Transplantation of Allogeneic CD34+ Blood Cells

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Pluripotent stem cells of hematopoietic and lymphopoiesis are among the CD34+ cells in blood or bone marrow. After granulocyte-colony stimulating factor (G-CSF) treatment, 1% to 2% of the mononuclear cells in blood are CD34+ cells, which can be procured by leukapheresis. We investigated the potential of CD34+ blood cells for reconstituting hematopoiesis and lymphopoiesis after allogeneic transplantation. HLA-identical sibling donors of 10 patients with hematologic malignancies were treated with G-CSF (filgrastim), 5 μg/kg subcutaneously twice daily for 5 to 7 days. CD34+ cells were selected from the apheresis concentrates by immunoadsorption, concomitantly the number of T cells was reduced 100- to 1,000-fold. After transplantation, five patients received cyclosporine A for graft-versus-host disease (GvHD) prophylaxis (group I); five patients additionally received methotrexate (group II). G-CSF and erythropoietin were given to all patients. Mean numbers of 7.45 x 10^6 CD34+ and 1.2 x 10^6 CD3 cells per kilogram were transplanted. In group I, the median times of neutrophil recovery to 100, 500, and 1,000 per mm^3 were 10, 10, and 11 days, respectively. Group II patients reached these neutrophil levels after 10, 14, and 15 days, respectively. Platelet transfusions were administered for a median of 18 days in group I and 30 days in group II, and red blood cells for 9 and 12 days, respectively. Between day 30 and 60, lymphocytes reached levels of 353 ± 269 cells per mm^3. The median grades of acute GvHD were III in group I and I in group II. Two patients in group I died from acute GvHD. Two leukemic relapses occurred in group II. Complete and stable donor hematopoiesis was shown in all patients with a median follow up of 370 (45 to 481) days. Allogeneic blood CD34+ cells can successfully reconstitute hematopoiesis and lymphopoiesis. Reduction of T cells by CD34+ blood cell enrichment and cyclosporine A alone might not be sufficient for prophylaxis of severe acute GvHD.

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PATIENTS AND METHODS

Informed consent was obtained from patients and donors using the protocols and forms approved by our institution’s ethical committee.

Patients. Ten consecutive patients with hematologic malignancies and HLA identical sibling donors were considered for allogeneic transplantation with purified CD34+ blood cells (see Table 1).

Donors. All donors (6 men, 4 women, median 31.5 years old, range: 20 to 63), were healthy, HLA-A, -B, -C, -DR, ABO, and Rh blood group completely identical siblings, except for three cases with blood group mismatches. Sex mismatch existed in three cases (Table 1). The mixed lymphocyte reaction between patients and their donors was negative in all cases.

Mobilization and collection of CD34+ blood cells. A dose of 4 x 10^6 CD34+ cells per kilogram body weight of the recipient was considered the minimum required for successful engraftment. The CD34+ cells were immunoselected from the granulocyte-CSF (G-CSF) mobilized blood preparations as recently published. Thus, accelerated hematopoietic recovery without any significant exacerbation of acute GvHD. Because that study strongly suggested that purified CD34+ cells contain both committed and pluripotent stem cells, the present study investigated the effectiveness of selected blood CD34+ cells alone for allogeneic transplantation.
G-CSF (filgrastim; Amgen, Munich, Germany) was administered as follows: 5 μg/kg body weight subcutaneously twice daily for 5 to 7 days at 12-hour intervals. In addition to our previous protocol,platelets were separated from the leukapheresis concentrates by "soft spin" centrifugation (200g for 10 minutes with brake switched off) and reinfused to the donor immediately after completing theapheresis.

Immunofluorescence selection of CD34+. CD34+ cell immuno-selection with a CD34+ specific monoclonal antibody was performed on all leukapheresis preparations, according to the manufacturer's instructions with the Ceprate SC system (CellPro Inc, Bothell, WA) and as described.

The selected blood CD34+ cells had to be stored in liquid nitrogen in five cases (Table 1) because the coordination of stem cell harvest and transplantation was not possible for organizational reasons. The blood CD34+ cells for immediate transplantation were washed and resuspended in 50 mL phosphate buffered saline supplemented with 10 U/mL heparin.

Transplantation of CD34+ blood cells. The thawed or fresh cells were slowly injected with a 50 mL syringe into the recipient. Two graft portions were provided in this way: one from mobilization days 4 and 5, and the other from the next day or days. The frozen CD34+ cells were thawed in a water bath at 38°C for 5 minutes, diluted slowly to 30 mL with phosphate buffered saline supplemented with 10 U/mL heparin and subsequently injected into the recipient. The stored CD34+ cells were also transplanted in two portions (at days 0 and 2).

