Peripheral Blood CD34+ Cells Differ From Bone Marrow CD34+ Cells in Thy-1 Expression and Cell Cycle Status in Nonhuman Primates Mobilized or Not Mobilized With Granulocyte Colony-Stimulating Factor and/or Stem Cell Factor

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Granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF) have been shown to stimulate the circulation of hematopoietic progenitor cells in both mice and nonhuman primates. We evaluated the immunophenotype and cell cycle status of CD34+ cells isolated from the bone marrow (BM) and leukapheresis product of cytokine-mobilized nonhuman primates. CD34+ cells were isolated from rhesus macaques that had received no cytokine therapy, 100 μg/kg/d G-CSF, 200 μg/kg/d SCF, or a combination of both 100 μg/kg/d G-CSF and 200 μg/kg/d SCF as a subcutaneous injection for 5 days. BM was aspirated before (day 0) and on the last day (day 5) of cytokine administration. On days 4 and 5, peripheral blood (PB) mononuclear cells were collected using a novel method of leukapheresis. Threefold more PB mononuclear cells were collected from animals receiving G-CSF alone or G-CSF and SCF than from animals that had received either SCF alone or no cytokine therapy. CD34+ cells were positively selected using an immunoadsorptive system from the BM, PB, and/or leukapheresis product. Threefold and 10-fold more CD34+ cells were isolated from the leukapheresis product of animals receiving G-CSF or G-CSF and SCF, respectively, than from animals receiving no cytokine therapy or SCF alone. The isolated CD34+ cells were immunophenotyped using CD34-allophycocyanin, CD38-fluorescein isothiocyanate, and Thy-1-phycoerythrin. These cells were later stained with 4',6-diamidino-2-phenylindole for simultaneous DNA analysis and immunophenotyping. BM-derived CD34+ cells did not differ significantly in cell cycle status and Thy-1 or CD38 phenotype before or after G-CSF and/or SCF administration. Similarly, CD34+ cells isolated from the leukapheresis product did not differ significantly in immunophenotype or cell cycle status before or after G-CSF and/or SCF administration. However, there were consistent differences in both immunophenotype and cell cycle status between BM- and PB-derived CD34+ cells. CD34+ cells isolated from the PB consistently had a smaller percentage of cells in the S+G2/M phase of the cell cycle and had a higher percentage of cells expressing Thy-1 than did CD34+ cells isolated from the BM. A greater proportion of PB-derived CD34+ cells were in the S+G2/M phase of the cell cycle after culture in media supplemented with interleukin-6 and SCF.

The identification and characterization of CD34+ hematopoietic stem cells from peripheral blood (PB) and bone marrow (BM) is of both clinical and biological interest. Initially identified by a monoclonal antibody (MoAb) raised against a human erythroleukemia cell line, KG-1a, CD34 has been identified as a ligand for L-selectin and has been found to be expressed by vascular endothelium and virtually all hematopoietic progenitor cells detected by in vitro assays. Antibodies that recognize CD34 have frequently been used for hematopoietic stem cell enrichment. Approximately 0.2% of normal PB mononuclear cells (PMBCs) and 1% to 4% of human BM cells express CD34. With chemotherapy and/or hematopoietic growth factor mobilization, the number of circulating CD34+ cells increases. The percentage of CD34+ cells in the leukapheresis product of patients receiving chemotherapy alone, the cytokine granulocyte colony-stimulating factor (G-CSF) alone, or the combination of chemotherapy and G-CSF is approximately 0.6%, 0.4%, and 2%, respectively. Investigators have shown that hematopoietic growth factors, such as interleukin-3 (IL-3), G-CSF, granulocyte/macrophage CSF, and stem cell factor (SCF) can effectively increase the absolute number of circulating progenitor and CD34+ cells. This increase in circulating CD34+ cell number has allowed clinicians to obtain sufficient quantities of CD34+ cells from the PB of mobilized donors so as to be able to perform transplants. Mobilized PB cells in human patients have proven quite effective in accelerating reconstitution after myeloablative therapies. Cytokine-mobilized PB cells have also been capable of contributing to the hematopoietic reconstitution of myeloablated mice, dogs, and primates. The combination of G-CSF and SCF has proven to be quite effective in mobilizing PB progenitor cells capable of hastening engraftment of irradiated animals. The combination of G-CSF and SCF was superior to G-CSF alone in mobilizing PB progenitor cells and reconstituting baboons treated with a single dose of 1.070 cGy total body irradiation.

