Autologous transplantation of ex vivo expanded bone marrow cells grown from small aliquots after high-dose chemotherapy for breast cancer

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The collection of small aliquots of bone marrow (BM), followed by ex vivo expansion for autologous transplantation may be less morbidity, and more cost-effective, than typical BM or blood stem cell harvesting. Passive elimination of contaminating tumor cells during expansion could reduce reinoculation risks. Nineteen breast cancer patients underwent autotransplants exclusively using ex vivo expanded small aliquot BM cells (900-1200 x 10^6). BM was expanded in media containing recombinant fit3 ligand, erythropoietin, and PIXY321, using stromal based perfusion bioreactors for 12 days, and infused after high-dose chemotherapy. Correlations between cell dose and engraftment times were determined, and immunocytochemical tumor cell assays were performed before and after expansion. The median volume of BM expanded was 36.7 mL (range 15.8-87.0). Engraftment of neutrophils greater than 500/µL and platelets greater than 20 000/µL were 16 (13-24) and 24 (19-45) days, respectively; 1 patient had delayed platelet engraftment, even after infusion of back-up BM. Hematopoiesis is maintained at 24 months, despite posttransplant radiation therapy in 18 of the 19 patients. Transplanted CD34+/Lin− (lineage negative) cell dose correlated with neutrophil and platelet engraftment, with patients receiving greater than 2.0 x 10^5 CD34+/Lin− cells per kilogram, engrafting by day 28. Tumor cells were observed in 1 of the 19 patients before expansion, and in none of the 19 patients after expansion. It is feasible to perform autotransplants solely with BM cells grown ex vivo in perfusion bioreactors from a small aliquot. Engraftment times are similar to those of a typical 1000 to 1500 mL BM autotransplant. If verified, this procedure could reduce the risk of tumor cell reinoculation with autotransplants and may be valuable in settings in which small stem cell doses are available, eg, cord blood transplants. (Blood. 2000; 95:2169-2174) © 2000 by The American Society of Hematology
tumor cell depletion, and the reliability of this system for BMSC expansion.

Materials and methods

Selection of patients

Patients with stage II, with more than 10 involved axillary nodes and stage III/IV carcinoma of the breast, were eligible for high-dose chemotherapy in this Institutional Review Board approved clinical trial. Up to 2 regimens of chemotherapy and radiotherapy were permitted before entry. Patients with stage IV disease were required to have chemotherapy responsive, low tumor burden disease. Histologically normal bilateral BM aspirates and biopsies with a minimum cellularity of 30%, no comorbid illnesses and a normal cardiac ejection fraction were required. A delay of 4 weeks from previous chemotherapy or radiotherapy was also required.

Bone marrow harvesting and cryopreservation

BM was aspirated from both the anterior/posterior iliac crests in the operating room, with initial aspirates collected for expansion. For these aspirates, 2.5 to 3.0 mL of BM was collected from each bone puncture, comprised of 2 aspirations at different depths. Approximately 75 mL of BM was collected in this fashion for expansion processing. Then, 1.5 x 10^6 additional nucleated cells/kg was harvested and cryopreserved as a back-up, to be infused should neutrophil or platelet engraftment be delayed.