Quality control. Nucleated cell and platelet counts, the numbers of CD34+, CD3+, CD4+, CD8+, CD20+, and CD56+ cells, as well as the number of colony-forming units-granulocyte macrophage (CFU-GM) and burst-forming units-erythroid (BFU-E) were monitored in the blood of all donors as follows: before the start of G-CSF mobilization, on the evening of day 1 (after the second subcutaneous G-CSF injection), and each morning on days 2 through 7 immediately before G-CSF application. The same parameters were measured in the leukapheresis products, before and after positive selection of CD34+ cells. Post thawing recovery and viability of CD34+ blood cells were controlled by cell counting and trypan blue dye exclusion.

Clinical transplantation protocol. The day of the first CD34+ blood cell transplantation was standardized as of day 0. The second portion was transplanted on day +2. All patients received 5 μg/kg G-CSF (filgrastim) per kilogram body weight as a short intravenous daily infusion, starting at day +1 and continuing until 500 neutrophils per mm³ were detected for 3 consecutive days. Recombinant human erythropoietin (Boehringer-Mannheim, Mannheim, Germany) was administered at a dose of 150 IU/kg as a continuous intravenous daily infusion, starting at day +7 and continuing until patients remained erythrocyte transfusion-independent for 1 week.

GVHD prophylaxis consisted of 3 to 5 mg/kg cyclosporine A as a daily continuous intravenous infusion, starting by day -1 and adapted to a target whole blood level of 200 ng/mL. Cyclosporine A was administered orally as soon as oral mucositis had disappeared and stopped by day 180 after transplantation. Five patients (patients A through E—group I) received only cyclosporine A prophylaxis. Five patients (patients F through J—group II) additionally received intravenous methotrexate at 15 mg/m² on day +1 and 10 mg/m² on days +3 and +6. Because of liver toxicity, the day 6 methotrexate injection was omitted in patient F and postponed until day 11 in patient I. Because of severe mucositis, patient J did not receive methotrexate at day +1. The intention was initially to use cyclosporine A alone, because we thought that the reduction of T cells in the graft would be sufficient for GVHD-prophylaxis. With cyclosporine A alone, four of five patients developed grade III-IV acute GVHD. Therefore the second group of five patients received...
methylotrexate in addition. Acute GvHD was graded according to standard criteria. 21

Supportive care was performed as published previously. 19 Red blood cells were transfused to maintain a hemoglobin level above 8.0 g/dL and single donor platelet transfusions were used to keep platelet counts above 20,000 per mm3. The time to engraftment was assessed by determining the number of days after transplantation for patients to achieve 100, 500, and 1,000 neutrophils per mm3, 50,000 platelets per mm3 without transfusion for 7 days, and 10,000 reticulocytes per mm3. Control BM aspirations were routinely performed between days +8 and +28 posttransplant. Successful engraftment more than 3 weeks posttransplantation was proven by DNA analyses of patients' BM or blood nucleated cells. DNA polymorphism was analyzed by polymerase chain reaction for the β-globin gene 20 and at the HGM-locus D17S30 with variable numbers of tandem repeats (VNTR) at the pYNZ22-region. 21 If no differences between donors and recipients were found with either test, then the DS180-locus was checked for VNTR differences (AmpliFLP D1S80 PCR amplification kit; Perkin Elmer, Weiterstadt, Germany). Fluorescence in situ hybridization (FISH) for X and Y chromosomes on BM or blood cells was performed in the three cases of sex mismatch transplants (human Y chromosome DNA probe, Boehringer Mannheim; human X chromosome DNA probe; Dianova, Hamburg, Germany).

Data analysis. The data were analyzed with descriptive statistics, using standard statistical software (SPSS 6.01, Munich, Germany).

RESULTS

Mobilization. PB progenitor cells and CD34+ blood cell immunoisolation. Six of the 10 donors required four leukaphereses to obtain the target minimum quantity of 4 × 106 CD34+ cells after immunoisolation per kilogram body weight. Three leukaphereses were required in two further donors and only two aphereses in the remaining two donors. The following cell counts are calculated for a median of 72 kilogram of body weight of the recipients. Evaluating all aphereses (n = 34) before further manipulation, they contained a mean (±SD) of 599 ± 228 × 106 nucleated blood cells per kilogram, 89% of which were MNCs. Per apheresis the total numbers of the MNC subsets were (mean ± SD): 4.56 ± 2.7 × 108 CD34+ cells per kilogram, 4.97 ± 3.53 × 107 CFU-GM per kilogram, 8.53 ± 6.79 × 106 BFU-E per kilogram, 200 ± 90 × 106 CD3+ cells per kilogram with a CD4+ to CD8+ cell ratio of 1.57.

