Functional Heterogeneity of Human CD34+ Cells Isolated in Subcompartments of the G_0/G_1 Phase of the Cell Cycle

André Gothot, Robert Pyatt, Jon McMehel, Susan Rice and Edward F. Srour
Functional Heterogeneity of Human CD34+ Cells Isolated in Subcompartments of the G0/G1 Phase of the Cell Cycle

By André Gothot, Robert Pyatt, Jon McMehel, Susan Rice, and Edward F. Srour

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, DNA/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. Multipotent 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 PKH2+ population whereby LTHC-IC frequency was higher for CD34+ cells reselected in G0 after in vitro division than for CD34+ cells reisolated in G0, or in S/G2+M. However, the highest LTHC-IC frequency was found in quiescent PKH2+bright 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.

© 1997 by The American Society of Hematology.
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 ng/kg (maximum, 480 ng/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 allopbyocyanin (APC)-conjugated anti-CD34 (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 FACSStar 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 × 10^6 cells/mL in 1.5 mL 1.67-mMol/L solution of Hst (Molecular Probes, Eugene, OR) in Hst buffer. Hst buffer consisted of Hank’s balanced salt solution (Biowhittaker, Walkersville, MD), 20 mM/L HEPES (Biowhittaker), 1 g/L glucose, and 10% fetal calf serum (IFCS) Hyclone, Logan, UT). After incubation at 37°C for 45 minutes, Pyronin Y (IPY Sigma, St Louis, MO) was present in Hst buffer and was added at a final concentration of 1 ng/mL and the cells were further incubated for another 45 minutes at 37°C. The cells were washed once, resuspended in Hst buffer, and sorted on a FACStar Plus equipped with an argon laser providing the 488-nm excitation for PY and a krypton laser providing the 350-nm excitation for Hst. The PY signal was selected with a 575 ± 13 nm band-pass filter, and Hst was detected with a 424 ± 22 nm band-pass filter. Sorting windows were constructed as depicted in Fig 1A. Since, by definition, RNA staining with PY yields a continuous histogram without demarcation between positive and negative cells, an arbitrary sorting window comprising 10% to 15% of cells displayed minimal PY staining and 2n DNA content was used to sort G0,CD34+ cells in all experiments (Fig 1A). Viability and purity of sorted cells always exceeded 98% and 90%, respectively.

Short-term culture and cell-cycle analysis. CD34+ cells used in these experiments were initially sorted with APC-conjugated anti-CD34 (Caltag) followed by Hst/PY fractionation. Cells were plated in culture medium consisting of IMDM, 10% FCS, 2 mM/L L-glutamine, and antibiotics, referred to hereafter as complete medium, supplemented with interleukin-3 (IL-3), IL-6, and stem cell factor (SCF) at 100 ng/mL each. After 24 to 144 hours in culture, cells were harvested and subjected to high-resolution cell-cycle analysis as recently described by Jordan et al., with minor modification. Cells were washed and resuspended in 1 mL phosphate-buffered saline (PBS) + 0.4% formaldehyde. After 30 minutes at 4°C, 1 mL PBS + 0.2% Triton X-100 was added, and the cells were left overnight at 4°C. The cells were then washed twice in PBS + 1% bovine serum albumin (BSA) and stained with FITC-conjugated anti–Ki-67 (clone MIB-1; Immunotech, Westbrook, ME) for 60 minutes at 4°C. Isotype controls were stained in parallel. Finally, cells were washed and resuspended in PBS + 1% BSA containing 5 μg/mL 7-aminoactinomycin-D (7-AAD) Sigma. After a 3-hour incubation on ice, samples were run on a FACSScan flow cytometer (BDIS) using FL-1 and FL-3 channels for Ki-67 and 7-AAD, respectively. Alternatively, conventional cell-cycle analysis by DNA staining with propidium iodide was used as previously described.

Long-term culture of CD34+ subpopulations and progenitor assays. CD34+ cells isolated in G0/G1 subcompartments were seeded in 24-well plates at a density of 10^6 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 ng/mL GM-CSF, and 2 μM 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. 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 530 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, all at 100 ng/mL. An aliquot of PKH2-stained cells was fixed in 1% paraformaldehyde and kept at 4°C for determination of day 0 PKH2 fluorescence. Cultures were fed with cytokines every 48 hours. Care was taken to avoid cell densities higher than 5 × 10^5 cells/mL by frequent splitting of the cultures to minimize dye transfer between cells. After 7 days, subpopulations of CD34+ cells were sorted in two steps as follows.

