Cord Blood G_0 $CD34^+$ Cells Have A Thousand-Fold Higher Capacity For Generating Progenitors In Vitro Than G_1 $CD34^+$ Cells

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Key Words. *CD34 · Cord Blood · Ex vivo expansion · Cell cycle · Serum-free · G_0*

ABSTRACT

We examined the functional differences between G_0 and G_1 cord blood $CD34^+$ cells for up to 24 weeks in serum-free suspension culture, containing Flt-3 ligand, thrombopoietin and stem cell factor. By week 24, there is more than a 1,000-fold difference in granulocyte, macrophage-colony-forming cells (GM-CFC) cumulative production between the two populations, with cultures initiated from G_0 demonstrating an amplification of 1.1×10^5-1.8×10^6 of GM-CFC compared to 45-2.7×10^3 for the G_1 cells. Cells from the initial G_0 population are able to produce about 250-fold higher numbers of BFU-E than those from G_1 which translates to 3×10^3-1.1×10^5-fold expansion and 25-390-fold expansion for G_0 and G_1, respectively. This amplification of the progenitor cells is reflected in finding that a greater proportion of the progeny of the G_0 population are $CD34^+$, resulting in a 600-fold expansion of $CD34^+$ cells at week 8. As in other in vitro systems, total cell expansion is less discriminatory of stem cell behavior than progenitor cells, and there is no significant difference in total cell numbers between G_0 and G_1 cultures with a mean fold expansion of 2×10^7 at 24 weeks. ***Stem Cells*** *2001;19:505-513*

INTRODUCTION

The characteristics that define a hemopoietic stem cell (HSC) are extensive proliferative capacity and ability to self-renew and to sustain long-term lymphomyeloid hemopoiesis.

HSCs are present in bone marrow (BM), umbilical cord blood (UCB) and mobilized peripheral blood (MPB). The clinical use of BM is declining, while MPB is now used routinely for autologous and allogeneic transplantation [1]. The use of UCB, as a source of hemopoietic repopulating cells has also been established [2]. As the number of stem cells available, especially from UCB, may be limited, a major goal of experimental and clinical hematology is the identification of ex vivo conditions which support self-renewal and expansion. This has broad potential clinical applications in autologous and allogeneic transplantation as well as gene therapy.

The published methods describing cell expansion in vitro have utilized a variety of conditions: stroma-free or stroma-containing protocols in the presence or absence of serum and different combinations of growth factors [3-12]. In most reports the increase in stem cell numbers, if observed, was small. Extensive amplification of the progenitor cell population over 6 months was described in a serum-containing system supplemented with the cytokines Flt-3 ligand (FL-3) and thrombopoietin (TPO) [13]. However, to date similar levels of amplification have not been reported under serum-free conditions.

Stem cells are proliferatively quiescent residing in the G_0/G_1 phase of the cell cycle [14]. In MPB cell transplants virtually all the $CD34^+$ cells are found in G_0/G_1 compared to 85%-90% of BM [15, 16]. Therefore, cell cycle status may be critical in defining strategies for HSC expansion. It is now possible to distinguish and isolate viable cells in G_0 or G_1 phase using a combination of the DNA and RNA binding dyes, Hoechst 33342 and Pyronin Y [17, 18]. Hence, in this study we used simultaneous DNA/RNA staining and flow cytometric cell sorting to isolate and characterize cord blood (CB) $CD34^+$ cells in G_0 or G_1 phases of the cell cycle. We compared the proliferative capacity and the generation

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of progenitor cells, CD34+ cells, and blast cells among the ex vivo-expanded progeny.

This study is the first report showing extensive expansion potential in vitro over 24 weeks of primitive cells isolated according to their cell cycle status. Prolonged generation of hemopoietic progenitors (defined as colony-forming cells, [CFCs]), up to 2×10^6-fold and significant expansion of CD34+ and blast cells were observed under serum-free conditions, thus defining a system that allows extensive amplification accompanied by limited maturation. Potential for further maturation, however, is expressed if cells are subcultured in colony assays.

Materials and Methods

Cell Preparation

Human UCB cells were obtained from full-term normal deliveries with informed consent. The mononuclear cell (MNC) fraction was separated on Ficoll-Hypaque (Lymphoprep, density 1.077 g/ml, Life Technologies; Paisley, UK; http://www.lifetech.com) by density centrifugation and CD34+ cells isolated using CD34-conjugated superparamagnetic microbeads and MiniMACS columns (Miltenyi Biotech; Bergisch Gladbach, Germany; http://www.miltenyibiotec.com) [19]. Briefly, MNCs were incubated with CD34 antibody conjugated to microbeads for 30 minutes at 4°C. After incubation, cells were washed in ice-cold PBE (phosphate-buffered saline [PBS] containing 1% BSA [bovine serum albumin] [weight by volume; w/v] and 5 mM EDTA). Cells were passed through a MiniMACS column retained in a magnetic field, and the column washed with PBE to remove unbound cells. Target CD34+ cells were recovered by releasing the magnetic field and flushing cells from the column.

