RAPID COMMUNICATION

Increased Gene Transfer Into Human Hematopoietic Progenitor Cells by Extended In Vitro Exposure to a Pseudotyped Retroviral Vector

By Christof von Kalle, Hans-Peter Kiem, Sondra Goehle, Boris Darovsky, Shelly Heimfeld, Beverly Torok-Storb, Rainer Storb, and Friedrich G. Schuening

Retroviral-mediated gene transfer is the most attractive modality for gene transfer into hematopoietic stem cells. However, transduction efficiency has been low using amphotropic Moloney murine leukemia virus (MoMLV) vectors. In this study, we investigated modifications of gene transfer using amphotropic MoMLV vectors in cell-free supernatant for their ability to increase the low transduction of both committed hematopoietic progenitors, granulocyte-macrophage colony-forming units (CFU-GMs), and their precursors, long-term culture-initiating cells (LTC-IC). First, based on the observation that bone marrow cells express more gibbon ape leukemia virus (GALV) receptor (GIVr-1) than amphotropic receptor (Ram-1), PG13/LN, which is a MoMLV vector pseudotyped with the GALV envelope, was compared with the analogous amphotropic envelope vector (PA317/LN). Second, progenitor cell transduction efficiency was compared between CD34 enriched and nonenriched progenitor populations. Third, the duration of transduction in vitro was extended to increase the proportion of progenitor cells that entered cell cycle and could thereby integrate vector cDNA. In 20 experiments, 1 × 10^6 marrow or peripheral blood mononuclear cells (PBMCs)/mL were exposed to identical titers of pseudotyped PG13/LN vector or PA317/LN vector in the presence of recombinant human interleukin

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In the present study, we analyzed human hematopoietic progenitor cell transduction using the PG13/LN retroviral vector, a pseudotyped MoMLV vector that carries the gibbon ape leukemia virus (GALV) envelope protein. We observed that gene transfer into hematopoietic progenitor cells was more efficient with the GALV pseudotyped vector PG13/LN than with the amphotropic envelope vector PA317/LN. Gene transfer also increased after CD34 enrichment. By extending the duration of the transduction culture to three weeks in the presence of recombinant human interleukin-1 (rhIL-1), IL-3, IL-6, stem cell factor (rh SCF, c-kit ligand) and cell-free high-titer PG13/LN vector supernatant, we obtained efficient gene transfer into most committed and immature hematopoietic progenitor cells.

**MATERIALS AND METHODS**

**Target cells.** Bone marrow (BM) or PBMC samples were obtained from patients and healthy volunteers who were undergoing stem cell harvests for marrow or peripheral blood stem cell transplants after informed consent as approved by the Institutional Review Board of the Fred Hutchinson Cancer Research Center. BM cells were harvested by needle aspiration from the posterior iliac crest under general anaesthesia. PBMCs were obtained by apheresis on a Fenwal CS3000 Blood Cell Separator after 4 days of treatment with subcutaneous recombinant human granulocyte colony-stimulating factor (rh G-CSF, Amgen, Thousand Oaks, CA) at 10 μg/kg/d. Mononuclear cells were purified by Ficoll-gradient centrifugation (Lymphocyte Separation Media [LSM]; Organon Teknika-Cappell, Durham, NC) at 400g and 25°C for 30 minutes after 1:3 dilution in Hank’s balanced salt solution (HBSS) supplemented with 1% fetal bovine serum (PBS; GIBCO BRL, Bethesda, MD) and 10 μg/mL of preservative-free heparin (Fujisawa USA Inc, Deerfield, IL). Samples of mononuclear cells were resuspended in HBSS/1% PBS for CD34 cell enrichment or set up in long-term culture for retroviral transduction. Long-term marrow culture medium consisted of Iscove’s modified Dulbecco’s medium (GIBCO BRL) supplemented with 12.5% of horse serum (GIBCO BRL), 12.5% of FCS (GIBCO BRL), 10⁻⁶ mol/L hydrocortisone (Sigma Chemical Co, St Louis, MO), 10⁻¹⁰ mol/L 2-mercaptoethanol (Sigma), 1% L-glutamine, and 10 μM penicillin/streptomycin (GIBCO BRL). Cultures were maintained at 37°C in humidified air at 8% CO₂.

