Ex Vivo Expansion and Selection of Human CD34+ Peripheral Blood Progenitor Cells After Introduction of a Mutated Dihydrofolate Reductase cDNA via Retroviral Gene Transfer

By Michael Flasshove, Debabrata Banerjee, Shin Mineishi, Ming-Xia Li, Joseph R. Bertino, and Malcolm A.S. Moore

Retroviral gene transfer into human myeloid precursor cells allows introduction of marker genes as well as genes conferring resistance to chemotherapeutic drugs. We transduced a human mutant dihydrofolate reductase (DHFR) cDNA into CD34 antigen-positive peripheral blood cells from patients with breast or ovarian cancer obtained after treatment with chemotherapy and granulocyte colony-stimulating factor (G-CSF). This mutant DHFR has been shown to confer resistance to methotrexate (MTX) in murine bone marrow. We established a transduction protocol that permitted ex vivo expansion and selection of transduced early progenitor cells. The number of progenitor cells from transduced CD34-positive cells increased 50-fold after cytokine prestimulation with interleukin-1 (IL-1), c-kit ligand (KL; stem cell factor), and IL-3 and 2 weeks in liquid culture. Transduced colony-forming unit–granulocyte-macrophage (CFU-GM), assayed directly after the transduction procedures, were protected completely against 2 × 10−4 mol/L MTX, a concentration that significantly reduced the CFU-GM detected in the control population. Gene transfer of the mutant DHFR led to a twofold selective advantage for a pre-CFU population after exposure to MTX in liquid culture (P < .001). Polybrene, in contrast with protamine, significantly inhibited the expansion of progenitors. The presence of proviral DNA was monitored by polymerase chain reaction (PCR) and was detected in greater than 80% of CFU-GM and ex vivo expanded pre-CFU. We have demonstrated that human hematopoietic precursor cells can be expanded extensively after retroviral gene transfer. The same population of early progenitors can be selected ex vivo with low-dose MTX. As long-term expression of transduced genes in human hematopoietic cells remains a problem in vivo, these results may have implications for future clinical trials, especially for the introduction of nonselectable genes.

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dose chemotherapy as compared with bone marrow samples. Thus, CD34-selected peripheral blood cells represent the safest target population for gene transfer of drug resistance genes into human hematopoietic cells. Ex vivo expansion of transduced, hematopoietic progenitor cells would provide enough cells for reinfusion, whenever necessary. This might permit intensification of chemotherapy after introduction of a drug resistance gene by shortening the intervals and reducing the chances of critical cytopenia, which is likely to occur even with support from autologous peripheral blood stem cells (PBSC) unless wash-out-periods are included after each application of chemotherapy.

We have recently demonstrated the efficacy of a retroviral vector including either the murine or the human cDNA of a mutated DHFR gene to confer resistance against MTX in murine bone marrow in vitro and in vivo. The human DHFR containing the Phe → Ser mutation at codon 31 leads to a marked decrease of the MTX binding capacity without significant loss of enzymatic activity. We now report that the retroviral-mediated introduction of the mutant DHFR into CD34-enriched progenitor cells from human peripheral blood harvested after mobilization with granulocyte colony-stimulating factor (G-CSF) and cyclophosphamide increased the level of drug resistance and allowed for positive selection of transduced and ex vivo expanded progenitor cells.

**MATERIALS AND METHODS**

**Patients.** Leukapheresis product (2 mL) was collected from 13 patients with untreated ovarian cancer (n = 6) or stage IV breast cancer (n = 7) after informed consent. The mobilization regimens consisted of either G-CSF 5 μg/kg/d subcutaneously (SC) for 7 days (n = 2), cyclophosphamide 3 g/m2 intravenously (IV) followed by G-CSF for 14 days (n = 6), or cyclophosphamide 3 g/m2 plus taxol 200 μg/m2 followed by G-CSF for 14 days (n = 5).

**Sample preparation.** Mononuclear cells after Ficoll-Paque centrifugation (Pharmacia, Uppsala, Sweden; d = 1.077 g/mL) were enriched for CD34-positive cells using the CellPro Ceprate System (CellPro, Bothell, WA), following the manufacturer's recommendation. Alternatively, immunomagnetic beads were used for separation of CD34-positive cells. Briefly, mononuclear cells were washed twice with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA; Boehringer, Mannheim, Germany), adjusted to a concentration of 1 × 10^6 cells per milliliter, and incubated on ice for 30 minutes with 50 μg/mL of a murine anti-CD34–IgG1 developed in our laboratory (Cl.11.1.6, under license with Oncogene Science, Uniondale, NY). Cells were washed three times and incubated with sheep-anti-mouse IgG1(Fc)m immunomagnetic beads (Dynal, Oslo, Norway) for 30 minutes on ice, and the positive fraction was recovered with a magnetic particle separator. After 16 to 20 hours' incubation at 37°C in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, NY) containing 20% fetal bovine serum (FBS; Gemini Bioproducts, Calabasas, CA), 7.3 × 10^−3 M L-monomethylglycerol (Sigma, St. Louis, MO), and 50 μg/mL gentamicin (Gibco), the beads detached, and CD34-positive cells were harvested. One sample was stored at −180°C before further processing.

