Blood-Derived Autografts Collected During Granulocyte Colony-Stimulating Factor–Enhanced Recovery Are Enriched With Early Thy-1+ Hematopoietic Progenitor Cells

By Rainer Haas, Robert Möhle, Margit Pförsich, Stefan Frueheuf, Barbara Witt, Hartmut Goldschmidt, and Werner Hunstein

It was the objective of the study to characterize CD34+ hematopoietic progenitor cells from peripheral blood (PB) and bone marrow (BM) in a group of 24 cancer patients. After cytotoxic chemotherapy, R-metHu granulocyte colony-stimulating factor (R-metHuG-CSF; filgrastim, 300 μg daily, subcutaneously) was given to shorten the time of neutropenia as well as to increase the rebound of peripheral blood progenitor cells (PBPC) for harvesting. The proportion of CD34+ cells in the leukapheresis products (LPs) was 1.4-fold greater than in BM samples that were obtained at the same day (LP: median, 1.4% v BM: median, 1.0%, P < .01). Two- and three-color immunofluorescence showed that blood-derived CD34+ cells comprised a greater proportion of a particular early progenitor cell than CD34+ cells of bone marrow. Blood-derived progenitor cells tended to have a higher mean fluorescence intensity of CD34 and expressed significantly lower levels of HLA-DR (mean fluorescence intensity of HLA-DR: 442.6 ± 44.9 [LP] v 681.5 ± 64.6 [BM], mean ± SEM, P < .01). Furthermore, the blood-derived CD34+ cells comprised a 1.7-fold greater proportion of Thy-1+ cells (LP: median, 24.4% v BM: median, 14.4%, P < .001) and expressed significantly less c-kit (LP: median, 20.5% v BM: median, 31.0%, P < .01). Three-color analysis showed that high levels of Thy-1 expression were restricted to CD34+/HLA-DRdim or CD34+/HLA-DR+ cells confirming the early developmental stage of this progenitor cell subset. The proportion of CD34+/CD45RA+ cells representing early colony-forming unit granulocyte-macrophage (CFU-GM) was smaller in LPs compared with BM (P < .05). For an examination of BM CD34+ cells before the mobilization chemotheraphy, samples of 16 patients were available. The mean proportion of c-kit expressing CD34+ cells in the bone marrow during G-CSF-stimulated reconstitution decreased 1.8-fold compared with baseline values. There was no difference in the proportion of BM-derived CD34+/Thy-1+ cells and CD34+/CD45RA+ cells between steady-state hematopoiesis and G-CSF-supported recovery. Our data suggest that during G-CSF-enhanced recovery, CD34+ cells in the PB are enriched with more primitive progenitor cells to evenly replenish the BM after the chemotherapy-related cytotoxic damage.

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THE MECHANISMS underlying the mobilization and circulation of hematopoietic progenitor cells in the peripheral blood (PB) are still poorly understood. Trafficking in the PB may be an inherent property of hematopoietic stem cells, because embryonic hematopoiesis evolves in the yolk sac and develops later in the liver and the spleen.2–12 The bone marrow (BM) becomes the major hematopoietic organ around the sixteenth week of gestation, when a network of sinusoids, stromal cells and extracellular matrix is formed within the bone.3–4 Pathologic conditions such as myelofibrosis, myeloid leukemia, or severe anemia can be associated with hematopoiesis in the spleen or liver recapitulating features of early embryonic life.5–7 Extramedullary hematopoiesis in cynomolgus monkeys was also observed in spleen, lymph nodes, and the thymus after the administration of granulocyte colony-stimulating factor (G-CSF).8 Because G-CSF and other cytokines are known to increase the number of hematopoietic progenitor cells in PB, extramedullary hematopoiesis may result from immigrating stem cells rather than from dormant cells in situ.9–12

To address the question of hematopoietic progenitor cell mobilization and migration, the immunophenotype of blood-derived CD34+ cells in patients who received G-CSF after cytotoxic chemotherapy was examined. We were especially interested whether the circulation of progenitor cells is restricted to a particular CD34+ cell subset or should be understood as a random process. Therefore, PB progenitor cells (PBPCs) were harvested during the G-CSF–enhanced rebound phase and compared with samples from BM that were obtained at the PBPC collection day. Samples from BM before chemotherapy were available for 16 patients. The study, which included double- and three-color immunofluorescence, showed significant differences between BM and blood-derived CD34+ cells.