Isolation of G_0 and G_1 Cells

Cells were labeled using a method previously described by *Gothot* [20]. CD34+ cells were washed and then incubated in Hoechst buffer (Hank's balanced salt solution, 0.1% [w/v] D-glucose; 20 mM HEPES, 10% [volume by volume; v/v] fetal calf serum; FCS) at a concentration of 5×10^6 cells/ml. Hoechst 33342 dye (Molecular Probes; Eugene, OR) was added to give a final concentration of 1 μg/ml and the cells incubated for 45 minutes at 37°C. After this incubation period Pyronin Y (Sigma; St. Louis, MO; http://www.sigma-aldrich.com) was added to a final concentration of 0.5 μg/ml and incubation continued at 37ºC for a further 45 minutes. During the final 10 minutes of the incubation period 10 μl per 10^6 cells CD34-fluorescein isothiocyanate (FITC) (HPCA-2, Becton Dickinson; San José, CA; http://www.bd.com) or a mouse IgG_1-FITC (Becton Dickinson) as an isotype-matched control was added. The cells were then washed once and resuspended in ice-cold Hoechst buffer and sorted on a fluorescence-activated cell sorter (FACS) Vantage equipped with an argon laser providing excitation in the ultraviolet spectrum at 357 nm (Stabalite 2017) and an argon laser providing excitation in the visible spectrum (Innova 70C/2, Coherent) (Fig. 1).

Serum-Free Cultures

The G_0 or G_1 cells were cultured in 24-well plates (Becton Dickinson) in a volume of 1 ml, at 5×10^3 cells/ml, in StemSpan serum-free medium (Stem Cell Technologies; Meylan, France; http://www.stemcell.com). Previous experiments showed similar results when a range of cell concentrations was used (10^3-10^4 cells/ml up to 8 weeks of culture, data not shown). Cultures were supplemented with stem cell factor (SCF) at 300 ng/ml, FL-3 at 100 ng/ml, and TPO at 100 ng/ml and maintained at 37°C in 5% CO_2 and 5% O_2 in nitrogen. All cytokines were obtained from R&D Systems (Oxon, UK). Each week cultures were demi-depopulated and replaced with fresh medium containing SCF, FL-3, and TPO at the concentrations indicated. This method has been shown to give comparable results to doubling the culture volume each week [21]. The harvested cells were counted, and progenitor cell content was assessed in standard clonogenic assays. At specified time points, cells were analyzed for CD34 expression by flow cytometry. Cell morphology was determined after May-Grünwald/Giemsa staining.

Progenitor Cell Assays

To assay granulocyte-macrophage colony-forming cells (GM-CFC) and BFU-E, 1-10 $\times$ 10^3-harvested cells were plated in a 1 ml mixture containing a final concentration of 30% (v/v) FCS, 1% (w/v) deionized BSA, 10% (v/v) 5,637 conditioned medium (from the EJ bladder carcinoma cell line), two units erythropoietin, and 1.35% (w/v) methylcellulose. Cultures were plated in triplicate. Plates were incubated in a humidified atmosphere of 5% CO_2, 5% O_2 in nitrogen at 37°C for 14 days. Colonies were scored according to standard criteria [22].

CD34 Expression in Cultured Cells

Aliquots of cultured cells harvested at specified time points were washed and resuspended in PBS. After a 15-minute incubation at room temperature with a CD34-phycoerythrin (PE) antibody (HPCA-2, Becton Dickinson) or a mouse IgG_1-PE as an isotype-matched control, cells were washed and analyzed on a FACScan flow cytometer. CD34+ cells were gated on low side scatter and isotype controls ensured specificity.