**S-phase analysis.** After CD34-selection, cells were stimulated with different cytokine combinations for up to 72 hours using the same conditions described for ex vivo expansion. Cells were then analyzed for S-phase distribution using a cell proliferation kit (Amer sham, Little Chalfont, UK) with minor changes in the manufacturer's instructions. Cells were incubated at 37°C in 1:500 diluted labeling reagent containing 5-bromo-2'-deoxyuridine (BrdU) for 120 minutes. Cytosins were fixed in acetic ethanol and incubated with a murine anti-BrdU antibody for 120 minutes at 20°C. A secondary peroxidase anti-mouse antibody was added for 30 minutes, and positive cells were visualized with hydrogen peroxide and 3,3'–diaminobenzidine tetrahydrochloride. A minimum of 500 cells was scored to determine the percentage of cells in S-phase.

**Progenitor assay.** A range of 0.1 × 10^5 to 2 × 10^5 cells per milliliter were plated in triplicate in IMDM/20% FBS containing 0.36% agarose (FMC Bioproducts, Rockland, ME), 150 U/mL rhIL-1β (Syntex, Palo Alto, CA), 20 ng/mL recombinant human (rh) kit ligand (KL; Immunex, Seattle, WA), 50 ng/mL rh interleukin-3 (rhIL-3; Genetics Institute, Cambridge, MA), 6 U/mL rh erythropoietin (Epo; Amgen, Thousand Oaks, CA), and 1,000 U/mL rh-CSF (Amgen). For three experiments cells were plated only in IL-1, IL-3, and Epo. Colonies (greater than 50 cells) showing the morphology of colony-forming unit–granulocyte-macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E) were scored after incubation for 14 days at 37°C with 5% CO₂.

**Ex vivo expansion of cells and progenitors in a serial delta assay.** CD34-positive cells (4 × 10^5/mL) were incubated in liquid culture for 7 days at 37°C in IMDM/20% FBS containing IL-1, KL, and IL-3 at the same concentrations described for the progenitor assay. Cells were harvested weekly, washed once in IMDM/20% FBS, counted, resuspended at 4 × 10^6/mL with fresh cytokines, and incubated again for 7 days. Cumulative cell numbers were calculated for up to 28 days. The number of progenitors was determined using the agarose assay described above, with escalating numbers of cells being plated each week (0.1 × 10^5 to 2 × 10^5 cells per milliliter). Cumulative increase of progenitors was calculated by multiplying the cumulative number of cells from each week with the respective plating efficiency, and comparing it with the number of progenitors in the initial unexpanded CD34-positive population.

**Titration of polybrene/protamine.** After preincubation for 3.5 days in IL-1, KL, and IL-3, ex vivo expansion of cells and progenitors was determined as described above. During the first week of expansion in liquid culture, cells were exposed to 2 to 16 μg of polybreyne or protamine sulfate (Sigma).

**Retroviral vector design, producer line, and viral supernatant collection.** The retroviral vector producer and line were constructed as described. A human DHFR cDNA of 655 bp with the mutation Phe → Ser at codon 31 was cloned into the N2A-based double construct vector under control of an internal SV40 promoter. Clones of the amphotrophic packaging cell line GP+envAM12 producing a viral titer of 2 × 10^9 to 5 × 10^8 CFU/μL per milliliter after transfection from the ecotropic packaging cell line GP+E-E6 were selected in G418 (Gibco BRL, Gaithersburg, MD) and MTX. The producer clone DC/SVs31 was grown in Dulbecco's modified Eagle's medium (DMEM)/10% FBS in roller bottles until 70% confluent. Medium was changed to IMDM/20% FBS (for two experiments fresh DMEM/10% FBS was used instead), harvested 24 hours later, depleted of cell debris by centrifugation, frozen immediately at −80°C, and used only once after thawing. Supernatant from GP+envAM12 packaging cell line was harvested from T75 flasks under the same condition as the producer line and used for mock transduction. Absence of helper virus was confirmed as described.