MATERIALS AND METHODS

Patients. Since November 1993, 24 patients with hematologic malignancies or solid tumors were enrolled into this study. All patients received cytotoxic chemotherapy followed by R-metHuG-CSF 24 hours later (filgrastim, 300 μg daily, subcutaneously; Amgen, Thousand Oaks, CA). The patients were treated under the ethical guidelines of the Joint Committee on Clinical Investigation of the University of Heidelberg. Informed consent was obtained from each patient. The patient characteristics are detailed in Table 1. The cut-off date of this report is May 31, 1994.

PBPC were harvested during G-CSF–supported marrow recovery. The concentration of circulating CD34+ cells was determined from samples of whole blood using fluorescence-activated cell sorting (FACS) analysis. We started the leukaphereses as soon as a distinct population of CD34+ cells was detectable. At the day of leukapheresis, the median white blood cell (WBC) count and absolute neutrophil count were 17.05 X 10⁹/L (range, 17.05 to 60.97 X 10⁹/L) and 13.11 X 10⁹/L (range, 1.31 to 60.97 X 10⁹/L), respectively.

The two- and three-color immunofluorescence analyses of blood-derived CD34+ cells were performed on samples from leukapheresis products (LPs) that provide a CD34+ cell enriched mononuclear cell

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THY-1+ PROGENITORS ARE ENRICHED IN PB AUTOGRRAFTS

Immunofluorescence staining and flow cytometry. For the immunofluorescence analysis, 1 x 10^6 MNC or 20 to 50 L of whole blood (EDTA) were incubated for 30 minutes at 4°C with the fluorescein (FTTC)-conjugated monoclonal antibody (MoAb) HPCA-2 (CD34) and one of the following phycoerythrin (PE)-conjugated MoAbs: HLA-DR (clone L243) and c-kit Phyco. CD34 subsets were further analyzed using PE-labeled HPCA-2 (CD34) and one of the fluorescein isothiocyanate (FTTC)-labeled MoAb HLE-1 (CD45), Leu-18 (CD45RA), and Thy-1 (clone 5E10). Isotype-specific antibodies served as control. To further characterize the population of more primitive hematopoietic progenitor cells, three-color immunofluorescence was performed in five patients using HLA-DR-PerCP (clone L243). Except for c-kit Phyco (Immunotech SA, Marseille, France) and Thy-1 (PharMingen, San Diego, CA), all MoAbs were obtained from Becton Dickinson (Heidelberg, Germany). After the MoAb incubation, the cells were washed twice with PBS, and red blood cells (RBCs) were removed using a FACS lysing solution (Becton Dickinson, USA). After the MoAb incubation, the cells were washed twice with PBS, and resuspended in phosphate-buffered saline (PBS) supplemented with bovine albumin (Sigma Chemical Co, St Louis, MO) at a concentration of 0.5%.

Immunofluorescence analysis was performed using a five-parameter FACSscan (Becton Dickinson) equipped with an argon-ion laser tuned at 488 nm and 0.3 W. Emission from FITC and PE was measured using filters of 530 nm and 585 nm, respectively. PerCP fluorescence was determined using a 650-nm filter. Compensation levels were set by gating on the lymphoblastoid population identified by forward- versus side-scatter characteristics (FSC vs SSC) and aligning the mean channels of the single-stained CD45+ populations for each color with the corresponding unstained control.

