Circulation of CD34⁺ Hematopoietic Stem Cells in the Peripheral Blood of High-Dose Cyclophosphamide-Treated Patients: Enhancement by Intravenous Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor

By Salvatore Siena, Marco Bregni, Bruno Brando, Fernando Ravagnani, Gianni Bonadonna, and A. Massimo Gianni

We report that hematopoietic progenitor cells expressing the CD34 antigen (CD34⁺ cells) transiently circulate in the peripheral blood (PB) of cancer patients treated with 7 g/m² cyclophosphamide (HD-CTX) with or without recombinant human granulocyte macrophage-colony stimulating factor (rHuGM-CSF). In adult humans, CD34⁺ cells represent a minor fraction (1% to 4%) of bone marrow (BM) cells, comprising virtually all hematopoietic colony-forming progenitors in vitro and probably also stem cells capable of restoring hematopoiesis of lethally irradiated hosts. We show that CD34⁺ cell circulation is fivefold enhanced by rHuGM-CSF 5.5 μg/kg/day by continuous intravenous infusion for 14 days after HD-CTX. During the third week after HD-CTX (i.e., when CD34⁺ cells peak in the circulation), large-scale collection of PB leukocytes by three to four continuous-flow leukaphereses allows the yield of 2.19 to 2.73 x 10^8 or 0.45 to 0.56 x 10^8 CD34⁺ cells depending on whether or not patients receive rHuGM-CSF. The number of CD34⁺ cells retrieved from the circulation by leukaphereses exceeds the number that can be harvested by multiple BM aspirations under general anesthesia. Thus, after therapy with HD-CTX and rHuGM-CSF, PB represents a rich source of hematopoietic progenitors possibly usable for restoring hematopoiesis after myeloablative chemoradiotherapy. To determine whether CD34⁺ cells found in the PB are equivalent to their marrow counterpart, we evaluated their in vitro growth characteristics and immunological phenotype by colony assays and dual-color immunofluorescence, respectively. We show that PB CD34⁺ cells possess qualitatively normal hematopoietic colony growth and high cloning efficiency comparable to that observed with BM CD34⁺ cells. In addition, PB CD34⁺ cells display heterogeneous surface membrane differentiation antigens analogous to BM CD34⁺ cells. The availability of large quantities of CD34⁺ cells by leukapheresis is relevant to the field of stem cell transplantation and possibly to genetic manipulations of the hematopoietic system in humans.

© 1989 by Grune & Stratton, Inc.
CSF for 14 days after HD-CTX; (c) the yield of CD34+ cells from PB by leukapheresis is higher than the yield from conventional BM harvest for autologous transplantation, and this difference is more striking when patients are also treated with rHuGM-CSF; and (d) surface membrane differentiation antigens as well as in vitro clonal growth characteristics of CD34+ cells from PB are similar to those from BM.

MATERIALS AND METHODS

Patients and treatment protocol. As shown in Table 1, five patients entered this study. In all cases BM involvement was ruled out by morphologic and immunologic analysis of bilateral iliac crest aspirates and biopsies. After patients had given written informed consent, they were treated according to high-dose sequential chemotherapy protocols approved by the institute's committee for clinical investigation. On day 0 of therapy, these protocols implied administration of 7 g/m2 cyclophosphamide (HD-CTX) divided into five doses IV for 1 hour every 3 hours. Thereafter, patients C, D, and E received glycosylated rHuGM-CSF (Sandoz, Basel, Switzerland) 5.5 protein μg/kg body weight per day by continuous IV infusion through a dedicated lumen of a right atrial catheter. Patient C received rHuGM-CSF for 10 days from day +5 to +14 after HD-CTX. Patients D and E received rHuGM-CSF for 14 days from day +1 to day +14. Patients A and B did not receive rHuGM-CSF.

Large-scale collection of peripheral blood and bone marrow hematopoietic cells. During hematopoietic recovery after HD-CTX-induced pancytopenia, PB and BM buffy-coat cells were collected and cryopreserved to be used as a source of hematopoietic stem cells to support subsequent myeloablative treatments (ie, total body irradiation and high-dose melphalan), according to a previously described technique. Starting on the first day that leukocyte counts reached ≥1,000/μL and platelet counts reached ≥70,000/μL, patients underwent PB leukaphereses on 4 (patients A through D) or 3 (patient E) consecutive days with a continuous-flow blood cell separator (IBM-COBE 2997, MEDAS, Genoa, Italy). The centrifuge speed was adjusted to 800 rpm. The total blood volume processed in each leukapheresis was between 7.2 and 9.9 L at a flow rate of 30 to 45 mL/min, which resulted in harvested cell suspensions of 200 to 250 mL.

