Evaluation of Ex Vivo Expansion Potential of Cord Blood and Bone Marrow Hematopoietic Progenitor Cells Using Cell Tracking and Limiting Dilution Analysis

By Christie M. Traycoff, Steven T. Kosak, Susan Grigsby, and Edward F. Srou"r

In the absence of conclusive assays capable of determining the functionality of ex vivo expanded human hematopoietic progenitor cells, we combined cell tracking with the membrane dye PKH2, immunostaining for CD34, and limiting dilution analysis to estimate the frequency of long-term hematopoietic culture-initiating cells (LTHC-ICs) among de novo-generated CD34+ cells. Umbilical cord blood (CB) and bone marrow (BM) CD34+ cells were stained with PKH2 on day 0 and cultured with stem cell factor (SCF) and interleukin-3 (IL-3) in short-term stromal cell-free suspension cultures. Proliferation of CD34+ cells in culture was tracked through their PKH2 fluorescence relative to day 0 and the continued expression of CD34. As such, it was possible to identify cells that had divided while maintaining the expression of CD34 (CD34-PKH2<dub>) and others that expressed CD34 but had not divided (CD34+PKH2<sup>dim</sup>). In all such cultures, a fraction of both BM and CB CD34+ cells failed to divide in response to cytokines and persisted in culture for up to 10 days as CD34+PKH2<sup>bright</sup> cells. Between days 5 and 7 of culture, CD34-PKH2<sup>bright</sup> and CD34+PKH2<sup>dim</sup> cells were sorted in a limiting dilution scheme into 96-well plates prepared with medium, SCF, IL-3, IL-6, granulocyte-macrophage colony-stimulating factor, and erythropoietin. Cells proliferating in individual wells were assayed 2 weeks later for their content of clonogenic progenitors and the percentage of negative wells was used to calculate the frequency of LTHC-ICs in each population. Among fresh isolated BM and CB CD34+ cells, the frequencies of LTHC-ICs were 2.01% ± 0.98% (mean ± SEM) and 7.56% ± 2.48%, respectively. After 5 to 7 days in culture, 3.00% ± 0.56% of ex vivo-expanded BM CD34+PKH2<sup>bright</sup> cells and 4.46% ± 1.10% of CD34+PKH2<sup>dim</sup> cells were LTHC-ICs. In contrast, the frequency of LTHC-IC in ex vivo expanded CB CD34+ cells declined drastically, such that only 3.87% ± 2.06% of PKH2<sup>bright</sup> and 2.28% ± 1.75% of PKH2<sup>dim</sup> cells were determined to be initiating cells after 5 to 7 days in culture. However, when combined with a calculation of the net change in the number of CD34+ cells in culture, the sum total of LTHC-ICs in both BM and CB cells declined in comparison to fresh isolated cells, albeit to a different degree between the two tissues. These data suggest that the number of LTHC-ICs declines among de novo-generated CD34+ cells in culture and that for a relatively long period of time a substantial number of LTHC-ICs remain unresponsive to cytokine stimulation. Furthermore, these studies demonstrate the utility of this approach in investigating the hematopoietic potential of ex vivo-expanded progenitor cells and may provide a simple in vitro assay for the assessment of engraftment potential of hematopoietic tissues.

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The conceptual use of ex vivo-expanded human bone marrow (BM) or cord blood (CB) hematopoietic stem cells (HSCs) in clinical BM transplantation (BMT) is rapidly changing from being a bold idea to becoming a possible reality. If successful, ex vivo expansion of stem cells promises to play a major role in marrow transplantation because of its potential to generate clinically relevant grafts from a limited number of stem cells. Obviously, ex vivo expansion of HSCs for this purpose can be considered successful only when progeny cells retain both the pluripotential differentiation and self-renewal capabilities of the original stem cells. In addition, ex vivo expansion of HSCs may be applied to produce large numbers of committed or terminally differentiated cells to be used as adjunct support of neutropenia after BMT or as the first step in priming HSCs for efficient retrovirus-mediated gene transfer. The lack of suitable assay systems for the evaluation of the hematopoietic potential of candidate human HSCs, or their ex vivo expanded progeny, makes the goal of realizing ex vivo expansion of stem cells rather difficult.

Attempts at expanding purified populations of HSCs have generally led to a progressive loss of self-renewal capacity coupled with increasing lineage commitment. However, a number of murine in vivo studies, as well as others in which fetal sheep as an in vivo model for human hematopoiesis were used have shown the capacity of ex vivo-expanded hematopoietic progenitor cells (HPCs) to contribute to long-term in vivo hematopoiesis. However, data gleaned from murine studies cannot be automatically translated into the human setting. On the other hand, although the use of animals as surrogates approximates human in vivo conditions, the adequacy of large animal models for such studies is limited.