**Retroviral transduction.** For the initial coculture experiment, CD34-positive peripheral blood cells were incubated on a subconfluent stromal cell layer of either virus-producer line DC/SVs31 or GP+envAM12 packaging cell line after irradiation of the stromal cells with 15 Gy. Hematopoietic cells (2 × 10^6) were cocultured in IMDM/20% FBS in the presence of IL-3, KL, Epo, 20 ng/mL rhIL-6 mutein (Imclone, New York, NY), and 8 μg/mL polybrene.
Fig 1. Experimental design for selection of progenitor cells after retroviral transduction. Prestimulation (for 72 hours), transduction, and liquid culture were performed in the presence of IL-1, KL, and IL-3. Progenitors were grown in agarose containing IL-1, KL, IL-3, Epo, and G-CSF. Polybrene or protamine was added during transduction. Progenitors directly after transduction and cells of the first week of expansion were grown in the liquid or in the presence of 2 x 10^{-8} mol/L MTX. Inhibition or selection was compared for retroviral (DC/SV311-ver)-transduced cells and progenitors expanded without selection in MTX and mock-transduced cells. PCR for proviral integration was performed where indicated. For more details see Materials and Methods.

(Sigma). After 4 days, hematopoietic cells were harvested, washed, and counted, and cell and progenitor expansion was determined as described above. For cell-free retroviral transduction, 4 x 10^{4} CD34-positive cells per milliliter were resuspended once in retroviral or control supernatant in the presence of IL-1, KL, and IL-3. Either 8 \mu g/mL polybrene or 4 \mu g/mL protamine was added. Transduction was started directly after CD34-selection or after 72 hours' prestimulation with IL-1, KL, and IL-3. Cells were harvested after 24 to 36 hours after transduction. Progenitors directly after transduction and cells of the first week of expansion were grown in the presence of 2 x 10^{-8} mol/L MTX. Inhibition or selection was compared for retroviral (DC/SV311-ver) versus mock-transduced cells. PCR for proviral integration was performed where indicated. For more details see Materials and Methods.

MTX resistance and selection. After retroviral or mock transduction, cells were plated as described in 0.36% agarose plus IL-1, KL, IL-3, Epo, and G-CSF. MTX (2 x 10^{-4} mol/L) was added directly to three plates, and another three plates were incubated without MTX as a control. Mean colony number from both sets was determined after 14 days, and the percentage of inhibition was calculated as [100 x (colonies + MTX/colonies − MTX) − 100] for retroviral- and mock-transduced population of each sample. To select for successfully transduced cells, both retroviral-transduced and mock-transduced cells from the same sample were exposed to 2 x 10^{-8} mol/L MTX during the first week in liquid culture (Fig 1). Expansion of MTX-selected cells and progenitors was compared for both populations in a serial delta assay. One aliquot from retroviral-transduced and mock-transduced cells was expanded without selection in MTX to standardize for differences in the composition of supernatant from either DC/SV311 or GP+envAM12 (eg, serum batch variation, endogenous cytokine levels, etc). Values were expressed as percentages of progenitors expanded in the presence versus absence of MTX. Results were only included when the mean colony number per plate was greater than 5 for all conditions. FBS was treated for 5 minutes with 1 U/ml thymidine phosphorylase at 37°C before MTX was added to reduce the background of MTX-resistant colonies.

Transduction efficiency. Directly after retroviral transduction, cells were seeded into 96-well plates (10 cells per well) in IMDM/20% FBS containing IL-1, KL, and IL-3. Colonies were grown for 14 days, then supplemented with fresh medium and cytokines, and harvested after another 7 to 14 days when the size of the colonies was sufficient for analysis of proviral integration.

Analysis of proviral integration. After retroviral transduction hematopoietic cells—either aliquots from the weekly passaged suspension cells or individual colonies grown in 96-well-plates—were pelleted and stored at −80°C until further use. DNA preparation for PCR was performed according to the technique described by Hi-guchi. Samples were thawed under 100 \mu L proteinase K-containing lysis buffer, resuspended, and incubated at 55°C for 12 hours. CFU-GM, elicited after expansion in liquid culture, were lysed directly in proteinase K-containing buffer after isolation from agarose plates. DNA template (2 \mu L) was added to 8 \mu L PCR buffer containing dNTPs, MgCl2, Taq polymerase, gelatin, and KCl and amplified in a thermocycler (Perkin Elmer 9600, Norwalk, CT) for 40 cycles, each cycle 94°C (30 seconds), 58°C (30 seconds), and 72°C (30 seconds) using primers (5'-GGA AGC CGG TCT TGT CGA TC-3'; 5'-CGA AAT CTC GTG ATG GCA GG-3'), specific for a 415-bp fragment of Neo*. Products were analyzed by electrophoresis in 1% agarose gels, which were then blotted onto nylon membranes (Nytran; Schleicher & Schuell, Keene, NH) by Southern transfer and hybridized for 16 hours with a 32P-labeled NeoR probe at 42°C in formaldehyde-containing hybridization solution, washed, and visualized by autoradiography according to standard procedures. The same amount of DNA was amplified with primers (5'-TAA TAC GAC TCA TCA TAT GAT GTA TAT GCA GGG-3'; 5'-GAA GAT CAC ATG ATG TTG TCA GGC-3') specific for a 185-bp fragment of the β-globin gene and processed as described to serve as internal control for the presence of DNA in the samples. Mock-transduced colonies were used as negative control.