The day after completion of leukaphereses, patients underwent harvesting of autologous BM by multiple needle aspirations from posterior iliac crests under general anesthesia with the technique previously described. Aspirations were continued until at least 2 x 10^8 BM nucleated cells per kilogram of body weight were collected.

For the purpose of this study, small samples of mononuclear and polymorphonuclear cells from heparinized unprocessed PB, leukapheresis, and BM were isolated by density gradient (1.114 g/cm³) centrifugation over Mono-Poly resolving medium (Flow Laboratories, Settimo Milanese, Milan, Italy) as described previously. After centrifugation, the two erythrocyte-depleted fractions of low-density mononuclear cells and high-density polymorphonuclear cells were pooled together and washed twice before evaluating the frequency of CD34+ cells, the frequency of hematopoietic progenitors, and the phenotype of CD34+ cells.

Flow cytometry analysis of CD34+ cells. Cells expressing the surface membrane CD34 antigen were identified with the anti-HPCA-1 (My10) murine monoclonal antibody (Becton Dickinson, Mountain View, CA). The percentage of cells reactive with anti-HPCA-1 antibody (CD34+ cells) was determined by indirect immunofluorescence staining and flow microfluorometric analysis. Cells (10⁵) isolated by mono-polyseduring medium were sediend in phosphate-buffered saline (PBS) with 1% fetal calf serum and 0.02% sodium azide (PBS-azide). Cells were incubated with 20 μL anti-HPCA-1 or irrelevant negative control antibody for 30 minutes at 4°C, washed four times in PBS-azide, and incubated for 10 minutes in 50 μL fluorescein (FITC) conjugated goat anti-mouse Ig reagent (Technogenetics, Trezzano sul Naviglio, Italy). After three washes with PBS-azide, the cells were fixed and stored in 100 μL 1% formalin. Before analysis, samples were resuspended in PBS. CD34+ cells were further characterized by a dual-color immunofluorescence technique using the following phycoerythrin-conjugated murine monoclonal antibodies: MY9 (CD33) and MY7 (CD13) purchased from Coulter, Hialeah, FL; Leu-17 (CD38), Leu-15 (CD11b), Leu-1 (CD5), anti-HLA-DR, Leu-4 (CD3), and Leu-12 (CD19) purchased from Becton Dickinson. For the sake of clarity, only the cluster designation (CD) designation of the antibodies is reported in this article. CD33 antibody recognizes PB monocytes and BM CFU-mix, CFU-GM, and BFU-E but not their precursors; CD13 antibody recognizes peripheral blood granulocytes, monocytes, and a fraction of CFU-GM; CD38 antibody recognizes a fraction of myeloblasts, promyelocytes, large granular lymphocytes, activated lymphocytes, and plasma cells; CD11b antibody recognizes the C3bi complement receptor present on a fraction of myeloblasts, PB lymphocytes, large granulocytic lymphocytes, neutrophils, eosinophils, and monocytes; CD5 antibody recognizes most PB T lymphocytes and a subset of B lymphocytes; CD3 antibody recognizes most PB T lymphocytes and subsets of B lymphocytes; CD22 antibody recognizes many of the Fcγ receptors; CD19 antibody recognizes virtually all PB B lymphocytes, and fluorescein-conjugated goat anti-mouse Ig

### Table 1. Patient Characteristics and Yield of Hematopoietic Cells Harvested by PB Leukaphereses or Multiple Aspirations From Iliac Bones