Ex vivo expansion

The BM mononuclear cells were expanded for 12 days in single pass, stromal-based AastromReplicell closed-system perfusion culture chambers, using a computer-directed process of gas and medium flow, determined by preclinical studies to maximize hematopoietic cell growth and stromal layer development. The growth surface area of the culture chamber is 750 cm². From 2.25 to 4.5 x 10^6 BM mononuclear cells, separated using Ficoll-Paque PLUS (Amersharm Pharmacria Biotech AB, Uppsala, Sweden), were placed into each of 3 or 4 single-use cassettes (culture chamber and disposable fluid pathway). Oxygen (20%), carbon dioxide (5%), and nitrogen (75%) were continuously exchanged through a gas-permeable, liquid-impermeable membrane covering each culture area. The cells were expanded in long-term bone marrow culture medium (Life Technologies; Grand Island, NY), consisting of IMDM, 10% fetal bovine, and 10% horse serum supplemented with medium-199 (Gibco Laboratories) containing 10% heparin and supplemented with 25% fetal bovine serum (HyClone Laboratories, Logan, UT) at a concentration of 2.5 x 10^6 cells/mL. Cytospins of 5 x 10^5 cells were prepared using a Cyto-Tek centrifuge (Miles Scientific, Elkhart, IN). After fixing in acetone/methanol/formalin (4%/4%/10%) for 20 minutes, slides were stained with the Br-3 antibody, which targets mucin-a expressed by breast cancer cells plus the anticytokeratin monoclonal antibody cocktail (AE1/AE3, Signet Laboratories, Dedham, MA). The slides were then counterstained with hematoxylin. A total of 10 stained slides were examined per specimen using a standard binocular light microscope.

High-dose chemotherapy and transplantation of expanded cells

All patients were treated with the STAMP V high-dose chemotherapy regimen (800 mg/m²), thiopeta (500 mg/m²), and cyclophosphamide (6000 mg/m²) as previously described. The expanded cells were infused over 60 minutes, unfiltered, 72 hours after the chemotherapy was completed. All patients received G-CSF (Amgen) subcutaneously at a dose of 10 µg/kg daily starting 4 hours after cell infusion and continuing until the neutrophil count rose above 1 x 10^9/L for 3 consecutive days. Supportive care consisted of prophylactic fluconazole, norfloxacin, and acyclovir, as well as platelet transfusions for counts less than 20 x 10^9/L. The back-up cryopreserved cells were to be infused if the absolute neutrophil count (ANC) had not reached 500/µL by day 21 or the platelet count had not reached 20 000/µL by day 28 after transplant. Hospital discharge occurred once the ANC had reached 500/µL; or an ANC greater than 100/µL, with the patients being afebrile for 48 hours. Radiation therapy was permitted per center routine as consolidation therapy after transplant.

Analysis of engraftment correlates

Primary endpoints were reliability of the culture system, time to engraftment of neutrophils and platelets, toxicities from the infused cells, the number of platelet transfusions, and days of fever with neutropenia. Engraftment of neutrophils and platelets was defined respectively as the first of 7 days that the ANC rose above 0.5 x 10^9/L, and the first day the platelet count rose above 20 x 10^9/L, without transfusions. Analyses of time to engraftment were correlated to nucleated and stem cell dose per kilogram, both before and after expansion, using curve-fitting methods that gave the best correlation coefficient. The significance of the correlation was determined at 95% confidence interval using a 2-tailed Student t test.
**Results**

**Patient characteristics**

The patient characteristics are shown in Table 1. We treated a total of 19 patients, of whom only 2 had high-risk stage II disease. Of the 10 patients with stage IV disease, 8 had relapsed after prior adjuvant chemotherapy and 2 presented with metastatic disease. None had bone scan abnormalities in their pelvic bones, and as per center selection policy for breast cancer transplants, all were transplanted in either a complete remission (CR) or near CR. Their median BM cellularity was 30% and none had histologic evidence of tumor involvement at entry.

**Analyses of expanded cells**

Analyses of the BM cells collected for expansion are shown in Table 2. Twelve of the 19 patients received the expanded cells from a starting inoculum of $9 \times 10^6$ BM mononuclear (MNC) cells, and the remainder from $12 \times 10^6$ MNC cells in an attempt to explore cell dose per engraftment interactions. This represents a median starting marrow aliquot for the entire patient group of 36.7 mL (range 15.8-87.0) and a medium cell dose of $13.0 \times 10^6$/kg (range 9.2-21.4). The mean percentage of CD34+/Lin- cells in the inoculum was 2.6%, and the preexpansion CD34 dose per kilogram was 3.5 (range 1.5-7.2) $10^6$/kg.