Statistical methods. Student's t test for paired and unpaired samples was applied to test for significance.

| Table 1. Ex Vivo Expansion of Cells and Progenitors From CD34-Positive Peripheral Blood Samples in a Serial Delta Assay |
|---|---|---|---|
| n | Day 7 | Day 14 | Day 21 | Day 28 |
| Cells | 11 | 20.3 ± 1.7 | 157.1 ± 27.9 | 1,109.3 ± 210.1 | 1,305.7 ± 158.7 |
| Progenitors | 9 | 13.2 ± 4.1 | 59.0 ± 22.9 | 58.7 ± 32.8 | 36.8 ± 19.4 |

Data represent mean fold-expansion ± SEM from 11 and 9 independent experiments, respectively. Cells were grown in IL-1 + KL + IL-3 in suspension cultures with weekly dilution of the cell concentration to 4 x 10^4 cells per milliliter and 4 x 10^4 cells per milliliter. Progenitors were determined in an agarose assay with IL-1 + KL + IL-3 + Epo ± G-CSF.
PBSCs AFTER DHFR TRANSDUCTION

Fig 2. Ex vivo expansion of progenitor cells after prestimulation with IL-1 + KL + IL-3 and exposure to increasing concentrations of polybrene or protamine (μg/mL) for 7 days in liquid culture. Numbers of progenitors in the expanded cell population (starting from $8 \times 10^6$ CD34-positive cells initially) are determined as mean numbers of colonies in triplicates in an agarose assay containing IL-1 + KL + IL-3 + Epo + G-CSF.

RESULTS

Ex vivo expansion. Pheresis samples from six patients with ovarian cancer and four patients with breast cancer were selected for CD34-positive cells with immunomagnetic beads; column-separation was used for one patient with ovarian cancer and three patients with breast cancer. The mean (±SEM) plating efficiency of CD34-positive cells in an agarose assay including IL-1, KL, IL-3, and Epo ± G-CSF directly after selection was 6.3% (±1.4%). The expansion of progenitors (mean ± SEM) reached a maximum of 59.0 ± 22.9-fold at day 14; expansion of cell numbers reached a maximum of 1,305.7 ± 158.7-fold at day 28 (Table 1 and Fig 3, untransduced control). The maximal expansion of progenitors from patients with breast cancer was observed directly after selection was 6.3% (mean ± SEM) and protamine-exposed cells. The exposure of prestimulated CD34-positive cells to polybrene resulted in significant inhibition of progenitor expansion in a dose-dependent manner ($P < .05$; Fig 2).

Table 2. Percentage of Cycling Cells in CD34-Positive Population From Peripheral Blood After Stimulation With Various Cytokines

<table>
<thead>
<tr>
<th>Day 0</th>
<th>IL-1 + KL</th>
<th>IL-1 + KL + IL-3</th>
<th>IL-3 + KL + IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3 ± 3.2</td>
<td>13.6</td>
<td>24.6</td>
<td>32.8</td>
</tr>
<tr>
<td></td>
<td>41.5</td>
<td>49.8</td>
<td>54.9</td>
</tr>
</tbody>
</table>

Data show percentage of cells in S-phase (for day 0, mean ± SD, n = 4) after CD34-selection with immunomagnetic beads and 16 hours’ incubation in IMDM/20% FBS at 37°C (day 0) and after prestimulation with cytokine combinations for 2 and 3 days.

Table 3. Progenitor Expansion of CD34-Positive Peripheral Blood Cells After Coculture With DC/SVs31 or GP+envAM12 for 4 Days and Selection With MTX for 7 Days

<table>
<thead>
<tr>
<th>MTX Concentration (μmol/L)</th>
<th>DC/SVs31 Fold-Expansion ± SD</th>
<th>Unselected %</th>
<th>GP+envAM12 Fold-Expansion ± SD</th>
<th>Unselected %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.6 ± 3.0</td>
<td>100 ± 0</td>
<td>10.7 ± 3.0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>6 x 10⁻⁸</td>
<td>0.4 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>3.5 ± 0.2</td>
<td>32.8 ± 0.2</td>
</tr>
<tr>
<td>4 x 10⁻⁸</td>
<td>6.8 ± 0.8</td>
<td>62.4 ± 0.8</td>
<td>5.0 ± 0.5</td>
<td>46.5 ± 0.5</td>
</tr>
<tr>
<td>2 x 10⁻⁸</td>
<td>12.7 ± 0.7</td>
<td>119.5 ± 0.7</td>
<td>6.9 ± 0.5</td>
<td>64.5 ± 0.5</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>8.8 ± 0.8</td>
<td>82.7 ± 0.8</td>
<td>6.9 ± 0.5</td>
<td>64.5 ± 0.5</td>
</tr>
</tbody>
</table>

CD34-positive cells from peripheral blood were cocultured for 4 days on either the virus-producer line DC/SVs31 or the nonproducing packaging line GP+envAM12 as negative control in the presence of IL-3 + KL + IL-6 + Epo + polybrene and selected in MTX for 7 days. Progenitors grown in an agarose assay with IL-1 + KL + IL-3 + Epo were compared with initial numbers of progenitors. Percent unselected column shows percentage of selected population compared with the unselected control.