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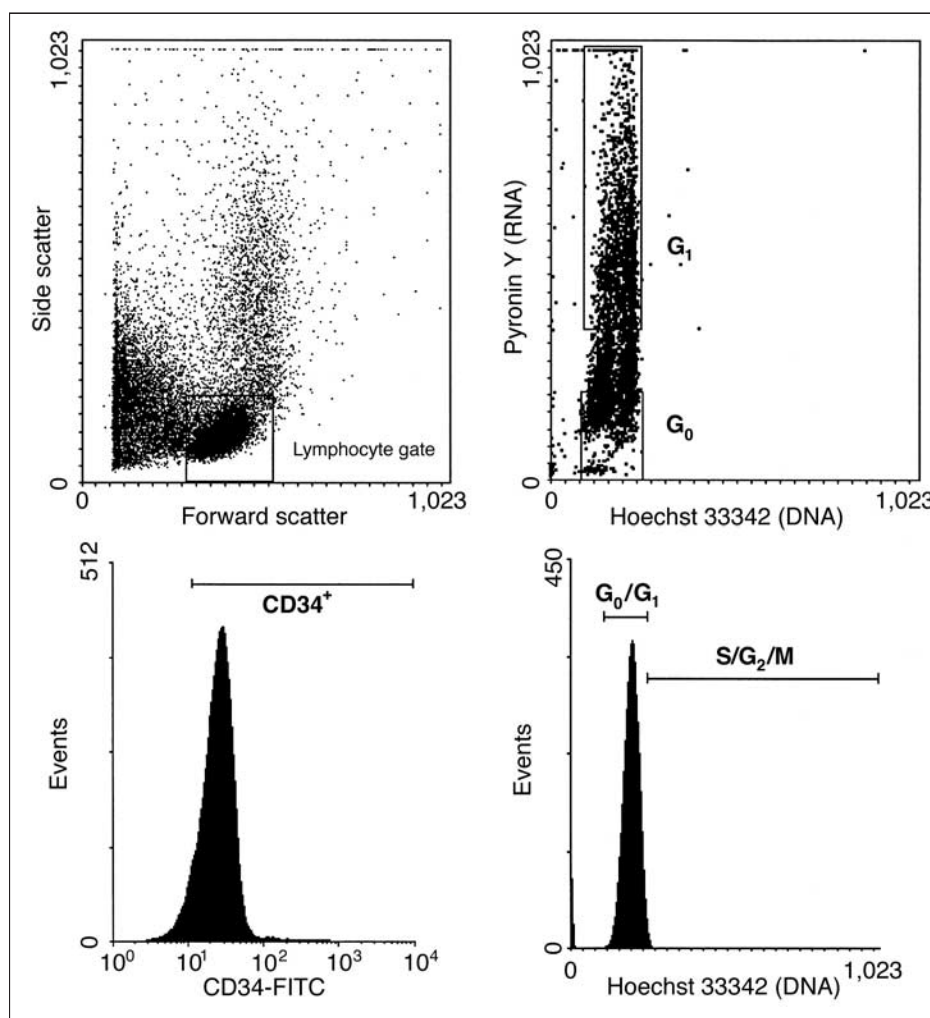

Figure 1. Flow cytometric selection of G_0/G_1 cells. *A lymphocyte gate was defined according to forward and side scatter, as shown on the top left panel, and cells within this gate were also selected for CD34 positivity (lower left panel). Simultaneous staining of RNA and DNA with Hoechst and Pyronin Y, respectively, allows cells in G_0 to be distinguished from those in G_1, by virtue of their RNA content. Cells with no more than "2n" DNA and low RNA content (Pyronin Y fluorescence 220 or less) are gated as G_0 and those with greater RNA content (Pyronin Y fluorescence 400 or more) are gated as G_1 (top right panel). A 180 fluorescence-channel gap is left between the two gates in order to avoid contamination in the selected populations. The lower right panel shows cell cycle analysis based on DNA content alone where greater than 99% of the CD34$^+$ CB cells are in the G_0/G_1 phase of the cell cycle.*

Statistical Analysis

Data were analysed using a two-tailed paired Student *t*-test. *p* values <0.05 designated significant differences between test points.

Results

We examined the quantity and quality of expansion for cells residing in G_0 or G_1 phase of the cell cycle in serum-free, stromal cell-free cultures supplemented with SCF, FL-3, and TPO. Four parameters were examined: total nucleated cells numbers, numbers of progenitors produced, production of cells expressing CD34, and proportion of blast cells present in the culture. Extensive amplification of total nucleated cells is achieved for up to 24 weeks. Figure 2A demonstrates that substantial cellular proliferation is seen: already 10,000-fold the input cell number by week 8, which continues and reaches 2×10^7-fold expansion by week 21. Figures 2B and 2C show, respectively, the cumulative fold increase in GM-CFC and BFU-E generated in up to 6 months of culture. There is a significant difference in CFC production between the G_0 and G_1 populations. The initial G_0 population is able to produce at least 100-fold higher numbers of GM-CFC than the G_1 population from about week 14 onwards. Furthermore, as these are cumulative plots the leveling out of the curve indicates that the G_1 cultures have stopped producing CFCs at 8 weeks, as compared to 18 weeks for the G_0 cultures. It can therefore be observed in all four experiments that the G_0 cultures produce greater numbers of CFCs and that they go on producing CFCs for on average of 10 weeks longer. At 20 weeks, there is a mean 7×10^5 (range 1.1×10^5-1.8×10^6)-fold expansion in GM-CFC in the G_0 starting population, compared to 500 (range 45-2,700)-fold expansion for cells initially in G_1. Similar striking differences are seen when examining erythroid colony formation: after 20 weeks of culture, cells from the initial G_0 population are able to produce about 250-fold higher numbers of BFU-E than those from G_1, which translates to a mean expansion of 5×10^4-fold (range 3×10^3-1.1×10^5) and 200-fold (range 25-390) for G_0 and G_1, respectively. When comparing BFU-E to GM-CFC, the magnitude of the expansion is higher (14-fold) and the time span in which the progenitor numbers expand is 2 weeks longer for the latter.