We have previously postulated that the expansion of CD34+ cells or subpopulations of CD34+ cells in vitro may be used as a parameter for the evaluation of the hematopoietic potential of cultured cells. Although helpful, such an approach provides a quantitative assessment of the number of ex vivo expanded CD34+ cells but falls short of evaluating the functional properties of such cells. Among the various functional properties of fresh or cultured cells that can be investigated in vitro, determination of the frequency of long-term hematopoietic culture-initiating cells (LTHC-ICs) using a limiting dilution analysis (LDA) approach may provide the most accurate evaluation of the primitive nature of a test sample of HPCs. Although LDA assays could be applied to calculate the frequency of LTHC-ICs among ex vivo-expanded cells, our understanding of the functional capabilities of such cells may be further enhanced if the hematopoietic potential of ex vivo-expanded HSCs or their progeny can be investigated in vivo.

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etic potential of de novo-generated CD34 cells in culture could be evaluated. However, a more meaningful frequency of LTHC-ICs among de novo-generated CD34 cells may be obtained if calculated separately without any interference from original CD34 cells that remain quiescent for a period of time after cytokine stimulation.14-15

Determination of frequencies of LTHC-ICs requires the use of LDA procedures.16 The methodology for such an assay, as originally described by Sutherland et al.,17 for human HSCs is rather cumbersome because of the need of pre-established stromal cell layers. To avoid the shortcomings of stromal cell layers, we developed an LDA assay based on a modification of that originally described by Sutherland et al. and used it to study the hematopoietic potential of ex vivo-expanded CD34+ cells. To track cultured CD34+ cells, we stained fresh, isolated CD34+ cells with the membrane dye PKH2 before their use in short-term suspension cultures and observed their proliferation via the gradual loss of PKH2 fluorescence in the intensity.

In the present study, we report on the combined use of cell tracking for the isolation of de novo-generated CD34+ cells in culture and the application of our new LDA assay for the evaluation of the hematopoietic potential of these ex vivo-expanded BM and CB progenitor cells. Our data indicate that numbers of LTHC-ICs declined in culture after ex vivo expansion of HPCs. However, sizable differences were noted in the degree of LTHC-IC loss between CB and BM CD34+ cells, suggesting that more in-depth investigations of the hematopoietic potentials of ex vivo-expanded HPCs from CB and BM are needed before the eventual clinical use of such cells.

MATERIALS AND METHODS

Collection and fractionation of umbilical CB and adult BM cells. CB samples from full-term deliveries were collected in sterile tubes containing 20 U/mL of preservative-free heparin. CB samples were destined to be discarded; therefore, obtaining informed consent was an indication for 15 minutes at 37°C with 1% human serum albumin. Immediately after staining, cells were sorted as previously described18 on either a Coulter Epics 753 dual-laser (Coulter Electronics, Hialeah, FL) or a FACStar plus (BDIS) flow cytometer, both of which were equipped with Cicer high speed computers (Cytomation Inc, Fort Collins, CO). For LDA assays, an Autoclone device (Coulter Electronics) was used with the Epics 753 flow cytometer to deliver a given cell number per well. In some experiments, a multichannel pipette was used to deliver, by hand, a given number of sorted cells according to a limiting dilution scheme. Viability and purity of sorted cells always exceeded 98% and 95%, respectively.

Sorted CD34+ cells were stained with PKH2 (Sigma Immunochemicals, St Louis, MO) before their use in short-term culture, as per the manufacturer’s instructions. Briefly, cells were suspended in 1 mL of diluent (Sigma Immunochemicals) and immediately transferred into a polystyrene tube containing 1 mL of 4 x 10^7 mol/L PKH2 in diluent at room temperature. After 5 minutes of incubation with frequent agitation, 2 mL of fetal calf serum (FCS; Hyclone, Logan, UT) was added to the suspension for 1 minute. The total volume was brought up to 8 mL with Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% FCS, L-glutamine, and antibiotics (complete medium) and the cells were washed three times in complete medium. All of the complete medium ingredients (except for FCS) were obtained from BioWhitaker (Walkersville, MD). Antibiotics consisted of penicillin and streptomycin, which were added at 100 U/mL and 100 μg/mL, respectively. After the first wash, cells were transferred to a new tube. After the third wash, cells were resuspended in complete medium and cultured with cytokines as described below.