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age (yr)/Diagnosis</th>
<th>Days* of rHuGM-CSF</th>
<th>No. of Leukaphereses</th>
<th>Timing*</th>
<th>Mononuclear Cells (x 10⁶)</th>
<th>Timing*</th>
<th>Mononuclear Cells (x 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient A</td>
<td>88-00987</td>
<td>29/HD</td>
<td>None</td>
<td>4</td>
<td>15-18</td>
<td>8.3</td>
<td>22</td>
</tr>
<tr>
<td>Patient B</td>
<td>88-11865</td>
<td>39/BC</td>
<td>None</td>
<td>4</td>
<td>17-21</td>
<td>10.2</td>
<td>22</td>
</tr>
<tr>
<td>Patient C</td>
<td>88-11313</td>
<td>40/BC</td>
<td>5-14</td>
<td>4</td>
<td>17-21</td>
<td>12.1</td>
<td>22</td>
</tr>
<tr>
<td>Patient D</td>
<td>88-11442</td>
<td>47/NHL</td>
<td>1-14</td>
<td>4</td>
<td>14-17</td>
<td>24.2</td>
<td>22</td>
</tr>
<tr>
<td>Patient E</td>
<td>88-09160</td>
<td>20/NHL</td>
<td>1-14</td>
<td>3</td>
<td>13-15</td>
<td>7.6</td>
<td>18</td>
</tr>
</tbody>
</table>

Abbreviations: UPN, unique patient number; HD, Hodgkin’s disease; NHL, non-Hodgkin’s lymphoma; BC, breast carcinoma.

On day 0, patients were treated with HD-CTX. Thereafter, patients C, D, and E received rHuGM-CSF 5.5 protein μg/kg/day IV continuous infusion as indicated.

*Time in days after HD-CTX (day 0).
CIRCULATING HUMAN HEMATOPOIETIC STEM CELLS

(Becton Dickinson) for 15 minutes at 4°C. Cells were then washed once, incubated with 20 μL monoclonal murine Ig to saturate all sites on the goat anti-mouse Ig, and washed again before second labeling with phycoerythrin-conjugated monoclonal antibodies. Quantitative analyses were performed using a FACSCANT cell sorter (Becton Dickinson). The frequency of CD34+ cells was determined by subtracting the percentage of cells positively staining with the control reagents (≤2%) from the percentage of cells positively staining after labeling with anti-HPCA-1 and FITC-conjugated anti-mouse Ig. A minimum of 20,000 events were collected in list mode on a consort 30 data management program. This allowed reanalysis of the data by contour plot diagrams to demonstrate the correlation between green FITC and red phycoerythrin colors of immunofluorescence for populations of cells identified also by forward- and right-angle light scattering.

A comparative estimate of the overall number of circulating CD34+ cells between different patients was made by comparing the areas under the curve of CD34+ cell counts per day. Areas under the cell count curve were calculated by the trapezoidal rule from first day to last day of sample analysis.25 Results were expressed as fold increase in comparison to the area under the CD34+ cell curve of control patient A not receiving rHuGM-CSF after HD-CTX.

Hematopoietic colony-forming assays. The frequency of CFU-mix, BFU-E, and CFU-GM hematopoietic progenitor cells was evaluated by clonal assays as previously described.26 In two experiments, PB cells from patients B and E were sorted into CD34+-enriched and CD34+-depleted populations and then cultured to determine the frequency of hematopoietic progenitor cells in these two populations. Technique for cell sorting implied incubation of PB nonadherent mononuclear cells with anti-HPCA-1 antibody for 30 minutes at 4°C, three washes, mixture with sheep anti-mouse Ig-conjugated magnetic beads (Dynabeads, Dynal, Oslo, Norway) at a ratio of 1:20 (cell to bead), and separation of CD34+ cells by a cobalt-samarium magnet-equipped device (Dynal). To obtain countable hematopoietic colonies, PB CD34+ cells were cultured at very low cell concentrations (100 to 1,000 cells/mL).

RESULTS

Circulation of CD34+ cells into peripheral blood. Peripheral blood samples were analyzed daily from day −1 before HD-CTX until further cancer chemotherapy was administered, usually the day after BM harvesting. Starting on day +7 (median) after HD-CTX, patients experienced HD-CTX–induced pancytopenia which lasted for 4 to 8 days, depending on whether they received rHuGM-CSF therapy.

