Positively Selected Autologous Blood CD34+ Cells and Unseparated Peripheral Blood Progenitor Cells Mediate Identical Hematopoietic Engraftment After High-Dose VP16, Ifosfamide, Carboplatin, and Epirubicin

By Wolfram Brugger, Reinhard Henschler, Shelly Heimfeld, Ronald J. Berenson, Roland Mertelsmann, and Lothar Kanz

To investigate the feasibility of peripheral blood CD34+ cell selection and to analyze CD34+ cell-mediated engraftment after high-dose chemotherapy, we performed a phase I/II trial in 21 patients with advanced malignancies. The rationale for the selection of CD34+ cells from peripheral blood progenitor cell (PBPC) collections is based on the observation that contaminating tumor cells can be depleted approximately 3 logs using this procedure. CD34+ cells from chemotherapy + granulocyte colony-stimulating factor-mobilized PBPCs were positively selected with an avidin-biotin immunoadsorption column (CEPRATE SC system). One leukapheresis product with a median number of 2.8 × 10^6 CD34+ cells/kg was labeled with a biotinylated anti-CD34 monoclonal antibody and subsequently processed over the column. The yield of selected CD34+ cells was 73% ± 24.8%. The purity of the CD34+ cell fraction was 61.4% ± 18.7%. CD34+ cells were shown to represent predominantly committed progenitors coexpressing CD33, CD38, and HLA-DR molecules (lin-). They gave rise to myeloid as well as erythroid and multilineage colonies in vitro. In addition, positively selected CD34+ cells also comprised early hematopoietic progenitor cells, as shown by the presence of CD34+/lin- cells. Transfusion of positively selected CD34+ cells (2.5 × 10^6 CD34+/kg; range, 0.45 to 5.1) after high-dose VP16 (1,500 mg/m²), ifosfamide (12 g/m²), carboplatin (750 mg/m²), and epirubicin (150 mg/m²) (VIC-E) in 15 patients resulted in a rapid and stable engraftment of hematopoiesis without any adverse events. As compared with 13 historical control patients reconstituted with a comparable number of unseparated PBPCs, time to neutrophil and platelet recovery was identical in both groups (absolute neutrophil count >600 µL, day +12; platelet count >50,000/µL, day +15). These data indicate that autologous peripheral blood CD34+ cells and unseparated PBPCs mediate identical reconstitution of hematopoiesis after high-dose VIC-E chemotherapy. Because positive selection of CD34+ cells from mobilized blood results in a median 403-fold depletion of T cells, allogeneic CD34+ cells from mobilized blood should be investigated as an alternative to bone marrow cells for allotransplantation.

PERIPHERAL BLOOD progenitor cells (PBPCs) are increasingly used for autografting after high-dose chemotherapy. These cells provide several advantages over the use of bone marrow stem cells, including a more rapid reconstitution of hematopoiesis. Moreover, another advantage might be a reduced risk of tumor cell contamination within PBPC collections as compared with autologous bone marrow. However, we have recently shown that there is a risk of comobilization of tumor cells and hematopoietic progenitor cells on recruitment of PBPCs. Although the biologic significance of these findings is unknown at present, we currently would prefer to transplant patients with PBPCs that have been depleted of potentially contaminating tumor cells. One possibility to purge PBPC preparations is the positive selection of CD34+ hematopoietic progenitor cells, resulting in an approximately 3 log depletion of tumor cells. CD34+ cells derived from the bone marrow have previously been shown to comprise committed as well as early hematopoietic stem cells, allowing hematopoietic engraftment in lethally irradiated baboons as well as in humans undergoing high-dose chemotherapy.

In this study, we investigated the feasibility of CD34+ cell selection from peripheral blood as well as the efficacy of these cells to reconstitute hematopoiesis after high-dose VP16, ifosfamide, carboplatin, and epirubicin (VIC-E) in patients with advanced malignancies. Our data show that a single leukapheresis is enough to select CD34+ cells for rapid and stable engraftment without additional bone marrow support.