Before immunoisolation a mean number of 15.3 × 106 CD34+ cells per kilogram body weight was available. The processing of the leukapheresis concentrates resulted in a yield of 50% CD34+ cells (range 23% to 84%, n = 18). The preparations for transplantation contained a mean of 67.7% CD34+ cells. The yields for CFU-GM and BFU-E were 28% and 26%, respectively. A description of the transplanted blood cells per patient is provided in Table 1.

In one case (patient B), the target dose of 4 × 106 immunoisolated CD34+ cells per kilogram was not achieved, although four leukaphereses were performed and processed with the CellPro system (see Table 1). Patients D, F, G, H, and I received stored CD34+ cells with more than 90% recovery of viable cells after thawing.

By CD34+ cell concentration, the total blood CD3+ cells were reduced from 683 × 106 cells per kilogram body weight by 2.1 to 3.4 log to 1.19 × 106 CD3+ cells per kilogram.

The CD4+ versus CD8+ cell ratio was reversed to 0.57. The CD56+ blood cells were reduced from 59 ± 24 × 106 per kilogram to 7.4 ± 0.4 × 107 per kilogram.

Patient data. The data on hematopoietic recovery for individual patients are given in Table 2. The data on GvHD, chimerism, and clinical outcome are summarized in Table 3 Complete chimeric engraftment was proven in all patients within 27 to 223 days after transplantation.

As of January 16, 1996, complete and stable donor hematopoiesis was shown in all patients in remission by DNA analysis with a median observation period of 370 (45 to 481) days. No host DNA from leukocytes or BM cells was found after transplantation, except for the time of leukemic relapse.

The three living patients of group I were in complete remission and had stable and complete hematologic parameters between 13 and 16 months posttransplant. All patients were independent from hematopoietic growth factors and blood transfusions. The median grade of acute GvHD was III. Two patients (D and C) have died, both from acute GvHD and multiorgan failure.

Three patients in group II had grade I acute GvHD, one patient had grade III acute GvHD, while one patient did not develop GvHD. As of January 16, 1996, three patients are in complete remission with stable and complete hematologic parameters, 9 to 13 months posttransplant. All patients were free from support with hematopoietic growth factors or blood transfusions. One patient (G) with CML had a hematologic relapse of his disease 5 months after transplantation and died. Another patient (I) who was transplanted with relapsed AML had a recurrence of leukemia 8 months after transplantation.

BM smears were assessable 9 to 22 days after transplantation in all but patient E, showing regenerating granulopoiesis, erythropoiesis, and megalakaryopoiesis. The mean (±SD) number of lymphocytes from day 30 through day 60 was 353 ± 269 per mm3 with the following distribution of subgroups: CD3+ 38 ± 21%, CD56+ 39 ± 22%, CD20+ 1 ± 6.8%. The ratio of CD4+ to CD8+ cells was 0.87 ± 0.97.

DISCUSSION

This study shows that CD34+ stem cells selected from the blood of an HLA-identical sibling donor can successfully reconstitute hematopoiesis and lymphopoiesis. The human CD34+ cell fraction can establish full hematopoietic activity and stable hematopoietic recovery after allogeneic transplantation. This is in line with the experience in autologous transplantation, in which hematopoiesis recovers if CD34+ blood cells are used.10,11

The critical number of allogeneic CD34+ cells from blood for durable lymphohemopoietic engraftment is not yet known. However, if the cell numbers from BM transplantation (BMT) are calculated for comparison, then 200 × 106 nucleated cells per kilogram body weight are considered to be sufficient,21 which contain approximately 1% CD34+ cells.19 This results in 2 × 106 CD34+ BM cells per kilogram. In autologous blood stem cell transplantation, the standard number of CD34+ cells for reliable hematopoietic recovery is 2.0 to 5.0 × 106 per kilogram.24,25 However, it is not clear yet whether this figure is also valid for allogeneic blood
transplantation, CD34+ cells represent pluripotent and committed stem cells of hematopoiesis. Therefore, analysis of CD34+ subgroups, describing the probable stem cell compartment, will be necessary to define the critical amount of the most primitive stem cells.

The selected CD34+ blood cells engrafted after a median of 11.5 days for neutrophils. This was shorter than in patients transplanted with BM cells alone, which was 14 days, despite transplantation, comparable with conventional and T-cell-depleted BMT.30,31 T lymphocytes (CD3+) and natural killer cells (CD56+) recovered more rapidly than B lymphocytes (CD20+), which were rather scarce until 60 days after transplantation.

