CONCISE REPORT

High-Efficiency Gene Transfer and Expression in Normal Human Hematopoietic Cells With Retrovirus Vectors

By Pierre Laneuville, Wayne Chang, Suzanne Kamel-Reid, Axel A. Fauser, and John E. Dick

Retroviral vectors containing the selectable bacterial gene for G418 resistance (neo) were used to demonstrate gene transfer into primary human bone-marrow progenitor cells. To obtain populations of cells in which a high proportion of cells were expressing the neo gene, several important modifications were made to earlier procedures. Cells from normal donors were infected in vitro, were exposed to high concentrations of G418 for two days in liquid culture to enrich for cells expressing the neo gene, and were plated in semisolid medium. Gene transfer and expression were detected in colonies arising from progenitors of granulocyte-macrophage and erythroid lineages. Survival curves indicated that a high proportion of progenitor cells, approaching 100%, were G418 resistant. Furthermore, addition of growth factors contained in 5637-conditioned medium to the bone marrow improved the recovery of G418-resistant progenitors twofold to threefold. In addition to these biological measurements of gene expression in progenitor cells, significant levels of neo-specific RNA, similar to the levels of RNA expression in the virus-producing fibroblast cell line, were detected in the bone marrow cells after preselection. These results demonstrate that retrovirus vectors can be used successfully to transfer genes at high efficiency into progenitor cells in the human blood-forming system.

The ability to transfer genetic information into hematopoietic cells provides a new and promising approach to addressing questions concerning stem cell commitment and proliferation. The introduction of developmentally regulated genes, lineage-specific genes, growth factor genes, or oncogenes into various hematopoietic cells will provide new insights into gene expression and regulation during hematopoietic differentiation. Furthermore, the correction of certain human genetic defects may be possible by the insertion of a functional gene into the primitive bone-marrow stem cells of the human hematopoietic system.

The low frequency of stem cells in hematopoietic tissues has necessitated the development of high efficiency gene transfer techniques. At present retroviral vectors provide the most efficient method for introducing genes into committed hematopoietic progenitor cells and pluripotent stem cells of the mouse. Gene transfer also has been reported into human hematopoietic progenitor cells. In the present study we have developed a procedure for obtaining a high proportion of human hematopoietic cells that express the neo gene following infection with retroviral vectors.

MATERIALS AND METHODS

Retrovirus vector. Details of the construction of the N2 vector used in this study have been described. This vector contains the neo gene activated by the 5'LTR in the sense orientation. Infectious replication-defective neo virus was produced by first transfecting the vector into p-2 helper cells. The virus-containing supernatants were then used to infect the helper cell line PA317. The resulting cell lines produced amphotrophic neo virus at a titer of 3 x 10^6 cfiu/mL, as assayed by the ability to produce G418-resistant colonies of HOC-7 human ovarian carcinoma cells.

Method of infection. The infection protocol was adapted from that previously shown to give high efficiency gene transfer into mouse hematopoietic stem cells. Bone marrow was obtained from hematologically normal individuals after informed consent was obtained. Light-density (sp

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were applied onto Zeta-probe membrane using a Bio-Rad spot blot manifold. The cells were lysed with sodium dodecyl sulfate (SDS) and washed in 2 x SSC, after which the membrane was baked, prehybridized in 50% formamide and 0.5% BLOTTO, and hybridized in the same solution with a 1.4-kb single-stranded–neo-specific RNA probe. The membrane was washed at 1% SSC, 0.1% SDS 60°C, and exposed to x-ray film at −70°C with screens.

RESULTS

The efficiency of gene transfer into human progenitor cells was determined using a biological assay based on in vitro colony formation in the presence and absence of G418. Following cocultivation of bone marrow cells with fibroblasts producing neo vector, several groups have found that approximately 5% to 20% of the colony-forming cells were G418 resistant. To obtain populations of bone marrow cells in which a higher proportion of cells expressed the neo gene, in vitro selection was applied by exposing infected cells to 2 μg/mL of G418 for 48 hours in liquid culture. The inclusion of a similar step in murine bone-marrow infections resulted in approximately 5% survival curves were generated by plating cells from liquid culture in increasing concentrations of G418 (Fig 1). Several points can be made from these curves. First, the survival of uninfected colony-forming cells was reduced by greater than 2 × 10^−2 at concentrations of G418 above 600 μg/mL. Although there was a considerable batch-to-batch variation in the toxicity of G418 making it imperative to pretest new lots of drug, the 600 μg/mL dose in this experiment was clearly nonpermissive for growth of wild-type cells. Second, in the absence of preselection in the shape of the survival curve suggests the presence of two populations of cells; one that is drug sensitive and killed at the same rate as the uninfected cells and the other that is drug resistant. Survival is approximately 20% at a dose of 600 μg/mL. Third, following preselection the survival curve was a straight line with 60% survival at 600 μg/mL. This represents a threefold enrichment over nonpreselected cells, and extrapolation of the curve to a dose of zero suggests that the entire population of preselected cells was G418 resistant.