Cells harvested weekly were analyzed by flow cytometry for CD34 surface expression. G_0 cultures produce more CD34$^+$ cells than G_1 cultures. Furthermore, the fraction of cells expressing CD34 on the cell surface declines more rapidly in the progeny of the G_1 population, compared to that of the G_0 population (Fig. 3). Figure 4 shows that the

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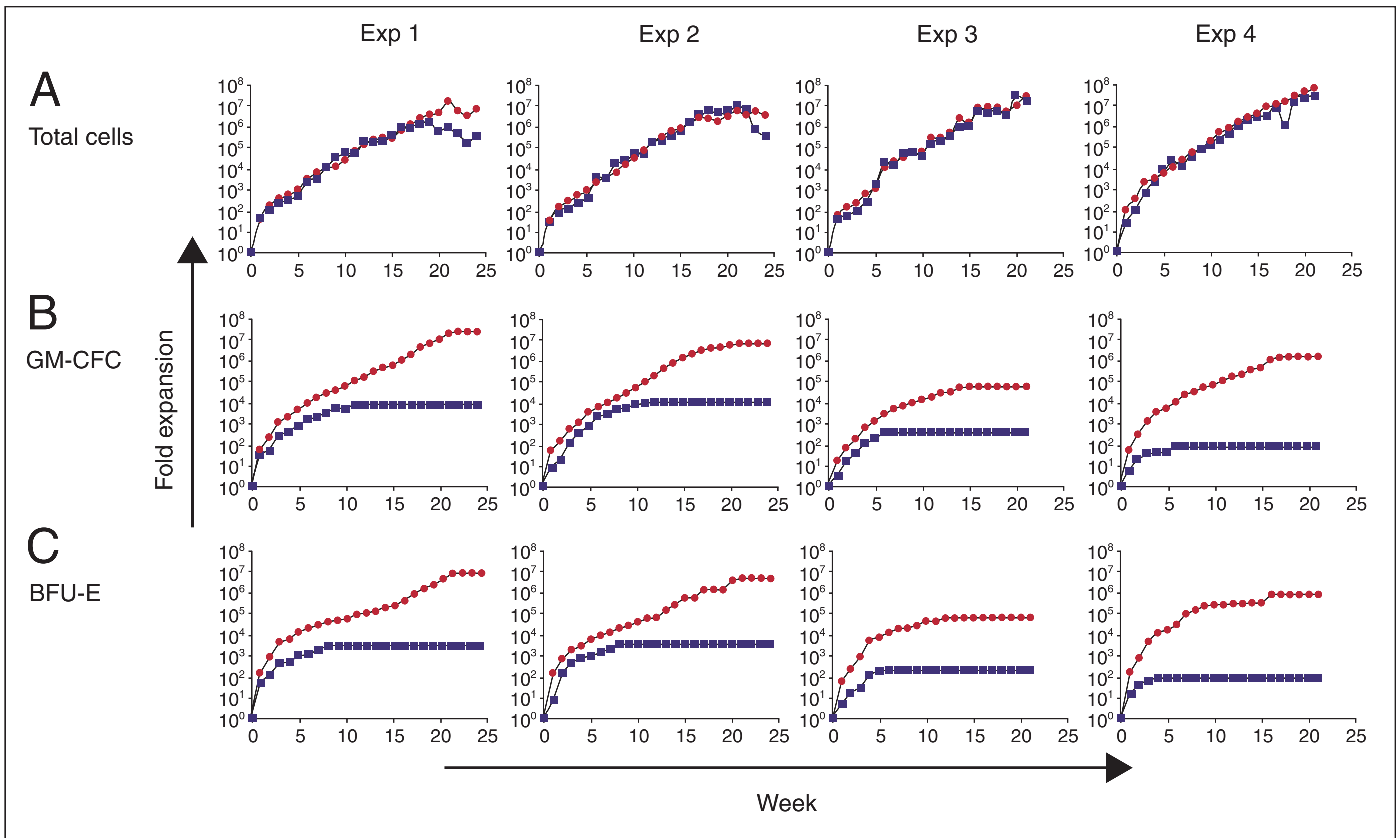

*Figure 2. **Expansion of total nucleated cells and progenitors.** Expansion of total nucleated cells is shown on the top row (A). The weekly totals were determined by taking the number of cells in the harvested proportion (half the culture volume) and making the calculation of how many cells would have been present if weekly demi-depopulation had not occurred (multiplied by 2^n where n = the week of demi-depopulation). The total number of cells was then divided by the starting number to calculate the fold expansion. G_0 cultures are represented by red symbols and G_1 by blue symbols. Cumulative production of GM-CFC is shown in the middle row (B). Each week the number of colonies present in an aliquot of harvested cells was assessed by progenitor cell assay. The number of colonies present in the total population was determined by taking into account the total number of cells present if demi-depopulation had not occurred (see above). Fold expansion was calculated by dividing by the number of GM colonies in the starting cultures. Significant differences in cumulative production of GM-CFC are seen between G_0 and G_1 ($p < 0.05$) from week 6 onwards. Cumulative production of BFU-E is illustrated in the bottom row (C). The number of erythroid colonies was calculated as in (B). Differences in cumulative production of BFU-E between G_0 and G_1 cultures are significant from week 2 onwards ($p < 0.05$).*