Multimarker flow cytometry and cell cycle analysis has shown that human CD34+ cells can be subdivided into a number of distinct cell populations. Two cell surface antigens that have been used to subdivide CD34+ cells have been CD38 and Thy-1. Human cells that express CD34, but not CD38, appear to give rise to primitive hematopoietic colonies that can be replated up to five sequential generations. Similarly, human CD34+ cells that coexpress Thy-1 have been shown in vitro to initiate long-term hematopoiesis. In preclinical studies, human fetal CD34+Thy-1+ BM cells have been shown to engraft human thymus transplanted in severe combined immunodeficiency (SCID) mice. In addition, CD34+Thy-1+ cells from human umbilical cord blood have been shown to have functional properties of prim-
itive hematopoietic progenitor cells, and CD34+Thy-1+Lin- isolated from the PB of cancer patients mobilized after chemotherapy plus granulocyte-macrophage CSF or G-CSF possess long-term hematopoietic activity both in vitro (using a 7-week cobblestone area-forming assay) and in vivo (using a SCID-hu mouse model). CD38 appears to function as an adenosine 5'-diphosphate ribosyl cyclase, whereas the function of Thy-1 remains unknown. It has been speculated that Thy-1 may mediate a negative signal that results in the inhibition of primitive cell proliferation. The expression of Thy-1 and CD38 on rhesus macaque CD34+ cells has not been well characterized, although the cross-reactivity of some MoAbs directed against human antigens has been evaluated in nonhuman primates.

There is an interest in determining the in vitro and in vivo cell cycle status of subpopulations of CD34+ cells isolated from the BM and PB to improve our ability to expand quiescent hematopoietic stem cells and use viral vectors that require cell cycling for viral integration. Recently, cell cycle differences have been identified in BM CD34+ subsets. The CD34+CD38hi subset appeared to have more cells in the S and G2/M than did the CD34+CD38lo subset. After 2 days in cytokine-supplemented culture, the CD34+CD38lo cells showed increased numbers of cells in the S and G2/M phases. In another study, the combination of IL-3 and SCF was found to increase the percentage of BM-isolated CD34+ cells to enter cycle. Because nonhuman primates have been used to evaluate cytokine, transplantation, and gene therapy protocols before human clinical trials, we were interested in examining the immunophenotype and cell cycle status of BM and PB CD34+ cells derived from rhesus macaques that were either mobilized or not mobilized with high doses of G-CSF and/or SCF.

**MATERIALS AND METHODS**

**Animals.** The young adult rhesus macaques (Macaca mulatta) that were used in these studies were serologically negative for simian T-cell lymphotropic virus, simian immunodeficiency virus, and simian AIDS-related type D virus. Animals with blood type B were selected and had an indwelling central catheter established. Experimental animals were quarantined and housed in accordance with the guidelines set by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Committee on Care and Use of Laboratory Animals, DHHS Public #NIH85-23, Revised 1985) and the policies set by the Veterinary Research Program of the National Institutes of Health (NIH, Bethesda, MD). The protocols evaluated were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute.

**Cytokine administration.** Rhesus macaques received no cytokines, 100 μg/kg/d recombinant human G-CSF, 200 μg/kg/d recombinant human pegylated SCF, or a combination of both 100 μg/kg/d G-CSF and 200 μg/kg/d SCF (all provided by Amgen, Inc, Thousand Oaks, CA) as a subcutaneous injection for 5 days. Growth factor doses were based on previous studies. Purified material was stored at 4°C until used. All cytokines for this study were pyrogen-free.

