Functional Heterogeneity of Human CD34+ Cells Isolated in Subcompartments of the G0/G1 Phase of the Cell Cycle

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Using simultaneous Hoechst 33342 (Hst) and Pyronin Y (PY) staining for determination of DNA and RNA content, respectively, human CD34+ cells were isolated in subcompartments of the G0/G1 phase of the cell cycle by flow cytometric cell sorting. In both bone marrow (BM) and mobilized peripheral blood (MPB) CD34+ cells, primitive long-term hematopoietic culture-initiating cell (LTHC-IC) activity was higher in CD34+ cells isolated in G0 (G0/CD34+ cells) than in those residing in G1 (G1/CD34+ cells). However, as MPB CD34+ cells displayed a more homogeneous cell-cycle status within the G0/G1 phase and a relative absence of cells in late G1, RNA fractionation was less effective in segregating LTHC-IC in MPB than in BM. BM CD34+ cells belonging to four subcompartments of increasing RNA content within the G0/G1 phase were evaluated in functional assays. The persistence of CD34 expression in suspension culture was inversely correlated with the initial RNA content of test cells. Multipotential progenitors were present in G0 or early G1 subcompartments, while lineage-restricted granulomonocytic progenitors were more abundant in late G1. In vitro hematopoiesis was maintained for up to 6 weeks with G0/CD34+ cells, whereas production of clonogenic progenitors was more limited in cultures initiated with G1/CD34+ cells. To test the hypothesis that primitive LTHC-ICs would reenter a state of relative quiescence after in vitro division, BM CD34+ cells proliferating in ex vivo cultures were identified from their quiescent counterparts by a relative loss of membrane intercalating dye PKH2, and were further fractionated with Hst and PY. The same functional hierarchy was documented within the PKH2bright population whereby LTHC-IC frequency was higher for CD34+ cells reisolated in G0 after in vitro division than for CD34+ cells reisolated in G1, or in S/G2 + M. However, the highest LTHC-IC frequency was found in quiescent PKH2bright CD34+ cells. Together, these results support the concept that cells with distinct hematopoietic capabilities follow different pathways during the G0/G1 phase of the cell cycle both in vivo and during ex vivo culture.

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THE CELL-CYCLE STATUS and responsiveness to in vitro cytokine stimulation is critical in defining strategies for hematopoietic progenitor/stem cell expansion1-3 and somatic gene therapy.4,5 It is generally believed that primitive hematopoietic progenitor cells (HPCs) lie dormant within the bone marrow (BM) microenvironment.6-8 Supporting this hypothesis is evidence that primitive HPCs, unlike committed progenitors, display a latency period before they respond to cytokine-driven cell-cycle activation5-11 and that they are resistant to exposure to antimitotic drugs like 5-fluorouracil.12-14 Cell-cycle analysis and sorting by direct measurement of DNA content with Hoechst 33342 (Hst) has been used to compare the hematopoietic function of cells in G0/G1 versus those in S/G2 + M phases.15 However, such a static “snapshot” measurement does not distinguish quiescent G0 cells from those in G1 that may represent cell populations with distinct hematopoietic capabilities. Although the suicide techniques using 'H-thymidine16-18 or cytosine arabinoside19 are dynamic procedures capable of determining the relative proportion of resting versus cycling HPCs, they rely on selective killing of actively cycling cells, therefore preventing their functional characterization. This prompted us to evaluate the use of multiparameter flow cytometry methods applicable to viable CD34+ cells and potentially capable of discriminating between subcompartments of the G0/G1 phase of the cell cycle.

The ability to correlate the DNA content of individual cells to other cell constituents such as total RNA or proteins has led to the description of different kinetic compartments within the traditional phases of the cell cycle. In particular, subcompartments of the G0/G1 phase were described based on simultaneous DNA/RNA determination.20-23 Cells with low RNA content maintain a state of deep dormancy in G0. As cells enter G1, they accumulate RNA, mainly in the form of ribosomal RNA, until they reach S phase. In a variety of cell systems, the rate of progression of individual cells through the cell cycle was found to be correlated with RNA content.24 Although Lajtha25 originally coined the term G0 to describe dormant hepatic cells responding to liver insult, other cell types residing in quiescent mitotic state characterized by a lower RNA content than G1 cells have been referred to as G0 or G0 cells.22 In a recent study from our laboratory, simultaneous DNA/RNA staining of BM CD34+ cells was used successfully to isolate, by flow cytometry, quiescent and primitive HPCs in the G0 phase of the cell cycle.26 The possibility to isolate viable HPCs with distinct DNA/RNA content was used for the present study to compare hematopoietic function of CD34+ cells isolated in subcompartments of the cell cycle.

