RAPID COMMUNICATION

Direct Demonstration That Autologous Bone Marrow Transplantation for Solid Tumors Can Return a Multiplicity of Tumorigenic Cells


Patients with solid tumors are increasingly being treated by autologous bone marrow transplantation (BMT). Although response rates appear to be increased, disease recurrence is the commonest cause of treatment failure. Whether relapse is entirely due to residual disease in the patient or arises also from infiltrating malignant cells contained in the autologous marrow transplant has not been resolved. If the latter explanation is correct, then purging would be required as part of the transplantation procedure. We used retrovirally mediated transfer of the neomycin-resistance gene to mark BM harvested from eight patients with neuroblastoma in clinical remission. The marked marrow cells were subsequently reinforced as part of an autologous BMT. At relapse, we sought the marker gene in malignant cell populations. Three patients have relapsed, and in each the marker gene was detected by phenotypic and genetic analyses of resurgent malignant cells at medullary and extramedullary sites. Analysis of neuroblast DNA for discrete marker gene integration sites suggested that at least 200 malignant cells, each capable of tumor formation, were introduced with the autologous marrow transplant and contributed to relapse. Thus, autologous BMTs administered to patients with this solid tumor may contain a multiplicity of malignant cells that subsequently contribute to relapse. The marker-gene technique we describe should permit evaluation of the mechanisms of relapse and the efficacy of purging in patients receiving autologous marrow transplantation for other solid tumors that infiltrate the marrow.

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AUTOLOGOUS bone marrow transplantation (BMT) permits intensified treatment for many solid tumors, including breast cancer, melanoma, and neuroblastoma.1-3 This approach to therapy has lead to higher tumor response rates than have been achieved with conventional treatment and may improve long-term survival rates.1,3 A limiting feature of autologous BMT is that many solid tumors are capable of hematogenous spread; hence, marrow collected from some patients may be contaminated with malignant cells, which could be inadvertently reintroduced at the time of transplantation.4,5 Different techniques have been used to purge BM putatively contaminated with malignant tumor cells.1,6,7 Although promising results have been reported,8 it is still unclear whether contaminating cells in the infused marrow contribute to disease recurrence, either in the marrow itself or in extramedullary sites.8 This uncertainty relates to the logistic problems inherent in the design of randomized clinical studies to show that purging is of benefit. For example, in patients receiving autologous BMT as treatment for neuroblastoma, demonstration that purging produced a 5% improvement in event-free survival would require 1,000 patients to be entered in each of the purging and control arms. To complete the accrual for such a study would require every eligible patient in North America to be recruited over a 20-year period. Nonetheless, without this critical information, the necessity and effectiveness of purging will remain in doubt.9

Retrovirally mediated gene marking techniques may offer the means to address these issues. If the harvested BM is exposed to a retroviral vector before reinfection, and the introduced gene is detected in the malignant cells at the time of relapse, one would have unequivocal evidence that residual malignant cells in marrow can contribute to disease recurrence.10,11 Here we report successful transfer of the neomycin-resistance gene into the harvested BM of patients with advanced neuroblastoma, and the detection of multiple marked neuroblastoma cells in each of the patients who relapsed in a medullary or extramedullary site.

MATERIALS AND METHODS

The protocol was approved by the institutional review board and biosafety committee of St Jude Children’s Research Hospital and the recombinant DNA advisory committee of the National Institutes of Health.12 Written informed consent was given by the patients’ parents.

We studied eight patients, 2 to 9 years of age, who were candidates for autologous BMT because of advanced neuroblastoma in first or second remission (Table 1). Each child had received 3 to 6 courses of chemotherapy that included cyclophosphamide, doxorubicin, etopo- side, and cisplatin. Marrow was harvested 2 to 5 weeks after completion of this therapy, when the peripheral blood neutrophil counts had increased to greater than 10³/mL. At that time, the marrow samples were judged to be free of malignant cells by standard clinical and marrow morphologic criteria. Nucleated cells (1.5 to 2.5 × 10⁸ kg of body weight) were harvested from the patients’ iliac crests and collected in sterile bags with acid citrate dextrose. Two-thirds of the harvested marrow was cryopreserved immediately. A monoclonal cell fraction of the remaining one-third was transduced with the LNL-6 or related GIN retroviral vector, both of which transfer the neomycin-resistance gene to target cells.12,13 The efficiency of
gene transfer with these vectors into clonogenic neuroblasts ranges from 0% to 16.5% (median, 3.5%). Because just one-third of the harvested marrow is exposed to the retroviral vector, this protocol should result in gene marking of approximately 1% of clonogenic neuroblasts present in the autologous marrow transplant. Both transduced and unmanipulated marrow cells were thawed and reinfused should result in gene marking of approximately ablative chemotherapy with carboplatin (700 mg/m²) neuroblasts present in the autologous marrow transplant. Both transgene transfer with these vectors into clonogenic neuroblasts ranges side was performed at 1, 3, months, and 6 months. and neuroblastoma. If relapse was morphologically or clinically evident, a purified population of malignant neuroblasts was prepared from a specimen of the excised material. PCR analysis was also used, with 25 cycles of amplification corresponding to linear signal strength.