**Rhesus leukapheresis procedure.** To collect PBMCs by leukapheresis from donors weighing less than 5 kg, modifications were made to the fluid path of a CS3000 Plus Blood Cell Separator (Baxter Healthcare Corp, Fenwal Division, Deerfield IL) to lower the extracorporeal volume requirement to 132 mL (see Fig 1). Collection was accomplished using a small S25A separation chamber and a shunt chamber (Fenwal no. 710700027) in the place of a collection chamber. A standard apheresis kit (Fenwal no. 4R2210) was installed in the CS3000. After autoprime, the roller clamps to the acid citrate dextrose-NIH formulation (ACD-A), saline, and vent prime lines were closed to prevent hemodiluting the donor when using hald/irrigate. The return line was modified by tightly rolling and tapping a 150-mL transfer pack (Fenwal no. 4R2001) and sterile-docking a male luer to the shortened outlet line. A blood component recipient set with a 170-μm filter and drip chamber (Fenwal no. 4C2100) was spiked into the modified 150-mL transfer pack and connected to the packed red blood cell line using a needle lock device (Fenwal no. 2C7831). The blood component recipient set was connected to a 20- to 18-gauge Angiocath placed in the saphenous vein of the donor. Hemostats were placed both on the standard, unused, return line and the inlet for the ACD line present on the draw line. The apheresis kit was primed with autologous blood that had been collected in citrate phosphate dextrose plus adsl 2 to 3 weeks before the leukapheresis procedure. The donor received a dose of 100 U/kg heparin immediately before the procedure. The inlet line was connected to an indwelling 6.6 French catheter placed in the right atrium of the heart. Blood was processed at the rate of 12 mL/min in automatic mode for a total of 2.5 times the animal’s calculated blood volume. At the completion of the procedure, the product was collected and 5 mL of ACD was added. The remaining cells were salvaged and either were used to prime the CS3000 for future leukapheresis procedures or were directly reinjected into the animal. PBMCs were collected by leukapheresis on day 4 and day 5 of cytokine administration. These days were selected based on evidence in rhesus macaques (data not shown) and in baboons that circulating progenitor numbers had increased by these time points. The number of PBMCs processed during the leukapheresis procedure was calculated by averaging the number of mononuclear cells in the complete blood cell count of samples taken immediately before, in the middle of, and at the end of the leukapheresis procedure, and then multiplying this average by the volume of blood processed. Cell counts were performed on a Coulter Model S5 electronic cell counter (Hialeah, FL) or on a Cell-Dyn5500 automated hematology analyzer (Abbott Laboratories, Abbott Park, IL). The number of PBMCs processed for each cytokine mobilization group was evaluated and the mean and standard error of the mean (SEM) was then determined. The number of PBMCs collected as the product was based on the complete blood cell count of the leukapheresis product multiplied by the total percentage of lymphocytes and monocytes within the product and the volume of the leukapheresis product. The PBMCs for each cytokine mobilization group was evaluated, and the mean and standard of deviation (SD) was then determined. The PBMCs collected from the leukapheresis products on day 4 and day 5 were processed and analyzed as independent samples.

**BM collection.** Before the administration of hematopoietic growth factors (day 0), 30 mL of heparinized BM was surgically harvested from one femur. Additional BM was harvested immediately before the second leukapheresis procedure (day 5) from the alternate femur. After BM harvest, the animal received a course of buprenorphine (0.1 to 0.3 mg/kg intramuscularly) for 3 days to alleviate any bone pain that may have been associated with the harvest.

**Immunoselection of CD34+ cells.** CD34+ cells from the BM and PB leukapheresis product were recovered by positive immunoselection using the Ceptrate LC-34-Biotin Kit (CellPro, Inc) according to the manufacturers instructions. An accurate determination of CD34+ cell yield after immunoselection was not determined, in part, because of the rarity of CD34+ cells in both the BM and leukapheresis product. No apparent difference was observed when comparing the immunophenotype of immunoselected CD34+ cells and CD34+ cells that were analyzed from the original blood sample. Cytospin prepara-
tions of the cells were prepared to document cellular morphology. Separate immunoselections were performed on each leukapheresis product collected on day 4 and day 5. The CD34+ cells collected on each day were stained and analyzed independently.

Culture of immunoselected CD34+ cells. CD34+ cells were immunoselected from the leukapheresis product of animals mobilized with SCF and G-CSF and were cultured in suspension for 3.5 days in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD) plus 15% fetal calf serum (HyClone Laboratories, Inc, Logan, UT) supplemented with 50 ng/mL of IL-6 and 100 ng/mL of SCF (both cytokines provided by Amgen, Inc).