We have previously shown10 that cytokine nonresponsive (CNR) cells, which resist initial cytokine stimulation and survive in culture for up to 7 days, are enriched for primitive
HPCs, while those undergoing in vitro proliferation appear to gradually lose their hematopoietic potential. We hypothesized that if proliferating cells were to maintain primitive function, they would reenter after cell division into a quiescent state characterized by minimal RNA content. Experiments were performed to investigate whether primitive HPCs could be reisolated from ex vivo expansion cultures by cell-cycle fractionation.

In the present studies, we assayed the hematopoietic function of CD34+ cells isolated from distinct subcompartments of the G0/G1 phase of the cell cycle by DNA/RNA staining. Such CD34+ cell subsets were evaluated in steady-state BM, in mobilized peripheral blood (MPB), and in the context of ex vivo expansion. Our results demonstrate that major functional differences could be attributed to the position of these cells in the G0/G1 phase, and suggest that primitive and committed progenitor cells follow different pathways in the G0/G1 phase of the cell cycle.

MATERIALS AND METHODS

CD34+ cell purification. BM and MPB samples were obtained from healthy adult volunteers according to guidelines established by the Human Investigation Committee of the Indiana University School of Medicine. Mobilization was achieved by daily G-CSF administration at 5 μg/kg (maximum, 480 μg/d) for 4 consecutive days. MPB cells were collected by apheresis on day 5. Low-density mononuclear cells from both tissues were isolated by centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and were enriched for CD34+ cells by immunomagnetic selection as previously described. All reagents for the immunomagnetic separation procedure were a generous gift from Baxter Healthcare (Santa Ana, CA). Immunomagnetically selected CD34+ cells were labeled with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 antibody (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) or in some cases with allophycocyanin (APC)-conjugated anti-CD34 antibody (Caltag, San Francisco, CA) to allow for further staining with FITC-conjugated anti–Ki-67 antibody. Total BM or MPB CD34+ cells were isolated on a FACStar Plus flow cytometer (BDIS). For some of the MPB samples in which CD34+ cell purity was greater than 90% after immunomagnetic selection, the flow cytometry purification step was omitted.

Cell-cycle fractionation with Hst and Pyronin Y. Total CD34+ cells were resuspended at 1 to 2 × 10^5 cells in 1.5 mL 1.67-μmol/L solution of Hst (Molecular Probes, Eugene, OR) in Hst buffer. Hst buffer consisted of Hanks’ balanced salt solution (Biowhittaker, Walkersville, MD), 20 mmol/L HEPES (Biowhittaker), 1 g/L glucose, and 10% fetal calf serum (IFCS; Hyclone, Logan, UT). After incubation at 37°C for 45 minutes, samples were run on a FACScan flow cytometer (BDIS). For some of the MPB samples in which CD34+ cell purity was greater than 90% after immunomagnetic selection, the flow cytometry purification step was omitted.

Cell-cycle fractionation with Hst and Pyronin Y. Total CD34+ cells were resuspended at 1 to 2 × 10^5 cells in 1.5 mL 1.67-μmol/L solution of Hst (Molecular Probes, Eugene, OR) in Hst buffer. Hst buffer consisted of Hanks’ balanced salt solution (Biowhittaker, Walkersville, MD), 20 mmol/L HEPES (Biowhittaker), 1 g/L glucose, and 10% fetal calf serum (IFCS; Hyclone, Logan, UT). After incubation at 37°C for 45 minutes, samples were run on a FACScan flow cytometer (BDIS).