MATERIALS AND METHODS

Study design. Criteria for entering this phase I/II study included patients (≤5 years) with advanced malignancy eligible for high-dose chemotherapy. Patients with central nervous system (CNS) metastases were excluded. The study protocol was approved by the institutional review board, and all patients gave written informed consent. Twenty-one patients were entered between December 1992 and September 1993 (Table 1). They were initially treated with two cycles of standard-dose VP16, ifosfamide, cisplatin (VIP) chemotherapy followed by granulocyte colony-stimulating factor (G-CSF) administration. Patients who responded with a complete or partial remission to the induction VIP chemotherapy (n = 15) were subjected to high-dose chemotherapy and transplanted with positively selected CD34+ cells. Six nonresponding patients (2 with progressive disease and 4 with no change) did not receive high-dose chemotherapy; one of them died because of tumor progression after the second cycle of induction chemotherapy.

The data of the CD34+ group of patients were compared with that of a historical group of patients (n = 13) who were treated according to the same high-dose chemotherapy protocol but were reconstituted with unseparated PBPCs. All patients received G-CSF after transplant. Both treatment groups were balanced with respect to age, prior treatment status, and type of disease (Table 1).

Standard-dose induction chemotherapy and mobilization of

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Positive selection of CD34+ cells from mobilized PBPCs. Harvested mononuclear cells (within a total volume of 50 mL) from one leukapheresis collection were incubated with a biotinylated anti-CD34 monoclonal antibody (MoAb; clone 12.8) for 20 minutes, washed in phosphate-buffered saline (PBS; Baxter, Munich, Germany), and passaged over a computer-driven avidin-immunoaffinity column device (CEPRATE SC System; CellPro Inc, Bothell, WA) with a loading capacity of more than 10^11 mononuclear cells. Adsorbed CD34+ cells (target cells) were removed from the avidin column, washed in PBS, resuspended in a final volume of 10 to 15 mL (2 x 10^8 cells/mL), and frozen in a controlled rate freezer in the presence of 7.5% dimethyl sulfoxide (DMSO; Sigma, Deisenhofen, Germany), 4% human serum albumin, and 10% U/L hep缩水 (Liquemin; Roche, Grenzach, Germany). Aliquots of the CD34+ target cell fraction and the nontarget cell fraction (unbound cells) were analyzed to assess the percentage of CD34+ cells as well as the antigenic phenotype and colony-forming capacity of cells in each fraction.

Clonogenic assay for committed hematopoietic progenitors. Unseparated or nonadsorbed PBPCs (1 x 10^6/mL) as well as positively selected CD34+ cells (5 x 10^6/mL, 1.5 x 10^7/mL, and 5 x 10^8/mL) were grown in 0.9% methylcellulose, as described. Cultures were performed in duplicates and stimulated with 100 ng/mL SCF (Amgen, Thousand Oaks, CA), 100 ng/mL interleukin-1β (IL-1β; Behringwerke AG, Marburg, Germany), 100 ng/mL granulocyte-macrophage-CSF (GM-CSF), 100 U/mL IL-3, 100 U/mL IL-6 (all from Sandoz, Nürnberg, Germany), and erythropoietin (EPO; 1 U/mL; Sulzbach/Taunus, Cilag, Germany). Formation of myeloid colony-forming units-granulocyte-macrophage (CFU-GM) erythroid-burst-forming unit-erythroid (BFU-E), and multilineage colonies (CFU-granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM)) was scored at day 12 to 14.

Flow cytometry. Cell fractions were incubated with phycoerythrin (PE)-conjugated anti-CD34 monoclonal or fluorescein isothiocyanate (FITC)-conjugated MoAbs (HPCA-2; Becton Dickinson, Rödermark, Germany) and/or anti-CD33-PE, anti-CD14-FITC, anti-CD11b-FITC, anti-CD15-FITC, anti-CD3-PerCP, or anti-HLA-DR-PerCP conjugated MoAbs (all from Becton Dickinson) for 30 minutes at 4°C. Dead cells were excluded by propidium iodide staining. Analysis was performed with a FACSscan analyzer (Becton Dickinson) equipped with a filter set for FITC-PE dual-color fluorescence. Data acquisition was performed with FACSVerse II research software and each measurement included 20,000 cells.

High-dose chemotherapy and stem cell support. High-dose chemotherapy with cumulative doses of 1,500 mg/m^2 VP16 (etoposide), 12 g/m^2 ifosfamide, 750 mg/m^2 carboplatin, and 150 mg/m^2 epirubicin was administered through a double lumen hickman catheter from days -4 through day -1. Unseparated PBPCs (control group) or positively selected CD34+ cells (study group) were rapidly rein fused 24 hours after the end of high-dose chemotherapy (day +1). All patients received G-CSF (Neupogen) from day +1 up to day +15 (5 μg/kg body weight subcutaneously). Supportive treatment included ondansetron and desamethasone, mesna uroprotection, parenteral nutrition, prophylactic oral antibiotics (ciprofloxacin), and fluconazole. Irradiated cytomegalovirus (CMV)-negative platelet transfusions were administered if platelet counts decreased to less than 20,000/μL blood. Packed red blood cells were administered if the hemoglobin level decreased to less than 8.0 g%.