Immunophenotyping of CD34 cells from BM and PB. The BM and PB leukapheresis CD34+ cells that were positively selected using the CellPro immunoadsorption system were immunophenotyped with CD34-allophycocyanin (APC) to evaluate CD34 purity and were also immunophenotyped with CD38-fluorescein isothiocyanate (FITC) and Thy-1 phycoerythrin (PE) to identify CD34+CD38 and CD34+Thy-1 subpopulations. The CD34 MoAb used (clone 563) was a gift from Dr. G. Gaudernack (Institution of Transplantation Immunology, Rikshospitalet, The National Hospital, Oslo, Norway). Clone 563 is a murine IgG1 that recognizes a different CD34 epitope from that of the CellPro CD34 MoAb clone (clone
Fig 2. (A) Cytokine mobilization of WBC into PB. The average (SD) circulating WBC/μL of blood from animals that had received a 4- and 5-day subcutaneous course of either no cytokines (n = 6), 200 μg/kg/d SCF (n = 6), 100 μg/kg/d of G-CSF (n = 6), or the combination of SCF and G-CSF (n = 11) before leukapheresis. (B) Total number of mononuclear cells processed and collected from PB by leukapheresis. The average (SEM) number of mononuclear cells processed through the CS3000 Plus Cell Separator and the average (SD) number (SEM) of PBMCs collected in the leukapheresis product from animals that received either no cytokines (n = 6), SCF (n = 6), G-CSF (n = 6), or the combination of SCF and G-CSF (n = 11). (C) Absolute number of CD34+ cells obtained after immunoselection from the leukapheresis product. The average (SD) absolute number of CD34+ cells immunoselected from the leukapheresis product from animals that received either no cytokines (n = 6), SCF (n = 6), G-CSF (n = 6), or the combination of SCF and G-CSF (n = 6). (D) The percentage of Thy-1 expressing CD34+ immunoselected cells from the leukapheresis product or BM. The average (SD) percentage of CD34+ cells that express Thy-1 from (1) the leukapheresis product of animals that received a 4- and 5-day subcutaneous course of either no cytokines (n = 6), SCF (n = 5), G-CSF (n = 6), or the combination of SCF and G-CSF (n = 6); or (2) the BM of animals that received either no cytokines (n = 6), SCF (n = 6), G-CSF (n = 6), or the combination of SCF and G-CSF (n = 6).

12.8) used in the immunoselection. Most antihuman CD34 MoAbs either do not cross react with rhesus macaque CD34+ cells or recognize the same epitope as that of the antibody used in the immunoselection. Fortunately, immunoselection for rhesus CD34+ cells with clone 12.8 did not interfere with subsequent CD34 staining with clone 563. For immunophenotyping, the CD34 clone 563 was directly conjugated to APC by Molecular Probes, Inc (Eugene, OR). The CD36-FITC MoAb used was the OKT10 clone (Ortho Diagnostics, Raritan, NJ). The Thy-1-PE clone used was a gift from Dr P. Lansdorp (Terry Fox Laboratories Vancouver, British Columbia, Canada). To evaluate adhesion proteins CD29-FITC and CDw49d-FITC antibodies were obtained from AMAC, Inc (Westbrook, ME).

Cells were incubated in the MoAbs or isotype controls for 30 minutes on ice and then washed 2 times with phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Fc receptors were blocked by preincubating the cells for 5 minutes in 10% human AB serum (Advanced Biotechnologies Inc, Columbia, MD) without washing before the addition of MoAbs. Cells were fixed in 1% paraformaldehyde in PBS and stored at 4°C. All tubes were first run to analyze for immunophenotyping patterns of CD34-APC, CD38-FITC, and Thy-1-PE before the cells were processed for DNA analysis. The remaining cells were then processed for DNA analysis using a nuclear DNA staining procedure that was modified for intact cells. The DNA-staining procedure involved taking a 300-μL aliquot of cells and slowly adding 700 μL of -20°C cold 100% ethanol to yield a final concentration of 70% ethanol. The cells were
or with no cytokine therapy. The WBC average (SD) measured for the control group without cytokines was 4.7 (0.7) X 10^9/l.