Analysis of ex vivo expansion with PKH2 cell tracking and cell-cycle fractionation. A total of 0.5 to 1 × 10^5 CD34+ cells were labeled with the membrane dye PKH2 (Sigma) for cell tracking per the manufacturer’s instructions. Although PKH26 (emission at 575 nm) has been reported to be a more reliable indicator of cell proliferation, PKH2 (emission at 550 nm) was used in this study to allow simultaneous use with PY. After staining, cells were counted and plated in 24-well plates at a density of 2 × 10^5 cells/mL in complete medium supplemented with IL-3, IL-6, and SCF, each at 100 ng/mL. Every week, half of the cells were removed, followed by replacement with fresh medium and cytokines. Aliquots of harvested cells were assayed for progenitor cell content in 1.3% methylcellulose, 30% FCS, 100 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-6, 5% FCS, 100 ng/mL FBS, 10 ng/mL IL-7, 10 ng/mL IL-11, 5 ng/mL GM-CSF, and 2 U/mL erythropoietin (EPO). Hematopoietic colonies were scored 2 weeks later according to standard criteria.

Persistence of CD34 expression in culture. After 2 weeks of long-term culture, aliquots of cultured cells were harvested, washed, and resuspended in 100 μL mouse serum (Sigma). After a 10-minute incubation at room temperature, FITC-conjugated anti-CD34 antibody or an isotype-matched control (BDIS) were added to the cell suspension. Staining was performed for 20 minutes on ice, after which cells were washed and analyzed on a FACScan flow cytometer. CD34 expression was measured only on cells with low forward- and side-scatter properties, and was defined as cells displaying a fluorescence greater than 99% of the isotype control.

Analysis of ex vivo expansion with PKH2 cell tracking and cell-cycle fractionation.

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Fig 1. General method of cell-cycle fractionation using DNA and RNA analysis with Hst and PY. (A) Dual-parameter dot plot showing analysis of BM CD34<sup>+</sup> cells stained with Hst and PY. Cells residing in G<sub>0</sub> (low PY uptake) appear at the bottom of the G<sub>0</sub>/G<sub>1</sub> region identified by low Hst staining. Cells in G<sub>1</sub> have a brighter PY signal within the G<sub>0</sub>/G<sub>1</sub> peak. Cells in S/G<sub>2</sub>/M display both high Hst and high PY staining. (B) Postsort analysis of BM CD34<sup>+</sup> cells sorted in G<sub>0</sub>. (C) Hst/PY fluorescence distribution of G<sub>0</sub> CD34<sup>+</sup> cells shown in B after 24 hours under IL-3-IL-6-SCF stimulation, showing G<sub>0</sub>-G<sub>1</sub> progression. (D) Same sample after 72 hours in culture, depicting cells moving into S/G<sub>2</sub>/M phase.

fore lost part of their original PKH2 fluorescence. Sorted CD34<sup>+</sup> PKH<sup>2</sup><sup>dim</sup> cells were further stained with Hst and PY as already described, and were sorted in a second step into cells residing in G<sub>0</sub>, G<sub>1</sub>, or S/G<sub>2</sub>/M.

Limiting dilution analysis of long-term hematopoietic culture-initiating cells. LTHC-IC frequencies were determined by a stroma-free limiting dilution analysis as described previously. LTHC-IC frequencies were calculated using Poisson statistics.

Growth factors. Human recombinant IL-3, IL-6, SCF, and GM-CSF were a kind gift from Amgen (Thousand Oaks, CA). Human recombinant EPO was obtained from Amgen.

Statistics. Comparisons of cell populations isolated in various phases of the cell cycle were made by analysis of variance (ANOVA). If P (ANOVA) was less than .05, pairwise multiple comparisons were made using Student-Newman-Keuls (SNK) tests. When only two groups were to be compared, Student's t-tests were used. All P values are two-sided. SigmaStat 2.0 software (Jandel, San Rafael, CA) was used for all calculations.

RESULTS

Fractionation of CD34<sup>+</sup> cells into subcompartments of the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle. Traditional cell-cycle analysis with a DNA probe such as Hst distinguishes between cells in the S/G<sub>2</sub>/M phase and those in the G<sub>0</sub>/G<sub>1</sub> phase. We have recently described the successful isolation of CD34<sup>+</sup> cells in the G<sub>0</sub> phase of the cell cycle by simultaneous DNA/RNA staining. In this method, cells determined to be in the G<sub>0</sub>/G<sub>1</sub> phase based on Hst fluorescence distribution can be further fractionated into subcompartments of varying cellular RNA content by staining with PY. Quiescent cells, in G<sub>0</sub>, have a low RNA content. As cells progress through G<sub>1</sub>, they accumulate RNA and finally move to the S/G<sub>2</sub>/M phase during which Hst staining increases (Fig 1).