**Enrichment of CD34⁺ cells.** Marrow and PBMCs were enriched for CD34⁺-antigen-positive cells with the Ceprate LC stem cell concentrator (CellPro, Bothell, WA). Mononuclear cells were incubated for 25 minutes at room temperature with 20 μg/mL of biotinylated mouse IgM antihuman CD34 antibody 12.8 in phosphate-buffered saline (PBS; GIBCO, Grand Island, NY) with 1% bovine serum albumin (BSA; CellPro), then passed over a sterile column of streptavidin-coated polyacrylamide beads (CellPro). After washing with PBS, CD34⁺ cells were resuspended by gentle mechanical agitation and eluted from the column with PBS/1% BSA with 10 U/mL of heparin (Fujisawa). Results were controlled by flow-cytometric analysis using a FACScan (Becton Dickinson, San Jose, CA) after re-staining with fluorescein isothiocyanate (FITC)-conjugated FITC-conjugated FITC-conjugated FITC-conjugated FITC-conjugated HPCA-2 antibody (Becton Dickinson) compared to an FITC-conjugated control (Caltag, San Francisco, CA).

**Retroviral vectors.** The retroviral vector LN, the PA317, and the PG13 pseudotyped producer cell lines were kindly provided by Dr A.D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). In

**Table 1. G418-Resistant CFU-GM From Marrow and PBMCs Transduced With PA317/LN and PG13/LN**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>PA317/LN</th>
<th>PG13/LN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marrow</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>56/747</td>
<td>93/723</td>
</tr>
<tr>
<td>2</td>
<td>4/219</td>
<td>3/153</td>
</tr>
<tr>
<td>3</td>
<td>2/722</td>
<td>3/51</td>
</tr>
<tr>
<td>4</td>
<td>7/133</td>
<td>5/1186</td>
</tr>
<tr>
<td>5</td>
<td>11/700</td>
<td>205/639</td>
</tr>
<tr>
<td>6</td>
<td>26/268</td>
<td>33/198</td>
</tr>
<tr>
<td>7*</td>
<td>9/133</td>
<td>10/116</td>
</tr>
<tr>
<td>8</td>
<td>25/93</td>
<td>23/107</td>
</tr>
<tr>
<td>9</td>
<td>13/317</td>
<td>29/154</td>
</tr>
<tr>
<td>10</td>
<td>5/74</td>
<td>12/103</td>
</tr>
<tr>
<td>Total†</td>
<td>657/6,134</td>
<td>1,107/6,468</td>
</tr>
<tr>
<td>PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>11/607</td>
<td>327/700</td>
</tr>
<tr>
<td>12</td>
<td>61/1,563</td>
<td>65/793</td>
</tr>
<tr>
<td>13</td>
<td>26/215</td>
<td>47/284</td>
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<tr>
<td>14</td>
<td>13/317</td>
<td>13/310</td>
</tr>
<tr>
<td>15</td>
<td>51/374</td>
<td>19/238</td>
</tr>
<tr>
<td>16</td>
<td>77/327</td>
<td>47/168</td>
</tr>
<tr>
<td>17</td>
<td>3/126</td>
<td>2/84</td>
</tr>
<tr>
<td>18*</td>
<td>355/3,687</td>
<td>439/3,733</td>
</tr>
<tr>
<td>19†</td>
<td>420/1,380</td>
<td>600,020</td>
</tr>
<tr>
<td>20†</td>
<td>420/1,913</td>
<td>173/747</td>
</tr>
<tr>
<td>Total†</td>
<td>3,233/23,643</td>
<td>3,897/18,173</td>
</tr>
</tbody>
</table>

*No stroma in transduction culture.
†Total number of colonies evaluated.
‡CD34⁺ cell-enriched samples.

Equal amounts of cells from each sample were transduced with retroviral vector supernatant containing PA317/LN or PG13/LN at a titer of 1 × 10⁶ CFU/mL. Fifty percent of fresh virus supernatant was added every 24 hours for 5 days. No colony growth was observed in G418-selected mock-infected controls. Colony numbers are quoted as number of G418-resistant colonies/total colonies per 10⁶ mononuclear cells plated. The transduction efficiency, expressed as the percentage of G418-resistant to total colonies, is quoted in parentheses. The transduction efficiency of PG13/LN is significantly higher compared with PA317/LN transduction efficiency by paired two-sample t-test; experiments 1 through 20, P = .011; marrow, P = .019, PBMC, P = .064.
the LN vector, the bacterial neomycin phosphotransferase (neo) gene is transcribed from the MoMLV long-terminal repeat. PG13/LN and PA317/LN cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L of glucose, 10% FBS, 1% of L-glutamine and 1% of penicillin/streptomycin (all Gibco BRL). To produce retrovirus-containing medium for hematopoietic progenitor cell transduction, long-term culture medium was incubated on a subconfluent retrovirus producer cell layer (0.16 × 10⁴ cells/cm²) for 16 hours at 37°C, then centrifuged at 2,500 g for 10 minutes to remove viable nonadherent cells. Retroviral vector titers were measured by a colony-formation assay with NIH 3T3 cells as targets for PA317/LN and HeLa cells as targets for PG13/LN as previously described. When PA317/LN and PG13/LN retroviral vectors were compared, both were used at a tier of 1 × 10⁴ colony-forming units (CFU)/mL.