An SSC versus CD45 fluorescence dot plot was used to discriminate between the smallest lymphohematopoietic cell population and erythrocytes or debris (Fig 1). The CD34+ cells were analyzed in a fluorescence versus SSC plot. Thus, only cells with lymphoid or lymphoblastoid appearance (low SSC) were counted as CD34+ cells. The percentage of false-positive events accepted in the controls was 0.05%. The percentage of false-positive events in the controls was subtracted from the percentage of CD34+ cells that were then calculated in relation to the percentage of CD45+ cells. To determine the concentration of CD34+ cells/L of peripheral blood before mobilization, the proportion of CD34+ cells was multiplied by the MNC count obtained after density centrifugation. At the time of G-CSF-enhanced recovery, samples from whole blood were used and the proportion of CD34+ cells was multiplied by the WBC count. The concentration of CD34+ cells/mL of bone marrow was determined as follows: the volume of the BM aspirate was recorded and the

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Table 1. Patient Characteristics

Abbreviations: NHL, non-Hodgkin lymphoma; ALL, acute lymphoblastic leukemia; CR, complete remission; PR, partial remission; DexamBEAM, dexamethasone, carmustine, etoposide, cytosine-arabinoside, melphalan; HAM, high-dose cytosine-arabinoside, mitoxantrone; IE, ifosfamide, etoposide, PEI, ifosfamide, etoposide, cisplatin.

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MNC count/mL after density centrifugation was multiplied by the percentage of CD34+ cells.

For two- and three-color analysis, an average (mean ± SEM) of 456.6 ± 61.9 (LP) and 456.6 ± 56.4 (BM) CD34+ cells were acquired in a life gate based on fluorescence intensity and SSC as shown in Fig 1. Using the isotype-specific control, we formed a second gate based on FSC versus SSC that further reduced the nonspecific background staining. A percentage of events in the control samples was calculated and only cells with low SSC were included (3). The negative control consisted of an isotype-specific MoAb (IgG1) (4).

Compared to steady-state hematopoiesis, there was no significant change in the plating efficiency of BM-derived CFU-GM and BFU-E at the time of G-CSF–stimulated recovery (Table 2). Still, the median concentration of both CFU-GM and BFU-E increased 1.9- and 3.2-fold (P < .05), respectively, because the MNC count in the reconstituting marrow was three times as much as during steady-state hematopoiesis. The colony data are mirrored by the immunologic results. The mean proportion of BM-derived CD34+ cells postchemotherapy (1.46% ± 0.30%) remained almost unchanged compared with steady-state hematopoiesis (1.57% ± 0.22%, not significant). However, because of the increased cellularity, the CD34+ cell numbers rose from a median baseline value of 27.85 ×10^6 to a median concentration of 54.75 ×10^6 during recovery (P < .05).

Fig 1. Measurement of CD34+ cells (HPCA-2 FITC) from a leukapheresis product (1). The pan-leukocyte antigen CD45 (HL-1) was used to discriminate between the smallest lymphohematopoietic cells and erythrocytes or debris (2). A lymphoblastoid gate was created and only cells with low SSC were included (3). The negative control consisted of an isotype-specific MoAb (IgG1) (4).
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Table 2. CD34+ Progenitor Cells, CFU-GM, and BFU-E in Bone Marrow Samples Before PBPC Mobilization and During Filgrastim-Enhanced Recovery (postchemotherapy)

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<tr>
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<td>%</td>
<td>Per 1 μl</td>
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<td>median (range)</td>
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<td>(2.6-332.3)</td>
<td>(60.0-3,335.0)</td>
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Abbreviation: MNC, mononuclear cells.

*P < .05 (premobilization v postchemotherapy).

Subset analysis of CD34+ cells. Major emphasis was put on the assessment of the antigenic profile of CD34+ cells from reconstituting BM and leukapheresis products. In all patients, samples from both sources were obtained at the same day. The co-expression analysis included HLA-DR, Thy-1, c-kit and CD45RA. The assessment was performed on CD34+ cells acquired into a life gate which was based on fluorescence activity and side scatter characteristics. Before the data of the subset analyses will be presented, it should be noted that the overall proportion of CD34+ cells in the LP products was 1.4-fold greater than in BM samples (LP: median, 1.4%, range 0.1% to 8.4% v BM: median, 1.0%, range, 0.1% to 5.8%, P < .01).