S-phase analysis. CD34-positive cells were prestimulated after immunobead selection with different cytokine combinations for up to 72 hours. The percentage of cells in S-phase as determined by incorporation of BrdU and in situ staining was 8.4% ± 3.2% (mean ± SD, n = 4) after separation with immunomagnetic beads and detachment of the beads after 16 hours’ incubation at 37°C in IMDM/20% FBS. Stimulation of the cells with cytokines, especially IL-1, KL, and IL-3 or IL-3, KL, and IL-6, increased the percentage of cycling cells to 49.8% and 54%, respectively, within 72 hours (Table 2).

Titrations of MTX and protection of transduced progenitors from MTX. We determined the optimal MTX concentration at which cells that acquired MTX resistance via retroviral transduction could be selected and expanded. In a pilot experiment, we cocultured CD34-positive cells with either the virus-producer line DC/SVs31 or the packaging cell line GP+envAM12 in the presence of IL-3, KL, IL-6, and Epo and found that $2 \times 10^{-8}$ mol/L MTX was the optimal concentration for selection of transduced cells. At this concentration, retrovirally transduced progenitors, as measured in an agarose assay, expanded 12.7-fold versus 5.0-fold for the control population, whereas the unselected controls of both populations expanded equally (10.6 ± 10.7-fold; Table 3).

In addition, exposure of untransduced CD34-positive cells to $2 \times 10^{-8}$ mol/L MTX during the first 7 days in liquid culture resulted in an inhibition of progenitor expansion of 43% (day 7), 78% (day 14), 51% (day 21), and 61% (day 29), respectively (data not shown).

The addition of $2 \times 10^{-8}$ mol/L MTX to agarose plates directly after prestimulation and retroviral transduction inhibited the colony number in control cultures: $-33.4%$ ±
Table 4. Ex Vivo Expansion of Cells and Progenitors From CD34-Positive Peripheral Blood Cells After Retroviral Transduction and Selection With MTX

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Unstimulated/ polybrene Cells</td>
<td>11.8 ± 2.4</td>
<td>91.7 ± 25.6</td>
<td>235.1 ± 50.0</td>
</tr>
<tr>
<td>Progenitors</td>
<td>7.0 ± 4.5</td>
<td>50.3 ± 29.3</td>
<td>40.9 ± 30.3</td>
</tr>
<tr>
<td>2. Prestimulated/ polybrene Cells</td>
<td>60.6 ± 18.6</td>
<td>184.2 ± 64.3</td>
<td>374.8 ± 87.0</td>
</tr>
<tr>
<td>Progenitors</td>
<td>5.4 ± 1.8</td>
<td>3.8 ± 1.6</td>
<td>0.3 ± 0.4</td>
</tr>
<tr>
<td>3. Prestimulated/ protamine Cells</td>
<td>114.9 ± 33.2</td>
<td>618.1 ± 122.9</td>
<td>1,580.5 ± 360.6</td>
</tr>
<tr>
<td>Progenitors</td>
<td>23.4 ± 10.4</td>
<td>50.0 ± 13.3</td>
<td>15.5 ± 4.6</td>
</tr>
</tbody>
</table>

Data represent mean fold-expansion ± SEM from three to seven independent experiments. The fold-increase includes 72 hours' pre-stimulation with IL-1 + KL + IL-3 for groups 2 and 3, and 24 to 36 hours' incubation in retroviral supernatant for all experiments. Polybrene or protamine were added only during viral incubation at a concentration of 8 μg/mL or 4 μg/mL, respectively. Liquid culture was performed with IL-1 + KL + IL-3; progenitors were determined in agarose containing IL-1 + KL + IL-3 + Epo = G-CSF. During the first week of expansion after the retroviral transduction, cells were exposed to 2 × 10^-4 mol/L MTX. Cumulative expansion of cells and progenitors was calculated.

16.4% (mean ± SD) versus 3.3% ± 10.3% for retroviral-transduced cells ($P < .0001$). The difference between protamine- or polybrene-facilitated transduction (−23.8% ± 13% or −36.9% ± 17.3% inhibition for the control cultures vs 3.1% ± 11.3% or 3.4% ± 10.6% for transduced cells) was not significant.