Statistics. The statistical significance of the data obtained was analyzed by the Wilcoxon’s rank sum test and by the Student’s t-test (StatSoft Inc, Tulsa, OK). A P value <.05 was considered significant.

RESULTS

Positive selection and in vitro characterization of peripheral blood CD34+ cells. CD34+ cells from each single leukapheresis product were selected by the CellPro immunoadsorption column, a procedure that could be completed in less than 3 hours. The selection data are shown in Table 2. The mean number of mononuclear cells loaded was 1.2 ± 0.4 x

<table>
<thead>
<tr>
<th>Table 1. Patient Characteristics</th>
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<tr>
<td><strong>Standard-Dose</strong></td>
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<tr>
<td><strong>VIP Induction Chemotherapy</strong></td>
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<tr>
<td><strong>(n = 21)</strong></td>
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<tr>
<td>Male/female</td>
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<tr>
<td>Median age in yr (range)</td>
</tr>
<tr>
<td>No. of patients</td>
</tr>
<tr>
<td>Cancer</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Prior chemotherapy (yes/no)</td>
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<tr>
<td>Treatment outcome</td>
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Abbreviations: CR, complete remission; PR, partial remission.

<table>
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<tr>
<th>Table 2. Positive Selection of Peripheral Blood CD34+ Cells From VIP Chemotherapy + G-CSF Mobilized PBPCs</th>
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<tr>
<td><strong>Starting Cells (leukapheresis)</strong></td>
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<tr>
<td><strong>Total mononuclear cells</strong></td>
</tr>
<tr>
<td>% CD34+ cells</td>
</tr>
<tr>
<td>CD34+ kg (x10^6)</td>
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<tr>
<td>Yield of CD34+ cells (%)</td>
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<tr>
<td>% CD34+ cells</td>
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<tr>
<td>CD3 kg (x10^6)</td>
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</table>

Data are presented as mean ± SD from 21 patients. The percentage of CD34+ cells and CD3+ cells was determined by flow cytometry, as described in Materials and Methods.
10^6/kg, with a mean of 2.3% ± 1.7% CD34+ cells. After column separation, the total number of positively selected cells was 2.4 ± 1.8 × 10^6 (3.6 ± 2.0 × 10^6/kg), the percentage of CD34+ cells within this fraction was 61.4% ± 19.7%. The purity of the CD34+ cell fraction was positively correlated with the percentage of CD34+ cells in the leukapheresis product. The more pretreated the patient was, the lower the percentage of CD34+ cells. Best results in terms of purity and absolute numbers of CD34+ cells were obtained in newly diagnosed patients, with a consistent purity of more than 75% and a maximum number up to 9.5 × 10^6 CD34+ cells/kg body weight. The overall yield of CD34+ cells after column separation was 73%, with a mean number of 2.2 ± 1.8 × 10^6 CD34+ cells/kg. Generally, one column separation from one single 2-hour leukapheresis was sufficient to obtain a threshold dose of 1 × 10^6 CD34+ cells/kg body weight. Only in 5 extensively treated patients two consecutive separations were required to harvest more than 1 × 10^6 CD34+ cells/kg. In the unadsorbed cell fraction, the percentage of CD34+ cells was less than 0.3%. The total cellular recovery in the bound and unbound fractions was 92% (range, 81% to 98%), indicating that almost no cells were lost during the selection procedure.

The number of CD3+ T cells was significantly depleted on positive selection of CD34+ cells. The absolute number of CD3+ cells decreased from 48.4 × 10^6/kg in the leukapheresis preparation to 0.12 × 10^6/kg in the adsorbed cell fraction, which represents a 400-fold decrease in the number of T cells (Table 2).

