reported to be a good predictor of engraftment in the human model.[14]

Our results show that addition of FLT3-L (100 ng/mL) to expansion cultures supplemented with SCF, IL-3 and IL-6 results in significantly greater expansion of committed progenitor cells, both in the presence and absence of MIP-1α. An increase in the proliferation rate and a higher recruitment of immature cells into the cell-cycle was suggested and could explain the results observed in our assays. These results are consistent with other observations regarding a potent effect of FLT3-L on the activation of primitive progenitor cells.[29-33]

Addition of MIP-1α in the presence of SCF, IL-3 and IL-6 did not have a significant effect on the expansion of either the more mature (NC and $CD34^+$ cells) or the more immature compartments ($CD34^{bright}$ cells and colony-forming-unit population). When a stimulatory cytokine (FLT3-L) was also present in the culture medium, the presence of MIP-1α resulted in significantly lower expansion in the more committed compartment (compared to the group with SCF, IL-3, IL-6 and FLT3-L) while no differences existed in the expansion of more immature populations. These results agree with the hypothesis suggesting that MIP-1α acts by preventing the differentiation of immature progenitors, leading them to accumulate, rather than affecting their proliferation. Thus, the presence of MIP-1α would result in slightly lower amounts of mature and precursor cells than when the chemokine is not present, while leaving the size of the immature compartment unaffected or increased.[21,34] Although it is still unclear how the effect of MIP-1α is achieved, it is believed that it prevents immature progenitors from going into the cell cycle.[35,36]

The results obtained from the week 5 CAFC assay show that both FLT3-L and MIP-1α have a positive effect on CAFC maintenance, an effect that is most beneficial when both cytokines are present, when up to 91% input CAFC can still be detected after 6 days culture. Moreover, CAFC maintenance in the presence of either FLT3-L or MIP-1α is higher than with SCF, IL-3 or IL-6 alone. Nevertheless, none of the combinations of cytokines analyzed was capable of expanding these primitive progenitors. Even though both Kögler *et al.*[7] and Moore *et al.*[37] demonstrated LTC-IC expansion in the presence of IL-3, it is possible that the presence of this cytokine in our cultures can explain, at least partly, our failure to expand week 5 CAFC. Piacibello *et al.*[8] obtained LTC-IC expansion in cultures supplemented with thrombopoietin and FLT3-L, but could only maintain input LTC-IC levels after 2 weeks of *ex vivo* culture when IL-3 was added to the cultures. Zandstra *et al.*[38] showed that *ex vivo* expansion of bone marrow LTC-IC required the use of high concentrations of FLT3-L (300 ng/mL); furthermore, they also showed that the presence of relatively high levels of IL-3 (60 ng/mL) when the concentration of FLT3-L in the medium was low (10 ng/mL) was detrimental for the maintenance of LTC-IC. Similar results on the negative effect of IL-3 over the expansion of immature progenitors were reported by Verfaillie *et al.*[21] using stroma-dependent cul-

tures. Thus, it is likely that failure of the stem cell compartment to expand in our culture system was due to the presence of IL-3 together with relatively low concentrations of FLT3-L.

Delayed engraftment of the megakaryocytic lineage is a common feature of CB transplants reported to date.[3,4] We sought to make a preliminary assessment of the megakaryocytic lineage analyzing cells co-expressing CD34 and CD61 antigens, a population which is very low in the initial CB CD34+ cell fraction. Interestingly, the combinations of cytokines tested show that it is possible to expand the megakaryocytic precursors present in CB *ex vivo* in a serum-free medium, in the absence of specific cytokines for this lineage (MGDF and IL-11). This finding suggests that combinations including early acting proliferative cytokines promote the expansion of progenitor cells representing all hematopoietic lineages (erythrocytic, myelo-monocytic and megakaryocytic) and can therefore be useful for clinically oriented *ex vivo* expansion protocols.[39,40] There are not, however, any data supporting a direct involvement of this population in the short-term repopulating potential.

Taken together these results suggest that, from those tested, the most appropriate cytokine combination for clinically oriented serum-free *ex vivo* expansion of cord blood-derived progenitor cells would be that containing SCF, IL-3, IL-6, FLT3-L and MIP-1α, since this is capable of generating considerable expansion in the mature compartment and can maintain the levels of stem cell candidates. It would be of interest to evaluate the progenitors generated *in vivo*, in order to define the role of cells thus obtained in short-term repopulating ability.