Short-term cell culture. On day 0, up to 10^6 cells/mL previously stained with PKH2 as described above were seeded in individual wells of 48-well tissue culture plates in complete medium and incubated at 37°C in 100% humidified 5% CO2 in air. At initiation and every 48 hours thereafter, cultures were supplemented with 500 ng/mL interleukin-3 (IL-3). To some cultures (as indicated in Results), 100 ng/mL of IL-6 was also added. These cytokines and cytokine combinations were chosen based on studies showing that SCF, IL-3, and IL-6 were quite effective, when used in retrovirus-mediated gene transfer studies, in inducing proliferation but not differentiation.19,20 All cytokines used in these studies were kind gifts from Amgen (Thousand Oaks, CA). Care was taken to avoid cell densities exceeding 10^6/mL. Whenever such densities were reached, cultures were split and fresh medium and cytokines were added. Cells were removed after 5 to 7 days, counted, washed, and stained with PE-conjugated CD34 for 20 minutes on ice. A sample of cultured cells not stained with any fluorochrome (except the original PKH2 from day 0) was used to determine the background PKH2 staining and to apply adequate compensation. Another sample was stained with PE-conjugated, isotype-matched, nonspecific myeloma protein and used to establish PE positivity. PKH2 fluorescence of cultured cells was compared with that of a sample fixed in 1% paraformaldehyde on day 0 immediately after staining of fresh sorted CD34+ cells to determine the green fluorescence intensity corresponding to cells that had not divided. This comparison allowed for the localization, within a dual parameter histogram (Fig 1C), of the group of CD34+ cells that have remained quiescent and are therefore CD34+PKH2+.
Fig 1. Cell tracking with PKH2 of CB CD34+ cells maintained in short-term cultures for 7 days. Fresh sorted CB CD34+ cells were stained with PKH2 and analyzed on day 0 (A) and then cultured in a short-term culture maintained with SCF and IL-3 as described in Materials and Methods. On days 3 (B), 5 (C), and 7 (D), aliquots of cultured cells were removed, stained with PE-conjugated CD34, and analyzed on day 0 (A) and then cultured in a short-term culture maintained with SCF, IL-3, and 5% CO2 and supplemented with cytokines as indicated above. Collected cells were used in hematopoietic progenitor cell assays as described below.

LDA. Fresh isolated CD34+ cells, CD34+ HLA-DR+, and CD34+ HLA-DR- cells, as well as cultured CD34+PKH2aw and CD34+PKH2aww cells, were seeded at 64, 32, 16, 8, 4, and 2 cells/well in 100 μL of complete medium supplemented with 200 ng/mL SCF, 25 ng/mL IL-3, 25 ng/mL IL-6, 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), and 2 μM erythropoietin into the wells of flat-bottomed 96-well plates. Depending on the type of cells used, only four of the cell dilutions mentioned above (starting at 64, 32, or 16) were used in one experiment. For every cell dilution, a total of 48 or 96 wells were prepared. Plates were incubated at 37°C in 100% humidified atmosphere containing 5% CO2 and fed with another 100 μL of complete medium supplemented with cytokines on day 7. After 14 days, a total of 120 μL of medium was removed from each well, followed by the addition of 150 μL of a mixture consisting of 3 parts FCS and 4 parts 3.3% methylcellulose and containing, at a final concentration, 5 × 10−5 mol/L 2-mercaptoethanol, 100 ng/mL SCF, 25 ng/mL IL-3, 25 ng/mL IL-6, 25 ng/mL GM-CSF, and 2 μM erythropoietin. Cells were mixed into the semisolid medium by gentle vortexing and the plates were incubated as described above. After 14 days, the plates were scored under an inverted microscope for the presence of burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte-macrophage (CFU-GM), or colony-forming unit-mixed (CFU-GEMM) in every well. Wells were considered positive, indicating the presence of at least one LTHC-IC in the original cell inoculum deposited into the well, only if one or more hematopoietic colonies per well were well delineated. The number of negative wells per cell dose input was then calculated and the data used in a chi-minimization assay to calculate a frequency of LTHC-ICs in a given test cell population.

In some experiments, fresh isolated CD34+ cells were seeded in limiting dilution in 200 μL of complete medium into flat-bottomed 96-well plates previously prepared with allogeneic stromal cells, as described by Brandt et al. Plates with semisolid to confluent monolayers of stromal cells were irradiated at 1,500 rad before their use. After their seeding with test cells, stromal plates were incubated for 5 weeks at 37°C in 100% humidified atmosphere containing 5% CO2 and half of the medium was removed and replenished weekly with complete medium. After 5 weeks, 120 μL of the medium was removed. Every well then received 150 μL of the same methylcellulose mixture described above and the plates were incubated for an additional 14 days under the same conditions. Scoring of positive wells and calculation of LTHC-IC frequency was performed as described above.

HPC assays. Fresh isolated cells (500 to 105/mL) or cultured cells (2 × 103 to 2 × 107/mL) were suspended in duplicates in plastic 35-mm tissue culture dishes containing 1 mL 30% FCS, 5 × 10−5 mol/L 2-mercaptoethanol, cytokines as indicated above, and 1.1% methylcellulose in IMDM. Cultures were incubated in 100% humidified 5% CO2 in air at 37°C. After 14 days, BFU-E, CFU-GM, and CFU-GEMM were enumerated using an inverted microscope according to previously reported criteria. Statistical analysis. All results are reported, where applicable, as the mean ± standard error of the mean (SEM). Statistical analysis
on groups of data was performed using two-sided t-test or the nonparametric Mann-Whitney test for independent measurements.