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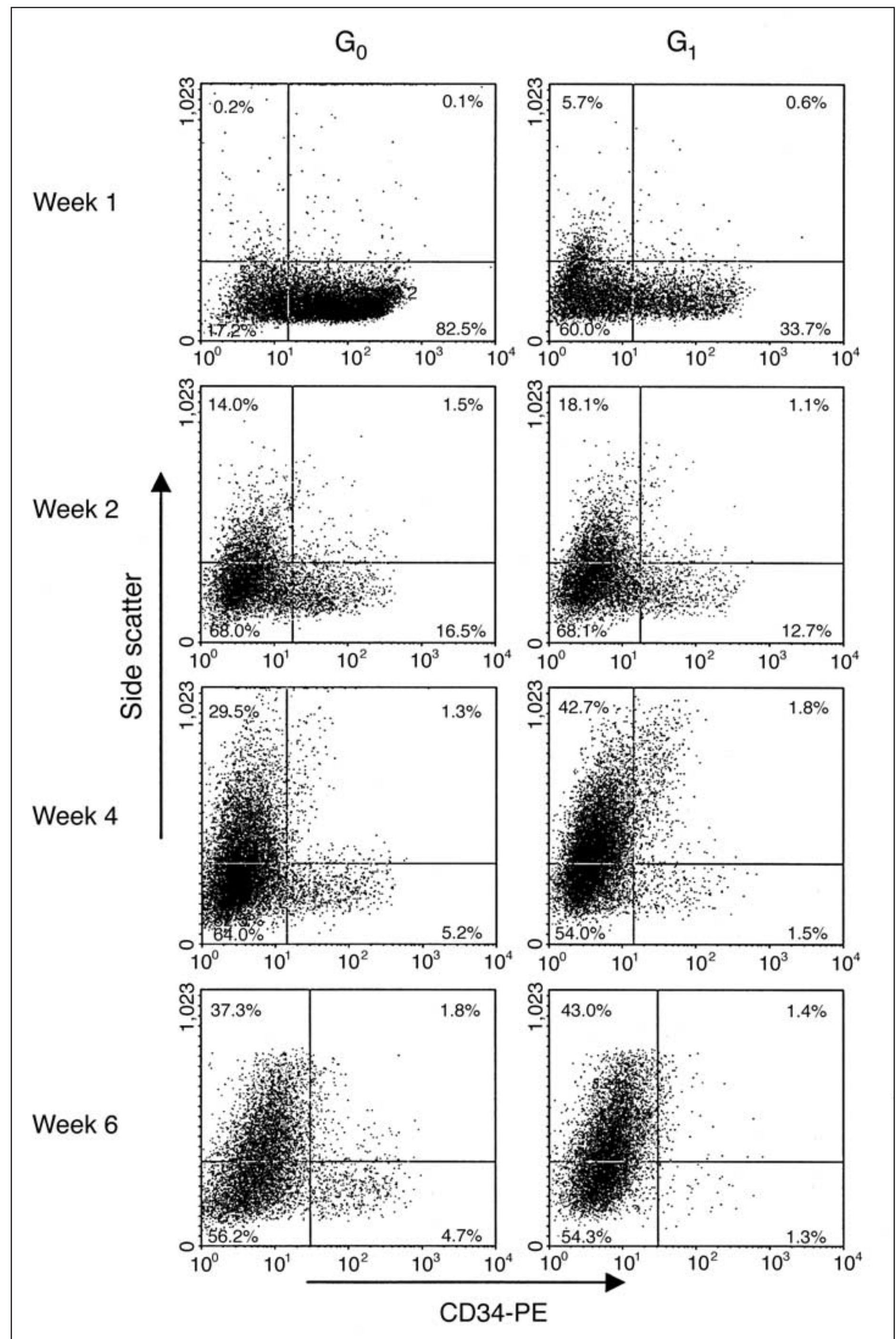
amplification of CD34+ cells in the first 8 weeks of culture is 20 times greater, reaching 600-fold at 8 weeks, in the progeny of the G_0 population compared to that of the G_1 population ($p = 0.04$). The number of CD34+ cells declines after week 12, and are then difficult to measure. However, generation of progenitor cells continues at least until week 19. There may be a change in the phenotype of progenitor cells generated in vitro, in agreement with previous reports [23] that the CD34 marker may be lost in cultured cells.