Transduction of marrow and PBMCs by exposure to retrovirus-containing medium. Nonenriched and CD34-enriched marrow cells or PBMCs were cultured in a 1:1 mixture of fresh and retrovirus containing long-term culture medium at a density of 1 × 10⁶ cells/mL in vented T75-cm² tissue-culture flasks (Costar, Cambridge, MA). Unless indicated otherwise, these long-term cultures of hematopoietic progenitors were cultured on a confluent layer of allogeneic marrow stroma cells irradiated at 1,500 cGy 1 day before the initiation of culture. Polybrene (Sigma) was added to the cultures at a final concentration of 4 µg/mL of rhIL-1, rhIL-3 (Amgen), rhIL-6 (Inmunex Corp, Seattle, WA), as well as stem cell factor (Steel factor, SCF, Immunex) were added on the first day at 50 ng/mL unless indicated otherwise. The supernatant was exchanged by pipetting off 50% of the culture media and centrifuging it at 400 g for 5 minutes. The cell pellet was resuspended in an identical volume of fresh vector-containing supernatant supplemented with 4 µg/mL of Polybrene and added back to the culture. Cultures were maintained for 1, 2, and 3 weeks. Vector-containing supernatant in cultures fed 1 time/week was added 1 day after weekly demidepopulation according to the protocol of Carter et al. Mock-infection control experiments were conducted in each experimental arm with supernatant harvested from NIH 3T3 cell layers under the conditions described above.

Clonogenic assays and detection of neo expression in CFU-GM. Mononuclear cells were harvested from mock-transduced and LN vector--transduced cultures and assayed in triplicate for granulocytic (CFU-GM) colonies. Cells were plated at 5 × 10⁴ for nonenriched populations or 1 × 10⁴ for CD34-enriched populations in 2 mL of DMEM/0.3% agarose (FMC, Rockland, ME) supplemented with 1% of L-glutamine and 1% of penicillin/streptomycin (all Gibco BRL). The pairs of bar graphs represent cumulative data from triplicate analyses of 3 marrow and 8 blood pairs of independent experiments with cells from 11 different donors. The average percentages of CD34⁺ mononuclear cells are indicated below the bar graphs. (B), nonenriched marrow or PBMC sample; (L), CD34-enriched sample; marrow, BM-derived CFU-GM; blood, PBMC-derived CFU-GM.

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Table 2. Effect of Length of Vector Exposure on Efficiency of Gene Transfer Into CFU-GM

<table>
<thead>
<tr>
<th>CFU-GM ± G418 (%)</th>
<th>1 wk</th>
<th>2 wks</th>
<th>3 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM 2⁺</td>
<td>ND</td>
<td>ND</td>
<td>26/276 (9)</td>
</tr>
<tr>
<td>BM 3⁻</td>
<td>1/190</td>
<td>61/243 (25)</td>
<td>12/238 (5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5 Feedings/wk</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>10/19</td>
<td>238/260 (91)</td>
<td></td>
</tr>
<tr>
<td>BM 1at†‡</td>
<td>90/1070</td>
<td>147/417 (35)</td>
<td></td>
</tr>
<tr>
<td>BM 1b†</td>
<td>33/1098</td>
<td>217/324 (67)</td>
<td></td>
</tr>
<tr>
<td>BM 1ct</td>
<td>20/1200</td>
<td>430/640 (67)</td>
<td></td>
</tr>
<tr>
<td>BM 2⁺</td>
<td>ND</td>
<td>162/221 (73)</td>
<td></td>
</tr>
<tr>
<td>BM 3⁻</td>
<td>19/111</td>
<td>268/361 (74)</td>
<td>1,477/1,680 (98)</td>
</tr>
<tr>
<td>BM 4</td>
<td>2/111</td>
<td>23/50 (46)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Summary of nine experiments analyzing neo gene transfer into CFU-GM after 1, 2, or 3 weeks of long-term transduction. PG13/LN vector containing supernatant was replaced either 1 or 5 times/wk. Colony assays were conducted in triplicate. Colony numbers are quoted as number of G418-resistant colonies/total colonies per 10⁴ mononuclear cells plated. The transduction efficiency, expressed as the percentage of G418-resistant to total colonies, is quoted in parentheses. Abbreviations: ND, not done; —, technical failure.