CD34+ cells were undetectable in PB of HD-CTX–treated patients from day −1 to +10. They appeared in the circulation during hematopoietic recovery after HD-CTX–induced pancytopenia (Fig 1). Administration of rHuGM-CSF for 14 days from day +1 to +14 (Fig 1, patients D and E) accelerated the recovery and increased the number of leukocytes and circulating CD34+ cells. In contrast, administration of rHuGM-CSF only for 10 days from day +5 to +14 (patient C, not shown in Fig 1) did not influence either the leukocyte recovery or the number of circulating CD34+ cells as compared with patients who did not receive rHuGM-CSF (Fig 1, patients A and B). In all patients, the number of CD34+ cells decreased throughout the third week after HD-CTX. In comparison to control patient A, the fold increase of the areas under the CD34+ cell count curves of patients D and E receiving rHuGM-CSF for 14 days was 4.7 and 5.1, respectively.

Large-scale collection of peripheral blood and bone marrow hematopoietic cells. Starting on the first day on which leukocyte counts reached ≥1,000/μL and platelet counts reached ≥70,000/μL, patients underwent PB leukaphereses on 4 (patients A through D) or 3 (patient E) consecutive days. The yield of mononuclear cells collected by leukapherase-
es is shown in Table 1. As shown in Fig 2 (top), for each patient the cumulative yield of circulating CD34+ cells was between $0.45 \times 10^9$ and $2.73 \times 10^9$ cells. In particular, the frequency of CD34+ cells represented 5.4%, 5.5%, and 4.6% of collected PB mononuclear cells for patients who were untreated (patients A and B) or 10-day treated (patient C) with rHuGM-CSF. For patients D and E who received rHuGM-CSF for 14 days after HD-CTX, the frequency of CD34+ cells was 11.2% and 28.5% of mononuclear cells collected by leukapheresis.

Patients underwent harvesting of autologous BM on the working day after completion of the leukapheresis program. The yield of BM mononuclear cells is shown in Table 1. As shown in Fig 2 (bottom), the cumulative yield of CD34+ cells from BM harvests of each patient was between $0.07 \times 10^9$ and $0.52 \times 10^9$ cells. In particular, the frequency of BM CD34+ cells represented 1.8%, 6.1%, 9.5%, 2.8%, and 4.6% of collected mononuclear cells for patients A through E, respectively.

Flow cytometry characterization of peripheral blood CD34+ cells. As shown in Fig 3, the flow cytometry analysis of low-density PB leukocytes collected during hematopoietic recovery after HD-CTX showed heterogeneous cell types markedly different from those usually found in steady-state PB. Similarly to normal BM, the cytogram of PB leukocytes we studied showed three operationally defined cell populations that could be isolated and morphologically identified. Window L (“lymph”) was the cytogram region defined by light scatter as the population containing lymphocytes and erythroid cells that were relatively small by forward-angle light scatter on vertical axis and agranular by right-angle light scatter on horizontal axis. Window B (“blast”) comprised a cell population larger by forward-angle light scatter and with moderate amounts of right-angle light scatter. This population contained mononuclear cells of predominant blastlike cell morphology with few or no cytoplasmic granules. Window G (“granulocytic”) comprised cells with high forward-angle and right-angle light scatter signals. The cells in this window were predominantly immature granulocytes, most mature granulocytes having been removed by previous density-gradient centrifugation.

To determine the flow cytometry characteristics of PB CD34+ cells, we selected an FITC-immunofluorescence and right-angle light-scatter analysis gate which included CD34+ cells and their negative counterpart regardless of the forward-angle light-scatter properties. This combined immunofluorescence right-angle light-scatter analysis showed that
CD34⁺ cells in cytogram window B and/or window L had marked patient-to-patient variability (Fig 4). The membrane phenotype of CD34⁺ cells from four different patients was further analyzed by dual-color immunofluorescence. As shown in Table 2, CD34⁺ cells displayed heterogeneous myeloid differentiation markers that correlated with the different cytograms observed in each case. When CD34⁺ cells fell within window L (analysis 1 in Table 2 and Fig 4), they were mostly CD33⁺, CD13⁺, CD38⁺, and CD11b⁻. As the physical features of CD34⁺ cells shifted toward window B, these cells variably coexpressed more differentiated myeloid surface markers. In analyses 2 and 3, CD34⁺ cells were CD33⁺ or CD33⁺, CD13⁺ or CD13⁺, CD38⁺ or CD38⁺, and CD11b⁻ or CD11b⁻. In analysis 4, only 6% of gated cells (1.8% of all analyzed cells) were CD34⁺; the remaining myeloid cells mostly expressed more differentiated antigens such as CD33, CD13, CD38, and CD11b. Representatively, a gradient of phenotype maturation from poorly differentiated (analysis 1) throughout more differentiated (analysis 4) cells for the two antigens CD34 and CD33 is shown in Fig 5.