Cultured cells were counted, washed, and stained with biotinylated anti-CD34 monoclonal antibody (Caltag) followed by streptavidin (SA)-APC (Molecular Probes). Controls consisted of biotinylated isotype-matched control IgG followed by SA-APC. Cells were washed and resuspended in PBS + 1% BSA and sorted into two groups. The first consisted of CD34+PKH2bright cells, designating those that had not divided in culture, and the second contained CD34+PKH2dim cells, representing those that had divided and there-
fore lost part of their original PKH2 fluorescence. Sorted CD34^+PKH2^dim cells were further stained with Hst and PY as already described, and were sorted in a second step into cells residing in G_0, G_1, or S/G_2 + 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^+ cells into subcompartments of the G_0/G_1 phase of the cell cycle. Traditional cell-cycle analysis with a DNA probe such as Hst distinguishes between cells in the S/G_2 + M phase and those in the G_0/G_1 phase. We have recently described the successful isolation of CD34^+ cells in the G_0 phase of the cell cycle by simultaneous DNA/RNA staining. In this method, cells determined to be in the G_0/G_1 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_0, have a low RNA content. As cells progress through G_1, they accumulate RNA and finally move to the S/G_2 + 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_0 cells. Consecutive measurements of Hst/PY and Ki-67/7-AAD were performed. MPB CD34^+ cells were sorted in either G_0 or G_1 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_0,CD34^+ cells were Ki-67−negative 24 hours after initiation. Expression of Ki-67 was progressively upregulated while cells entered G_1 and S/G_2 + 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_1,CD34^+ 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_0,CD34^+ cells traversed into active phases of the cell cycle 24 hours after exposure to cytokines, almost 50% of initial G_0,CD34^+ cells were detected in S/G_2 + M at
FUNCTIONAL HEMATOPOIETIC HETEROGENEITY IN CELL CYCLE

Fig 2. Serial high-resolution cell-cycle analysis of cytokine-stimulated G_0 and G_1 CD34^+ cells. MPB G_0 and G_1 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 G_0 and G_1 phases with Hst and PY. (B to D) Cell-cycle analysis of cells harvested from G_1 CD34^+ cell-initiated culture at 24, 72, and 144 hours, respectively. (E to G) Cell-cycle analysis of cells harvested from G_0 CD34^+ cell-initiated culture at 24, 72, and 144 hours, respectively. The percentage of cells in G_0, G_1, and S/G_2/M (abbreviated as G_2) is indicated. Cells in the lower right quadrant were not considered.

A statistical difference was observed between the LTHC-IC frequency of G_0 CD34^+ cells and G_1 CD34^+ cells for both tissues. However, although a ninefold difference in the frequency of LTHC-IC between G_0 CD34^+ cells and G_1 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-

Fig 3. LTHC-IC frequencies of G_0 and G_1 CD34^+ cells isolated from BM and MPB. Frequencies are given per 100 cells as the mean ± SEM, n = 8 BM and 7 MPB. Samples were compared with t tests. *P = .009 v BM G_1,CD34^+ cells; **P = .02 v MPB G_1,CD34^+ cells; ***P = .01 v BM G_1,CD34^+ cells.
The functional heterogeneity of BM CD34^+ cells was examined in more detail between cell subsets, and was 15.2% colony-forming unit (CFU) and LTHC-IC frequencies, respectively. 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_0/CD34^+ cells (P < .05), while CD34^+ cells isolated in late G_1 phases, G_1a and G_1b, 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. Multipotent 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_1/G_2 CD34^+ cells, while CFU production was exhausted after 2 weeks in cultures of CD34^+ subsets isolated in G_0 or G_1. 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 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_0/CD34^+ cells (P < .05), while CD34^+ cells isolated in late G_1 phases, G_1a and G_1b, 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. Multipotent 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_1/G_2 CD34^+ cells, while CFU production was exhausted after 2 weeks in cultures of CD34^+ subsets isolated in G_0 or G_1. 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.

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_1/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
and was highest in cultures initiated with CD34<sup>+</sup> cells displaying low RNA content (P < .05). It is interesting that after 2 weeks in LTC, the absolute number of CD34<sup>+</sup> cells had expanded threefold in cultures initiated with G<sub>i</sub>CD34<sup>+</sup> cells, while it had remained stable in G<sub>0</sub>CD34<sup>+</sup> cells and decreased approximately 80% and 88% in G<sub>0</sub>CD34<sup>+</sup> and G<sub>i</sub>CD34<sup>+</sup> cell–initiated cultures, respectively.