Expansion and MTX selection of transduced cells and progenitors. The mean expansion of progenitors after retroviral transduction and MTX selection in group 2 (prestimulated + 8 μg/mL polybrene; Table 4) reached a maximum of 5.4-fold after the first week posttransduction versus 50.3-fold for group 1 (unstimulated + 8 μg/mL polybrene) and 50.0-fold for group 3 (prestimulated + 4 μg/mL protamine) after two weeks, respectively (Fig 3). Cells and progenitors from group 2 expanded significantly less ($P < .01$) after 2 and 3 weeks in liquid culture than group 3 (Table 4). The maximal expansion of cells and progenitors observed in group 2 was significantly lower ($P < .02$ and $P < .05$) than the maximal expansion of cells and progenitors from the same samples without transduction.

The progenitor expansion (fold; mean ± SEM) for transduced cells without MTX selection in group 1 was 9.8 ± 5.7, 57.5 ± 34.7, and 25.6 ± 19.0 after the first, second, and third week, respectively. Group 2 expanded 3.6 ± 0.8, 2.8 ± 1.2, and 1.9 ± 1.9, and group 3 expanded 19.3 ± 9.3, 37.1 ± 9.8, and 5.3 ± 1.2 after 1, 2, and 3 weeks, respectively. The corresponding expansion for the control cells without MTX selection was 11.6 ± 8.2, 62.5 ± 38.6, and 23.5 ± 13.8 in group 1; 6.2 ± 1.7, 6.7 ± 4.0, and 0.8 ± 0.8 in group 2; and 18.0 ± 12.0, 23.4 ± 0.4, and 11.4 ± 8.3 in group 3.

The absolute number of progenitor cells (mean ± SEM) recovered from 8 × 10^6 CD34-positive cells after transduction with and without prestimulation was 4.135 ± 1.660 (control) and 6.211 ± 3.060 (transduced) in group 1, 21.367 ± 7.939 (control) and 17.786 ± 8.413 (transduced) in group 2, and 48.874 ± 18.342 (control) and 43.346 ± 18.222 (transduced) in group 3.

Cells were selected after retroviral- or mock-transduction in 2 × 10^-4 mol/L MTX, and progenitors derived from selected and further expanded population were compared (Fig 1). The percentage (mean ± SD) of MTX-selected over non-selected progenitors elicited at day 19 to 22 was 14% ± 30% versus 81% ± 22% for transduced and control populations, respectively (group 5 in Table 5; $P < .0001$), and 146% ± 33% versus 71% ± 20% for the optimal selection of each sample (group 6 in Table 5) independent of the time point ($P < .00001$), indicating a twofold selective advantage for the retroviral-transduced cells. For the subgroup of samples where transduction was performed without prestimulation (group 1 in Table 5), MTX selection led to an initial inhibition of progenitor expansion for control and transduced cells. After 2 additional weeks of in vitro expansion, only a nonsignificant growth advantage for transduced (146% ± 26%) versus control cells (98% ± 21%) was seen. When prestimulation with IL-1, KL, and IL-3 was included in the transduction protocol, we observed a significant selective advantage after 2 weeks of expansion in liquid culture ($P < .001$). Control cells had reduced their expansion capacity (70% ± 16%) after 7-day selection in 2 × 10^-4 mol/L MTX, whereas the progenitor cells from retroviral-transduced cells expanded to a greater extent (142% ± 36%) with selection than without (group 4 in Table 5). We observed the same

Fig 3. Ex vivo expansion of progenitors from untransduced control and retroviral-transduced CD34-positive cells. Each data point represents accumulated fold-expansion of progenitors (mean ± SEM) from three to nine independent experiments. Suspension culture was performed in IL-1 + KL + IL-3; progenitor (agarose) assay with IL-1 + KL + IL-3 + Epo = G-CSF.
PBSCs AFTER DHFR TRANSDUCTION

Table 5. Selection of Ex Vivo Expanded Progenitors From CD34-Positive Peripheral Blood After Retroviral Transduction

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated/polybrene</td>
<td>71 ± 15</td>
<td>76 ± 37</td>
<td>98 ± 21</td>
</tr>
<tr>
<td>Prestimulated/polybrene</td>
<td>72 ± 10</td>
<td>121 ± 27</td>
<td>146 ± 26</td>
</tr>
<tr>
<td>Prestimulated/protamine</td>
<td>61 ± 24</td>
<td>152 ± 27</td>
<td></td>
</tr>
<tr>
<td>Prestimulated/total</td>
<td>65 ± 17</td>
<td>146 ± 36</td>
<td></td>
</tr>
<tr>
<td>All samples/polybrene</td>
<td>81 ± 22</td>
<td>144 ± 30</td>
<td></td>
</tr>
<tr>
<td>All samples/protamine</td>
<td>71 ± 20</td>
<td>146 ± 33</td>
<td></td>
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</table>

Data denote percentages of progenitors derived from ex vivo expanded CD34-positive peripheral blood cells after incubation with retroviral (DC/Sv31) or mock (GP+envAM12) supernatant and selection in 2 × 10^{-6} mol/L MTX during the first week of expansion as compared with the same treatment without selection. All liquid culture was performed with IL-1 + KL + IL-3. Progenitors were assayed in triplicate agarose plates with IL-1 + KL + IL-3 + Epo ± G-CSF. Groups 1 to 3 represent mean values ± SD from three to four independent experiments. Group 4 combines all experiments with prestimulation. Group 5 consists of results from third week/unstimulated (day 19) and second week/prestimulated (days 21 and 22). In group 6 the values were combined at the time of best selection for each sample.