To assess the validity of Hst/PY cell-cycle fractionation, we compared it with a recently described method of high-resolution cell-cycle analysis, ie, the Ki-67/7-AAD method. In this procedure, a DNA histogram is generated by 7-AAD and plotted against expression of the nuclear antigen Ki-67, which is present in cycling cells but not in G<sub>0</sub> cells. Consecutive measurements of Hst/PY and Ki-67/7-AAD were performed. MPB CD34<sup>+</sup> cells were sorted in either G<sub>0</sub> or G<sub>1</sub> as defined by Hst/PY staining, plated in short-term cultures, and analyzed at different time intervals with Ki-67/7-AAD (Fig 2). Most of the cells (87% in the example shown in Fig 2) recovered from cultures initiated with G<sub>0</sub>CD34<sup>+</sup> cells were Ki-67–negative 24 hours after initiation. Expression of Ki-67 was progressively upregulated while cells entered G<sub>1</sub> and S/G<sub>2</sub>/M stages at 72 and 144 hours, but a significant percentage of cells, 14.3%, were still Ki-67–negative after 144 hours in culture. In G<sub>1</sub>CD34<sup>+</sup> cell–initiated cultures, the vast majority of cells entered into active phases of the cell cycle immediately after cytokine stimulation and were Ki-67–positive (>93%) at 24 hours after initiation of short-term culture. Interestingly, whereas only 3% of G<sub>0</sub>CD34<sup>+</sup> cells traversed into active phases of the cell cycle 24 hours after exposure to cytokines, almost 50% of initial G<sub>1</sub>CD34<sup>+</sup> cells were detected in S/G<sub>2</sub>/M at
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Fig 2. Serial high-resolution cell-cycle analysis of cytokine-stimulated G0 and G1 CD34+ cells. MPB G0 and G1 CD34+ cells were plated in complete medium supplemented with IL-3-IL-6-SCF. At indicated times, cells were harvested and analyzed for cell-cycle status with 7-AAD and Ki-67. (A) Dual-parameter plot showing fractionation of CD34+ cells in G0 and G1 phases with Hst and PY. (B to D) Cell-cycle analysis of cells harvested from G0 CD34+ cell-initiated culture at 24, 72, and 144 hours, respectively. (E to G) Cell-cycle analysis of cells harvested from G0 CD34+ cell-initiated culture at 24, 72, and 144 hours, respectively. The percentage of cells in G0, G1, and S/G2/M (abbreviated as G2) is indicated. Cells in the lower right quadrant were not considered.

A statistical difference was observed between the LTHC-IC frequency of G0 CD34+ cells and G1 CD34+ cells for both tissues. However, a ninefold difference in the frequency of LTHC-IC between G0 CD34+ cells and G1 CD34+ cells was documented for BM (P < .009), only a twofold difference (P = .02) was demonstrated for the same two fractions of MPB cells, indicating that increased homogeneity in the cell-cycle status of MPB CD34+ cells versus BM CD34+ cells was associated with a more pronounced func-

Assessment of LTHC-IC activity after cell-cycle fractionation of BM and MPB CD34+ cells. The ability of Hst/PY staining to achieve fractionation of primitive HPCs was compared for BM and MPB. Reports from several groups and from our laboratory indicated that MPB CD34+ cells consist of a population of noncycling cells, as compared with BM CD34+ cells. These observations were confirmed in the present study by standard propidium iodide DNA staining, such that 13.0% ± 2.0% of freshly isolated BM CD34+ cells were in S/G2/M phase versus 0.81% ± 0.66% for MPB CD34+ cells (P < .0001). We extended these previous findings by examining the relative abundance of PYlow (G0) and PPybright (G1) cells within the G0/G1 peak in both tissues. Representative dot plots in Fig 1A (BM) and Fig 2A (MPB) show that MPB CD34+ cells are enriched for cells displaying low RNA staining within the G0/G1 phase, suggesting that relative to BM CD34+ cells, MPB CD34+ cells are not only devoid of cells in S/G2/M but also cells in late G1 phase.