In the reports with blood cells for allogeneic transplantation, hematopoietic recovery was within the range of 8 to 10 days without methotrexate. In our study, using allogeneic CD34+ blood cells, an acceleration of erythropoiesis and thrombopoiesis was also suggested. Without methotrexate, there was a trend to reduced duration of transfusion dependence as compared with published data. Thus, transplantation of allogeneic CD34+ blood cells may be even more advantageous than BM cells for these cell lineages.29 T lymphocytes (CD3+) are depleted BMT.30,31 The selected CD34+ blood cells engrafted after a median of 11.5 days for neutrophils. This was shorter than in patients transplanted with BM cells alone, which was 14 days, despite the stimulation of granulopoiesis with G-CSF.26 BM cytology during the early phase showed regeneration of hematopoiesis. The hematopoietic recovery was similar in patients who received stored or fresh CD34+ blood cells. The five group I patients had a shorter regeneration of neutrophils and platelets, than the group II patients with methotrexate. Thus methotrexate may impair hematopoietic recovery, as after BMT.27,28

**Table 2. Hematopoietic Recovery, Transfusions**

<table>
<thead>
<tr>
<th>Recovery of Hematopoiesis (days)</th>
<th>Neutrophils × 1,000 per mm³</th>
<th>Platelets × 1,000 per mm³</th>
<th>Reticulocytes × 1,000 per mm³</th>
<th>Last Transfusion (days)</th>
<th>Transfusions (number)</th>
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<tr>
<td>Patients</td>
<td>100</td>
<td>500</td>
<td>1,000</td>
<td>20</td>
<td>50</td>
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<td>Group I</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>20</td>
<td>24</td>
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<tr>
<td>C</td>
<td>9</td>
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<tr>
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<td>9</td>
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<td>10</td>
<td>12</td>
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<td>Median group I</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>13</td>
<td>16</td>
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<td>I</td>
<td>18</td>
<td>20</td>
<td>20</td>
<td>13</td>
<td>32</td>
</tr>
<tr>
<td>J</td>
<td>14</td>
<td>16</td>
<td>17</td>
<td>14</td>
<td>27</td>
</tr>
<tr>
<td>Median group II</td>
<td>11</td>
<td>14</td>
<td>15</td>
<td>13</td>
<td>32</td>
</tr>
<tr>
<td>Median for all</td>
<td>10</td>
<td>11.5</td>
<td>13</td>
<td>13</td>
<td>28.5</td>
</tr>
</tbody>
</table>

* All patients received single donor apheresis platelet concentrates, each of them containing approximately 8 standard units of 5 × 10^10 platelets.

**Table 3. GvHD, Chimerism, and Outcome**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Grade Acute</th>
<th>Chimerism</th>
<th>Specimen</th>
<th>Day</th>
<th>Method</th>
<th>Probe</th>
<th>Status and Survival (days), as of January 16, 1996</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>III</td>
<td>lim</td>
<td>Blood</td>
<td>97</td>
<td>PCR</td>
<td>D1S80</td>
<td>CR (481)</td>
</tr>
<tr>
<td>B</td>
<td>IV</td>
<td>lim</td>
<td>Blood</td>
<td>223</td>
<td>PCR</td>
<td>pYNZ-22</td>
<td>CR (474)</td>
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<tr>
<td>C</td>
<td>IV</td>
<td>NA</td>
<td>Blood</td>
<td>27</td>
<td>FISH</td>
<td>Y,X-chromosomes</td>
<td>Died (45)</td>
</tr>
<tr>
<td>D</td>
<td>III</td>
<td>NA</td>
<td>Blood</td>
<td>28</td>
<td>PCR</td>
<td>pYNZ-22</td>
<td>Died (86)</td>
</tr>
<tr>
<td>E</td>
<td>II</td>
<td>lim</td>
<td>Blood</td>
<td>120</td>
<td>FISH</td>
<td>Y,X-chromosomes</td>
<td>CR (397)</td>
</tr>
<tr>
<td>F</td>
<td>I</td>
<td>lim</td>
<td>BM</td>
<td>34</td>
<td>PCR</td>
<td>D1S80</td>
<td>CR (383)</td>
</tr>
<tr>
<td>G</td>
<td>I</td>
<td>0</td>
<td>BM</td>
<td>77</td>
<td>PCR</td>
<td>D1S80</td>
<td>Rel, died (348*)</td>
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<tr>
<td>H</td>
<td>I</td>
<td>0</td>
<td>Blood</td>
<td>21</td>
<td>PCR</td>
<td>β-globin</td>
<td>CR (272)</td>
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<tr>
<td>I</td>
<td>0</td>
<td>0</td>
<td>Blood</td>
<td>54</td>
<td>PCR</td>
<td>pYNZ-22</td>
<td>Rel (293*)</td>
</tr>
<tr>
<td>J</td>
<td>III</td>
<td>lim</td>
<td>BM</td>
<td>51</td>
<td>PCR</td>
<td>D1S80</td>
<td>CR (272)</td>
</tr>
</tbody>
</table>