Flow cytometry data for FITC, PE, APC, DAPI, forward scatter and side scatter was collected in listmode. The DAPI fluorescence was measured as both linear-integrated and linear-peak signals and FITC, PE, and APC were collected as logarithmic signals. Cell doublets were excluded by gating using either DAPI-peak versus DAPI-integrated signals or using DAPI-peak versus ratio of DAPI-peak-integrated signals.

The absolute number of CD34-Thy-1' cells/mL of either BM or leukapheresis product was calculated by multiplying the percentage of CD34' cells that were Thy-1' by the absolute number of CD34' cells collected, and dividing this number by the volume of either BM or leukapheresis product collected for each animal. These numbers were then averaged based on group, and an SD was determined.

RESULTS

Rhesus apheresis of cytokine-mobilized PB cells. The effectiveness of different human cytokines to mobilize the release of rhesus leukocytes from BM into the PB was evaluated. White blood cell (WBC) counts measured immediately before leukapheresis (Fig 2A) show that G-CSF alone and the combination of G-CSF and SCF both increased the WBC count significantly above the levels achieved with SCF alone or with no cytokine therapy. The WBC average (SD) measured for the control group without cytokines was 4.7 (0.7) X 10^9/l (n = 6); for those treated with SCF alone, the WBC count was 11.0 (7.1) X 10^9/l (n = 6); for those treated with G-CSF alone, the WBC count was 82.0 (30.2) X 10^9/l (n = 6); and for those treated with the combination of G-CSF and SCF, the WBC count was 61.6 (19.3) X 10^9/l (n = 6). No significant difference in WBC count was observed between G-CSF-mobilized and G-CSF- and SCF-mobilized animals at day 4 and day 5. This is consistent with a similar rise in baboons in which, at these early time points, there was little difference in WBC count between the two groups. The relative effectiveness of G-CSF or the combination of G-CSF and SCF to increase mobilized PB cells was also observed in both the PBMC fraction processed and collected (Fig 2B). The average (SD) PBMCs collected after leukapheresis was 1.3 (0.3) X 10^9 PBMCs (n = 6) for the control group, 1.5 (0.2) X 10^9 PBMCs (n = 6) for the SCF alone group, 5.5 (3.5) X 10^9 PBMCs (n = 6) for the G-CSF alone group, and 3.6 (2.1) X 10^9 PBMCs (n = 11) for the SCF and G-CSF group (Fig 2B).

The average collection efficiency for PBMCs using the CS3000 was 40.4% (12.5%) (n = 24), with no apparent difference in efficiency between mobilized and nonmobilized donors.

CD34+ cell mobilization and recovery. CD34+ cells were positively selected from the leukapheresis product or BM, and the purities of the recovered CD34+ cells were evaluated. Purities after immunoselection for CD34+ cells were consistently better for BM than for the leukapheresis product. Immunoselected CD34+ cells from mobilized and nonmobilized BM had CD34 purities averaging 88.7% (7.4%) (n = 23). Leukapheresis products processed using the same immunoadsorptive system had CD34 purities averaging 60.9% (21.8%) (n = 23). Cytokine mobilization with G-CSF and SCF clearly increased the absolute number of CD34+ cells recovered from the leukapheresis product following the CD34 immunoselection (Fig 2C).

The absolute number of CD34+ cells was greater in the leukapheresis products obtained from animals mobilized with the combination of G-CSF and SCF (2.3 [1.3] X 10^7 CD34+ cells; n = 6) than the leukapheresis products obtained from animals mobilized with G-CSF alone (0.7 [0.4] X 10^7 CD34+ cells; n = 6; see Fig 2C). Still fewer CD34+ cells were collected from leukapheresis products obtained from animals mobilized with SCF alone (0.2 [0.1] X 10^7 CD34+ cells; n = 5) and from nonmobilized animals (0.1 [0.1] X 10^7 CD34+ cells; n = 6; see Fig 2C). Leukapheresis products from day 4 and day 5 yielded similar numbers of CD34+ cells. The absolute number of CD34+ cells collected per milliliter of BM was 2.7 (1.3) X 10^5 (n = 12), 7.3 (1.7) X 10^5 (n = 3), 4.3 (1.3) X 10^5 (n = 3), and 5.7 (3.3) X 10^5 (n = 3) for the nonmobilized, SCF-mobilized, G-CSF-mobilized, and SCF plus G-CSF-mobilized animals, respectively. The absolute number of CD34+ cells collected per milliliter of leukapheresis product was 0.4 (0.4) X 10^5 (n = 6), 0.4 (0.2) X 10^5 (n = 5), 1.8 (1.2) X 10^5 (n = 6), and 5.8 (3.5) X 10^5 (n = 6) for the nonmobilized, SCF-mobilized, G-CSF-mobilized,
A. RQ826 PRE-MOBILIZATION BONE MARROW

