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></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6/F</td>
<td>Stage D 2nd Remission</td>
<td>LNL6</td>
<td>Well—12 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>

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

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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 technique\textsuperscript{14} 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,\textsuperscript{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,\textsuperscript{15,16} we detected two integrants among $10^6$ 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 al\textsuperscript{15}), 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.

DISCUSSION

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 Neo\textsuperscript{6} 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.\textsuperscript{18}

The cell marking technique we describe transfers the neomycin-resistance gene with relatively low efficiency,\textsuperscript{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

DR Rill, VM Santana, WM Roberts, T Nilson, LC Bowman, RA Krance, HE Heslop, RC Moen, JN Ihle and MK Brenner