The phenotypical characterization of selected CD34+ cells showed that a median of 40% (range, 23% to 84%) coexpressed CD33, 98% coexpressed CD38 (range, 94% to 100%), and 94% coexpressed HLA-DR (range, 86% to 99%). The percentage of CD34+/CD38+ and CD34+/HLA-DR+ cells was 1.7% (range, 0.5% to 13%) and 4.5% (range, 0.5% to 15%), respectively. The number of CD34+/CD19+ cells was 0.4% (range, 0.2% to 5%). The number of CD34+/glycophorin A+ cells was 9.4% (range, 1% to 28%). Clonogenic assays of positively selected CD34+ cells gave rise to myeloid, erythroid, and multilineage colonies, with a median of 98 CFU-GM (range, 46 to 272), 68 BFU-E (range, 36 to 213), and 27 CFU-GEMM (range, 3 to 61) per 1.5 × 10^6 adsorbed cells. The median number of colonies from the unadsorbed cell fraction was consistently less than 5 CFU-GM per 1.5 × 10^6, whereas BFU-E and CFU-GEMM colonies were grown only occasionally. CD34+-selected PBPCs were able to maintain long-term bone marrow cultures for up to 8 weeks (data not shown).

**Use of peripheral blood CD34+ cells after high-dose chemotherapy.** Fifteen patients previously shown to respond to the induction chemotherapy were transplanted with positively selected peripheral blood CD34+ cells. A median of 2.5 × 10^6 CD34+ cells/kg body weight (range, 0.45 to 5.1) were infused into the patients after high-dose VIC-E chemotherapy. Only 1 patient, a very intensively pretreated woman with metastatic osteosarcoma, was transplanted with less than 1 × 10^6 CD34+ cells/kg (0.45 × 10^6/kg).

No adverse events were associated with the infusion of positively selected CD34+ cells. The total volume of the selected CD34+ cells varied between 5 and 30 mL. No premedication was administered before infusion of progenitor cells.

The reconstitution pattern of neutrophils and platelets after high-dose VIC-E chemotherapy and CD34+ cell transplantation are shown in Fig 1. The median time to neutrophil recovery greater than 0.1 × 10^9/L was 11 days and the time to more than 0.5 × 10^9 neutrophils/L was 12 days. Time to platelet recovery greater than 20 × 10^9/L was 12 days and the time to platelet counts greater than 50 × 10^9/L was 15 days. When compared with 13 historical control patients who received unseparated PBPCs (median number of 2.7 × 10^6 CD34+ cells; range, 0.8 to 5.6) after application of the identical high-dose chemotherapy regimen, the engraftment data for both neutrophils and platelets were not different (Fig 2). The 95% confidence interval for the mean differences between the study group and the control group was +0.6 days (−1.85 to +0.65 days) for the time to greater than 0.5 × 10^9 neutrophils/L and −0.3 days (−2.22 to +2.82 days) for the time to greater than 50 × 10^9 platelets/L, respectively. Engraftment indices and hospital morbidity data of the two patient groups are shown in Table 3. Although this was not a randomized study, the data indicate that there were no significant differences in the duration of febrile episodes, days on intravenous antibiotics, or transfusion requirements. There were no deaths in either of the two patient groups. None of the patients has shown any signs of secondary graft failure after high-dose VIC-E chemotherapy and transplantation of selected CD34+ or unseparated PBPCs.

The treatment outcome of the patients treated with high-dose chemotherapy is summarized in Table 1. However, the median follow-up is too short at this point to get any conclusive results. In the CD34+ group of patients, 3 of 15 patients died between 5 and 9 months after high-dose chemotherapy because of recurrent disease.

**DISCUSSION**

We have shown that the positive selection of CD34+ cells from VIP chemotherapy + G-CSF mobilized peripheral blood is feasible using the CellPro immunoadsorption device. This selection procedure is simple and reproducible, resulting in a highly enriched fraction of peripheral blood CD34+ cells with an average of 2.2 × 10^6 CD34+ cells/kg from one single 2-hour leukapheresis. Positively selected peripheral blood CD34+ cells are clonogenic, as shown by the in vitro formation of myeloid, erythroid, and multilineage colonies. Moreover, selected peripheral blood CD34+ cells included long-term culture-initiating cells, indicating that very early progenitors are not lost during the selection procedure (Henschler et al, unpublished data). Our clinical data show that peripheral blood CD34+ cells are capable of rapidly reconstituting hematopoiesis after high-dose VP16, ifosfamide, carboplatin, and epirubicin. In addition, the infusion of selected CD34+ cells was not associated with any adverse reactions. The rates of neutrophil and platelet engraftment were equivalent when compared with those of a historical control group of patients transplanted with unseparated PBPCs. This observation suggests that the CD34+ cells are...
the cells responsible for hematopoietic reconstitution and that accessory cells from peripheral blood are not required.