Cytospins prepared at 4 and 12 weeks of culture showed a high percentage of blast cells in both G_0 and G_1 cultures, indicating that the balance of cell production is biased towards the immature end of the spectrum (Figs. 5A and 5B, Table 1). However, when the cells from either culture type are transferred to clonogenic assays, maturation to segmented neutrophils and benzidine-positive normoblasts is comparable to that seen in colonies derived from freshly isolated CD34+ cells (data not shown), suggesting that the progenitors generated in vitro possess normal differentiation capacity. It is of interest that while the proportion of blasts present at 12 weeks is still high, especially in the G_0 cultures, cell cycle analysis at the same time showed that 33.5% of the cells were in G_0 (Figs, 5C-E).

Discussion

We sought to determine whether CD34+ CB cells could be maintained in serum-free, stromal cell-free culture conditions over a prolonged period of time and whether differences exist in the proliferative potential of cells isolated from G_0 and G_1 phases of the cell cycle. G_0 and G_1 sorted cells were set up in suspension cultures with a combination of three cytokines (SCF, FL-3, and TPO) known to act on primitive hemopoietic cells [24-27]. Other investigators have included interleukin-3 (IL-3) in their expansion protocols. However, as a number of recent reports have suggested that IL-3 induces differentiation

__Figure 3. Expression of CD34 on expanded cells.__ FACS plots of CD34 expression from a representative experiment are shown. CD34+ cells have low side scatter and are found in the lower right quadrant (as defined by isotype controls). It is clear that G_0 cultures show a greater proportion of CD34+ cells, at all the time points analyzed, than G_1 cultures.



rather than expansion of progenitors [23, 28], it was omitted from our cytokine cocktail. Similarly, IL-6 was not used, as our earlier work [29] showed that addition of IL-6 to cultures was not advantageous. We avoided the use of serum, as most sera contain both inhibitors and stimulators of growth, and constituents can vary, even within the same batch. This, in turn, may lead to problems with reproducibility. Moreover, the risks of using animal sera, which may contain allergens or infectious agents, like bovine spongiform encephalitis or other prion-type diseases, for expansion protocols with potential clinical use are unacceptable. Even the use of human serum may present risks. Thus, the use of serum-free conditions is crucial if expanded cells are to be used in the clinical setting.

Our expansion of total nucleated cells (2×10^7-fold the input number at 21 weeks) compares favorably with the published data by other investigators examining expansion of CFC in cultures extended beyond 3 weeks, who have achieved expansion of nucleated cells for up to 3-8 weeks of culture, but thereafter, have seen a rapid decline of cell numbers [6, 30]. More recently, *Gilmore et al*. examined the ex vivo expansion potential of CD34+ cells from CB [11]. In conditions containing 10% FCS, FL-3, and TPO, they achieved an average time in culture of 8.9 weeks, a mean expansion of nucleated cells of 1,385× the input number. This compares to a mean expansion of nucleated cells of 38,500-fold presented here (Fig. 2A) at 9 weeks. *Gilmore et al*. also showed a 300-fold expansion of CD34+ cells for up to 14 weeks compared to 600-fold here after 8 weeks. Furthermore, we achieved 2×10^7-fold amplification of nucleated cells in cultures started with either G_0 or G_1 cells, and routine and reproducible cell expansion to 20 weeks and beyond. *Piacibello et al*. [13] showed comparable total cell expansion in cultures of CD34+ CB cells, but their cultures contained 10% FCS. Thus, to our knowledge, we have for the first time achieved a 2×10^7-fold expansion in serum-free, stromal cell-free cultures.

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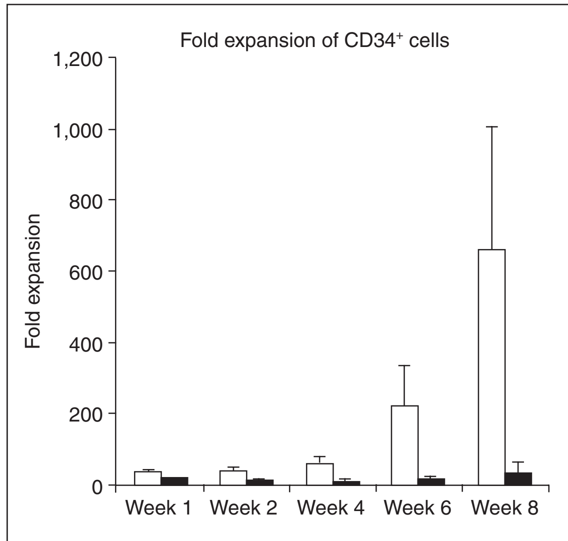

Figure 4. Fold expansion of CD34+ cells. *Expansion of CD34+ cells is expressed as fold expansion compared to input number.* n = *4 for all time points except week 1 where* n = *2 and week 2 where* n = *3. G_0 and G_1 cultures are represented by open and closed symbols, respectively. The greater ability of G_0 cultures to produce CD34+ cells becomes more pronounced with time in culture.*

We have shown that G_0 cultures produce substantially greater numbers of progenitors than G_1 cultures (7×10^5-fold expansion in GM-CFC and 5×10^4-fold expansion of BFU-E compared to 500-fold and 200-fold expansion, respectively), and that production of progenitors continues for longer in G_0 cultures. Others have shown that freshly harvested cells residing in G_0 contain more BFU-E and CFU-mix progenitors than those from G_1, where the majority of colonies are of the granulocyte-macrophage lineage [20]. We have confirmed these findings (data not shown). It should be noted that, in our experiments, production of erythroid progenitors ceases sooner and at lower numbers than that of granulocyte-macrophage progenitors. A possible reason may be that the culture conditions favor the generation of GM-CFC, a possibility that may be tested.