Table 1. Neuroblastoma Patient Characteristics

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)/Sex</th>
<th>Diagnosis at Transplant</th>
<th>Vector</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4/M</td>
<td>Stage D 1st Remission</td>
<td>G1N</td>
<td>Well—18 mo</td>
</tr>
<tr>
<td>2</td>
<td>6/M</td>
<td>Stage D 1st Remission</td>
<td>LNL6</td>
<td>Marrow relapse at 9 mo</td>
</tr>
<tr>
<td>3</td>
<td>2/M</td>
<td>Stage D 1st Remission</td>
<td>G1N</td>
<td>Well—16 mo</td>
</tr>
<tr>
<td>4</td>
<td>3/M</td>
<td>Stage D 1st Remission</td>
<td>G1N</td>
<td>Well—15 mo</td>
</tr>
<tr>
<td>5</td>
<td>4/F</td>
<td>Stage D 1st Remission</td>
<td>G1N</td>
<td>Well—12 mo</td>
</tr>
<tr>
<td>6</td>
<td>6/F</td>
<td>Stage D 2nd Remission</td>
<td>LNL6</td>
<td>Well—6 mo</td>
</tr>
<tr>
<td>7</td>
<td>4/M</td>
<td>Stage D 1st Remission</td>
<td>G1N</td>
<td>Liver metastasis at 6 mo</td>
</tr>
<tr>
<td>8</td>
<td>9/F</td>
<td>Stage C 2nd Remission</td>
<td>LNL6</td>
<td>Marrow relapse at 6 mo</td>
</tr>
</tbody>
</table>

Marrow samples were taken at 1 month, 3 months, 6 months, 9 months, and 1 year after autologous BMT. Computed tomography was performed at 1, 3, and 6 months and at 1 year to detect recurrent neuroblastoma. If relapse was morphologically or clinically evident, a purified population of malignant neuroblasts was prepared from a mononuclear cell preparation of marrow or tumor biopsy by fluorescence flow cytometry, using GD2 antibody as a positive marker and CD45 antibody as a negative marker to provide distinction from hematopoietic progenitor cells. Reanalysis of the sorted population showed less than 0.1% leukocytes. In clonogenic cultures, the selected neuroblastoma population produced no hematopoietic colonies from 10⁵ plated cells. To determine whether these cells were positive for the marker gene, we extracted DNA directly from one sample and then attempted to grow the remaining cells in methylcellulose, with or without the neomycin analogue G418 at 1 mg (active)/mL, a concentration that inhibits the growth of nontransduced cells. DNA was then extracted from individual colonies with the distinctive neuroblastoma morphology and phenotype. All DNA samples were amplified by the polymerase chain reaction (PCR) with use of primers specific for the neomycin-resistance gene, as previously described. Semiquantitative PCR analysis was also used, with 25 cycles of amplification corresponding to linear signal strength.

Proviruses integration sites were analyzed by an adaptation of the inverted PCR reaction. In brief, RNAase treated DNA from GD2-CD45- purified neuroblastomas was completely restricted with TaqI endonuclease and then ligated with T4 ligase at 12°C overnight. Nested PCR with a Perkin-Elmer kit (Norwalk, CT) was used to amplify the ligated fragments. For amplification, we used the primers derived from LTR sequences. Internal primers were as follows: A, 5'-AGGAACGTCTTACCACA, and A', 5'-CTGGTCCTGGGAAGGT; followed by the external primers B, 5'-TCC-TGACCTTGATCTGA, and B', 5'-CTGAGGTATGGTACTCC to extend the PCR product. The products were electrophoresed on a 1.8% agarose gel, blotted, and analyzed by hybridization with an end-labeled oligonucleotide probe specific for the vector LTR. We used the following formula to estimate the minimum number of malignant cells present in the patients' marrow that contributed to relapse: minimum number of cells = (1/efficiency of marking × proportion of marrow exposed to vector) × number of integrants.

RESULTS

Table 1 shows treatment outcomes for the eight patients whose harvested marrow cells were transduced with the LNL6 or G1N retroviral vector. Five patients have remained in remission for 5 to 18 months after transplantation, and three patients have relapsed.

The recurrence of neuroblastoma in patients no. 2 and 8 was detected in routine marrow biopsies taken at 6 and 9 months posttransplantation. These samples contained 15% and 45% neuroblastoma cells, respectively. In each case, the marker gene was detected by PCR analysis in a DNA sample from 10⁵ GD2-CD45- neuroblasts and from clonogenic cells grown from the neuroblast population (Fig 1).