GvHD, chimerism and clinical outcome; in group I, the median grade of acute GvHD was II; in group II it was grade I and for all 10 patients it was grade II. Abbreviations: PCR, polymerase chain reaction; lim, limited; CR, complete remission; Rel, relapse.

* Relapses occurred in patient G with CML at day 150 and in patient I with AML at day 225 after transplantation.
four blood-apheresis products would result in more than $2\times 10^9$ CD3$^+$ cells per kilogram, which is ten times more CD3$^+$ cells compared with a standard BM harvest.19

Allogeneic BM containing more than $1 \times 10^5$ clonable T cells20 or more than $1 \times 10^6$ total T cells per kilogram can increase the incidence and severity of acute GvHD.15,32,33 Despite prophylaxis with combinations of methotrexate, cyclosporine A or prednisolone, acute GvHD in its more intensive forms, grade II-IV, limits the success of BMT by significant morbidity and a high incidence of death.34 In previous studies transplantation of viable, donor buffy-coat, blood cells after BMT to enhance the graft versus leukemia effect significantly increased the incidence of grade II-IV acute GvHD to 82% and led to a higher rate of 64% of nonrelapse deaths.35 Chronic GvHD may develop in a large proportion of recipients of unmodified blood with large numbers of T cells. The transfusion of donor buffy coat cells in addition to BM in patients with severe aplastic anemia resulted in an incidence of chronic GvHD of 68% and a mortality rate of 27%, whereas without donor buffy coat cells only 33% developed chronic GvHD, without lethal complications.16 In adoptive immunotherapy with blood MNC for relapsed chronic myeloid leukemia, a high incidence of acute and chronic GvHD has been reported.39,40 The CD3$^+$ selection and comonimation T-cell reduction might be advantageous in preventing chronic GvHD.

Depletion of T cells from the marrow graft, efficiently reduces the incidence of severe acute GvHD and subsequent chronic GvHD.36,37 T-cell depletion may lead to a risk of rejection, graft failure, and leukemic relapse.36,37 although this is not confirmed by others.38 At the time of BMT, at least $1 \times 10^6$ T cells per kilogram body weight are considered to be the minimum number for maintaining the graft-versus-leukemia (GvL) effect and for preventing graft failure or rejection.17

We hypothesized that the positive selection of CD3$^+$ cells with a concomitant 2 to 3 log reduction of T cells may reduce both the GvHD and rejection risks and preserve the GvL effect. The reduced quantity of transplanted T cells offered the possibility to omit the use of methotrexate for GvHD prophylaxis, and to avoid its toxic effects. However, in the five patients with only cyclosporine A for GvHD prophylaxis, four of them developed grades III and IV of acute GvHD and two died from GvHD-associated infections. Furthermore, the three surviving patients have chronic GvHD.

In the second group of patients who received cyclosporine A and methotrexate for GvHD-prophylaxis, only one patient experienced grade III acute GvHD and the others had none or only grade I. Chronic GvHD was observed in two patients, at 4 to 8 months after transplantation. Therefore, we suggest that CD3$^+$ cell reduction by CD3$^+$ cell selection and cyclosporine A are not sufficient for prevention of severe acute and chronic GvHD. This contrasts with the preliminary experiences in unmodified allogeneic blood transplants, using combination immunosuppression with cyclosporine A and other agents, in which the incidence of acute GvHD was not higher than after the transplantation of BM.

However, prospective comparative studies are necessary with the transplantation of enriched CD3$^+$ and unmodified blood cells, which are mobilized by G-CSF. Then the utility of CD3$^+$ blood cell selection for the prevention of acute and chronic GvHD can be assessed.

We conclude that the selection of hematopoietic progenitor cells from blood provides a new approach to obtain engrafting hematopoietic and lymphopoietic cells. Blood stem cell mobilization and collection is more comfortable and convenient for the donors than marrow harvesting. The present data prove that CD3$^+$ cells, immunoselected from the blood of the HLA-identical sibling donors, provide rapid and durable engraftment after allogeneic transplantation.

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REFERENCES