RESULTS

Tracking of cellular proliferation in vitro. Initial experiments were designed to examine if PKH2 could be used for tracking proliferating CD34+ cells in vitro. Figure 1 depicts an experiment in which isolated CB CD34+ cells were stained with PKH2 and then cultured for 7 days with SCF + IL-3. Aliquots of proliferating cells were removed from culture on days 3, 5, and 7 and were stained with PE-conjugated CD34. Flow cytometric analysis of these cells indicated that a gradual decline in the percentage of CD34+ cells occurred over a period of 7 days (Fig 1A, B, C, and D). Also evident in Fig 1 is the loss of PKH2 fluorescence intensity among CD34+ cells (and CD34- cells as well) such that, by day 7, the majority of CD34+ cells (>95%) displayed a "dim" PKH2 staining (Fig 1D) relevant to the intensity of PKH2 fluorescence observed on day 0 (Fig 1A). It is important to point out that a population of CD34+ cells (upper right hand quadrant in Fig 1B, C, and D) that had maintained a "bright" PKH2 fluorescence intensity did not proliferate over the 7-day period and were therefore referred to as cyto-kine nonresponsive (CRN) cells.

Although PKH2 has been previously shown to be a stable membrane dye25 with no adverse effects on progenitor cell survival and function,4 negative effects of PKH2 staining on selected CD34+ cells, if any, were examined in both progenitor cell and LDA assays. In direct comparison of unstained and PKH2-stained CB CD34+ cells, a total of 109 colonies (41 ± 0.98% BFU-E, 55 ± 1.5 CFU-GM-, and 13 ± 1.5 CFU-GEMM-derived colonies) were generated from 105 unstained cells, whereas a total of 124 colonies (49 ± 2.8 BFU-E, 58 ± 5.6 CFU-GM, and 17 ± 2.1 CFU-GEMM-derived colonies) were cloned from an equivalent number of PKH2-stained cells. Frequencies of LTHC-ICs among fresh sorted unstained and PKH2-stained CB CD34+ cells were 1.4% (with 0.9% and 1.8% as the 95% confidence limits) and 1.9% (with 1.4% and 2.4% as the 95% confidence limits), respectively. Similar observations were made in experiments involving BM CD34+ cells (data not shown). These data showed that staining with PKH2 did not adversely affect early and late progenitor cell activities in selected CD34+ cells.

Identification of the type of primitive HPCs measured by limiting dilution analysis. Definition and measurement of the frequency of the primitive HPCs identified as long-term culture-initiating cells was first introduced by Sutherland et al.6 We compared the experimental approach of Sutherland et al6 with our system as described in Materials and Methods. Figure 2 outlines data generated using BM CD34+ cells in such assays. LTHC-IC frequency in purified BM CD34+ cells measured in our stromal cell-free method was 1 per 52 cells assayed or 1.92% (with 1.58% and 2.26% as the 95% confidence limits). In a 5-week stromal cell-free assay followed by a 14-day clonogenic assay), this frequency was determined to be 1 per 55 cells or 1.81% (with 1.42% and 2.19% as the 95% confidence limits), indicating that a similar, if not identical, primitive HPC is detected by both assays. Furthermore, values for the frequency of LTHC-ICs calculated from both assays were in close agreement with those reported by Sutherland et al6 for similar cells.