Contributions and Acknowledgments

GC carried out the laboratory work and did the data analysis. SQ participated in the design, analysis and discussion. JAC carried out the flow cytometry determination and participated in the discussion of the draft. JG was responsible for the establishment of the study and critical evaluation.

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References

1. Gluckman E, Broxmeyer HE, Auerbach AD, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical cord blood from an HLA-identical sibling. N Engl J Med 1989; 321: 1174-8.
2. Rubinstein P, Carrier C, Scaradavou A, et al. Outcomes among 562 recipients of placental-blood transplants from unrelated donors. N Engl J Med 1998; 329:1565-77.
3. Wagner JE, Rosenthal J, Sweetman R, et al. Successful transplantation of HLA-identical and HLA-1 and 2-antigen mismatched unrelated umbilical cord blood: Analysis of engraftment and acute graft-versus-host disease. Blood 1996; 88:795-802.
4. Kurtzberg J, Laughlin M, Graham ML, et al. Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients. N Engl J Med 1996; 335:157-66.
5. Rubisntein P, Carrier C, Adamson J, et al. New York Blood Center's program for unrelated placental/ umbilical cord blood (PCB) transplantation: 243 transplants in the first 3 years [abstract]. Blood 1996; 88 (suppl 1):142a.
6. Abboud M, Xu F, LaVia M, Laver J. Study of early hematopoietic precursors in human cord blood. Exp Hematol 1992; 20:1043-7.
7. Kögler G, Callejas J, Hakenberg P, et al. Hematopoietic transplant potential of unrelated cord blood: critical issues. J Hematother 1996; 5:105-16.
8. Piacibello W, Sanavio F, Garetto L, et al. Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood. Blood 1997; 89:2644-53.
9. Shapiro F, Pytowsky B, Rafii S, et al. The effects of Flk-2/flt3 Ligand as compared with c-kit Ligand on short-term and long-term proliferation of CD34+ hematopoietic progenitors elicited from human fetal liver, umbilical cord blood, bone marrow and mobilized peripheral blood. J Hematother 1996; 5:655-62.
10. De Bruyn C, Delforge A, Bron D, et al. Ex vivo expansion of CD34+CD38- cord blood cells. J Hematother 1997; 6:93-102.
11. Mayani H, Dragowska W, Lansdorp P. Characterization of functionally distinct subpopulations of CD34+ cord blood cells in serum-free long-term cultures supplemented with hematopoietic cytokines. Blood 1993; 82:2664-72.
12. Cardoso AA, Li M-L, Batard P, et al. Release from quiescence of CD34+CD38- human umbilical cord blood cells reveals their potentiality to engraft adults. Proc Natl Acad Sci USA 1993; 90:8707-11.
13. Srour EF, Brandt JE, Briddell RA, et al. Long-term generation and expansion of human primitive hematopoietic progenitor cells in vitro. Blood 1993; 81:661-9.
14. Breems DA, Blokland EAW, Ploemacher RE. Stroma-conditioned media improve expansion of human primitive hematopoietic stem cells and progenitor cells. Leukemia 1997; 11:142-50.
15. Rappold I, Ziegler BL, Köhler I, et al. Functional and phenotypic characterization of cord blood and bone marrow subsets expressing FLT3 (CD135) receptor tyrosine kinase. Blood 1997; 90:111-25.
16. Hannum C, Culpepper J, Campbell D, et al. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of hematopoietic stem cells and is encoded by variant RNAs. Nature 1994; 368:643-68.
17. Haylock DN, Horsfall MJ, Dowse TL, et al. Increased recruitment of hematopoietic progenitor cells under-