To compare the efficiency of Hst/PY staining to separate primitive HPCs in BM and MPB, G0 and G1 CD34+ cells were sorted from both sources and assayed for LTHC-IC activity (Fig 3). For these experiments, G0 and G1 CD34+ cells were defined for both tissues as having minimal and maximal PY staining within the G0/G1 peak, respectively.
tional homogeneity. In addition, $G_{1c}$ CD34$^+$ cells isolated from BM had a lower LTHC-IC frequency (0.7% ± 0.2%) than their counterparts isolated from MPB (5.7% ± 2.0%, $P < .01$), whereas no statistical difference was observed between $G_0$ CD34$^+$ cells isolated from these two sources ($P = .17$). This suggests that a specific subpopulation of CD34$^+$ cells residing in late $G_1$ in the BM microenvironment, and whose primitive HPC function is largely compromised, is not recovered after mobilization into the PB.

Hematopoietic function of BM CD34$^+$ cells isolated in subcompartments of the $G_0/G_1$ phase of the cell cycle. Additional experiments were performed to examine in more detail the functional heterogeneity of BM CD34$^+$ cells as a function of their position within the prereplicating phase of the cell cycle. Using Hst/PY staining, we arbitrarily defined four subcompartments of increasing RNA content within the $G_0/G_1$ phase (Fig 4), sorting windows for $G_{1c}$, $G_{1b}$, and $G_{1a}$ CD34$^+$ cells were defined as depicted in Fig 1A. BM CD34$^+$ cells isolated in these subcompartments were used for subsequent functional assays.

The relative contribution of each of the CD34$^+$ cell subsets to late and primitive progenitor populations was assessed by colony-forming unit (CFU) and LTHC-IC assays, respectively. Overall CFU frequency was not significantly different between cell subsets, and was $15.2% ± 3.4\%$ in $G_0$ CD34$^+$ cells, $18.3% ± 5.8\%$ in $G_{1a}$ CD34$^+$ cells, $17.4% ± 3.8\%$ in $G_{1b}$ CD34$^+$ cells, and $8.3% ± 3.8\%$ in $G_{1c}$ CD34$^+$ cells ($n = 3$, $P > .05$). However, different types of progenitors were detected in these cell populations such that $G_0$ CD34$^+$ cells had a significantly higher frequency ($P < .05$) of multipotential progenitors (CFU-MIX) than $G_{1a}$, $G_{1b}$, and $G_{1c}$ CD34$^+$ cell populations (Fig 5A), but a significantly lower frequency ($P < .05$) of myeloid committed progenitors (CFU-GM) than $G_{1a}$ and $G_{1b}$ CD34$^+$ cells.

LTHC-IC frequencies were measured in our stroma-free limiting dilution analysis assay as previously described (Fig 5B). LTHC-IC frequencies were inversely correlated with the initial RNA content of test cells. The highest LTHC-IC content was found in the $G_0$ CD34$^+$ cell subset. $G_0$ CD34$^+$ cells had 50% less LTHC-IC than $G_{1b}$ CD34$^+$ cells ($P > .05$), while CD34$^+$ cells isolated in late $G_1$ phases, $G_{1b}$ and $G_{1c}$ CD34$^+$ cells, had low or nearly undetectable LTHC-IC frequencies ($P < .05$ v $G_0$ CD34$^+$ cells). Altogether, these results indicate that in steady-state hematopoiesis, functionally distinct populations of HPCs can be identified along the $G_0/G_1$ pathway of the cell cycle. Multipotential CFU and CFU precursors reside in $G_0$ and to a lesser degree in early $G_1$ phase, while late $G_1$ subcompartments appear to contain mainly committed progenitors.

