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 reinfused 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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MARKED TUMOR CELLS AFTER ABMT

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—5 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>

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 reinjected into patients through a central venous line after they had received ablative chemotherapy with carboplatin (700 mg/m² x 3) and etoposide (500 mg/m² x 3).

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 methicellulose, 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. Semi quantitative PCR analysis was also used, with 25 cycles of amplification corresponding to linear signal strength.

Provirus integration sites were analyzed by an adaptation of the inverted PCR reaction. In brief, RNAase treated DNA from GD2-CD45 purified neuroblastoma cells was completely restricted with TaqI endonuclease and then ligated with T4 ligase at 0°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'-AGGAACTGCTTACCACA, and A', 5'-CTGTTCCTTGGAAGGGT; followed by the external primers B, 5'-TCCGTACCTTGTACCTGA, and B', 5'-CTGAGTGATGACTCC 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 neuroblastoma 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 of the isolated neuroblasts showed

![Fig 1. PCR analysis of GD2-CD45 neuroblastoma cells and colonies at the time of relapse PCR amplification was performed on DNA from 10⁵ sorted GD2-CD45 cells from the site of relapse and from individual, G418-resistant colonies grown in methicellulose (see Materials and Methods). Patient no. 2: lane 1, marrow tumor; lane 2, G418-resistant colony; lane 3, negative control. Patient no. 8: lane 4, marrow tumor; lane 5, G418-resistant colony; lane 6, negative control. Patient no. 7: lane 7, liver tumor; lanes 8 and 9, G418-resistant colonies (liver and marrow); lane 10, negative extraction control (nontransduced neuroblastoma cells). The band shown is the 720-bp neomycin-resistance gene amplification product.]
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 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, 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, 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, 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.

If the principles we have demonstrated extend to other solid tumors that also disseminate by hematogenous spread (e.g., 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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Direct demonstration that autologous bone marrow transplantation for solid tumors can return a multiplicity of tumorigenic cells

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