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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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.

Determination of frequencies of LTHC-ICs requires the use of LDA procedures. The methodology for such an assay, as originally described by Sutherland et al., 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 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 obtained from CB and BM are needed before the eventual clinical use of such cells.

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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 deemed unnecessary. Human BM aspirates were obtained from normal adult volunteers after informed consent was obtained according to guidelines established by the Human Investigation Committee of the Indiana University School of Medicine. Low-density CB and BM cells were separated over Ficoll/Hypaque (Pharmacia, Piscataway, NJ) and then fractionated by immunomagnetic selection to obtain CD34+ cells, as previously described. When 6 x 10^6 or less low-density CB cells were recovered from density centrifugation, these cells were stained and sorted directly without intermediate fractionation. For immunomagnetic selection, cells were first incubated on ice with 0.5 µg/mL chymopapain. The enzymatic reaction was stopped by the addition of cold media, and the released CD34+ cells were removed after collecting the beads using a permanent magnet. All reagents for the immunomagnetic separation procedure were kindly provided by Baxter Healthcare (Santa Ana, CA).

Immunofluorescence staining and flow cytometric cell sorting. Low-density CB cells and immunomagnetically enriched BM or CB CD34+ cells were stained on ice for 20 minutes with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (SG12 MoAb; kind gift from Baxter Healthcare). When needed, phycoerythrin (PE)-conjugated HLA-DR (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) was added to the staining protocol to yield HLA-DR+ and HLA-DR- fractions of CD34+ cells. Control MoAbs consisted of fluorochrome-conjugated, isotype-matched, nonspecific myeloma proteins. Cells were washed and resuspended for flow cytometric cell sorting in phosphate-buffered saline (PBS) supplemented with 1% human serum albumin. Immediately after staining, cells were sorted as previously described on either a Coulter Epics 753 dual-laser (Coulter Electronics, Hialeah, FL) or a FACStar plus (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) flow cytometer, both of which were equipped with Cicer high speed computers (Cytomation Inc, Fort Collins, CO). For LDA assays, an Autoloc 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 polypropylene tube containing 1 mL of 4 x 10^7 mL 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 used 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 stem cell factor (SCF) and 100 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. All cytokines used in these studies were kind gifts from Amgen (Thousand Oaks, CA). Care was taken to avoid cell densities exceeding 10^4/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+PKH2low. After
determining the relative size of this group of cells, a group of approxi-

mately equal size of CD34+ cells displaying low-intensity PKH2
fluorescence was identified as CD34+PKH2int (Fig 1C). In all experi-
ments, CD34+PKH2mwh cells constituted less than 5% of total cells
(Fig 1C) and therefore both populations of CD34+PKH2int and CD34+PKH2mwh were smaller than 5% of total cultured cells.

Long-term cell cultures. Secondary long-term cultures of
CD34+PKH2mwh and CD34+PKH2mwh cells sorted on day 5 to 7 from cultured primary cells were established in 1 mL of complete
medium in flat-bottomed 48-well plates, as previously de-
scribed. At initiation and every 48 hours thereafter, secondary
cultures were supplemented with 300 ng/mL SCF, 100 ng/mL IL-3,
100 ng/mL IL-6, 10 ng/mL granulocyte-macrophage colony-stimu-

lating factor (GM-CSF), and 2 U/mL erythropoietin. Cells were
removed by demidepopulating the medium followed by the
addition of fresh medium and supplementation with cytokines.

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+PKH2mwh and
CD34+PKH2mwh 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 U/mL eryth-
ropoietin 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 supple-
mented 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 U/mL 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 de-
tected. The number of negative wells per cell dose input was then
calculated and the data used in a chi-minimization assay to cal-

culate 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 semicofluent to conflu-
en 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 methylcellu-
lose 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 de-
scribed above.

HPC assays. Fresh isolated cells (500 to 10^5/mL) or cultured
cells (2 × 10^4 to 5 × 10^7/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% humidi-

fied 5% CO2 in air at 37°C. After 14 days, BFU-E, CFU-GM, and
CFU-GEMM were enumerated using an inverted microscope ac-
cording 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 cytokine nonresponsive (CNR) cells.

Although PKH2 has been previously shown to be a stable membrane dye\(^\text{25}\) with no adverse effects on progenitor cell survival and function,\(^\text{4}\) 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 BFU-E-, 55 ± 1.5 CFU-GM-, and 13 ± 1.5 CFU-GEMM-derived colonies) were generated from 103 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.\(^\text{10}\) We compared the experimental approach of Sutherland et al.\(^\text{10}\) 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 5-week stromal cell-based assay this frequency was determined to be 1.81% (with 1.42% and 2.19% as the 95% confidence limits).

Fig 2. Limiting dilution analysis of data from a representative experiment in which decreasing numbers of fresh isolated BM CD34+ cells were plated over pre-established irradiated stromal cells (○) for 5 weeks or in suspension (■) for 14 days in flat-bottomed 96-well plates. At the end of the respective culture period, all wells were overlaid with semisolid medium as described in Materials and Methods. The percentage of negative wells was then calculated 2 weeks later after all individual wells were scored for the presence of hematopoietic colonies. Care was taken to exclude from calculation any stromal cell-containing well that at the end of the initial culture period did not contain a semiconfluent monolayer of stromal cells. In this experiment, the LTHC-IC frequency measured by the stromal cell-free method was 1.92% (with 1.58% and 2.26% as the 95% confidence limits) and in the 5-week stromal cell-based assay this frequency was determined to be 1.81% (with 1.42% and 2.19% as the 95% confidence limits).