Recent work shows that during fetal development and at birth, most of the stem cells are CD34+ [31]. Our data on

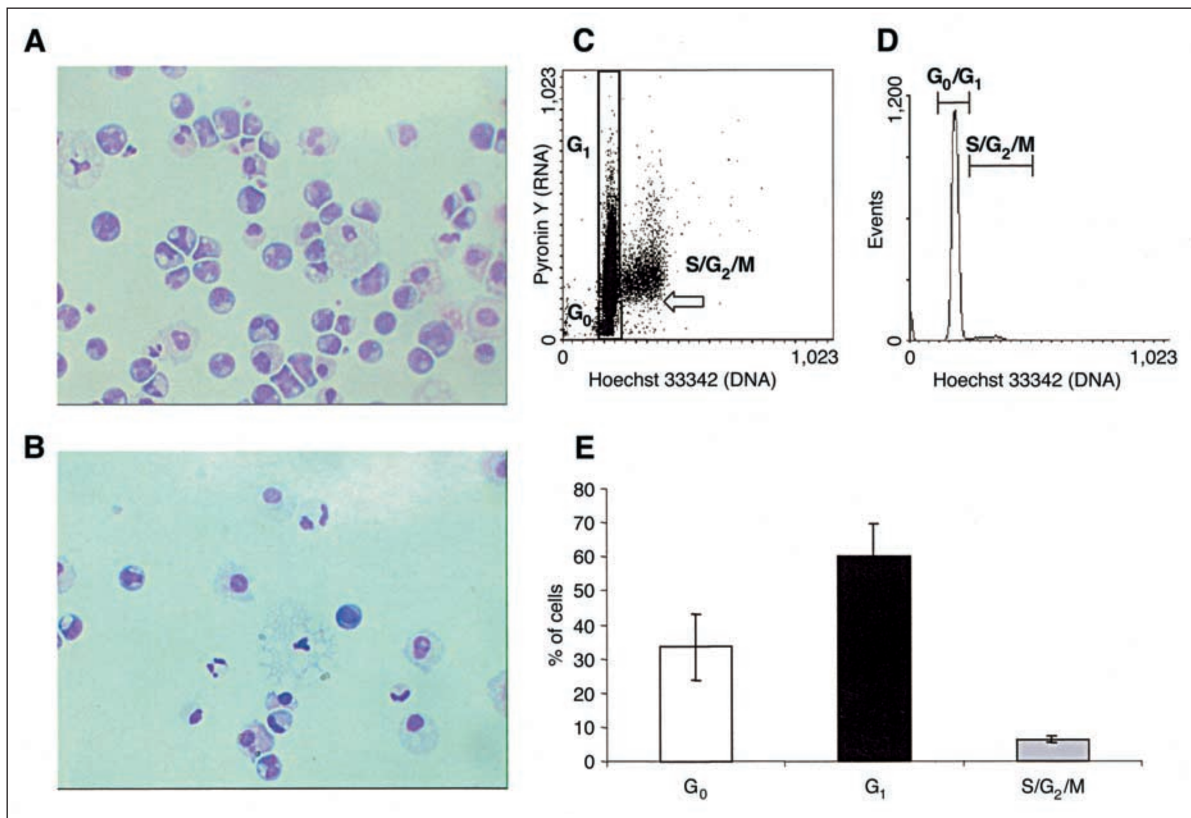

Figure 5. Cell cycle analysis and morphology of expanded cells. *Cell morphology of G_0 and G_1 cultures after 12 weeks is shown in (A) and (B), respectively. Representative DNA/RNA plot after 12 weeks expansion (C). Note that in contrast to the original gate in Figure 1 set to select purified G_0 or G_1 populations, the lower limit of the G_1 gate here has been brought down to the top of the G_0 gate for analysis of the cultured cells, as indicated by the arrow. To determine where G_1 starts, the lower limit of the gate has been set on a level with the cells in $S/G_2/M$ [17]. A histogram of DNA staining at 12 weeks is shown in (D). Note a greater proportion of cells are in $S/G_2/M$ compared to initial cultures. The proportions of cells in different phases of the cell cycle after a 12-week expansion are shown in (E)* (n = *4).*