As shown in Table 2, PB CD34⁺ cells were predominantly DR⁺, only a minor fraction of CD34⁺ cells not coexpressing the DR antigen. By dual-color fluorescence analysis, CD34⁺ cells exhibited a wide range of DR antigen expression from high to undetectable densities (Fig 6). The CD34⁺/DR⁻ subpopulation was more frequent in analyses 1 and 2 (2.1% to 17.5%) in which sizable fractions of CD34⁺ cells were also CD33⁺, CD13⁺, CD38⁺, and CD11b⁻ (ie, lacked other more differentiated myeloid markers).

We also evaluated CD34⁺ cells for coexpression of the CD5 antigen, a known marker of mature T lymphocytes and a fraction of B lymphocytes. As shown in Table 2, CD34⁺ cells were CD5⁻ except in one case (analysis 2) in which about 25% of cells coexpressed this antigen. CD34⁺ cells have been reported to comprise lymphoid progenitor cells expressing intranuclear terminal deoxynucleotidyl transferase. Therefore, PB CD34⁺/CD5⁺ cells may represent an early step of lymphoid differentiation that deserves further investigation.

Nonmyeloid CD34⁺ cells were mostly lymphocytes of the T lineage; B cells were less than 6% in all analyses.

**Culture characterization of peripheral blood CD34⁺ cells.** To determine whether CD34⁺ cells from PB included hematopoietic progenitors as BM CD34⁺ cells do, low-density mononuclear cells from leukaphereses of patients B and E were separated into CD34⁺-enriched and CD34⁻-depleted fractions. As shown in Table 3 for a representative case, CD34⁺-enriched cell suspensions comprised the majority of hematopoietic progenitors and, taking into account the percentage of CD34⁺ cells in the assayed cell suspension, the cloning efficiency of CFU-GM, BFU-E, and CFU-mix represented approximately 15%, 5%, and 0.6% of cultured cells, respectively.

Furthermore, samples of all leukaphereses and BM grafts were evaluated in parallel for their content of CD34⁺ cells as well as CFU-GM progenitor cells. There was a linear correlation (r = .83 and .85) between the number of CD34⁺ cells and day 14 CFU-GM hematopoietic progenitors present in the same samples (Fig 7). These data indirectly
confirmed that evaluation of the frequency of CD34+ cells in PB as in BM reflected that of hematopoietic progenitors. In addition, the same data indicated that determinations of the frequency of heterogeneous (CD34 cells) and specifically committed (CFU-GM) hematopoietic progenitors were correctly performed using two quite different assays and that results were not biased by manipulations involved with such large cell suspensions.

**DISCUSSION**

In this study, we documented that many hematopoietic CD34+ cells are induced to circulate in the PB of adult cancer patients during that period of rapid leukocyte recovery after therapy with HD-CTX. In addition, after HD-CTX, IV administration of rHuGM-CSF for 14 days at a dosage associated with occasional mild side effects resulted in a fivefold enhancement of the number of circulating CD34+ cells. It has been known since 1976 that myelosuppressive cancer chemotherapy induces an overshoot of circulating committed hematopoietic progenitors after an initial pancytopenia. In particular, HD-CTX therapy produces an extraordinary increase of circulating hematopoietic progenitors corresponding to a 50- to 100-fold increase of CFU-mix and CFU-GM, as well as a 10- to 20-fold increase of BFU-E over normal steady-state values. Earlier reports have also shown...
shown that rHuGM-CSF alone or in combination with standard chemotherapy can induce a significant expansion of the circulating hematopoietic progenitor cell compartment in patients with solid tumors. By combining HD-CTX and 14-day rHuGM-CSF therapy, we could further amplify the number of circulating hematopoietic progenitors by approximately five- to tenfold in comparison to values relative to HD-CTX therapy without rHuGM-CSF. The knowledge that circulating hematopoietic progenitors are CD34+ can be useful for their rapid enumeration, biological characterization, and fractionation for experimental and possibly clinical utilization.

