

RECONSTITUTION OF HEMATOPOIESIS AFTER HIGH-DOSE CHEMOTHERAPY BY AUTOLOGOUS PROGENITOR CELLS GENERATED EX VIVO

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Abstract *Background.* Autologous peripheral-blood progenitor cells can restore hematopoiesis after high-dose chemotherapy in patients with solid tumors or hematologic cancers. We investigated the ability of peripheral-blood progenitor cells generated ex vivo to restore hematopoiesis in patients with cancer who have undergone high-dose chemotherapy.

Methods. Ten patients who had received high-dose chemotherapy were given transplants of autologous progenitor cells that had been generated ex vivo. We used 11 million CD34+ hematopoietic progenitor cells as the starting population for the cell growth. This number corresponds to less than 10 percent of the usual preparation of peripheral-blood CD34+ mononuclear cells used in leukapheresis. The CD34+ cells were grown in medium containing autologous plasma, recombinant human stem-cell factor, interleukin-1 β , interleukin-3, interleukin-6, and erythropoietin.

Results. No toxic effects were observed with the infu-

sion of the generated cells. The cells promoted a rapid and sustained hematopoietic recovery when transplanted after treatment with high-dose etoposide (1500 mg per square meter of body-surface area), ifosfamide (12 g per square meter), carboplatin (750 mg per square meter), and epirubicin (150 mg per square meter). The pattern of hematopoietic reconstitution was identical to that in historical controls treated with unseparated mononuclear cells or positively selected CD34+ cells.

Conclusions. A small number of peripheral-blood CD34+ cells, when grown ex vivo, can supply a population of hematopoietic precursors that have the ability to restore blood formation in patients treated with high doses of chemotherapy. This method, which requires only a small volume of the patient's blood, may reduce the risk of tumor-cell contamination, circumvent the need for leukapheresis, and allow repeated cycles of high-dose chemotherapy. (N Engl J Med 1995;333:283-7.)

HEMATOPOIETIC progenitor cells in the peripheral blood are used increasingly to restore the formation of blood after high-dose chemotherapy for solid tumors or hematologic cancers.¹ As compared with rescue by autologous bone marrow transplantation, restoration with such cells shortens the period of pancytopenia and reduces the risks of infection and bleeding.²⁻⁴ However, the collection of peripheral-blood progenitor cells requires the removal of a large volume of blood by leukapheresis.

To minimize the contamination of collections of peripheral-blood progenitor cells by tumor cells,^{5,6} we have developed a method of growing the progenitor cells ex vivo from a relatively small volume of blood. A combination of stem-cell factor, interleukin-1 β , interleukin-3, interleukin-6, and erythropoietin promotes the growth of clonogenic progenitor cells and maintains primitive hematopoietic stem cells ex vivo.^{7,8} Pre-clinical studies have suggested that hematopoietic progenitor cells in peripheral blood, identified by the CD34 cell-surface marker, could be grown in cytokine-supported tissue culture and then used for hematopoietic reconstitution after high-dose chemotherapy.

In this study, we investigated the ability of CD34+ peripheral-blood progenitor cells generated ex vivo to restore hematopoiesis in patients undergoing high-dose chemotherapy for solid tumors. Ten patients received transplants of autologous progenitor cells that had been grown ex vivo from 11 million peripheral-blood

CD34+ cells, a number that corresponds to less than 10 percent of the usual preparation of such cells used in leukapheresis.⁹ We show the feasibility of this method and demonstrate that progenitor cells grown ex vivo can mediate rapid and sustained hematologic recovery when administered after a high-dose combination of etoposide (1500 mg per square meter of body-surface area), ifosfamide (12 g per square meter), carboplatin (750 mg per square meter), and epirubicin (150 mg per square meter). The pattern of reconstitution was identical to the recovery of hematopoiesis in historical controls who were treated at our institution with unseparated mononuclear cells or positively selected CD34+ cells.⁴

METHODS

Selection of Patients

Ten patients with advanced cancer who were eligible for high-dose chemotherapy were included in this phase 1-2 trial. The protocol was approved by the institutional review board as well as by local governmental authorities (the Regierungspräsidium of Freiburg, Germany). The characteristics of the patients are shown in Table 1. An evaluation of the patients' pretreatment status indicated that 3 of the 10 had had previous radiotherapy or chemotherapy, but the hematologic recovery of these patients after high-dose chemotherapy did not differ from the recovery in the other 7 patients.