Patient no. 7 had evidence of metastatic disease in the liver on computed tomography scans performed 6 months after marrow transplantation. The hepatic lesion was removed surgically. Immunohistology confirmed the presence of a neuroblastoma deposit and GD2-CD45- cells were separated from a specimen of the excised material. PCR analysis of DNA from 10⁵ or more of the isolated neuroblasts showed

![PCR Analysis](image-url)
Fig 2. Limiting cycle (semiquantitative) PCR analysis of GD2+CD45- tumor cells at the time of relapse. Dilution curves (lanes 1 through 7) were obtained by diluting a neomycin-resistance gene-transduced K562 clone with a single integrant in nontransduced K562 cells at the following concentrations: 50%, 10%, 5%, 1%, 0.1%, 0.01%, and 0%. Lane 8, patient no. 2; lane 10, patient no. 7; lane 12, patient no. 8. Lanes 9 and 11, negative control DNA. One microgram of DNA from each specimen was amplified. The band shown is the 720-bp neomycin-resistance gene amplification product.

The presence of the marker gene. Histologic examination of a concurrent marrow biopsy failed to show tumor cells; however, the patient subsequently developed a marrow relapse and GD2+CD45- neuroblastoma colonies containing the neomycin-resistance gene were grown in methylcellulose (Fig 1).

We used a limiting cycle PCR technique14 to estimate the proportion of resurgent neuroblasts that were marker gene positive in each of these three patients (Fig 2). The neomycin-resistance gene was present in 0.05% to 1% of the GD2+CD45- cell population at sites of relapse (marrow and liver). Because only 1% of clonogenic neuroblasts present in the autologous marrow transplant were genetically marked (Materials and Methods), this level of tumor marking in vivo is consistent with a significant contribution to relapse from malignant cells present in the autologous marrow transplant.

We also estimated the number of reinfused marrow cells that likely contributed to relapse. Because retroviral vectors integrate into the host cell genome at many different sites,17 unique integration bands are generated by using restriction enzymes that cut once within the proviral genome and once in adjacent DNA. Using an inverted PCR technique, which can discriminate vector integration events by virtue of the size of the amplified PCR product,15,16 we detected two integrants among 106 purified neuroblasts from patient no. 2 as well as patient no. 8 (Fig 3). If we assume that neuroblasts are marked with an overall efficiency of 1% (Materials and Methods and Rill et al13), then a minimum of 200 "clonogenic" tumor cells were reinfused with the autologous marrow transplant, although the precise number depends on the efficiency with which tumorigenic cells are marked.

We have used gene marking to determine directly whether BM from patients with advanced neuroblastoma in remission contains residual malignant cells capable of contributing to disease recurrence. In each of the three patients who relapsed, a proportion of the resurgent neuroblasts contained the marker gene. We feel confident that the marker signal did not originate from contaminating normal leukocytes, because there was coexpression of the malignant marker and the transferred Neo5 gene could be readily discerned in individual colonies of G418-resistant neuroblastoma cells grown in methylcellulose from an FACS-sorted malignant population. Even in the bulk GD2+CD45- cell population, the low efficiency of gene transfer to normal cells means that too few marked leukocytes could have been present to account for the observed gene transfer levels.18

The cell marking technique we describe transfers the neomycin-resistance gene with relatively low efficiency,12 and only one-third of the harvested marrow is exposed to the retroviral vector before infusion. Despite these constraints, the massive expansion of the malignant cell population near the time of overt relapse, coupled with the sensitivity of PCR analysis, makes this procedure adequate for use with clinical samples.

The technique also allows quantitation of the clinically
relevant disease infiltrate in the autologous marrow transplant. Using PCR-based analysis of provirus integration sites,15,16 we were able to estimate that at least 200 malignant cells from the infused population contributed to relapse. Although the precision of this estimate is dependent on the accuracy of the assumption that we mark neuroblasts that are tumorigenic in vivo with the same frequency that we mark neuroblasts that form colonies ex vivo,17 the data establish that a multiplicity of cells in the marrow transplant contribute to disease recurrence. Moreover, the proportion of marked malignant cells in vivo at the time of relapse was similar to the proportion marked ex vivo and infused with the autologous marrow transplant,12 showing that residual tumor cells in marrow have considerable clonogenic potential in vivo and suggesting that these cells make a substantial contribution to resurgent disease. Although we could not formally assess the contribution of residual, unharvested neuroblasts to disease recurrence, our results strongly suggest that effective methods of purging will be a prerequisite to improve the outcome of intensified treatment followed by autologous BMT in patients with advanced neuroblastoma.19

If the principles we have demonstrated extend to other solid tumors that also disseminate by hematogenous spread (eg, breast cancer), then re-examination of purging techniques will likely be required for these neoplasms as well. It should be possible, using our gene marking strategy, to assess the efficacy of conventional and new marrow purging methods in small-scale clinical trials. Because integrant analysis would allow one to quantify residual disease, the technique could also be used to identify purging technologies that are only partially effective by themselves, but that in combination with other methods become highly effective with minimal toxic effects on hematopoietic stem cells.

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