* No growth factors added.
† Mononuclear marrow cells that had been cryopreserved after enrichment to 68% CD34⁺ cells.
‡ Long-term transduction culture initiated on irradiated allogeneic stroma cell feeder layer. All other transduction cultures were initiated without stromal support.
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with 20% of prescreened, heat-inactivated FBS (GIBCO) and 50 ng/mL each of IL-1, IL-3, IL-6, SCF, granulocyte-macrophage colony-stimulating factor (GM-CSF; ImmuneX) and G-CSF in 35-mm Petri dishes (Costar, Cambridge, MA) with and without G418 (Sigma, St Louis, MO) at 3 mg/mL, twice the pretested inhibitory concentration. Cultures were maintained in a 100% humidified atmosphere at 37°C and 10% CO2. After 14 days, CFU-GM colonies were counted. The ratio of G418-resistant colony formation to total colony formation was used to measure the transduction efficiency. Isolated colonies were picked in 20 μL of agar, washed twice in HBSS at 50°C, and submitted to polymerase chain reaction (PCR) analysis for the detection of neo cDNA (Fig 1). No colony growth was observed in the triplicate G418-containing dishes of mock-infected control cells from any sample.

To evaluate gene transfer into more immature progenitor cells,56 1 x 106 nonenriched or 1 x 106 CD34-enriched cells were transferred onto a second irradiated stromal feeder layer. This culture was maintained for 5 weeks by weekly replacing 50% of the long-term culture media. Cells retrieved from these cultures were then assayed for CFU-GM with and without G418 as described above, using 1 x 105 viable mononuclear cells from nonenriched or 2.5 x 105 from CD34-enriched progenitor cell cultures.

Detection of neo cDNA in CFU-GM colonies by PCR. For direct PCR amplification of neo cDNA, a modification of a previously described protocol57 was used. Colony cells obtained after washing were digested with proteinase K in lysis buffer (KCl 50 mmol/L, TRIS-HCL 10 mmol/L, MgCl2 2.5 mmol/L, 0.45% NP40, 0.45% Tween 20, Proteinase K 400 μg/mL) at 55°C for 3 hours. The following oligonucleotides specific for neo cDNA were used to generate an 821-bp product: sense primer neo 350: 5’ AAG AGA CAG GAT GAA GGA TCG 3’, antisense primer neo 1027: 5’ GCCAACGCTATGTCC-TGATA 3’ under the amplification conditions described above.

Giemsa and Wright staining of slides. Cells from single colonies were mounted on slides with a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA), air-dried, and stained with Wright-Giemsa hematology staining solution (Curtin Matheson Scientific Inc, Houston, TX).

Statistical evaluation. The mean, median, and standard deviation of colony scores were calculated for each experiment. Data were analyzed using Student’s t-test. Significant differences are labeled with the appropriate P values.

RESULTS

Efficiency of gene transfer into hematopoietic progenitor cells with the GALV envelope PG13/LN vector. A series of 20 experiments compared the efficiency of gene transfer obtained in marrow and peripheral blood—derived CFU-GM with the GALV-pseudotyped PG13/LN vector with that of the amphotropic envelope vector PA317/LN. Both vectors were used at a titer of 1 x 106 CFU/mL using an identical 5-day transduction protocol. As shown in Table 1, the transduction of hematopoietic progenitor cells as measured by G418-resistant CFU-GM colony formation was more efficient in PBMC/LN than in PA317/LN in marrow (17.9% vs 10.6%, respectively, P = .019) and PBMCs (20.8% vs 12.4%, respectively, P = .064). Long-term hematopoietic culture initiating cells (LTC-IC) were analyzed in two experiments using marrow and PBMC. After 5-day transduction with virus from PG13/LN cells, 4 of 14 colonies (28%) grown from marrow-derived LTC-IC, and 7 of 20 colonies (35%) grown from PBMC-derived LTC-IC were G418 resistant. Using PA317/LN supernatant, 4 of 21 (19%) of marrow-derived and 4 of 22 (18%) PBMC-derived colonies grown from LTC-IC were G418 resistant.