Frequency of LTHC-ICs in fresh isolated CD34+ cells and CD34+ subsets. And others have previously shown that BM CD34+ HLA-DR- cells represent a population of cells highly enriched for primitive HPCs, whereas the majority of committed progenitor cells are found among BM CD34+ HLA-DR+ cells.11 We have also shown recently that the distinction between committed and primitive CB HPCs based on the expression of HLA-DR yields different results than those obtained for BM cells.17 The majority of primitive CB HPCs were found to reside within CD34+ HLA-DR+ cells and not CD34+ HLA-DR-.7 These results have been recently corroborated by Dugan et al.24 We therefore applied this new LDA assay to examine whether primitive BM (CD34+ HLA-DR+) and CB (CD34+ HLA-DR+) cells display, as expected, higher frequencies of LTHC-ICs than populations of committed progenitor cells derived from both tissues. As shown in Table 1, 7.56% ± 2.48% and 2.01% ± 0.98% (mean ± SEM) of fresh isolated CB and BM CD34+ cells were found to be LTHC-ICs, respectively. We
next analyzed fractions of CB and BM CD34+ cells identified by the expression or lack thereof of HLA-DR. Populations of both hematopoietic tissues traditionally identified as being enriched for primitive HPCs contained the highest frequencies of LTHC-ICs. Whereas 13.80% ± 3.62% of CB CD34+ HLA-DR+ cells were identified as LTHC-ICs, only 8.34% ± 3.63% of CD34+ HLA-DR- cells were found to be LTHC-ICs. A reversed trend was documented for BM CD34+ HLA-DR+ cells, with a higher frequency of such cells within the BM than among total BM CD34+ HLA-DR- cells (3.63% ± 2.44%). Table 1 shows a higher frequency of LTHC-ICs among BM CD34+ HLA-DR- cells than among total BM CD34+ cells while indicating similar frequencies of LTHC-ICs between CB CD34+ and CD34+ HLA-DR- cells. These somewhat unexpected results may be explained by the relative degree of CD34 expression on sorted cells, assuming that increased CD34 expression is associated with increased hematopoietic potential. Whereas total selected CD34+ cells (ie, those within the sorting window) included cells with "dim" CD34 expression, such cells were excluded when HLA-DR fractions of CD34+ cells were selected. Figure 3 depicts two representative experiments highlighting the differences between the plots generated from the limiting dilution analysis of data obtained from CB and BM CD34+ HLA-DR+ and CD34+ HLA-DR- cells.

**Table 1. Frequency of LTHC-ICs Among Fresh Isolated BM and CB CD34+ Cells and Their Subsets**

<table>
<thead>
<tr>
<th>Phenotype of Cells</th>
<th>Frequency of LTHC-ICs per 100 Cells of</th>
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<tbody>
<tr>
<td>CB</td>
<td>BM</td>
</tr>
<tr>
<td>Day 0 CD34+</td>
<td>7.56 ± 2.48 (8)</td>
</tr>
<tr>
<td>Day 0 CD34+ HLA-DR+</td>
<td>13.80 ± 3.62 (5)</td>
</tr>
<tr>
<td>Day 0 CD34+ HLA-DR-</td>
<td>8.34 ± 3.63 (5)</td>
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</table>

Values are the mean ± SEM. The number of separate samples analyzed for every group of cells is indicated in parentheses. Significant statistical difference (P < .05) was detected between CB CD34+ and BM CD34+ cells.

Frequency of LTHC-ICs among ex vivo-expanded CD34+ cells. To investigate whether ex vivo expansion of CD34+ cells results in the generation of progeny cells possessing functional properties attributed to primitive HPCs, a series of experiments was designed in which the frequencies of LTHC-ICs were measured for CD34+PKH2dim and CD34+PKH2bright cells and in some experiments for total CD34+ and CD34- cells. No culture-initiating cells were detected among cultured CB or BM CD34+ cells, indicating that all LTHC-ICs were restricted to those cells expressing CD34. In the case of cultured CB cells, a demonstrable decline of 49% and 70% in the frequency of LTHC-ICs was observed in PKH2bright and PKH2dim fractions of CD34+ cells, respectively (Table 2). In contrast, a 66% increase in the frequency of LTHC-ICs among marrow CD34+PKH2bright cells, accompanied by a substantially higher (146%) frequency of such cells within the PKH2dim fraction of ex vivo-expanded marrow CD34+ cells, was demonstrable. The number of CD34+ cells in culture and the frequency of LTHC-ICs detected in all assayed fractions of cultured cells were used to calculate the total number of LTHC-ICs both on day 0 and after ex vivo expansion (Table 3). Only 49% and 87% of LTHC-ICs detected in fresh isolated CB and BM CD34+ cells, respectively, were detectable after 5 to 7 days of ex vivo expansion (Table 3), suggesting that expansion of CD34- cells did not result in a net increase in the number of LTHC-ICs.

Of interest in this regard is that the ability of LTHC-ICs detected within cultured CD34+ PKH2bright cells to sustain in vitro hematopoiesis in secondary suspension long-term cultures was different from that of LTHC-ICs present within CD34+PKH2dim cells. Compared with secondary long-term cultures established with CD34+ PKH2dim cells, those initiated with CD34+ PKH2bright cells sustained the production of hematopoietic cells for a longer duration and produced...
an overall larger number of assayable clonogenic progenitors (Fig 4). Not only were CD34+ PKH2bright cells capable of generating erythroid as well as myeloid progenitors, these cells gave rise to multipotential clonogenic cells for a period of 3 weeks (Fig 4).