The cell expansion data are shown in Table 3. There was a median 4.6-, 11.0-, and 0.94-fold expansion of nucleated cells, CFU-GM and CD34+/Lin- cells in the expanded product. Of the 19 patients, 7 had expansion of CD34+/Lin- cells that averaged 2.7-fold. No unique clinical features, eg, stage or amount of prior therapy were noted in this patient subgroup. There was a 43.1-fold increase in stromal progenitor cells as measured by CFU-F. LTCIC cells were also expanded by a median value of 20%. The mean number of nucleated cells and CD34+/Lin- cells infused per kilogram were 55(32-98) $\times 10^6$/kg and 3.4(0.42-11.8) $\times 10^6$/kg, respectively. There was a decline in absolute numbers of B and T cells during expansion to 68% ± 15% and 82% ± 23%, respectively, of inoculation numbers.

**Clinical outcome**

All 19 patients received their expanded cells as planned, with all expansions exceeding minimum expansion and viability requirements. All microbiologic cultures of the expanded cells were negative and there were no WHO grade 2 or greater toxicities from their infusion. No patient had sepsis before engraftment and there were no grade 2 or greater nonhematopoietic transplant-related toxicities seen in any patient.

The median (range) times to ANC and platelet engraftment were 16 (13-24) and 24 (18-45) days, respectively (Table 4). Engraftment of neutrophils was sustained in all patients at follow-up times up to 30 months. One patient (patient 7) failed to engraft platelets by day 35 after transplant and received her cryopreserved marrow cells without incident. Despite the infusion of these cells, she failed to engraft platelets to greater than $100 \times 10^9$/L. Three others without ANC (1 patient) and/or platelet (3 patients) engraftment occurring before day +21 and +28, respectively, refused their back-up BM infusions. All engrafted within a week of these endpoints, and have had durable engraftment of all cell lineages. Eighteen (95%) of the patients received planned consolidative radiotherapy to the ipsilateral chest wall and axilla (stage II and III disease), or to single sites of metastatic disease after transplant, starting approximately day +60 after transplant. In several patients, a temporary drop in platelets, never requiring transfusions, was seen after the radiotherapy was completed. No patient has lost her graft at a median follow-up time of 24 months (range 20-30 months) for the entire group. Fourteen patients remain alive, with deaths due to progressive disease in patients with stage IV disease (4/10) and stage III (1/7) at 5 to 15 months after transplant.

Circulating neutrophils were seen for the first 2 to 3 days after the infusion of the expanded cells in most patients, and may have contributed to the fact that 68% had 2 or fewer days of posttransplant fever, despite the median time to ANC engraftment of 16 days. In fact, all but 1 patient had an ANC on day +1 after transplant greater than 100/µL, and in several patients, the ANC on the day after transplant was higher than on the actual day of transplant (median [ranges] for day 0 and day +1 were 604 [152-1710] and 282 [79-2000] µL/mL). The median time to discharge from the hospital was 12 days after transplant (range 9-16) at which time all patients were afebrile.

All 12 patients receiving greater than $2 \times 10^6$ CD34+/Lin- cells per kilogram of expanded cells engrafted platelets by day 28 in contrast to only 4/7 of those who received at least $2 \times 10^6$/kg. The most rapid platelet engraftment occurred in those who received either at least $5 \times 10^6$ CD34+/Lin- expanded cells per kilogram (median 20 days) or those in whom any expansion of CD34+/Lin- was seen (median 21 days). There was no correlation of either pre-and postexpansion nucleated cells, CFU-GM, multilineage committed progenitor cells, or LTCIC cell doses per kilogram to time to engraftment of neutrophils. There was a correlation between ANC engraftment and postexpansion CD34+/Lin- cell dose per kilogram ($P = .0005$)(Figure 1). Platelet engraftment correlated only with CD34+/Lin- postexpansion cell dose ($P < .0005$) and total nucleated cell dose ($P = .03$) infused, and there was a borderline correlation to the number of stromal cells infused (CFU-F; $P = .08$). Although engraftment correlated with the number of postexpansion CD34+/Lin- cells per kilogram, it did not correlate with the number of preexpansion CD34+/Lin- cells per kilogram.