The enrichment of neo-expressing–bone-marrow cells after preselection was confirmed by whole-cell RNA blotting of cells infected with the PA317N2 vector (Fig 2). Equal numbers of cells were loaded into the first well of each row, followed by doubling dilutions permitting direct comparison of the signal intensity at each dilution. A faint signal was detected in cells that had been infected but not preselected (Fig 2, lane b), while a very strong signal was seen in the preselected group (Fig 2, lane c). No signal was detected in mock-infected cells that had been cocultivated with the nonvirus-producing PA317 fibroblasts (Fig 2, lane a). For comparison equal numbers of PA317 and PA317N2 fibroblasts were also analyzed at the same time. The signal intensity from the PA317N2 virus-producing cell line (Fig 2, lane d) was essentially equivalent or slightly lower than the preselected bone marrow cells.

![Fig 1. Survival curves of normal human bone-marrow progenitor cells infected with a neo vector. Bone marrow cells were either infected with the N2 vector or were mock infected according to the protocol outlined in “Materials and Methods.” After infection the cells were incubated in the presence or absence of 2 μg/mL G418. Cells also were cultured in the presence or absence of 5637-conditioned medium. Following these treatments the bone marrow cells were plated in methylcellulose culture in the absence and presence of 200 to 1,000 μg/mL G418. After 14 days the number of BFU-E and CFU-GM were scored. The relative colony-forming ability was calculated as the total colonies in the presence of G418 over the number of colonies in the absence of drug. The legend is given in the figure.](image1)

![Fig 2. Whole-cell RNA blotting of human bone-marrow cells infected with a neo vector. Equal numbers of cells (10⁶) were loaded into the first well in each row; doubling dilutions were added to each subsequent well in the row. Row a contains mock-infected bone marrow cells cocultured with PA317 cells; Row b contains PA317N2-infected bone marrow cells that were not preselected; Row c contains PA317N2-infected bone marrow cells that were preselected; Row d contains PA317N2 virus-producing fibroblasts; and Row e contains PA317 parental cells. The membrane was probed with a neo-specific RNA probe.](image2)
Since retroviruses require DNA synthesis to integrate, one might expect that early progenitor cells, which are generally out of cell cycle, would be more easily infectable if they were stimulated to divide. Conditioned medium from 5637 cells contains growth factors that maintain the viability and proliferative potential of hematopoietic progenitor cells. Therefore this conditioned medium was added to bone marrow cells to stimulate cell division and to enhance cell viability during cocultivation and preselection. Survival curves were generated as described above, and the data are also presented in Fig 1. The shape of the curves is essentially the same as those generated in the absence of added factors, indicating that at least at the level of this analysis there were no large increases in the frequency of gene transfer or expression. However, it was also important to determine whether the total recovery of G418-resistant–colony-forming cells was improved by the addition of these conditioned media. The total recovery of colony-forming cells and G418-resistant–colony-forming cells was calculated for each group +/- growth factor and +/- preselection. The data in Table I indicate that the addition of 5637-conditioned medium to the cells during liquid culture increased the recovery of all progenitors and in particular the G418-resistant progenitors by twofold to threefold.

**DISCUSSION**

The experiments described above demonstrate that retrovirus vectors containing the dominant selectable neo gene can infect human hematopoietic progenitor cells. Approximately 20% of the in vitro colony-forming cells expressed the neo gene following cocultivation of normal human bone marrow cells with virus-producing fibroblasts. Preselection of these infected cells in high concentrations of G418 resulted in a threefold increase in the number of expressing progenitor cells. The shape of the curve suggested that the entire preselected population of progenitor cells was drug resistant. Whole-cell RNA blotting of the infected cells revealed a similar conclusion; there was a significant increase in the level of neo RNA in the preselected population as compared to nonpreselected cells. In addition, the level of expression was similar to that observed in the virus-producing fibroblasts. This preselection step was previously shown in the mouse to eliminate progenitor cells and CFU-S that did not express the neo gene. The inclusion of this step prior to in vivo reconstitution of mice with infected stem cells is important to ensure that only infected stem cell clones participate in reconstitution. This will also be an important consideration in any future human reconstitution experiments, since uninfected stem cell clones could compete against the infected clone.