Another major difference between CB and BM cultures was the total number of CD34+ cells generated after ex vivo expansion and, consequently, the fold increase in such cells in vitro. The number of CD34+ cells in CB cultures was demonstrably higher after 5 to 7 days in culture (increases ranged between 140% and 886% of the original number of cells) such that a 5.24 ± 1.06-fold increase in the number of CD34+ cells over that present on day 0 was detected (Fig 5). On the other hand, a decline in the number of CD34+ cells was detected in similar BM cultures assayed at equivalent time points (Fig 5). In these cultures, only a 0.74- ± 0.19-fold increase in the total number of CD34+ cells generated in vitro was observed (Fig 5). Although it is logical to correlate the loss of CD34+ cells with an analogous loss of LTHC-ICs in BM cultures, it is not clear at present why a sizable increase in the number of CB CD34+ cells in culture was associated with a more than 50% loss of LTHC-ICs.

**DISCUSSION**

*Paramount in our inability to critically evaluate the hematopoietic potential of ex vivo-expanded human HPCs is the lack of a suitable assay system with which the primitive nature of a test population can be estimated.*

This is further complicated by the knowledge that, among cultured HPCs, some cells remain quiescent and, as such, may constitute the fraction of cells containing the majority of detectable LTHC-ICs, a phenomenon that may falsely suggest the generation of LTHC-ICs in vitro. To avoid this and to ensure that in vitro-generated CD34+ cells can be evaluated separately, we tracked cells in culture using PKH2 and estimated, the hematopoietic potential of ex vivo-expanded progenitors, the accuracy and adequacy of such an assay had to be verified. To that end, we estimated the frequency of LTHC-ICs in populations of BM and CB cells previously shown to contain more committed progenitor cells, namely marrow CD34+ HLA-DR-24,27,30 and CB CD34+ HLA-DR+17,32 cells. When assayed using our new procedure, these two populations of HPCs contained higher frequencies of LTHC-ICs than populations previously shown to contain more committed progenitor cells (marrow HLA-DR+31 and CB HLA-DR-17 fractions of CD34+ cells). These findings therefore validated the accuracy of this LDA assay and its applicability for the measurement of primitive progenitor cells, specifically, in this case, LTHC-ICs. In addition, LTHC-IC frequencies determined in stromal cell-based assays and in this new approach were very similar (Fig 2). Furthermore, values of LTHC-ICs for BM CD34+ HLA-DR+ and CD34+ HLA-DR- cells reported in this study were in close agreement with those reported by Sutherland et al for similar phenotypes of BM cells. These data indicated that similar LTHC-IC frequencies were determined in both assays, suggesting that committed progenitor cells are depleted in shorter periods of time (2 weeks) in suspension cultures than in stromal cell based cultures (5 weeks), possibly because of the high degree of cellular proliferation in stromal cell-free suspension cultures. A similar approach to the LDA method described here was previously implemented by Brandt et al to examine the in vitro survival of pre-colony-forming cells, a group of primitive HPCs, most likely very similar in their functional properties to LTHC-ICs. However, it is important to point out that, although similar frequencies of LTHC-ICs were determined with both the stromal cell-based and stromal cell-free LDA assays, it is possible that these two procedures may not be measuring the same primitive HPCs, but, instead, two classes of HPCs present at similar frequencies.

The use of PKH2 for cell tracking allowed us to identify, in culture, a group of cells that failed to respond to cytokine stimulation, referred to here as CNR cells. Such cells (CD34+ PKH2bright) were present in culture for a number of days regardless of the degree of ex vivo expansion achieved during this time period. Such cells have been previously reported in studies examining ex vivo expansion of BM cells as well as mobilized peripheral blood cells. Given the deep dormant state of these cells, it is possible that they represent a very primitive subpopulation of candidate HSCs, which for an extended period of time remain CNR. In fact, the persistence of LTHC-ICs among CNR cells supports this notion and suggests that continued in vitro hematopoiesis in long-term cultures may be supported by the slow recruitment of LTHC-ICs from within CNR cells in a process analogous to the in vivo situation. This is best exemplified by the ability of PKH2bright cells to support in vitro hematopoiesis in sec-

**Table 2. Frequency of LTHC-ICs Among Fractions of Ex Vivo-Expanded BM and CB CD34+ Cells**

<table>
<thead>
<tr>
<th>Phenotype of Cells</th>
<th>CB Frequency of LTHC-ICs per 100 Cells</th>
<th>Percent Change†</th>
<th>BM Frequency of LTHC-ICs per 100 Cells</th>
<th>Percent Change†</th>
</tr>
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<tbody>
<tr>
<td>Day 0 CD34+</td>
<td>7.56 ± 2.49* (8)</td>
<td>—</td>
<td>2.01 ± 0.96 (6)</td>
<td>—</td>
</tr>
<tr>
<td>Day 5 to 7 CD34+PKH2bright</td>
<td>3.87 ± 2.06 (3)</td>
<td>−49%</td>
<td>3.00 ± 0.56 (4)</td>
<td>+66%</td>
</tr>
<tr>
<td>Day 5 to 7 CD34+PKH2dim</td>
<td>2.29 ± 1.75 (3)</td>
<td>−70%</td>
<td>4.46 ± 1.10 (4)</td>
<td>+146%</td>
</tr>
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</table>