Induction Chemotherapy and Mobilization of Progenitor Cells

All the patients received two cycles of induction chemotherapy three weeks apart, consisting of etoposide (500 mg per square meter), ifosfamide (4 g per square meter), cisplatin (50 mg per square meter), and epirubicin (50 mg per square meter), a regimen previously shown to be active against a variety of cancers.¹⁰ Twenty-four hours after the second cycle of chemotherapy, the patients received filgrastim (granulocyte colony-stimulating factor, or G-CSF [Neupogen, Amgen, Munich, Germany]) at a dose of 5 μ g per kilogram of body weight subcutaneously to treat chemot

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ed neutropenia and mobilize peripheral-blood progenitor cells,⁹ which were collected in a single leukapheresis in which 6 liters of blood was processed.⁹ CD34+ cells were positively selected by immunoadsorption columns (Ceprate SC system; CellPro, Bothell, Wash.), as described elsewhere.⁴ A median of 2.8 million CD34+ cells per kilogram of body weight were recovered; the mean (\pm SD) purity of the selected fraction of CD34+ cells was 72.1 ± 9.3 percent, with a yield of 64 percent.

Ex Vivo Growth of Positively Selected CD34+ Cells

The starting population for ex vivo growth consisted of 15 million cells after selection for the CD34 cell-surface marker, corresponding to a median of 11 million CD34+ cells (fewer than 2×10^5 CD34+ cells per kilogram of the patient's body weight). These cells were grown in RPMI 1640 medium (Seromed-Biochrom, Berlin, Germany), 2 percent autologous plasma, recombinant human stem-cell factor (Genzyme, Rüsselsheim, Germany; 10 ng per milliliter of solution), recombinant human interleukin-1 β (Genzyme; 3 ng per milliliter), recombinant human interleukin-3 (provided by L. Färber, Sandoz, Nuremberg, Germany; 100 ng per milliliter), recombinant human interleukin-6 (provided by L. Färber; 100 ng per milliliter), and recombinant human erythropoietin (1 unit per milliliter; Cilag, Sulzbach, Taunus, Germany).⁷ The CD34+ cell fraction was cultured in five tissue-culture flasks (175 cm²; Falcon, Heidelberg, Germany) with 30,000 cells per milliliter, for a total volume of 100 ml per flask. The flasks were incubated at 37°C in a humidified atmosphere containing 5 percent carbon dioxide, and the cells were fed with 100 ml of fresh cytokine-supported medium on the seventh day of culture. On the 12th day, nonadherent cells were collected from the flasks, washed in 0.9 percent saline, and resuspended in 100 ml of normal saline supplemented with 1 percent human albumin (Cutter, Cologne, Germany) for reinfusion. Because of the timing of the preparative regimen, the CD34+ cells selected from 7 of the 10 patients were cultured without freezing. For the remaining three patients, CD34+ cells were frozen and thawed before culture. When the results obtained with the two groups of cells were compared, it was found that freezing and thawing did not influence the ex vivo results or the subsequent engraftment.

In Vitro Analyses of Progenitor Cells Generated ex Vivo

Assays for clones of the myeloid, erythroid, and multipotent lineages were performed as described elsewhere.⁴ CD34+ cells and the cells grown in culture were analyzed by flow cytometry (FACSscan analyzer, Becton Dickinson, Heidelberg, Germany) with monoclonal antibodies to CD1a, CD3, CD14, CD15, CD33, CD34, CD38, HLA-DR, glycoprotein IIIa (CD61), glycophorin A, and CD36.⁴

Levels of cytokines (interleukin-1 β , interleukin-6, interleukin-8, stem-cell factor, granulocyte colony-stimulating factor, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulat-

ing factor, and tumor necrosis factor-alpha) were analyzed by enzyme-linked immunosorbent assay (Quantikine, R&D Systems Europe, Abingdon, United Kingdom) in the supernatants of the cultures of progenitor cells on the 12th day of culture, as well as in plasma samples collected before the administration of high-dose chemotherapy and at various times thereafter.