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Table 1. Percentage of cells expressing CD34 and morphology of cultured cells

		Exp 3		Exp 4		Exp 5		Exp 6	
		G_0	G_1	G_0	G_1	G_0	G_1	G_0	G_1
% of cells expressing CD34	Week 1	ND	ND	ND	ND	86.2	38.2	45.3	35.6
	Week 2	37.9	14.7	ND	ND	16.0	11.2	16.0	9.5
	Week 4	5.5	0.1	3.7	0.6	4.8	1.0	7.9	3.0
	Week 6	1.3	0.2	4.5	0.1	4.3	0.9	3.2	0
	Week 8	2.6	0.3	2.8	0	3.1	0.1	1.8	0
% blasts	Week 4	ND	ND	ND	ND	25	9	31	5
	Week 12	18	12	13	9	8	3	10	1

$CD34^+$ data assessed by flow cytometry. The % blasts were assessed on cytospins stained with May-Grünwald/Giemsa. At week 12 there were significantly more blast cells in G_0 cultures compared to G_1 ($p < 0.05$). See also Figures 5A and 5B. (ND = not done).

total cells and progenitor cell expansion show that $CD34^+$ CB cells residing in G_0 have a higher capacity for proliferation compared to G_1, and we may speculate that, in the culture conditions used, HSC are at least maintained and are likely to undergo self-renewal, since it is extremely unlikely that the large numbers of CFC observed for up to 20 weeks are achieved in the absence of stem cells. This interpretation is also supported by previous work [21], which shows that in serum-containing cultures of $CD34^+$ CB cells showing a similar expansion of CFC over 12 weeks, nonobese diabetic/severe-combined immunodeficient (NOD/SCID) repopulating cells had increased for up to 12 weeks. Our data support the contention that expansion of the stem cell population is likely to be found in the G_0 cultures. This hypothesis is currently being tested.

The difference in numbers of progenitor cells, however, is not reflected in higher numbers of total cells. This is not surprising for two reasons: A) as in other in vitro and in vivo systems, and in chemotherapy-treated patients, total nucleated cell expansion is a less discriminatory parameter of stem cell behavior than progenitor cells [32], and B) the cytokine cocktail used does not allow full differentiation and maturation. However, morphological examination of the cells shows that there are greater numbers of macrophages and other more mature cells present in the G_1 cultures (Figs. 5A and 5B).

In our system G_0 cultures produce greater numbers of $CD34^+$ cells than G_1 cultures. *Gothot et al*. [20] saw a similar proportion of cells expressing CD34 after 2 weeks of culture of G_0 and G_1 cells, in IL-3, IL-6, and SCF, but did not examine later time points. The proportion of cells expressing CD34 was lower in their system (7.8% compared to 23.3% $CD34^+$ here at week 2 in G_0 cultures) and corresponding levels of expansion of $CD34^+$ cells were only 2.9-fold compared to 40.6-fold here, at week 2. These differences may reflect the source of HSC (BM versus CB) as it has been reported that CB usually shows a greater amplification capacity in vitro [33, 34].

BM HSCs capable of long-term repopulation of the marrow in murine experiments are mainly in the G_0/G_1 phases of the cell cycle [35, 36], as opposed to $S/G_2/M$. Recently, *Gothot et al*. described a higher frequency of long-term hemopoietic culture-initiating cells, and CFC content, of unexpanded $CD34^+$ BM and MPB cells separated into the G_0 compared to the G_1 phase of the cell cycle [20, 37]. This group subsequently went on to demonstrate that MPB $CD34^+$ G_0 cells show superiority over G_1 cells in their ability to engraft NOD/SCID mice [38], but identically selected G_0 CB cells showed only slightly higher (1.6-fold compared to G_1 cells) levels of reconstitution potential when assessed 8 weeks after transplant. Here, the differences in numbers of CFC at 6 weeks of culture between G_0 and G_1 populations are already significant ($p < 0.05$ for both GM-CFC and BFU-E). Examination of later time points in our culture confirms that, at least in vitro, cells selected for G_0 status show a much higher capacity to generate progenitor cells. It would be of interest to compare the engraftment capacity of the progeny of the cultured G_0 and G_1 CB cells.

In summary, we have shown that G_0 $CD34^+$ CB cells are capable of extensive expansion over 24 weeks and that substantial differences occur in primitive hemopoietic cells according to their cell cycle status. The extended duration over which G_0 cells are able to maintain production of progenitors and the magnitude of such production allow us to speculate that HSC may be undergoing self-renewal. We are testing this in transplantation experiments. The differences observed between cells isolated into G_0 and G_1 phases of the cell cycle suggest that as in BM [14, 20], HSC in CB reside preferentially in the G_0 phase of the cell cycle.

These results have important implications for ex vivo expansion and transplantation, and for the selection of cells for genetic manipulation. In addition, the mechanistic investigation of the phenomena relating to the balance between self-reproduction and differentiation should be feasible in this setting.

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