Maintenance of hematopoietic function in ex vivo–expanded BM CD34<sup>+</sup> 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<sup>10</sup> 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 G<sub>i</sub> after in vitro proliferation, we combined PKH2 cell tracking with DNA/RNA fractionation (Fig 7). Following ex vivo expansion for 7 days, CD34<sup>+</sup> cells were first separated into PKH2<sub>bright</sub> and PKH2<sub>dim</sub> subsets as quiescent and proliferating cells, respectively (Fig 7A and B). PKH2<sub>dim</sub> cells were further fractionated by Hst/ PY staining (Fig 7C) into three subpopulations, namely G<sub>i</sub>, G<sub>i</sub>, and S/G<sub>2</sub> + M. All cell populations were assayed for LTHC-IC content.

In accordance with our previous studies,<sup>10,33</sup> the total number of LTHC-ICs contained in ex vivo cultures 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.

### Table 1. Persistence of CD34 Expression in Cultures Initiated With BM CD34<sup>+</sup> Cells Isolated in Subcompartments of the G<sub>i</sub>/G<sub>0</sub>

<table>
<thead>
<tr>
<th>Cultures Initiated on Day 0 With</th>
<th>% CD34&lt;sup&gt;+&lt;/sup&gt; Cells on Day 14</th>
<th>Total No. of CD34&lt;sup&gt;+&lt;/sup&gt; Cells on Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>G&lt;sub&gt;i&lt;/sub&gt;CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>7.83 ± 0.85&lt;sup&gt;+&lt;/sup&gt;</td>
<td>29,383 ± 14,756</td>
</tr>
<tr>
<td>G&lt;sub&gt;0&lt;/sub&gt;CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>4.31 ± 0.39&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10,667 ± 2,462</td>
</tr>
<tr>
<td>G&lt;sub&gt;i&lt;/sub&gt;G&lt;sub&gt;0&lt;/sub&gt;CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.21 ± 0.57</td>
<td>6,167 ± 1,999</td>
</tr>
<tr>
<td>G&lt;sub&gt;i&lt;/sub&gt;CD34&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.53 ± 0.23</td>
<td>1,869 ± 1,255</td>
</tr>
</tbody>
</table>

A total of 10<sup>5</sup> BM CD34<sup>+</sup> cells isolated from G<sub>i</sub>/G<sub>0</sub> 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 G<sub>0</sub>, G<sub>i</sub>, and G<sub>i</sub>CD34<sup>+</sup> cells.

† Total number of CD34<sup>+</sup> cells calculated by multiplying the percentage of CD34<sup>+</sup> cells by the total cell number present in each culture.
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 the IL-3-IL-6-SCF combination. Cells from paired cultures were subjected to cell-cycle analysis at initiation and at 24-hour intervals (Table 3). Day 7 G0,CD34+ cells quickly progressed into active phases of the cell cycle (14.24% of cells in S/G2 + M at 24 hours) compared with freshly isolated G0,CD34+ cells (2.61% of cells in S/G2 + M, P < .05), indicating that after in vitro division under our culture conditions, G0,CD34+ cells not only had a reduced frequency of LTHC-ICs but were unable to reacquire their initial degree of cell-cycle dormancy.

**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...
of our staining procedure to discern between mitotically dor-
mant cells (G_0 CD34^+ cells) and those in which cell-cycle
activation had already been triggered (G_1 CD34^+ cells).

Discrimination between G_0 and G_1 phases is a controver-
sial subject. Our definition of G_0 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 G_0 phase, but rather essayed the functional properties of
all subsets isolated relative to one another using combined
DNA/RNA staining. While expression of other markers such as
Ki-67 or D-type cyclins may be more absolute criteria for
G_0/G_1 discrimination, staining for these markers requires
cell permeabilization and therefore prevents functional char-
acterization of cells isolated in separate cell-cycle subcom-
partments. Since the studies of Darzynkiewicz and Tra-
ganos, total RNA measurements have been used to describe
the progression of cells from a resting state, ie, G_0, to an
active prereplicating state, G_1. In addition, the appearance
of cell-cycle stage-specific markers such as Ki-67 or D-
type cyclins requires RNA synthesis, an event that may be
detectable with DNA/RNA staining before the actual appear-
ance of these moieties.