High-Dose Chemotherapy and Transplantation of Cultured Progenitor Cells

High-dose chemotherapy was administered three weeks after the second cycle of induction treatment. It consisted of 1500 mg of etoposide per square meter, 12 g of ifosfamide per square meter, 750 mg of carboplatin per square meter, and 150 mg of epirubicin per square meter.^{4,9} The progenitor cells were given to the patients in infusion 24 hours after the end of this therapy (i.e., on day 1). Immediately before the reinfusion of the cultured progenitor cells, antihistamines (2 mg of clemastine and 20 mg of famotidine) and dexamethasone (8 mg) were administered. Supportive care included prophylactic oral ciprofloxacin, fluconazole, and granulocyte colony-stimulating factor (5 μ g per kilogram once daily subcutaneously from day 1 through day 12).

RESULTS

In Vitro Analyses of Cultured CD34+ Progenitor Cells

After the ex vivo culture of CD34+ cells for 12 days, immunophenotyping demonstrated less than 5 percent CD14+ monocytic cells, less than 10 percent CD15+ granulocytic cells, and less than 0.5 percent CD3+ T cells. The counts of glycoprotein IIIa+ megakaryocytic cells, CD36+ erythroid cells, and CD1a+ dendritic cells ranged from 0.5 percent to 4 percent. More than 90 percent of the cells expressed HLA-DR strongly, and 85 percent of all cells were positive for CD33, a marker of immature myelomonocytic progenitor cells. CD34 expression was detected in less than 0.5 percent to 2.5 percent of the cells generated ex vivo.

The median number of cells generated ex vivo was 11.8 million per kilogram of the donor's body weight (range, 4.3 million to 23.1 million), which corresponds to a median increase by a factor of 62.4 (range, 33.4 to 115.5) in the number of total nucleated cells. In vitro assays showed that the cultured cells gave rise to erythroid and granulocyte-macrophage progenitor cells, as well as to multilineage colonies, with a median increase in the number of all clonogenic cells by a factor of 50.3 (range, 14.4 to 92.5). A median of 123,000 colony-forming cells of all types per kilogram (range, 64,000 to 155,000) could be generated ex vivo (Table 2) and then given to the original donor in transplantation after high-dose chemotherapy.

Production of Cytokines by Cells Generated ex Vivo

The cells generated ex vivo produced large amounts of macrophage colony-stimulating factor (median, 1952 pg per milliliter; range, 761 to 4220) and interleukin-8 (median, 534 pg per milliliter; range, 317 to 3742), as measured on day 12 of culture. However, these cells secreted only low amounts of tumor necrosis factor-alpha (median, 13 pg per milliliter; range, 9 to 68), granulocyte colony-stimulating factor (median, 20 pg per milliliter; range, 12 to 35), and granulocyte-macrophage

Table 1. Characteristics of the Patients.

PATIENT No.	AGE (YR)	TYPE OF METASTATIC CANCER*	PRIOR THERAPY	PATIENT RECEIVED UNCULTURED CD34+ CELLS AS WELL AS CELLS GROWN EX VIVO
1	38	Lung, non-small-cell	No	Yes
2	48	Lung, non-small-cell	No	Yes
3	41	Lung, non-small-cell	No	Yes
4	32	Nasopharyngeal	Yes	Yes
5	47	Unknown primary site	No	No
6	54	Unknown primary site	No	No
7	44	Lung, non-small-cell	Yes	No
8	26	Breast	Yes	No
9	57	Lung, non-small-cell	No	No
10	25	Soft-tissue sarcoma	No	No

*The patients with non-small-cell lung cancer had stage IIIA or IIIB cancer, and the patient with breast cancer had stage IV cancer.

Table 2. Numbers of Progenitor Cells Grown ex Vivo.