Transduction of human nonenriched and CD34-enriched marrow and peripheral blood cells. To test the influence of CD34+ cell enrichment on progenitor cell transduction, nonenriched and CD34-enriched mononuclear cells from each of three marrow and eight peripheral blood donors were transduced with the PA317/LN vector in a 5-day protocol
at the same target cell density and under identical conditions. CD34 enrichment increased the transduction efficiency in marrow-derived CFU-GM from an average 8.3% to 16.7% (P < .01) and in peripheral blood–derived CFU-GM from an average 16% to 27.9% (P < .01, Fig 2).

Gene transfer into hematopoietic progenitor cells by extended long-term transduction culture. We hypothesized that prolonging the transduction culture over time should increase the frequency of transduction in the more immature progenitor cells. In seven experiments, cultures of marrow cells were maintained 1, 2, and 3 weeks with 50% cell-free PG13/LN vector-containing medium, exchanged either once weekly or five times per week. The increased duration of the transduction culture led to an increase in transduction efficiency both for CFU-GM (Table 2) and LTC-IC (Table 3).

With once weekly feeding of cell-free retrovirus supernatant, the transduction efficiency in CFU-GM did not increase beyond 2 weeks and led to a low recovery of LTC-IC after 3 weeks compared with the experiments with cells from the same donors using 5 weekly exchanges of supernatant. With 5 weekly feedings of cell-free vector-containing medium, G418 resistance increased in CFU-GM from 4.5% in week 1 (n = 4) and 60% in week 2 (n = 2) to 73.8% in week 3 (n = 4). Using these culture conditions, we observed an expanded yield of CFU-GM colony-forming cells (Fig 3A). The high transduction efficiency obtained in CFU-GM after 2 and 3 weeks was consistently observed after an additional 4-week long-term culture to assay for transduced LTC-IC. After 1 week of transduction, 66.7% (n = 3) of colonies derived from subsequent 4-week LTC-IC culture were G418-resistant, after 2 weeks 100% (n = 1) and after 3 weeks 81.5% (n = 2). The number of LTC-IC–derived colonies obtained after 1, 2, and 3 weeks was comparable (Fig 3B), indicating that LTC-IC were maintained by the culture conditions used. One sample of G-CSF–mobilized PBMC was submitted to long-term transduction as described, leading to 53% and 91% of G418-resistant CFU-GM after weeks 1 and 2, respectively.

DISCUSSION

Efficient gene transfer into hematopoietic stem cells is important for successful clinical gene therapy. Transduction with amphotropic vectors is hampered by the low affinity of retroviral vectors to hematopoietic target cells. Crooks and Kohn couldn’t observe quantitative binding of these vectors to immature CD34+/CD38− progenitor cells without growth factors. In line with their studies, which analyzed PA317/LN binding but not transduction, our results suggest that vector binding to the amphotropic receptor Ram-1 is limiting for the transduction of hematopoietic progenitor cells with cell-free retrovirus supernatant. Using two identical LN vectors with different envelope proteins, we observed a significant increase in the transduction of both marrow and peripheral blood–derived CFU-GM with the GALV-pseudotyped PG13/LN vector compared with the original PA317/LN vector. Similar results were observed in two experiments that evaluated LTC-IC. This finding is compatible with the observation of Kavanaugh et al that the GALV receptor Glvr-1, a sodium-dependent phosphate symporter, is expressed at much higher density on rat marrow cells compared with Ram-1. Our data suggest that the GALV-pseudotyped PG13 packaging system may allow more efficient ex vivo gene therapy of hematopoietic stem cells.

Enrichment for CD34+ cells reduces marrow to about 1% of its mononuclear cells while retaining most of the hematopoietic progenitor and stem cells, thus reducing the volume of cell culture for ex vivo gene transfer in primates and humans. Whereas efficient gene transfer into CD34-en-
We have identified conditions that led to efficient transduction and expansion of CFU-GM as well as efficient transduction and maintenance of LTC-IC, the most immature human hematopoietic progenitor cells that can be assayed in vitro. Transduction with PG13-pseudotyped retroviral vector, after stem cell enrichment with the CD34 antigen and by extended culture in the presence of PG13 vector, IL-1, IL-3, IL-6, and SCF would be compatible with a clinical application.

ACKNOWLEDGMENT

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