However, reconstitution data with CD34+ cells and unseparated PBPCs might not be the same in regimens containing total body irradiation or in patients transplanted with peripheral blood CD34+ cells from allogeneic donors. Although our data provide evidence that peripheral blood CD34+ cells are capable of durably reconstituting hematopoiesis, only gene marking of peripheral blood CD34+ cells would prove their definite role in long-term hematopoiesis.

Our engraftment data with peripheral blood CD34+ cells are in accordance with preliminary data from the University of Colorado19 in breast cancer patients receiving positively selected CD34+ cells from G-CSF mobilized blood. However, these patients required three aphereses to get a comparable quantity of CD34+ cells for autografting. These data suggest that the mobilization of PBPCs on chemotherapy + G-CSF administration results in larger quantities of PBPCs than mobilization with G-CSF only.

The question of whether exogenously administered hematopoietic growth factors contribute to the acceleration of hematopoietic engraftment after transplantation of PBPCs or CD34+ cells is still under debate. In our patients (who all received G-CSF posttransplant), platelet recovery occurred 3
days later than neutrophil recovery, suggesting that G-CSF alone might not be sufficient to induce a parallel increase of both neutrophils and platelets. Previous data at our institution in patients receiving sequential IL-3 and GM-CSF after high-dose chemotherapy and unseparated PBPCs suggested that this combination might be more effective with respect to platelet reconstitution.\(^{5,13}\) However, randomized trials with different growth factors or growth factor combinations are required to answer this question.

The rationale to positively select CD34 cells for clinical use is based on the observation that up to 4 logs of contaminating tumor cells can be depleted using this procedure.\(^{10,19}\) However, because contaminating tumor cells can still be detected at very low numbers within selected CD34\(^+\) cell fractions,\(^{19}\) additional purging procedures may be required. These procedures might include depletion of malignant cells with MoAbs using a secondary immunoadsorption procedure or the application of antisense technologies. Another possibility might be the ex vivo expansion of CD34\(^+\) cells in the presence of hematopoietic growth factors with the potential to “biologically purge” malignant cells during ex vivo culture.\(^{23,24}\)

Because the number of T lymphocytes was depleted 400-fold on positive selection of peripheral blood CD34\(^+\) cells, our data suggest that allogeneic peripheral blood CD34\(^+\) cell transplantations could be an alternative to allogeneic bone marrow transplantation in patients with hematologic malignancies. The actual number of T cells within selected peripheral blood CD34\(^+\) cell preparations would be lower than in a conventional allogeneic bone marrow graft, suggesting that the incidence of graft-versus-host disease would also be reduced. Pilot studies using G-CSF mobilized allogeneic peripheral blood CD34\(^+\) cell transplants are ongoing at our institution.

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**Table 3. Engraftment indices and Hospital Morbidity After High-Dose VCR-E Chemotherapy: Peripheral Blood CD34\(^+\) Cells (Study Group) Versus Unseparated PBPCs (Control Group)**

<table>
<thead>
<tr>
<th>Study Group (n = 19)</th>
<th>Control Group (n = 13)</th>
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<tbody>
<tr>
<td>No. of days</td>
<td></td>
</tr>
<tr>
<td>To PLT &gt;5.0 × 10(^9)/L</td>
<td>15 (10-20)</td>
</tr>
<tr>
<td>To ANC &gt;0.5 × 10(^9)/L</td>
<td>12 (8-16)</td>
</tr>
<tr>
<td>With ANC &lt;0.5 × 10(^9)/L</td>
<td>8 (6-11)</td>
</tr>
<tr>
<td>Days with fever</td>
<td>2 (0-6)</td>
</tr>
<tr>
<td>Days on intravenous antibiotics</td>
<td>8 (1-19)</td>
</tr>
<tr>
<td>No. of units of packed red blood cells</td>
<td>5 (3-7)</td>
</tr>
<tr>
<td>No. of platelet units transfused</td>
<td>24 (12-66)</td>
</tr>
</tbody>
</table>

Values are the median, with the range in parentheses. Abbreviations: ANC, absolute neutrophil count; PLT, platelet count.

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