lies the ex vivo expansion potential of FLT3 ligand. Blood 1997; 90:2260-72.
18. Broxmeyer HE, Lu SL, Cooper S, et al. Myelopoietic enhancing effects of murine macrophage inflammatory proteins 1 and 2 on colony formation in vitro by murine and human bone marrow granulocyte/macrophage progenitor cells. J Exp Med 1989; 170:1583-94.
19. Broxmeyer HE, Lu SL, Cooper S, et al. Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation in vitro by bone marrow myeloid progenitor cells. Blood 1990; 76:1110-6.
20. Keller JR, Bartelmez SH, Sitnicka E, et al. Distinct and overlapping direct effects of MIP-1α and TGF-β on hematopoietic progenitor/stem cell growth. Blood 1994; 84:2175-81.
21. Verfaillie CM, Catanzarro PM, Li W. Macrophage inflammatory protein 1α, interleukin 3 and diffusible marrow stromal factors maintain human hematopoietic stem cells for at least eight weeks in vitro. J Exp Med 1994; 179:643-9.
22. Querol S, Cancelas JA, Canals C, et al. Comparative expansion of CD34+ immunoselected cells from bone marrow, mobilised peripheral blood and cord blood: effects of G-CSF and GM-CSF. Bone Marrow Transplant 1996; 17 (Suppl 1):551 (abstr).
23. Cancelas JA, Querol S, Canals C, et al. CD34+ cell positive selection from mobilised peripheral blood by an indirect immunomagnetic method: effect of the type of mobilisation and assessment of tumor depletion ability. J Hematother 1995; 4:531-8.
24. Lu L, Xiao M, Shen R, Grigsby S, Broxmeyer H. Enrichment, characterization, and responsiveness of single primitive CD34+++ human umbilical cord blood hematopoietic progenitors with high proliferative and replating potential. Blood 1993; 81:41-8.
25. Di Giusto DL, Lee R, Moon K, et al. Hematopoietic potential of cryopreserved and ex vivo manipulated umbilical cord blood progenitor cells evaluated in vitro and in vivo. Blood 1996; 87:1261-71.
26. Breems DA, Blokland EAW, Neben S, Ploemacher RE. Frequency analysis of human primitive hematopoietic stem cell subsets using a cobblestone area forming cell assay. Leukemia 1994; 8:1095-104.
27. Ploemacher RE, Van der Sluijs JP, Voerman JSA, Brons NHC. An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood 1989; 74:2755-63.
28. Sandstrom CE, Miller WM, Papoutsakis ET. Serum-free media for cultures of primitive and mature hematopoietic cells. Biotechnol Bioeng 1994; 43:706-33.
29. Broxmeyer HE, Lu L, Cooper S, et al. Flt3 ligand stimulates/costimulates the growth of myeloid stem/progenitor cells. Exp Hematol 1995; 23:1121-9.
30. Hirayama F, Lyman SA, Clark SC, Ogawa M. The flt3 ligand supports proliferation of lymphohematopoietic progenitors and early B-lymphoid progenitors. Blood 1995; 85:1762-8.
31. Muench MO, Roncarolo MG, Menon S, et al. FLK-2/FLT-3 ligand regulates the growth of early myeloid progenitors isolated from human fetal liver. Blood 1995; 85:963-72.
32. Bhatia M, Bonnet D, Kapp U, Wang JC, Murdoch B, Dick JE. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. J Exp Med 1997; 186:619.
33. Conneally E, Cashman J, Petzer A, Eaves C. Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc Natl Acad Sci USA 1997; 94:9836.
34. Van Ranst PCF, Snoeck H-W, Lardon P, et al. TGF-ß and MIP-1a exert their main inhibitory activity on very primitive CD34++CD38- cells but show opposite effects on more mature CD34+CD38+ human hematopoietic progenitors. Exp Hematol 1996; 24:1509-15.
35. Lord BI, Dexter TM, Clements JM, et al. Macrophage inflammatory protein protects multipotent hematopoietic cells from the cytotoxic effects of hydroxyurea in vivo. Blood 1992; 79:2605-9.
36. Soma T, Yu JM, Dunbar CE. Maintenance of murine long-term repopulating stem cells in ex vivo culture is affected by modulation of TGF-ß but not MIP-1a activities. Blood 1996; 87:4561-7.
37. Moore AS, Hoskins I. Ex vivo expansion of cord blood-derived stem cells and progenitors. Blood Cells 1994; 20:468-79.
38. Zandstra PW, Conneally E, Petzer AL, et al. Cytokine manipulation of primitive human hematopoietic cell self-renewal. Proc Natl Acad Sci USA 1997; 94:4698-703.
39. Locatelli F, Burgio GR. Transplant of hematopoietic stem cells in childhood: where we are and where we are going. Haematologica 1998; 83:550-63.
40. Aglietta M, Bertolini F, Carlo-Stella C, et al. Ex vivo expansion of hematopoietic cells and their clinical use. Haematologica 1998; 83:824-48.