PATIENT NO.	ALL NUCLEATED CELLS (MILLIONS)	ALL COLONY-FORMING CELLS*	PROGENITOR CELLS†
<i>per kilogram of donor's weight</i>			
1	11.3	139,000	135,000
2	12.2	91,000	66,000
3	23.1	111,000	79,000
4	22.3	155,000	95,000
5	15.4	141,000	102,000
6	10.8	151,000	96,000
7	11.5	76,000	71,000
8	9.4	75,000	68,000
9	18.5	137,000	134,000
10	4.3	64,000	42,000
Median	11.8	123,000	88,000
Range	4.3–23.1	64,000–155,000	42,000–135,000

*Includes granulocyte-macrophage colony-forming units, erythrocyte burst-forming units, and multipotential colony-forming units (progenitors of granulocytes, erythrocytes, macrophages, and megakaryocytes).

†Includes granulocyte-macrophage colony-forming units only.

colony-stimulating factor (median, 42 pg per milliliter; range, 8 to 255). All concentrations of cytokines (including stem-cell factor, interleukin-1 β , and interleukin-6) dropped to undetectable levels after the cultured cells were washed, suggesting that very small amounts

of cytokines were returned to the patients in infusion with the cells generated ex vivo.

Safety and Clinical Efficacy of Transplantation with Progenitor Cells Generated ex Vivo

The ability of the progenitor cells generated ex vivo to mediate hematopoietic reconstitution after high-dose chemotherapy was tested in 10 patients. Four patients received uncultured CD34+ cells at the same time as the cells generated ex vivo, to avoid any impediment to hematopoietic recovery while possible toxic effects induced by the cultured cells were being evaluated. The infusion of up to 1.6 billion hematopoietic cells cultured ex vivo in a final volume of 100 ml was not associated with allergic, pulmonary, or renal side effects. Eight patients had stomatitis of grades II through IV (according to the classification system of the World Health Organization) after high-dose chemotherapy that required total parenteral nutrition for a median of 5 days (range, 2 to 11). One patient had neutropenic septicemia on day 6 and died of multiorgan failure 14 days after transplantation.

Hematopoietic recovery was rapid in the nine remaining patients (Fig. 1). In the four patients who had hematopoietic recovery after the transplantation of both progenitor cells generated ex vivo and uncultured