Frequency of LTHC-ICs in fresh isolated CD34+ cells and CD34- subsets. We 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.\(^\text{11-26}\) We have also shown recently that the distinction between committed and primitive BM HPCs based on the expression of HLA-DR yields different results than those obtained for BM cells.\(^\text{17,26}\) The majority of primitive CB HPCs were found to reside within CD34+HLA-DR+ cells and not CD34+HLA-DR-.\(^\text{7}\) These results have been recently corroborated by Dugan et al.\(^\text{22}\) 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
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>
<th>CB</th>
<th>BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 CD34+</td>
<td>7.56 ± 2.48 (8)</td>
<td></td>
<td></td>
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<tr>
<td>Day 0 CD34+ HLA-DR</td>
<td>13.80 ± 3.62 (5)</td>
<td></td>
<td></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 each group of cells is indicated in parentheses. Significant statistical difference (P < .05) was detected between CB CD34+ and BM CD34+ cells.

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 cells in that a higher frequency of LTHC-ICs (5.43% ± 2.44%) was detected in CD34+ HLA-DR+ cells than in CD34+ HLA-DR- cells (3.36% ± 2.22%). Table 1 shows a higher frequency of LTHC-ICs among BM CD34+ HLA-DR+ cells than among total 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.

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+PKH2high 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 PKH2high and PKH2dim fractions of CD34+ cells, respectively (Table 2). In contrast, a 66% increase in the frequency of LTHC-ICs among marrow CD34+PKH2high 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+PKH2high 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+PKH2high 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, using a new LDA assay, the frequency of LTHC-ICs among ex vivo-generated CD34+ cells.

Before the use of a newly designed assay to evaluate the hematopoietic potential of ex vivo-expanded progenitors, the accuracy and adequacy of such an assay had to be verified. We hypothesized that, if data generated from the use of this assay corroborated previous findings regarding the phenotypic nature of primitive CB and BM HPCs, the usefulness and accuracy of this assay could be established. To that end, we estimated the frequency of LTHC-ICs in populations of BM and CB cells previously shown to be enriched for primitive HPCs, 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 al9 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 al10 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 cells14 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</th>
<th>BM</th>
<th>Percent Change</th>
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<tbody>
<tr>
<td>Frequency of LTHC-ICs per 100 Cells</td>
<td>Frequency of LTHC-ICs per 100 Cells</td>
<td>Percent Change</td>
<td></td>
</tr>
<tr>
<td>Day 0 CD34+</td>
<td>7.56 ± 2.49 (8)</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>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>4.46 ± 1.10 (4)</td>
<td>+146%</td>
</tr>
</tbody>
</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.

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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 No. of LTHC-ICs in Culture</th>
<th>Percent Decline</th>
<th>BM No. of LTHC-ICs in Culture</th>
<th>Percent Decline</th>
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<tr>
<td>Day 0 CD34+</td>
<td>40,055 ± 15,826</td>
<td>—</td>
<td>23,376 ± 9,157</td>
<td>—</td>
</tr>
<tr>
<td>Day 5 to 7 CD34+</td>
<td>20,947 ± 13,902</td>
<td>51%</td>
<td>16,893 ± 5,337</td>
<td>13%</td>
</tr>
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Numbers of LTHC-ICs detected on day 0 are reported as mean ± SEM calculated from CB cultures initiated with $3.40 \times 10^5$ ± $1.50 \times 10^5$ (n = 3) and from BM cultures initiated with $9.50 \times 10^5$ ± $4.01 \times 10^5$ 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.

Secondary long-term cultures (Fig 4). The likelihood of such a process of maintenance of in vitro hematopoiesis has been previously examined and found to be possible.\(^1\) However, if this speculation were true, our data show that self-renewal among these cells is markedly diminished, or eliminated, under these culture conditions, even in the absence of demonstrable cellular proliferation.

Of interest in these studies is that, although the frequency of LTHC-ICs among newly formed BM CD34+ cells (CD34+PKH2\textsuperscript{dim}) 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+PKH2\textsuperscript{dim} (○) and CD34+PKH2\textsuperscript{bright} (●) 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.
been 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 cells with 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. Somewhat 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-
mented.\textsuperscript{3-7} Given the confirmed persistence of nonproliferating HSCs in culture,\textsuperscript{14,15} it might be possible that the capability of ex vivo-expanded cells to contribute to long-term hematopoiesis may be derived from CNR cells and not from de novo-generated progeny HPCs. Evidence for this contention may be gleaned from the fact that ex vivo-expanded cells failed to engraft more animals than was possible with the same number of fresh cells used to initiate the culture,\textsuperscript{7} indicating maintenance rather than augmentation of the marrow repopulating potential of expanded cells. However, such an argument falls short of reconciling between detecting LTHC-ICs among de novo-generated CD34\textsuperscript{+} cells in culture and the allegation that only CNR cells are capable of sustaining in vivo engraftment, unless two distinct classes of LTHC-ICs were enumerated in PKH2\textsuperscript{blue} and PKH2\textsuperscript{red} fractions of cultured cells.

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.\textsuperscript{4-17,21,30,39-41} 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.\textsuperscript{42,43} 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\textsuperscript{+} cells, the preferential response of classes of ex vivo-expanded CD34\textsuperscript{+} cells to cytokines and the differences in the functional properties of CD34\textsuperscript{+} 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\textsuperscript{+} 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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