We next determined the ability of $G_0/G_1$ subcompartments of BM CD34$^+$ cells to maintain in vitro hematopoiesis in long-term cultures. CD34$^+$ cells isolated with varying RNA content within the $G_0/G_1$ phase (Fig 4) were plated in stroma-free suspension cultures and stimulated by a combination of IL-3, IL-6, and SCF. At weekly intervals, aliquots of cells were replated in progenitor cell assays to determine CFU production (Fig 6). CFUs were detectable up to 6 weeks in cultures initiated with both $G_0$ CD34$^+$ cells and $G_{1b}$ CD34$^+$ cells, while CFU production was exhausted after 2 weeks in cultures of CD34$^+$ subsets isolated in $G_{1b}$ or $G_{1c}$. Total CFU output was maximal in $G_0$ CD34$^+$ cell-initiated cultures, and decreased in other cell subsets in inverse relationship to the initial RNA content of the test cells (Fig 6). The persistence of CD34 expression among cultured cells was monitored on day 14 in similar long-term cultures (Table 1). The percentage of CD34$^+$ cells in vitro correlated with CFU production.
and was highest in cultures initiated with CD34+ cells displaying low RNA content (P < .05). It is interesting that after 2 weeks in LTC, the absolute number of CD34+ cells had expanded threefold in cultures initiated with G0CD34+ cells, while it had remained stable in G1aCD34+ cells and decreased approximately 80% and 88% in G1bCD34+ and G1cCD34+ cell-initiated cultures, respectively.

Maintenance of hematopoietic function in ex vivo–expanded BM CD34+ cells isolated in different phases of the cell cycle. In a subsequent step, we reasoned that upon completion of cell division, the most primitive progenitors may reenter transiently into a state of quiescence while more committed progenitors remain mitotically active. Previous studies from our laboratory using PKH2 cell tracking had already pointed out that a significant proportion of LTHC-ICs contained in ex vivo expansion cultures belonged to a group of quiescent cells surviving in the absence of cell division, termed CNR cells. To distinguish nondividing LTHC-ICs from those reentering G0 after in vitro proliferation, we combined PKH2 cell tracking with DNA/RNA fractionation (Fig 7).

Table 1. Persistence of CD34 Expression in Cultures Initiated With BM CD34+ Cells Isolated in Subcompartments of the G0/G1 Phase of the Cell Cycle

<table>
<thead>
<tr>
<th>Cultures Initiated on Day 0 With</th>
<th>% CD34+ Cells on Day 14</th>
<th>Total No. of CD34+ Cells on Day 14</th>
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<tbody>
<tr>
<td>G0CD34+</td>
<td>7.83 ± 0.85*</td>
<td>29,383 ± 14,756</td>
</tr>
<tr>
<td>G1aCD34+</td>
<td>4.31 ± 0.39t</td>
<td>10,667 ± 2,462</td>
</tr>
<tr>
<td>G1bCD34+</td>
<td>1.21 ± 0.57</td>
<td>6,167 ± 1,999</td>
</tr>
<tr>
<td>G1cCD34+</td>
<td>0.53 ± 0.23</td>
<td>1,869 ± 1,255</td>
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</table>

A total of 106 BM CD34+ cells isolated from G0/G1 subcompartments (Fig 4) were plated in complete medium supplemented with IL-3-IL-6-SCF. After 14 days, the persistence of CD34 expression was determined by FACS analysis of cultured cells. Data are the mean ± SEM, n = 3. Statistical analysis was by ANOVA followed by SNK pairwise comparisons.

* P < .05 v cultures initiated with G1a, G1b, and G1c CD34+ cells.
† P < .05 v cultures initiated with G1a and G1b CD34+ cells.
‡ Total number of CD34+ cells calculated by multiplying the percentage of CD34+ cells by the total cell number present in each culture.

In accordance with our previous studies, the total number of LTHC-ICs (Table 2) slightly decreased over a 7-day period in suspension cultures supported with IL-3, IL-6, and SCF. Analysis of PKH2 fluorescence showed that approximately 30% of total LTHC-ICs recovered from ex vivo cultures did not respond to the cytokine stimulation used in these studies, and were still in the CD34+PKH2bright subset after 7 days in ex vivo expansion cultures.