* The number of separate samples analyzed for every group of cells is indicated in parentheses.
† Values are the mean ± SEM.
‡ Percent change refers to the relative decrease or increase in the frequency of LTHC-ICs when compared with day 0 values.
Table 3. Absolute Numbers of LTHC-ICs Detected in Fresh Isolated and Ex Vivo-Expanded CB and BM CD34+ Cells

<table>
<thead>
<tr>
<th>Phenotype of Cells</th>
<th>CB</th>
<th>BM</th>
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<tbody>
<tr>
<td></td>
<td>No. of LTHC-ICs in Culture</td>
<td>No. of LTHC-ICs in Culture</td>
</tr>
<tr>
<td>Day 0 CD34+</td>
<td>40,055 ± 15,826</td>
<td>23,376 ± 9,157</td>
</tr>
<tr>
<td>Day 5 to 7 CD34+</td>
<td>20,947 ± 13,902</td>
<td>16,893 ± 5,337</td>
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Numbers of LTHC-ICs detected on day 0 are reported as mean ± SEM calculated from CB cultures initiated with $3.40 \times 10^5 \pm 1.50 \times 10^5$ (n = 3) and from BM cultures initiated with $9.50 \times 10^5 \pm 4.01 \times 10^5$ cells (n = 3). Percent decline refers to the relative decrease in the total number of LTHC-ICs when compared with day 0 values. Percent decline in the number of LTHC-ICs reported in the text and shown above (51% for CB and 13% for BM) were calculated as the mean ± SEM of percent decline values obtained from single experiments and not from mean values of numbers of LTHC-ICs shown above for day 0 and day 5 to 7 CD34+ cells.

Of interest in these studies is that, although the frequency of LTHC-ICs among newly formed BM CD34+ cells (CD34+PKH26+) was higher than that documented for fresh isolated BM CD34+ cells, a 70% decline in the frequency of LTHC-ICs among de novo-generated CB CD34+ cells was observed. These findings may be explained by two separate theories. First, it is possible that, contrary to what has

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**Fig 4.** A representative experiment depicting the production of total cells (upper left panel), BFU-E (upper right panel), CFU-GM (lower left panel), and CFU-GEMM (lower right panel) in secondary long-term cultures initiated from CD34+PKH26+ (■) and CD34+PKH26+ (○) cells isolated on day 5 from ex vivo-expanded cultures of CB CD34+ cells. Cultures were maintained by the addition every 48 hours of 500 ng/mL SCF, 100 ng/mL IL-3, and 100 ng/mL IL-6. Every week, half of the cultured cells were removed and assayed for clonogenic progenitor cells as described in Materials and Methods. The total production of clonogenic progenitors at every time point was calculated using the formula $X = (\text{number of clonogenic cells per culture} \times (1/2)^n)$, where $X$ is the number of total colonies in culture and $n$ is equal to the number of previous demidepopulations.
be proposed, marrow HPCs possess a higher potential to undergo ex vivo expansion than CB CD34+ cells. Alternatively, it may be possible that because of the enhanced proliferative response of CB CD34+ cells and their ability to exit G0/G1 phases of cell cycle quicker than BM CD34+ cells, a rapid decline in the frequency of LTHC-ICs among cultured CB cells may occur over a period of 5 to 7 days. The enhanced proliferative capacity of CB cells is demonstrated by their superior fold expansion of CD34+ cells (Fig 5) over BM cells, an observation that indicates that the number of cellular divisions through which CB cells have been is higher than that for BM cells. Such an explanation suggests that the number of divisions of cultured CD34+ cells, rather than the duration of the culture, may be the critical parameter influencing the maintenance or expansion of LTHC-ICs in vitro. Preliminary studies in our laboratory aimed at investigating this issue seem to favor the second hypothesis (E.F. Srou, unpublished observations). If this postulate is correct, then any documented advantage in the ability of BM cells to generate LTHC-ICs in culture over CB cells would be negated if CD34+ cells from both tissues undergoing an equal number of cell divisions in vitro are compared head to head.