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**Table 1. Patient characteristics**

<p>| Number transplanted | 19 |</p>
<table>
<thead>
<tr>
<th>Stage of disease at transplant</th>
<th>II</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>-----------------------</td>
<td>2</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Median age (range)</td>
<td>45 (32-54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of prior chemotherapy regimens</td>
<td>1: 15</td>
<td>2: 4</td>
<td></td>
</tr>
<tr>
<td>Median number of chemotherapy cycles</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number receiving prior radiotherapy</td>
<td>2</td>
<td></td>
<td></td>
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</tbody>
</table>

**Table 2. Preexpansion bone marrow cell characteristics**

| % Marrow cellularity preharvest | 30 (30-50) |
| Nucleated cells/mL harvested ($\times 10^6$) | 47.8 (31.0-77.5) |
| Mononuclear cells | 50.8 (38.4-74.2) |
| CD34+/Lin- in inoculum | 2.5 (1.6-3.9) |
| Mononuclear cell dose/kg expanded ($\times 10^6$) | 13.0 (9.2-21.4) |
| Milliliters of marrow used for expansion | 36.7 (15.8-87.0) |

Values expressed as medians (ranges).
Table 3. Cell expansion data

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Postexpansion</th>
<th>Expansion Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mononuclear</td>
<td>900</td>
<td>4307 (2524-6277)</td>
</tr>
<tr>
<td>CFU-GM $\times 10^8$</td>
<td>3.1 (0.9-9.6)</td>
<td>33.4 (11.9-74.8)</td>
</tr>
<tr>
<td>CFU-F $\times 10^3$</td>
<td>55.6 (11.3-126.9)</td>
<td>2397.7 (244.5-5629)</td>
</tr>
<tr>
<td>CD34$^-$/Lin $\times 10^6$</td>
<td>26.5 (14.4-49.5)</td>
<td>24.7 (2.5-73.6)</td>
</tr>
<tr>
<td>LTCIC $\times 10^3$</td>
<td>74.0 (32.4-518)</td>
<td>91.0 (9.0-370.5)</td>
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</tbody>
</table>

*Twelve patients transplanted with starting inoculum of $900 \times 10^6$, 7 patients with $1200 \times 10^6$ cells.

Tumor contamination assays

Of the 19 preexpansion samples, 1 (5.2%) was positive at a concentration of 4 tumor cells per $10^6$ marrow mononuclear cells. This patient’s expanded cells and those of the remaining patients were tumor cell-free at the sensitivity of 3 tumor cells per $10^6$ (90% confidence level).

Discussion

Using this stromal-based, continuous perfusion method of BMSC expansion, we have been able to achieve engraftment in patients after high-dose chemotherapy with a thiopeta-based (500 mg/m$^2$) regimen, starting from a median volume of BM of only 36.7 mL. Although this regimen has not been conclusively demonstrated to be ablative, several trials have indicated that the maximally tolerated dose of thiopeta is 100 to 180 mg/m$^2$, due to prolonged myelosuppression.22,23 One of 19 patients required the infusion of her “back-up” BM cells, yet still did not engraft platelets to greater than 60 000/µL, suggesting preexisting stem cell damage. Engraftment times for neutrophils and platelets are similar to those of a typical 1000 to 1500 mL autologous BM transplant,24,25 despite the infusion of approximately 1 log fewer CD34$^+$ cells per kilogram. In addition, the days of febrile neutropenia appeared to be fewer than recent series of both autologous BM or PBPC transplant.24,25 This finding, initially noted in the phase I trial of ex vivo expansion using perfusion bioreactors27 may have been due to the small numbers of circulating neutrophils seen during the first days after transplant, resulting from the infusion of large numbers of committed myeloid progenitor cells.