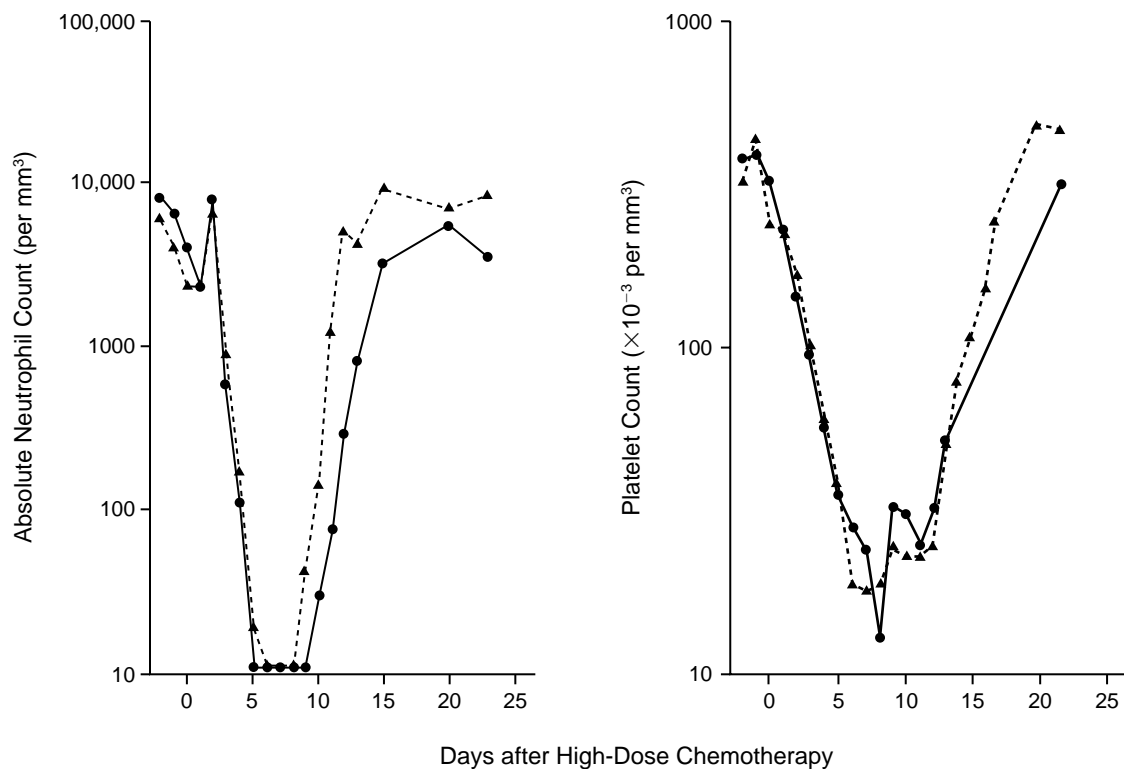


Figure 1. Hematopoietic Recovery in Nine Patients after Treatment with High-Dose Etoposide, Ifosfamide, Carboplatin, and Epirubicin and Transplantation of Progenitor Cells Generated ex Vivo.

Data are expressed as median absolute neutrophil counts and median platelet counts in the four patients who received both progenitor cells generated ex vivo and uncultured CD34+ cells (dashed lines) and in the five patients who received only progenitor cells generated ex vivo (solid lines).

CD34+ cells (median, 2.6 million CD34+ cells per kilogram; range, 1.4 million to 3.1 million), neutrophil counts greater than 500 per cubic millimeter first occurred on days 11 and 12. In the five patients who received only progenitor cells generated ex vivo, the neutrophil count was above 500 per cubic millimeter by day 13 (range, day 11 to day 15). The median duration of a neutrophil count below 100 cells per cubic millimeter was 5 days (range, 5 to 7) in the patients who received both uncultured CD34+ cells and progenitor cells generated ex vivo, as compared with 6 days (range, 3 to 11) in the patients who received only progenitor cells generated ex vivo. The time required to reach a platelet count above 20,000 per cubic millimeter was identical in both groups (median, 12 days; range, 11 to 15) (Fig. 1). The recovery of the platelet count to more than 50,000 per cubic millimeter occurred at a median of 14 days (range, 11 to 19). Analyses of the correlation between the number of colony-forming cells transplanted and the time to hematopoietic recovery (Table 3) suggest that a threshold dose of approximately 100,000 colony-forming cells per kilogram of body weight was needed for rapid engraftment. No patient had a secondary nadir of either neutrophils or platelets after transplantation, nor did infectious complications develop during a follow-up period ranging from 4 to 16 months.

Plasma Cytokine Levels

Before high-dose chemotherapy, the plasma levels of the various cytokines were in the normal range (Table 4). However, 10 days after transplantation there

Table 4. Levels of Cytokines in Samples of Plasma before and after High-Dose Chemotherapy and the Transplantation of Progenitor Cells Generated ex Vivo.*

CYTOKINE†	DAY -5	DAY 10	DAY 17
	<i>picograms per milliliter</i>		
Interleukin-1 β	1.2 (0.8 to 1.6)	1.1 (0.9 to 1.8)	1.2 (0.7 to 1.8)
Interleukin-6	11.8 (0.5 to 41)	111 (3.1 to 419)	5.1 (1.6 to 174)
Interleukin-8	58 (42 to 83)	424 (42 to 3668)	59 (44 to 460)
Stem-cell factor	847 (341 to 1153)	1072 (612 to 1705)	775 (536 to 1368)
G-CSF	43.2 (34 to 51)	8496 (38 to >10,000)	39 (24 to >10,000)
GM-CSF	1.5 (1.1 to 2.3)	4.8 (0.9 to 18.1)	1.3 (1.1 to 4.2)
TNF- α	1.1 (1.1 to 1.1)	2.6 (0.35 to >100)	1.3 (1.0 to 9.1)

*Data are medians (followed in parentheses by ranges), as determined by an enzyme-linked immunosorbent assay. Five patients were studied before treatment (on day -5), and plasma samples from all patients who could be evaluated were studied after treatment (on days 10 and 17).