HLA-DR was measured because the expression of this antigen on a CD34+ cell indicates lineage-commitment. The majority of CD34+ cells from BM samples and LP products were positive for HLA-DR (>95%). Intraindividual differences observed between CD34+ cells from both compartments were related to fluorescence intensity (Fig 2). This parameter correlates with antigen density and depends on the developmental stage of the progenitor cell. Compared with CD34+ cells from BM, the CD34 mean fluorescence intensity on blood-derived CD34+ cells tended to be higher (100.9 ± 8.9 v 91.7 ± 8.2, mean ± SEM, not significant). More important, the level of HLA-DR expression on blood-derived CD34+ cells was significantly lower (442.6 ± 44.9 v 661.5 ± 64.6, P < .01), suggesting that the proportion of more primitive progenitor cells in the PB is greater than in the BM.

The data on Thy-1 are consistent with this view. In 21 of 24 patients, the proportion of CD34+/Thy-1+ cells in LP products was greater than in BM samples. The median 1.7-fold difference is significant (LP: median, 24.4%; range, 0.6% to 49.6% v reconstituting BM: median, 14.4%; range 3.3% to 40.7%, P < .001). As the correlative display shows (Fig 2), Thy-1 is predominantly present on CD34b+gh' cells, supporting the notion of an early hematopoietic progenitor cell subset. Thereby, CD34+ cells from BM and LP products

**Fig 2.** The results of a two-color immunofluorescence analysis of CD34+ cells in a representative patient (no. 9) are shown. During filgrastim-supported recovery, an LP product and a BM sample were obtained. Immunophenotyping was performed using the following MoAbs: HPCA-2-FITC/PE (CD34), HLA-DR-PE (clone L243), c-kit-PE, Leu-18-FITC (CD45RA), and Thy-1-FITC (clone 5E10). For the analyses, 400 (BM and LP) cells were acquired into a life gate as shown in Fig 1. The CD34 mean fluorescence intensity tended to be higher on blood-derived CD34+ cells, whereas their level of HLA-DR expression was significantly lower compared with CD34+ cells from BM. Of note, Thy-1 expression was restricted to the CD34+gh' cell population in samples from both sources. On the basis of two-color immunofluorescence analysis, c-kit or C45RA expression could not be ascribed to a particular level of CD34 expression (see Fig 3).
Three-color immunofluorescence analysis of CD34+ cells in 5 patients. LPs and BM samples were obtained during filgrastim-supported recovery. Immunophenotyping was performed using the following MoAbs: HPCA-2-PE/FlTC (CD34), HLA-DR-PerCP (clone K43), Thy-1-FITC (clone 5E10), c-kit-PE, and Leu-18-FITC (CD45RA). For the analyses, a median of 500 cells (range, 300 to 1,000) were acquired into a life gate as shown in Fig 1. The dot plots show results from overlaying the corresponding individual dot plots of the five patients. Expression of Thy-1 is confined to CD34+/HLA-DRdim or CD34+/HLA-DR- cells (shaded area). The latter phenotype signifies a more primitive hematopoietic progenitor cell. In contrast, the BM samples contained a larger proportion of CD34+ cells, expressing high levels of c-kit and HLA-DR (shaded area). This pattern of coexpression indicates lineage commitment of a CD34+ cell. Although the percentage of CD34+ cells costaining for CD45RA was not significantly different between BM samples and LP products, CD34+/CD45RAbright cells with high levels of HLA-DR expression were predominantly found in samples from reconstituting BM (shaded area). This particular subset is thought to be enriched with late CFU-GM.