Selective advantage for the two subgroups (groups 2 and 3) of prestimulated samples: for polybrene-facilitated transfer, 142% ± 48% versus 69% ± 24% (P < .05), and for protamine, 143% ± 31% versus 71% ± 9% (P < .05).

Detection of proviral DNA. To determine the percentage of progenitors that were successfully transduced, we examined colonies grown for 2 to 4 weeks in liquid culture in 96-well plates seeded at a concentration of 10 cells per 0.1 mL. Colonies were positive for Neo as confirmed by Southern blot of PCR-amplified DNA at a frequency of 70.4% ± 27.9% (mean ± SD) for unstimulated samples, 86.9% ± 12.5% for prestimulated plus polybrene samples, and 86.7% ± 11.5% for prestimulated plus protamine samples (Table 6). Aliquots from the expanded populations remained positive for the time of expansion (data not shown). Moreover, late-appearing progenitors derived from retroviral-transduced cells, ie, colonies grown in agarose for 2 weeks from cells after 2 to 3 weeks expansion in liquid culture, were positive for the presence of proviral DNA, indicating that predominantly retroviral-transduced progenitors had been expanded (Fig 4).

DISCUSSION

Clinical trials have proven the feasibility of gene transfer but have shown major problems in translating in vitro gene transduction efficiency into in vivo efficacy. We have used a Moloney murine leukemia virus-based retroviral vector that encodes two genes: the transposon 5 of the neomycin phosphotransferase and a mutated DHFR cDNA. The mutant DHFR with a point mutation Phe → Ser at codon 31 is cloned downstream of an internal SV40-derived promoter into the 3'LTR of the vector leading to a "double copy" into integration into the host genomic DNA. We have reported increased resistance to MTX after gene transfer of this vector into murine bone marrow. We have developed a system to determine drug resistance and selection of human hematopoietic cells that have been transduced by the mutant DHFR gene. We chose to use CD34-positive cells mobilized into the peripheral blood of cancer patients after treatment with a combination of chemotherapy and cytokines, because high transduction efficiency into a pre-CFU population of chemotherapy-mobilized peripheral blood cells and into human CD34-enriched peripheral blood cells as well as long-term repopulation by retroviral-transduced peripheral blood cells in a canine model have been reported. Peripheral blood samples from breast cancer patients after high-dose chemotherapy contain less tumor cells than bone marrow specimens from the same patients, suggesting that hematopoietic progenitor cells from peripheral blood may be a safer target population for gene transfer than bone marrow.

Since drug resistance conferred by the mutant DHFR is not absolute, we titrated the concentration of MTX in a selection experiment using a coculture technique for gene transfer. Exposure to 2 × 10^{-4} mol/L MTX or higher concentrations for 1 week after retroviral transduction gave rise to the highest selection advantage of retroviral-transduced versus mock-transduced progenitors, and exposure at 2 × 10^{-4} mol/L MTX resulted in the highest expansion (Table

Table 6. Integration of Proviral DNA Into Progenitors From CD34-Positive Peripheral Blood Before and After Ex Vivo Expansion

<table>
<thead>
<tr>
<th>Protocol</th>
<th>% (mean ± SD)</th>
<th>No. of Tested Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonies before ex vivo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>expansion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated/polybrene</td>
<td>70.4 ± 27.9</td>
<td>25</td>
</tr>
<tr>
<td>Prestimulated/polybrene</td>
<td>86.8 ± 12.5</td>
<td>18</td>
</tr>
<tr>
<td>Prestimulated/protamine</td>
<td>86.7 ± 11.5</td>
<td>19</td>
</tr>
<tr>
<td>Colonies after ex vivo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>expansion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated/polybrene</td>
<td>57.1</td>
<td>7</td>
</tr>
<tr>
<td>Prestimulated/polybrene</td>
<td>87.5</td>
<td>16</td>
</tr>
<tr>
<td>Prestimulated/protamine</td>
<td>100</td>
<td>8</td>
</tr>
</tbody>
</table>