Within the CD34+PKH2bright cell population (Fig 8), cells...
reentering G0 retained a significantly higher frequency of LTHC-ICs (1.9 ± 0.2) compared with cells reisolated in G1 or S/G2 + M (0.8 ± 0.25 and 0.3 ± 0.2, respectively, P < .05), indicating that reacquisition of minimal RNA content after in vitro division was a distinct property of primitive hematopoietic cells assayed as LTHC-ICs. However, the LTHC-IC frequency of CD34+ cells reentering G0 after a 7-day ex vivo expansion was significantly lower than that detected among nonproliferating CD34+PKH2bright cells, harvested from the same cultures (4.1 ± 0.5, P < .05; Fig 8), adding further evidence that a prolonged quiescent state was associated with primitive hematopoietic function.10

The apparent difference in the LTHC-IC content of freshly isolated G0/CD34+ cells (6.5% ± 2.0%, Fig 3) versus cultured CD34+ cells reisolated with minimal RNA content (1.9% ± 0.2%; Fig 8) prompted us to compare the relative level of cell-cycle dormancy of these two groups of cells to investigate the relationship between cell-cycle quiescence and primitive hematopoietic function. The rate of cell-cycle activation of G0/CD34+ cells isolated before and after ex vivo expansion was measured in short-term cultures. Suspension cultures from paired BM samples were initiated with day 0 G0/CD34+ cells and 1 week later with day 7 G0/CD34+PKH2dim cells reisolated with minimal RNA content after ex vivo expansion. Both cultures were stimulated with 100 ng/mL each of IL-3, IL-6, and SCF in complete medium. At initiation and at 24-hour intervals, samples were assayed for cell-cycle analysis by the propidium iodide method. Results are expressed as the mean ± SEM. Statistical analysis was made by paired Student’s t-tests.


discussion
In this study, we used simultaneous DNA/RNA staining and flow cytometric cell sorting to isolate and characterize fresh CD34+ cells in different subcompartments of the G0/G1 phase of the cell cycle, and to examine the relationship between reacquisition of mitotic quiescence and maintenance of primitive hematopoietic potential among proliferating ex vivo—expanded CD34+ cells. Conventional cell-cycle analysis based on measurement of DNA content can classify cells in G0/G1, or S/G2 + M phases of the cell cycle as noncycling and cycling cell populations, respectively.15,32 This kind of static measurement categorizes all cells containing 2n DNA as G0/G1, and as such fails to address the kinetics of cell-cycle progression or heterogeneity of the cell-cycle rate within a given cell population. In addition, since the vast majority of primitive HPCs isolated in steady-state hematopoiesis reside in the G0/G1 phase,1 the simple distinction between S/G2 + M and G0/G1 cells is of limited interest. Data obtained with simultaneous DNA/RNA staining allow for further fractionation of G0/G1 cells and can provide additional insight into the cell-cycle kinetics of hematopoietic cells. Our studies demonstrate that CD34+ cells within the G0/G1 phase of the cell cycle can be separated into distinct subpopulations based on RNA content. In kinetic experiments reported here (Fig 2), we demonstrate the ability

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**Table 2. Absolute Number of LTHC-ICs Detected Among Freshly Isolated and Ex Vivo—Expanded CD34+ Cells**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. of LTHC-ICs*</th>
<th>Percent Cells†</th>
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<tbody>
<tr>
<td>Day 0</td>
<td>Total CD34+</td>
<td>3,750 ± 1,440</td>
</tr>
<tr>
<td>Day 7</td>
<td>Total CD34+</td>
<td>3,481 ± 617</td>
</tr>
<tr>
<td></td>
<td>CD34+ PKH2 bright</td>
<td>1,092 ± 764</td>
</tr>
<tr>
<td></td>
<td>CD34+ PKH2 dim</td>
<td>2,389 ± 558</td>
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</table>

Simultaneous CD34+/PKH2 cell sorting on day 7 was performed as illustrated in Fig 7B. Paired data from 4 BM samples are reported as the mean ± SEM. 

* Total number of LTHC-ICs normalized per 10⁶ initial CD34+ cells.
† Percent cells with indicated phenotype given relative to the total number of cells in culture on day 7.

**Table 3. Proliferation Rate of G0/CD34+ Cells Isolated Before and After Ex Vivo Expansion**

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>% Cells in S/G2 + M at Indicated Time (h) After Isolation</th>
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<tbody>
<tr>
<td>G0/CD34+</td>
<td>1.45 ± 0.52*</td>
</tr>
<tr>
<td>G0/CD34+ PKH2dim</td>
<td>2.55 ± 0.87*</td>
</tr>
</tbody>
</table>

Cultures were initiated with freshly isolated BM G0/CD34+ cells and G0/CD34+PKH2dim cells at day 7 isolated from paired samples as illustrated in Fig 7. Both cultures were stimulated with 100 ng/mL each of IL-3, IL-6, and SCF in complete medium. At initiation and at 24-hour intervals, samples were assayed for cell-cycle analysis by the propidium iodide method. Results are expressed as the mean ± SEM, n = 3.