†G-CSF denotes granulocyte colony-stimulating factor, GM-CSF granulocyte-macrophage colony-stimulating factor, and TNF- α tumor necrosis factor α .

were marked increases in the plasma concentrations of interleukin-6, interleukin-8, and granulocyte colony-stimulating factor, whereas plasma levels of stem-cell factor, interleukin-1 β , granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-alpha remained unchanged (Table 4). On day 17 after transplantation, all cytokines assayed were again in the normal range. For comparison, the plasma cytokine levels assayed in five historical control patients in whom hematopoiesis was reconstituted with positively selected, uncultured CD34+ cells after the same high-dose chemotherapy did not differ significantly from those in our study patients, although levels of interleukin-6 were slightly lower on day 10 (median, 46 pg per milliliter; range, 19 to 204).

DISCUSSION

This report documents the ability of autologous progenitor cells generated ex vivo to restore hematopoiesis after high-dose chemotherapy in patients with cancer. We have shown that ex vivo culture of 11 million CD34+ cells yields sufficient numbers of progenitor cells to allow rapid and sustained recovery of blood counts after high-dose chemotherapy in adults. The degree of hematopoietic reconstitution was similar to that in historical control patients who were treated with either unseparated mononuclear cells or CD34+ cells.⁴

We do not know which cell population mediates the rapid hematopoietic recovery after the transplantation of progenitor cells. It could consist of committed clonogenic progenitor cells; non-clonogenic CD33+ precursor cells, which make up 85 percent of the cells generated ex vivo in our system; or very early progenitor cells, such as cells that can initiate a long-term culture.

Our results do not allow firm conclusions about the long-term in vivo hematopoietic capabilities of the CD34+ cells cultured ex vivo. Reconstitution by endogenous precursors is likely to contribute to the long-term maintenance of hematopoiesis after high-dose chemotherapy. The introduction of a genetic marker into the transplanted cells or the use of allogeneic cells may

Table 3. Relation between the Number of Colony-Forming Cells Generated ex Vivo and Subsequently Transplanted and the Time to Recovery of the Platelet Count.*

PATIENT No.	No. OF CELLS/KILOGRAM	No. OF DAYS TO PLATELET RECOVERY†
1	139,000	12
2	91,000	14
3	111,000	12
4	155,000	11
5	141,000	12
6	151,000	15
7	76,000	14
8	75,000	15
10	64,000	17

*Patient 9 had neutropenic septicemia on day 6 after transplantation and died of multiorgan failure on day 14.

†Defined as a platelet count greater than 20,000 per cubic millimeter.

clarify this issue. One interesting clue is that very primitive human hematopoietic progenitor cells can persist under the culture conditions used here; 70,000 to 100,000 such cells have been detected after 12 days of culture.⁸ Extrapolation from experiments in mice indicates that a total of 10,000 to 30,000 cells capable of initiating long-term cultures may suffice for long-term reconstitution of the hematopoietic system after myeloablative therapy.⁸

The generation of progenitor cells *ex vivo* has advantages over the use of unseparated mononuclear cells or CD34+ cells for autografting. Hematopoietic progenitor cells can be generated *ex vivo* from small numbers of CD34+ cells. A starting population of 11 million CD34+ cells can be recovered from 100 to 200 ml of blood at the time of maximal mobilization of circulating peripheral-blood progenitor cells.⁷ We estimate that reducing the total volume of blood obtained from the patient and selecting CD34+ cells will reduce the load of tumor cells in the final transplant by about four orders of magnitude. This calculation has clinical relevance, because contaminating tumor cells in autologous marrow infusions can contribute to the recurrence of disease.¹¹⁻¹³ We cannot exclude the possibility that the cytokines used to generate CD34+ progenitor cells may also increase the clonogenic growth of tumor cells. However, neither primary nor xenograft-derived epithelial tumor cells increase in number during a 12-day coculture in serum-free medium (unpublished data).

Further strategies of *ex vivo* manipulation of hematopoietic progenitor cells should include the generation of immune effector cells. We have shown that functionally active antigen-presenting cells can be grown from the same starting population of CD34+ peripheral-blood progenitor cells by modifying the cytokine-supported conditions of culture.¹⁴ These cells can present soluble protein antigens to autologous T cells *in vitro*¹⁴ and thus offer new prospects for the immunotherapy of minimal residual disease after high-dose chemotherapy.

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