The addition of growth factors in the conditioned media from 5637 bladder carcinoma cells appeared to act by increasing the viability and recovery of the total number of progenitor cells and therefore also those progenitor cells expressing the neo gene by twofold to threefold. While the addition of the 5637-conditioned media did not appear to increase the gene transfer frequency, it will still be important to identify and to test other purified growth factors that act on early stem cells for their ability to increase the infection frequency.

The experiments presented here demonstrate that NEO vectors using the viral LTR promoter can be transferred into human hematopoietic progenitor cells and continue to be expressed for at least 14 days, during which a colony is formed in the presence of G418. It will be important to determine whether long term expression can be maintained in early progenitor cells. In the mouse, we and others have shown that there is considerable heterogeneity in expression of the neo gene in committed hematopoietic progenitor cells derived from more primitive stem cells infected with a retrovirus vector. Furthermore, retroviruses that contain promoters such as SV40 appear to be down regulated in murine and human hematopoietic progenitor cells (and unpublished observations). An understanding of this problem is essential before vectors can be used to study gene expression during differentiation or for gene therapy of human disease.

The various cells of the hematopoietic system are derived from the proliferation and differentiation of individual pluri-

### Table 1. Effect of Preselection and Growth Factors on the Recovery of G418<sup>+</sup> Progenitor Cells

<table>
<thead>
<tr>
<th>Vector</th>
<th>Growth Factor</th>
<th>Preselection</th>
<th>Cells Cultivated ( \times 10^7 )</th>
<th>Total CFU Recovered ( \times 10^5 )</th>
<th>Total G418&lt;sup&gt;+&lt;/sup&gt; CFU Recovered ( \times 10^5 )</th>
<th>Fold Increase in Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>—</td>
<td>—</td>
<td>1.0</td>
<td>9.0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>PA317N2</td>
<td>—</td>
<td>—</td>
<td>1.44</td>
<td>7.7</td>
<td>2.0</td>
<td>—</td>
</tr>
<tr>
<td>PA317N2</td>
<td>5637</td>
<td>—</td>
<td>1.40</td>
<td>11.6</td>
<td>3.6</td>
<td>1.9</td>
</tr>
<tr>
<td>PA317N2</td>
<td>—</td>
<td>+</td>
<td>3.56</td>
<td>1.1</td>
<td>0.57</td>
<td>—</td>
</tr>
<tr>
<td>PA317N2</td>
<td>5637</td>
<td>+</td>
<td>3.60</td>
<td>1.9</td>
<td>1.02</td>
<td>1.8</td>
</tr>
<tr>
<td>PA317N2</td>
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<td>+</td>
<td>4.2</td>
<td>0.47</td>
<td>0.28</td>
<td>—</td>
</tr>
<tr>
<td>PA317N2</td>
<td>5637</td>
<td>+</td>
<td>4.2</td>
<td>1.56</td>
<td>0.82</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Light-density bone marrow cells from normal donors were cocultivated with irradiated PA317 fibroblasts that produced the N2 vector (PA317N2) or PA317 alone (mock) according to the protocol outlined in "Materials and Methods." Cells were incubated in liquid culture for a further 48 hours in the absence (-) or presence (+) of 2 mg/mL G418. The liquid culture medium of the indicated groups was also supplemented with 10% 5637-conditioned medium. CFU-GM and BFU-E were scored after 14 days growth in methylcellulose culture in the absence or presence of 600 μg/mL G418. The combined counts were multiplied by the number of viable mononuclear cells to determine the total CFU recovered. The fold increase in recovery of G418<sup>+</sup> CFU following addition of 5637-conditioned medium was calculated by dividing the total G418<sup>+</sup> CFU recovered in the presence of 5637-conditioned medium by the total G418<sup>+</sup> CFU in its absence taking into account the starting number of cells cocultivated.
potent stem cells. The molecular mechanisms underlying this differentiation process are unknown. Introduction of new genes into human hematopoietic stem, using either retrovirus vectors or physical techniques of gene transfer, should facilitate molecular approaches with human marrow cells similar to that now possible in the mouse. As well, the correction of certain human genetic defects may be facilitated by the development of this gene transfer technology.

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REFERENCES

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