As has recently been noted for PBPC transplants, engraftment of both neutrophils and platelets was directly correlated to CD34 cell dose per kilogram transplanted.29,30 Unlike PBPC transplants, in which the minimum dose of CD34 positive cells leading to predictable platelet engraftment in patients with breast cancer by day 28 is 2 to 5 $10^6$/kg,27 the preliminary data in this trial indicated that only 2 $10^6$ CD34$^+$ cells per kilogram of expanded stem cells was needed to produce optimal platelet engraftment. The engraftment times seen here are at odds with the PBPC CD34$^+$ cell doses in the same range as indicated from several sources,28-30 and several hypotheses may explain our patients’ rapid engraftment times. First, STAMP V may not be sufficiently ablative to lead to profound aplasia for periods of longer than 28 days. As indicated above, data from several sources would indicate otherwise. Second, expansion in the perfusion bioreactors led to maintenance of the primitive stem cell pool as measured by the LTCIC and CD34$^-$/Lin$^-$ populations, which suggests that the same cell doses infused immediately after collection would produce the same results. We believe that this is unlikely because of a lack of correlation of engraftment to preexpansion CD34$^+$/Lin$^-$ cells, and the fact that this is a significantly smaller cell dose of unmanipulated BM cells than needed to reliably reconstitute animals.31-32 and again, we infused fewer CD34$^+$ stem cells than needed to reliably reconstitute hematopoiesis in PBPC clinical trials that have tested the CD34$^+$ doses in the range administered here.26-30 In fact, these data indicate that when less than 1.0 $10^6$ CD34/kg are infused as part of a PBPC transplant conditioned with non–total body irradiation (TBI) or busulfan-based regimens, including STAMP V chemotherapy, not only are median engraftment times prolonged but as many as 40% of patients do not achieve platelet engraftment by day 60 post transplant. Finally, it may be possible that more primitive stem cells, or possibly “facilitating cells,” including stromal cells33,34 that are infused with the expanded BM cells, are promoting the engraftment of a lower stem cell dose. Unique to these transplants is the infusion of expanded stromal progenitor cells as measured by the CFU-F assay,35 which increase during the 12-day culture period approximately 40-fold, which when infused with hematopoietic stem cells have been demonstrated to be important in facilitating engraftment in a murine model.36 Stromal cell damage exists before transplant,37,38 and recent data indicates that it is damaged as a result of high-dose therapy,30,41 perhaps because of a reduction of vascular cell adhesion molecule-1 (VCAM-1) on bone marrow stromal cells after chemotherapy exposure.38 Stromal cell contact appears critical for primitive hematopoietic cell expansion ex vivo.33,34 and these primitive cells maintain their surface expression of cell adhesion molecules unlike cells expanded in stromal-free cultures.42 Thus, it is possible that by infusion of large numbers of stromal progenitor cells with a transplant of hematopoietic stem

Table 4. Engraftment data

<table>
<thead>
<tr>
<th>Engraftment Parameter</th>
<th>Median Days to Engraftment (Range)</th>
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<tbody>
<tr>
<td>ANC $&gt;500/\mu L$</td>
<td>16 (13-24)</td>
</tr>
<tr>
<td>ANC $&gt;1000/\mu L$</td>
<td>18 (15-28)</td>
</tr>
<tr>
<td>Platelets $&gt;20 000/\mu L$</td>
<td>24 (19-45)</td>
</tr>
<tr>
<td>Platelets $&gt;50 000/\mu L$</td>
<td>33 (24-66)</td>
</tr>
<tr>
<td>Platelets $&gt;100 000/\mu L$</td>
<td>48 (26-364)</td>
</tr>
</tbody>
</table>

ANC = Absolute neutrophil count.

*18/19 received radiation therapy starting day +60.
cells that the same phenomena may be occurring in vivo. In fact, initial clinical trials of mesenchymal cell transplantation either alone or with a typical PBPC transplant are underway.43-44 Expanded mesenchymal cells were infused into 14 patients with advanced breast cancer along with PBPC appeared to shorten engraftment times of neutrophils (greater than 500/µL) and platelets (greater than 20 000) to 8 days each.45

Early hematopoietic engraftment of patients infused with cells grown from stromal cell–based cultures is in contrast to the G-CSF appear promising.51 Alternatively, preliminary results and higher BMSC doses collected after a short course of dose of greater than 5 cells expanded or in those patients receiving a total CD34

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