* P > .05, † P < .05: paired t-tests.

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Fig 8. LTHC-IC frequencies of ex vivo—expanded CD34+ cells isolated in different phases of the cell cycle. CD34+ cell subsets were sorted as described in Fig 7. LTHC-IC frequencies are given per 100 cells of each phenotype indicated. Paired data from four BM samples are reported as the mean ± SEM. Statistical analysis was made by ANOVA followed by SNK pairwise comparisons. *P < .05 v G0, G1, and S/G2 + M CD34+ PKH2bright cells; **P < .05 v G0, and S/G2 + M CD34+PKH2dim cells.
of our staining procedure to discern between mitotically dormant cells (G0/CD34+ cells) and those in which cell-cycle activation had already been triggered (G1/CD34+ cells).

Discrimination between G0 and G1 phases is a controversial subject. Our definition of G0 cells as those displaying minimal PY staining may appear arbitrary. In fact, we made no attempt to determine absolute counts of CD34+ cells in the G0 phase, but rather assessed the functional properties of cell subsets isolated relative to one another using combined DNA/RNA staining. While expression of other markers such as Ki-67 or D-type cyclins34 may be more absolute criteria division and loss of primitive hematopoietic function.27 To determine whether cells reisolated in G1 or S/G2 were enriched for multipotential CFUs ity exists that a cycling G1 LTHC-IC would reenter G0 after cell division in ex vivo expansion cultures, our data showed that CD34+ cells reentering G0 have a shorter prereplicating phase than freshly isolated G0/CD34+ cells. These findings suggest that under our ex vivo culture conditions, once HPCs have started to proliferate in vitro, they can no longer return to the same initial dormancy state, possibly as a result of a progressive loss of developmental capacity. Indeed, our group has recently demonstrated a correlation between cell division and loss of primitive hematopoietic function. To- analysis had already been triggered (G1/CD34+ cells).

These observations are in line with a recent report35 that the primitive hematopoietic potential of primitive HPCs undergoing proliferation in vitro was inferior to that of cells remaining quiescent in expansion cultures, referred to as CNR cells. The present results indicate that although the frequency of LTHC-ICs among proliferating cells was lower than that of CNR cells, the residual primitive hematopoietic activity of in vitro proliferating cells could be mostly attrib- uted to a specific identifiable subgroup of these cells, namely the composed cells that had reentered into a relative quiescent state. It remains to be established whether different culture conditions such as coculture with stromal layers or addition of cytokines such as Flt3 ligand36-38 or thrombopoiet- in39,40 which have been shown to affect activation and mainten- ance of primitive cells, would modify the distribution of LTHC-ICs among the different fractions isolated after ex vivo expansion as described here.

When viewed together, our results can be used to propose a model of the cell cycle of hematopoietic cells similar to that proposed by Gerdes et al16 for PHA-stimulated peripheral blood lymphocytes. In this model (Fig 9), primitive LTHC-ICs, initially residing in G0 in the BM microenvironment, can enter the cell cycle via start sequences leading to G1. In the event of a self-renewal cycle, cells may reenter the initial low RNA dormancy state after mitosis,
whereas during the process of differentiation and lineage commitment, cells remain in cycle with a shorter prereplicating phase. Furthermore, our results demonstrate that ex vivo–expanded CD34+ cells isolated with minimal RNA content have a higher degree of responsiveness to cytokine stimulation than their freshly isolated counterparts selected according to the same criteria. Since a direct relationship could be demonstrated between the hematopoietic potential of CD34+ cells and the level of mitotic quiescence both in vivo and during ex vivo culture, it is possible that the loss of LTHC-IC activity in cultured G0/CD34+ cells is a reflection of the higher turnover rate of these cells relative to similar cells in vivo. These findings may have important implications in designing strategies for ex vivo expansion and genetic transduction of primitive hematopoietic cells.

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REFERENCES


Functional Heterogeneity of Human CD34⁺ Cells Isolated in Subcompartments of the G₀/G₁ Phase of the Cell Cycle

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