- GRANULARITY
- SIZE
- CD38-FITC
- CD34-APC
- THY-1-PE
- DNA CONTENT

B. RQ826 POST-MOBILIZATION G-CSF+SCF BONE MARROW

- GRANULARITY
- SIZE
- CD38-FITC
- CD34-APC
- THY-1-PE
- DNA CONTENT

C. RQ826 POST-MOBILIZATION G-CSF+SCF LEUKAPHERESIS PRODUCT

- GRANULARITY
- SIZE
- CD38-FITC
- CD34-APC
- THY-1-PE
- DNA CONTENT

Fig 3.

RQ1111 POST-MOBILIZATION G-CSF+SCF

- BONE MARROW
- BLOOD
- APHERESIS
- CULTURED APHERESIS

Fig 5.
and SCF plus G-CSF–mobilized animals, respectively. For the BM, the absolute number of CD34+ cells collected using the Ceprate LC-34-Biotin column represented 1.6% (0.5%) (n = 12), 2.0% (0.8%) (n = 3), 0.4% (0.2%) (n = 3), and 1.0% (0.4%) (n = 3) of the mononuclear cells collected after ficoll-hypaque separation for the nonmobilized, SCF-mobilized, G-CSF–mobilized, and G-CSF plus SCF-mobilized animals, respectively. For the leukapheresis product, the absolute number of CD34+ cells represented 0.09% (0.08%) (n = 6), and Thy-1. A subpopulation of CD34+ cells isolated from the BM of SCF-mobilized animals, respectively. Interestingly, the cellular distribution of Thy-1 appears to be different between rhesus macaques and humans. Unlike human granulocytes, which are negative for Thy-1, granulocytes isolated from rhesus macaques strongly express Thy-1. Two populations of CD34+ Thy-1+ cells were identified (Fig 3) based on their CD38 expression, CD38-bright and CD38-dim. Using the OKT10 clone, we have been unable to identify a CD34+CD38+ cell population in rhesus BM or leukapheresis product. For animal RQ826, 84% of the backgated CD34+Thy-1+ BM cells were CD38-bright before cytokine mobilization, 77% of the BM CD34+Thy-1+ cells were CD38-bright after G-CSF and SCF administration, and 90% of the mobilized PB G-CSF and SCF CD34+Thy-1+ cells were CD38-bright (Fig 3). The population of cells seen in Fig 3 that were dimly staining for CD34 and Thy-1 were small in size and expressed low levels of CD38.

Because there were differences in Thy-1 expression between BM and PB, we were also interested in determining whether there were phenotypic differences in the expression of adhesion proteins between circulating PB- and BM-derived CD34+ cells. In particular, we evaluated the expression of the integrin α4β1 on CD34+ cells. This adhesion protein has been shown to play a role in hematopoietic stem cell and microenvironment interactions.28 CD34+ cells isolated from the BM and leukapheresis product were no different between either PB and CD34+ Thy-1+ cells in S+G2/M for cells gated on CD34+ or CD34+Thy-1+ immunophenotyping. The average (SD) percentage of CD34+ or CD34+Thy-1+ cells in S+G2/M was determined for BM from animals that received either no cytokines (n = 14), SCF (n = 3), G-CSF (n = 3), or the combination of SCF and G-CSF (n = 7), and the leukapheresis product from animals that received either no cytokines (n = 6), SCF (n = 6), G-CSF (n = 6), or the combination of SCF and G-CSF (n = 11).
cells was independent of cytokine mobilization. Similar to the G-CSF and SCF mobilization example, differences in cell cycle status between PB and BM CD34+ cells or CD34+ Thy-1+ cells were observed without cytokine treatment, with G-CSF treatment alone, or with SCF treatment alone. Administration of cytokines did not significantly alter the cell cycle profiles observed in premobilization and postmobilization BM. However, there was a slight increase in the percentage of cycling PB CD34+ cells observed with SCF+G-CSF mobilization, but this increase was not statistically significant for the data set. As one might expect, small CD34+ cells were not in the S+G2/M phases of the cell cycle.