We also present evidence that the number of CD34+ cells retrieved from the circulation by leukaphereses exceeds the number that can be harvested by multiple BM aspirations from iliac bones under general anesthesia. In particular, the highest numbers of CD34+ cells are found in the leukapheresis cell suspensions of patients treated for 14 days with rHuGM-CSF, in which they represent approximately 10% to 30% of all mononuclear cells. In the same patients, the yield of CD34+ cell is ten- to 20-fold higher from PB than from BM. Thus, these data indicate that appropriate combinations of HD-CTX and rHuGM-CSF can render PB another rich source of hematopoietic progenitor cells possibly usable for restoring hematopoiesis after myeloablative chemoradiotherapy. The advantages of using hematopoietic stem cells harvested from PB include the possibility of circumventing the need for general anesthesia and allowing collection of stem cells even if the BM is damaged by previous radiotherapy or infiltrated with malignant cells.

In the last 3 years, our group has been exploiting the use of combined transplantation of autologous BM and PB leukocytes to restore hematopoiesis after fully myeloablative cancer therapy (ie, 12.5 Gy total body irradiation and 120 to 180 mg/m² IV melphalan). In our initial clinical study, transplanted PB leukocytes were collected after therapy with HD-CTX. In particular, PB leukocytes were harvested by three to four leukaphereses during the period of rapid leukocyte recovery occurring after pancytopenia induced by HD-CTX (ie, when CD34+ cells circulate and peak in the PB as shown in this article). In comparison to control patients transplanted only with BM, those who received both PB and BM leukocytes underwent shorter periods of neutropenia and thrombocytopenia, required less platelet and erythrocyte

![Fig 6. Representative dual-color immunofluorescence analyses of the density distributions of CD34 and DR antigens on PB leukocytes collected during hematopoietic recovery after HD-CTX. Bivariate contour plots of CD34 and/or DR antigen(s) were generated as described in the Materials and Methods section. Left and right panels are relative to patients D and E of Table 2 analyses 4 and 1, respectively. A minor fraction of CD34+ cells did not express HLA-DR antigens (right lower quadrants).](image-url)

Table 3. Growth of Hematopoietic Progenitors From PB CD34+ Cells

<table>
<thead>
<tr>
<th>Cells Plated per Culture</th>
<th>Hematopoietic Progenitors</th>
<th>CD34+ Enriched</th>
<th>CD34+ Depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU-mix</td>
<td>BFU-E</td>
<td>CFU-GM</td>
</tr>
<tr>
<td>10</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>100</td>
<td>0.3 ± 0.5</td>
<td>3.2 ± 1.1</td>
<td>13.6 ± 3.0</td>
</tr>
<tr>
<td>1,000</td>
<td>7.3 ± 2.3</td>
<td>44.6 ± 4.5</td>
<td>99.5 ± 0.7</td>
</tr>
<tr>
<td>10,000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of colonies (mean ± SD) from triplicate cultures of PB leukocytes from a leukapheresis of patient E. CD34+ enriched and CD34+ depleted cell suspensions were obtained from positive immune fractionation as described in the Materials and Methods section. They contained 79.4% and 1.9% CD34+ cells, respectively.
transfusions and, most important, experienced overall reduced treatment-related morbidity. More recently, administration of rHuGM-CSF for 14 days after HD-CTX resulted not only in shorter duration of neutropenia but also in the possibility of harvesting larger quantities of PB leukocytes as well as hematopoietic progenitors. In a series of seven consecutive patients, combined transplantation of autologous BM and PB leukocytes harvested after HD-CTX and rHuGM-CSF allowed even more rapid and complete hematopoietic recovery of all hematopoietic lineages and better treatment tolerance than has been described previously with the same method but without rHuGM-CSF. A possible explanation for this further clinical achievement may be that committed hematopoietic progenitors as well as early pluripotent stem cells are retrieved from circulation and transplanted in addition to BM cells after myeloablation. Recent studies in rodents indicate that BM transplantation is followed by two phases of engraftment associated with hematopoietic progenitors at different stages of maturation. An initial phase corresponding to early hematopoietic recovery is produced by committed progenitors, and a second sustained engraftment phase is produced by the pluripotent stem cell. If this is true of humans also, our studies using PB and BM progenitor cells in autografting situations might reflect transplantation of committed progenitors and pluripotent stem cells producing an early engraftment phase and then sustained hematopoiesis, respectively. In this regard, the contribution of PB and/or BM CD34+ cells remains to be established.