Before ex vivo expansion: Cells were harvested after transduction and seeded into 96-well plates at a concentration of 10 cells per well and grown in IMDM/20% FBS plus cytokines for 2 to 4 weeks. PCR-amplified DNA was tested for presence of the Neo gene. After ex vivo expansion: Colonies from agarose plates after 19 to 26 days of ex vivo expansion were tested as described above.
For subsequent experiments, we used the $2 \times 10^{-8}$ mol/L MTX concentration in a stroma-free transduction protocol with high-titer viral supernatants instead of the coculture technique, which is difficult to translate into clinical trials and has been shown to compromise the repopulating abilities of murine bone marrow. Serial delta assays were performed to assess ex vivo expansion capacity of cells and progenitors in the presence and absence of retroviral transduction. When prestimulated CD34-positive cells were transduced in the presence of polybrene, a significant reduction in the expansion of progenitors was observed. This was not the case when the cells were exposed directly without prestimulation to polybrene-containing supernatants, possibly because the cycling, ie, sensitive population within the sample, was smaller. Cornetta and Anderson have reported protamine to be equally effective as polybrene in retroviral gene transduction, and recently published transduction protocols have used 4 to 5 $\mu$g/mL protamine. We exposed IL-1, KL, and IL-3– prestimulated CD34-positive cells to polybrene or protamine for a prolonged period of time. We found a marked decrease of progenitor expansion for all polybrene concentrations (2 to 16 $\mu$g/mL; $P < .05$), whereas the addition of protamine did not inhibit the expansion of cells and progenitors. As a consequence, the transduction protocol using prestimulation and protamine did not compromise the expansion of progenitor cells as compared with untransduced CD34-positive peripheral blood cells (maximal expansion, 50-fold vs 59-fold; Fig 3).

The initial transduction efficiency was determined by the presence of the Neo6 gene in colonies grown in suspension in 96-well plates. All samples investigated showed 44% to 100% Neo6-positive colonies without significant differences between the transduction protocols, although transfer efficiency into unstimulated CD34-positive cells was lower than into prestimulated ones (Table 6). The detection of Neo6-positive CFU-GMs derived from cells after 19 to 25 days’ expansion in liquid culture indicates that stable integration of the vector into early progenitor cells occurred with a high efficiency (Table 6). The cell population that elicits colonies after 3 weeks of exponential expansion under limiting dilution conditions is detecting an early hematopoietic precursor comparable with the long-term culture-initiating cell detected in stromal cocultures after 5 to 8 weeks of demipopulation.

The high transduction efficiency we found by PCR corresponded at a functional level to increased resistance to MTX. Retroviral-transduced CFU-GM/BFU-E were protected against MTX at a concentration that significantly reduced the number of control progenitors, indicating that the majority of the rapidly cycling cell population susceptible to $2 \times 10^{-8}$ mol/L MTX had been successfully transduced. When CD34-positive cells were prestimulated with IL-1, KL, and IL-3, transduced, and exposed to MTX for 1 week, we observed a significant twofold selective advantage after 2 to 3 weeks of expansion for the retroviral-transduced population (Table 5). This selection was achieved during a limited time in an in vitro system. Administration of MTX in vivo over a prolonged period of time might allow a much better selection of transduced cells. Transduction of samples without prestimulation did not confer a significant selective advantage. The number of CD34-positive peripheral blood cells in S phase was less than 10% at the beginning of the procedure and increased with different cytokine combinations, including IL-1, KL, and IL-3, up to 50% within 72 hours. All cytokines tested had previously been shown to increase retroviral transduction efficiency. Provil integration can only be performed in cycling cells. The transduction protocol using cytokine priming might be more effective by increasing the percentage of cycling cells and upregulating amphotrophic receptors.

We have been able to transduce human hematopoietic cells successfully and render them more drug resistant than their untransduced counterparts. The transduced population includes CFU-GM as well as pre-CFUs and can be expanded ex vivo. The progenitors we measure after 2 weeks in liquid culture are probably generated from the primitive stem cell compartment. Selection of transduced cells has been described using MDR1 and the murine Arg22 mutant DHFR in murine experiments and using Neo6 in human bone marrow cells. Introduction of a mutant human DHFR

![Fig 4. Detection of proviral DNA in retrovirally transduced CFU-GM before and after ex vivo expansion. Lanes 1 through 6 represent colonies grown in liquid culture 96-well plates directly after prestimulation and transduction in the presence of IL-1 + KL + IL-3. Lanes 7 through 14 represent colonies after 14 days expansion in liquid culture and 14 days in agarose plates. DNA was amplified by PCR and probed to either NeoF or $\beta$-globin. Lane 15 represents DNA from untransduced CD34-positive cells.](image-url)
allows for in vitro selection of transduced human cells and may prove to be as effective in vivo, as a low-dose MTX therapy could be applied without major side effect. A gene of interest could thus be expressed at a significant level when delivered together with the mutant DHFR in association with MTX selection. This strategy may be useful to overcome the current problem of low in vivo transduction efficiency with retroviral vectors.

ACKNOWLEDGMENT

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Ex vivo expansion and selection of human CD34+ peripheral blood progenitor cells after introduction of a mutated dihydrofolate reductase cDNA via retroviral gene transfer

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