One potential explanation for the differences observed in Thy-1 expression and cell cycle status between BM-derived and leukapheresis-derived CD34+ cells was that the leukapheresis procedure itself may have selected for nondividing cells of this particular phenotype. Counterflow centrifugation is commonly used as a method for isolating cells in different phases of the cell cycle. To evaluate this possibility further, G-CSF and SCF-mobilized CD34+ cells were immunoselected both from the PB and the leukapheresis product and compared. No significant differences in phenotype or in cell cycle status were observed between CD34+ cells immunoselected directly from the PB versus the leukapheresis PBMC product (Figs 5 and 6).

To evaluate whether a greater percentage of PB CD34+ cells would be in cycle after culture, immunoselected PB CD34+ cells were placed in culture for 3.5 days in media supplemented with SCF and IL-6. Over the 3.5 days, the cells increased 1.6-fold (0.5-fold) in number (n = 4). After culture, the cell cycle status and immunophenotype were reevaluated. The phenotype of the circulating CD34+ cells was altered after culture, with a substantial loss in the percentage of CD34+ Thy-1+ cells (Fig 5). In addition, both the CD34+ cells and the CD34+Thy-1+ subset following culture had a greater percentage of cells in S+G2/M from a baseline value of 14.3% (3.7%) and 9.1% (3.3%) to postculture values of 39.6% (7.9%) and 39.2% (12.3%) for CD34+ cells and CD34+Thy-1+ cells, respectively (Fig 6).

**DISCUSSION**

No alterations in phenotype were observed for either the BM or PBMC CD34+ cells with cytokine mobilization. The proportions of cells expressing CD34, Thy-1, and CD38 all remained fairly constant. What did change was the absolute number of CD34+ cells immunoselected from the leukapheresis product. Despite comparable numbers of PBMCs being collected from animals receiving G-CSF alone and the combination of G-CSF and SCF, greater numbers of CD34+ cells were collected from animals immobilized with the combination of G-CSF and SCF. This observation may explain why there was a significant difference in WBC count between G-CSF–mobilized and G-CSF and SCF–mobilized baboons after approximately 1 week of cytokine therapy.

The immunoselected CD34+ cells obtained from the PB, BM, and leukapheresis product were characterized for purity and for CD34 and Thy-1 expression. Unlike human CD34+ cells, which have a distinct CD38 subset, rhesus macaque CD34+ cells express CD38 on all CD34+ cells. The absence of a CD34+CD38+ population of cells in rhesus macaques, however, does not prevent multilineage reconstitution of rhesus macaques after BM transplantation, because immunoselected CD34+ cells from rhesus macaques have previously been shown to contribute to multilineage hematopoietic reconstitution.

The distribution of Thy-1 on rhesus macaque CD34+ cells collected from the leukapheresis product and BM are quite similar to that observed for human CD34+ cells. Human CD34+ cells isolated from the BM and the leukapheresis product of cancer patients treated with cytotoxic chemotherapy and hematopoietic growth factors have been found to differ in Thy-1 expression. These immunophenotypic differences between PB- and BM-derived CD34+ cells may account for the accelerated hematopoietic engraftment observed in patients receiving mobilized PB when compared with that for those receiving BM. An increase in the absolute number of CD34+ cells and CD34+Thy-1+ cells collected by leukapheresis was observed with either G-CSF or the combination of G-CSF and SCF therapy. Thus, the increased frequency of Thy-1+ cells in the circulation may be caused by either a failure of a subpopulation of BM CD34+ cells to migrate into the circulation or a predilection for CD34+Thy-1+ cells to circulate.