The overall number of LTHC-ICs among ex vivo expanded BM and CB CD34+ cells was lower than that derived from fresh isolated cells, even though, in the case of CB cells, an increase in the absolute number of CD34+ cells in culture was documented. Previously, we reported that, when using a highly purified population of HPCs, the number of CD34+ HLA-DR- cells increases in vitro and suggested that such an observation, accompanied with a documented increase in the number of assayable high proliferative potential colony-forming cells, may constitute a valid measurement of ex vivo expansion. In a similar approach, Lansdorp and Dragowska demonstrated the maintenance but not the expansion of CD34+ cells in the initial phenotype of CD34+ CD45RA- CD71+. In the murine system, a substantial increase in the number of cultured cells expressing the phenotype of the original cell input (Sca-1+ Lin- WGA+) was documented, even though in vivo reconstituting data indicated that only maintenance of long-term repopulating cells and not expansion was accomplished in such cultures. Some-what contradictory results have been recently reported when Li and Johnson showed that survival, but not the increase, of murine HSCs occurred in culture. Even though only SCF was used in the studies of Li and Johnson, which allowed for only a minimal increase in cell number, reconstitution of lethally irradiated mice indicated that no net increase in the number of HSCs occurred in culture. Taken together, these observations suggest that, although helpful, monitoring ex vivo expansion of HPCs through the assessment of cells with the original phenotype only, especially in the case of CB, may not be accurate. In the absence of an in vivo assay for human stem cells, it becomes essential to assay simultaneously as many different functional properties attributable to HSCs as possible to evaluate the hematopoietic potential of ex vivo-expanded human HPCs.

We have previously demonstrated a substantial increase in the number of CD34+ cells generated in vitro over a period of 21 days in culture. Although such a fold increase in the number of BM CD34+ cells may be viewed as contradictory to our current results, two important differences between our previous and present data should be stressed. First, Srou et al used a highly purified population of BM CD34+ HLA-DR- CD15+ rhodamine 123dim cells, thus eliminating committed CD34+ cells that could quickly lose CD34 expression in vitro. Second, the previously reported increase in the number of CD34+ cells was accumulated over a period of 21 days. In fact, during the first 7 days of culture, a minimal increase in the number of CD34+ cells was observed. Given these, and results from other laboratories, it therefore appears that substantial increases in total number of CD34+ cells are observed in cultures maintained for periods of time exceeding 10 days, whereas shorter periods of incubation result in minimal increases in the number of CD34+ cells in cultures initiated with BM or peripheral blood CD34+ cells or their subsets.

Documentation of the potential of ex vivo-expanded human HSCs to sustain long-term engraftment remains elusive. Our laboratory has demonstrated the capacity of such cells to engraft in an animal model, albeit to a limited degree compared with fresh isolated cells. However, in the murine system, the capacity of ex vivo-expanded HPCs to contribute to long-term in vivo hematopoiesis has been clearly docu-
ment.

Our present findings appear to shed some light on a rather controversial parameter usually used for the assessment of ex vivo expansion of hematopoietic progenitor cells, namely the long-term generation of clonogenic cells in culture. A number of laboratories, including ours, have previously implied that production of assayable progenitor cells in long-term cultures could be viewed as a direct evaluation of ex vivo expansion. Although such evaluations reflect the ability of LTHC-ICs to give rise to assayable progenitor cells, they certainly do not measure the production of functional LTHC-ICs in vitro. In fact, our present data indicate that, even though an increase in the number of CFU-GM could be achieved in 5 to 7 days (data not shown), the number of LTHC-ICs in these cultures decreased over the same period of time, suggesting that the enumeration of progenitor cells may not be an appropriate method for the assessment of successful ex vivo expansion.

Given the fact that cellular proliferation is a prerequisite for retroviral-mediated gene transfer, it is essential when transducing hematopoietic stem cells to achieve a minimal degree of cell division without inducing differentiation. Induction of cellular proliferation has been traditionally accomplished through the use of cytokines, especially in the case of primitive BM progenitors. Data presented here seem to cast doubt on our ability to stimulate proliferation of primitive HPCs without incurring major losses in the numbers of LTHC-ICs. On the other hand, it also appears that a major compartment of cultured LTHC-ICs, namely CNR cells, do not respond to cytokine stimulation and, as such, may remain resistant to infection by retroviruses. However, our results may be instructive in tailoring strategies aimed at increasing the efficiency of retroviral-mediated gene transfer into primitive HPCs.

In conclusion, these studies describe a combination of two fairly simple approaches (cell tracking and LDA) with which it was possible to examine the hematopoietic potential of ex vivo-expanded HPCs. Identifying a reliable in vitro assay capable of evaluating the marrow repopulating potential of candidate HSC populations or ex vivo-expanded progenitor cells remains controversial. Given the heterogeneity of selected CD34+ cells, the preferential response of classes of ex vivo-expanded CD34+ cells to cytokines and the differences in the functional properties of CD34+ cells derived from different hematopoietic tissues, our data suggest that the use of LDA coupled with ways capable of distinguishing between the different groups of ex vivo-expanded CD34+ cells may be one of the most informative assays suitable for the examination of the functional properties of ex vivo-expanded HSCs. Furthermore, these studies suggest that more detailed investigations of the nature and hematopoietic potentials of ex vivo-expanded HPCs are needed before the potential use of such cells in the clinic.

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Evaluation of ex vivo expansion potential of cord blood and bone marrow hematopoietic progenitor cells using cell tracking and limiting dilution analysis

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