Coexpression of c-kit on CD34+ cells was inversely related to Thy-1 (Fig 4). A median of 31.0% marrow-derived CD34+/c-kit+ cells (range 0.05% to 88.2%) was noted during cytokine-enhanced recovery. The proportion of CD34+/c-kit+ cells in LP products was significantly smaller with a range of 4.1% to 100% (median, 20.5%; P < .01). Three-color analysis showed that CD34+/c-kit+ cells in the BM comprised a distinct population expressing high levels of HLA-DR (Fig 3). On the other hand, c-kit was less expressed or negative on CD34+/HLA-DRdim or CD34+/HLA-DR- cells. The results imply that c-kit expression on CD34+ cells is associated with lineage-commitment.

There was no difference between BM samples and LP products in the percentage of CD34+ cells staining positive for CD45RA. In 17 patients, samples from both sources contained a distinct population of CD34+/CD45RAbright cells. Compared with LP products, the median number of cells of this particular subset was 1.5-fold greater in BM samples (P < .05). Three-color analysis showed that CD34+ cells staining brightly for CD45RA also expressed higher levels of HLA-DR (Fig 3). In contrast, the CD34+/CD45RAdim cells were not related to a particular HLA-DR fluorescence intensity.

Subset analyses of CD34+ cells from BM samples before mobilization are available for 16 of the 24 patients. An important finding of this analysis relates to the expression of c-kit. As shown in Fig 5, BM during steady-state contains a mean 1.8- and 2.3-fold greater proportion of CD34+/c-kit+ cells compared with BM samples obtained during G-CSF–enhanced recovery and LP products, respectively (P < .01). As far as the coexpression of Thy-1 and CD45RA is concerned, no significant differences were noted between BM samples before the mobilization therapy and after chemotherapy.

DISCUSSION

During G-CSF–supported recovery after cytotoxic chemotherapy, CD34+ cells from PB are different from those in BM. Therefore, it appears unlikely that mobilization and trafficking of hematopoietic progenitor cells result from a
THY-1+ PROGENITORS ARE ENRICHED IN PB AUTOGRAFTS

Fig. 1. Correlative display of CD34+/Thy-1+ cells and CD34+/c-kit cells in 24 BM and LP samples. The figure shows an inverse relationship between both progenitor cell subsets with respect to their distribution in BM samples and LP products. Symbols located within the upper triangle indicate that the proportion of the respective CD34+ cell subset for a given patient is larger in the LP product than in the BM sample. In 21 of 24 patients, the proportion of CD34+/Thy-1+ cells was greater in LP products than in BM samples. In contrast, a larger proportion of CD34+/c-kit+ cells was found in the reconstituting BM in 18 of the 24 patients studied.

random release into the PB. The antigenic profile of the CD34+ cells suggests that the PB harbors a larger proportion of a particular early progenitor cell than the BM. This conclusion is not only true for the comparison with BM during cytokine-enhanced recovery, but also for BM during steady-state hematopoiesis. The data presented here are consistent with findings of an animal study showing that treatment with cyclophosphamide plus G-CSF leads to an increase of more primitive progenitor cells (day 8 spleen CFCs and day 7 cobblestone area-forming cells) in the PB, whereas their proportion markedly decreased in the marrow.13

Our line of argument begins with the data on HLA-DR coexpression. We found that blood-derived CD34+ cells tended to have a higher mean fluorescence intensity of CD34 that was associated with a significantly lower level of HLA-DR expression. Because the level of antigen expression is related to the developmental stage of a CD34+ cell, the results imply that PB is enriched with more primitive progenitors during G-CSF-supported marrow recovery.14 The data on Thy-1 support this view. As shown by Craig et al,15 early hematopoietic progenitor cells from fetal liver and BM are characterized by CD34 and Thy-1 coexpression. This can be concluded from their ability to initiate long-term marrow cultures and to establish human hematopoiesis in SCID mice.16 More recently, Mayani and Lansdorp showed that cord blood-derived CD34+/Thy-1+ cells expressing low undetectable levels of CD45RA and CD71 were enriched for primitive progenitor cells including high proliferative potential (HPP)-CFC.17 It is therefore of relevance that the blood-derived CD34+ cells of our patients comprised a significantly greater proportion of CD34+/Thy-1+ cells compared with CD34+ cells from reconstituting and steady-state BM. Although our study does not include functional assessment of the proliferative and self-renewal capacity, the early nature of the CD34+/Thy-1+ cells is very likely considering that this phenotype has been associated with a stem cell candidate when human fetal liver and BM or cord blood cells were examined. Moreover, as shown by the three-color immunofluorescence analysis, Thy-1+ cells were preferentially detected within the CD34+high/HLA-DRdim cell population. A