In addition to differences between BM and PB CD34+ cell immunophenotype, there was a consistent difference between the cell cycle status of PB and BM CD34+ cells. The percentage of noncycling CD34+ cells was consistently higher for the PB or leukapheresis product than that for the BM. Preliminary results suggest that human PB CD34+ cells may also have fewer cells in S-phase under steady state conditions and after mobilization with chemotherapy and cytokines than CD34+ cells isolated from BM. Both observations are consistent with earlier studies that found that circulating granulopoietic and erythropoietic progenitor...
cells were more resistant to \(^3\)H-thymidine suicide, and a more recent study that found that PB progenitor cells mobilized by G-CSF and other cytokines were resistant to \(^3\)H-thymidine suicide. All these results suggest that there is a selective difference in cell cycle status between CD34\(^+\) cells found in the circulation and CD34\(^+\) cells found within the BM, despite the presence of high levels of circulating hematopoietic growth factors. Potential reasons for this difference are that noncycling CD34\(^+\) cells may have a lower affinity for binding to the hematopoietic microenvironment than do cycling CD34\(^+\) cells and, therefore, would have a greater tendency to circulate, or that, for CD34\(^+\) cells to cycle, adherence to the BM stroma is required. No phenotypic difference in a4\(\beta1\) function or in the expression of other adhesion molecules, such as lymphocyte function-associated antigen-1, between BM-derived and circulating CD34\(^+\) cells were not precluded.

Failure to cycle when exposed to a combination of hematopoietic growth factors has been used as a criteria for identifying primitive, hematopoietic stem cells. Recent examples include retention of the membrane label PKH26 on a subset of CD34\(^+\) cells when cultured in serum-free medium supplemented with IL-3, IL-6, SCF, and erythropoietin and resistance of a subset of CD34\(^+\) cells to the antimetabolite 5-fluorouracil when stimulated with IL-3 and SCF. Although IL-3 was not used in our study, G-CSF and the combination of G-CSF and SCF have been used in mice, dogs, nonhuman primates, and humans to mobilize progenitors that contain BM repopulating cells. This suggests that mobilized CD34\(^+\) cells contain a population of primitive cells that are not in cycle in the presence of G-CSF and SCF and have the capacity to repopulate the BM of a myeloablated host. Because cellular repopulation is required for efficient retroviral infection, it would appear that immunoselected CD34\(^+\) cells from the leukapheresis product would be more difficult to transduce than BM. However, this does not appear to be the case in that immunoselected CD34\(^+\) cells from the leukapheresis product could be induced to proliferate in suspension culture, and in that the absolute number of CD34\(^+\) cells that were collected per milliliter of the leukapheresis product from the SCF and G-CSF–mobilized animals was greater than that collected from BM. These observations are consistent with murine studies that have shown that retrovirus transduction efficiency was higher for PB from splenectomized mice mobilized with G-CSF and SCF than for BM from mice treated with 5-fluorouracil, and that the culturing of murine BM cells in IL-3, IL-6, and SCF enhances retroviral transduction efficiencies in murine hematopoietic cells.

The leukapheresis procedure developed has permitted the collection of large numbers of PB leukocytes from 3- to 5-kg donors without the need for exposing the donor to allogeneic blood products in priming the instrument. This prevents allo-sensitization before myelosuppression, thus minimizing the opportunity for developing antibodies to erythrocyte, leukocyte, and platelet antigens and subsequent destruction of transfused blood products because of antibody formation. In addition, this leukapheresis procedure has permitted the collection of large quantities of other PB cells, such as lymphocytes and/or monocytes, for further study. Evaluation of cytokine-mobilized PB and BM stem cells from 3- to 5-kg donors may have application in pediatric and veterinary medicine in which cell transfusion, cell therapy, and genetic therapy are practiced. Future studies may examine why there is a predilection for noncycling CD34\(^+\) cells to circulate in the PB and may further delineate the role of the subpopulations of CD34\(^+\) cells in hematopoiesis.

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REFERENCES

10. Sheridan WP, Begley CG, Juttner CA, Szer J, To LB, Maher D, McGrath KM, Mostyn G, Fox RM: Effect of peripheral-blood...
progenitor cells mobilized by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. Lancet 339:640, 1992
38. Miller DG, Adam MA, Miller AD: Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 10:4239, 1990
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