In steady-state BM CD34^+ cells, the relationship between
position within the G_0/G_1 phase and primitive hematopoietic
function is striking. Whereas late committed progenitors be-
longed to immediate prereplicating phases, CD34^+ cells iso-
lated in G_0 or early G_1 were enriched for multipotential CFUs
and for LTHC-ICs. These observations support the concept
that in steady-state hematopoiesis, the turnover of BM primi-
tive hematopoietic cells is low compared with that of more
committed progenitors, and that a direct relationship exists
between the rate of cycling and the degree of lineage com-
mitment. These observations are in line with a recent report
showing a correlation between the proportion of cells in S/
G_2 + M and the proportion of committed progenitors in
murine BM.

The same method of cell-cycle fractionation was less ef-
ective in separating primitive LTHC-ICs when applied to
MPB CD34^+ cells, thus confirming previous reports demon-
strating the cell-cycle status homogeneity of MPB CD34^+ cells,
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 presence of LTHC-ICs among proliferating cells
is largely compromised are not recovered in the MPB of G-CSF–treated donors.

The application of membrane dye cell tracking coupled
with DNA/RNA fractionation allowed us to examine for the
first time whether primitive hematopoietic cells (LTHC-ICs)
proliferating in vitro are capable of reentering into a G_0 state.
A relationship between cycling status and hematopoietic
function similar to that observed among freshly isolated
CD34^+ cells was demonstrated for ex vivo–expanded
CD34^+ cells belonging to different phases of the cell cycle.
Indeed, CD34^+ cells reisolated with minimal RNA content
(G_0 ) were enriched for LTHC-IC activity compared with
CD34^+ cells reisolated in G_1 or S/G_2 + M. However, when
comparing the level of quiescence of freshly isolated
G_0 CD34^+ cells with that of G_1 CD34^+ cells recovered after
cell division in ex vivo expansion cultures, our data showed
that CD34^+ cells reentering G_0 have a shorter prereplicating
phase than freshly isolated G_1 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.

Together, these observations may be interpreted to suggest that
primitive cells reenter a state of transient quiescence after
in vitro division, thereby allowing their reisolation in G_0,
while less primitive HPCs may bypass the G_0 phase and
return immediately to a prereplicating stage. These results
reveal the notion that “the self-renewal process is associated with renewed dormancy
in the cell cycle, while the differentiation process is char-
cterized by continuous cell doubling.”

The initial cell-cycle position before ex vivo expansion
of LTHC-ICs reselected in G_0 after in vitro proliferation was
not directly determined in the present study. Our data (Figs
3 and 5B) show that the majority of LTHC-ICs originate
in G_0. However, a significant proportion of these primitive
progenitor cells arise from early G_1. Therefore, the possi-
ble existence of a cycling G_1 LTHC-IC would reenter G_0 after
expansion culture. However, the higher generation of CD34^+ cells (Table 1) and CFUs (Fig 6) in identical expansion
cultures by G_0 LTHC-ICs suggests that secondary LTHC-
ICs detected in the PKH2^dim fraction belong initially to the
G_0 fraction.

The findings of this study shed new light on previous data reported by our laboratory. We previously
demonstrated in both the human and murine systems
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 presence of LTHC-ICs among proliferating cells
is largely compromised and are not recovered in the MPB of G-CSF–treated donors.

The application of membrane dye cell tracking coupled
with DNA/RNA fractionation allowed us to examine for the
first time whether primitive hematopoietic cells (LTHC-ICs)
proliferating in vitro are capable of reentering into a G_0 state.
A relationship between cycling status and hematopoietic
function similar to that observed among freshly isolated
CD34^+ cells was demonstrated for ex vivo–expanded
CD34^+ cells belonging to different phases of the cell cycle.
Indeed, CD34^+ cells reisolated with minimal RNA content
(G_0 ) were enriched for LTHC-IC activity compared with
CD34^+ cells reisolated in G_1 or S/G_2 + M. However, when

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 al for PHA-stimulated periph-
eral blood lymphocytes. In this model (Fig 9), primitive
LTHC-ICs, initially residing in G_0 in the BM microenvironment,
can enter the cell cycle via start sequences leading to
G_1. In the event of a self-renewal cycle, cells may reenter
the initial low RNA dormancy state after mitosis,
factor confers on umbilical cord blood CD34+ cells an enhanced ex vivo expansion potential. Exp Hematol 22:1264, 1994