Fig. 5. Two-color immunofluorescence analysis of CD34+ cells. Twenty four samples of LPs and BM obtained during filgrastin-supported recovery (post-chemotherapy) are shown. BM samples before PBPC mobilization are available for 16 patients (day 0). The horizontal line represents the mean. The differences found for the coexpression of c-kit were statistically significant (P < .01). LPs contained the largest proportion of CD34+/Thy-1+ hematopoietic progenitor cells.
small CD34+/Thy-1+/HLA-DR− subset was also observed in the LP products and may comprise very primitive hematopoietic progenitor cells. Interestingly note, Mayani and Lansdorp found that addition of anti-Thy-1 to cultures from selected CD34+ cord blood cells resulted in an inhibition of HGF-CFC proliferation. Therefore, it is conceivable that the G-CSF–stimulated CD34+ cells in the PB may be deprived of a negative growth signal that is otherwise effective during steady-state hematopoiesis, when the progenitor cells lodge within their usual microenvironment.17

On the other hand, the proportion of CD34+/c-kit+ cells was significantly lower in LP products than in BM samples. This finding provides indirect evidence that PB during G-CSF–enhanced recovery is enriched with early progenitor cells. Because three-color analysis ascribed high levels of c-kit to BM-derived CD34+/c-kit+ cells, the data indicate that the majority of BM CD34+/c-kit+ cells are lineage-committed. It also implies a role of c-kit for the adhesion of CD34+ cells within the marrow microenvironment, considering that membrane-bound stem cell factor (c-kit ligand) is constitutively expressed by stromal cells from long-term BM cultures, endothelial cells and diploid fibroblasts.18 In addition, an earlier report showed that this receptor-ligand pair is involved in the homing of two other migratory cell lineages, eg, primordial germ cells and melanocytes.19 The proportion of BM-derived CD34+/c-kit+ cells in our patients was significantly smaller than premobilization values, which is probably a result of the preceding cytotoxic chemotherapy. This may be confirmed from data of Katayama et al,20 who showed that during steady-state, most of the murine BM progenitor cells expressed high levels of c-kit, whereas the majority of BM progenitors after cytotoxic treatment with 5-fluorouracil were c-kitlow.

The results of CD45RA coexpression likewise suggest that BM-derived CD34+ cells contain a greater proportion of more mature progenitor cells. As shown by Fritsch et al,21 CD34+/CD45RA− cells are enriched with “late” CFU-GM. In 17 of the patients studied, we observed a median 1.5-fold greater proportion of this subset in BM samples compared with LP products.

As far as the quantitative aspect is concerned, the proportion of CD34+ progenitor cells and the plating efficiency for CFU-GM and BFU-E in the reconstituting BM were not significantly different from steady-state BM. At the same time, the number of CD34+ cells in the PB increased remarkably and comprised a greater proportion of early CD34+/Thy-1− progenitor cells.

The comparison between both compartments provides a strong argument for the use of blood-derived progenitor cells rather than BM for transplantation. The significant proportion of early CD34+/Thy-1− hematopoietic progenitor cells is an important aspect considering long-term hematopoiesis after PBPC-supported myeloablative high-dose therapy.22 Other implications relate to gene therapy, if a pluripotent stem cell is the ex-vivo target for stable integration of a gene. Further studies should address the question, whether a similar antigenic profile of CD34+ cells will be observed when G-CSF or other cytokines are administered during steady-state hematopoiesis.

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Blood-derived autografts collected during granulocyte colony-stimulating factor-enhanced recovery are enriched with early Thy-1+ hematopoietic progenitor cells

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