The main question is whether CD34+ cells in PB are equivalent to their marrow counterpart. In an attempt to address this issue we evaluated the in vitro growth characteristics and the immunologic phenotype of PB CD34+ cells by colony assays and dual-color immunofluorescence, respectively. We showed that PB CD34+ cells can be cultured at 100 to 1,000 cells/mL while qualitatively normal colony growth and high cloning efficiency comparable to that observed with BM CD34+ cells is maintained. Furthermore, PB CD34+ cells possess heterogeneous differentiation antigens and light-scatter properties analogous to those of BM CD34+ cells. Compelling experimental evidence suggests that early hematopoietic progenitors with some characteristics of pluripotent stem cells exist among BM CD34+ cells and that they are distinguishable from more differentiated committed progenitor cells. In particular, BM CD34+ cells can be divided into functionally distinct progenitor populations based on their expression of the major histocompatibility class II (HLA-DR) and the CD33 antigens. The density of HLA-DR determinants increases with maturation from the most immature CFU-blast progenitors (CD34+/DR-) through CFU-mix and BFU-E (CD34+/ weakly DR+) to CFU-GM (CD34+/strongly DR+). Recently, Andrews et al show that CD34+/CD33+ cells with low right angle and high forward light scattering account for virtually all committed colony-forming progenitors, whereas CD34+/CD33- cells with low right-angle and low forward-angle light scattering are earlier hematopoietic progenitors (ie, CFU-blast and precursors of committed colony-forming cells in Dexter-type long-term culture). Consistent with these findings, PB CD34+ cells comprise CD34+/CD33- and CD34+/DR- as well as CD34+/CD33+ and CD34+/DR+ cells. The former phenotypes are associated with undifferentiated (CD34+/CD13-,CD34+/CD38-, CD34+/CD11b-) cells with light-scatter properties of small lymphocytes, whereas the latter phenotypes are associated with more differentiated (CD34+/CD13+, CD34+/CD38+, CD34+/CD11b+) cells with light-scatter properties of blast-like cells. Although the frequency of CFU-blast colonies among circulating CD34+ cells remains to be evaluated, it is noteworthy that PB CD34+/CD33- and CD34+/DR- cells are present mostly in cell samples containing cells with flow cytometry characteristics of small agranular “lymphocyte-like” cells somehow resembling those composing CFU-blast colonies. Together, these data suggest that CD34+ cells in PB blood after HD-CTX with or without rHuGM-CSF are analogous to those in the BM of adult humans in the steady state.
CIRCULATING HUMAN HEMATOPOIETIC STEM CELLS

Our data show that an appropriately designed combination of HD-CTX cancer therapy and rhHuGM-CSF induces circulation of CD34+ hematopoietic progenitors. The possibility of harvesting large quantities of these cells by leukapheresis is relevant to the field of stem cell transplantation and possibly to genetic manipulations of the hematopoietic system in humans.

REFERENCES

22. Ross GD, Cain JA, Lachmann PJ: Membrane complement receptor type 3 (CR3) has lectin-like properties analogous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for eC3b. J Immunol 134:3307, 1985

ACKNOWLEDGMENT

We thank Drs Giuseppe Pellegris and Gian Alfredo Sciorelli for constant support, Dr Carmelo Bengalà for patient care, Drs Corrado Tarella and Dario Ferrero for interesting discussions, Drs Angelika Stern and Thomas C. Jones for invaluable cooperation, and Nadia Belli, Mario Avella, and Elisabetta Sommaruga for skillful technical assistance.


31. Bernstein ID, Leary AG, Andrews RG, Ogawa M: Blast colony and precursors of colony forming cells detectable in long term marrow culture express the same phenotype (CD33−CD34+). (submitted)
Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide-treated patients: enhancement by intravenous recombinant human granulocyte-macrophage colony-stimulating factor

S Siena, M Bregni, B Brando, F Ravagnani, G Bonadonna and AM Gianni