The clinical application of gene transfer is hindered by the availability of the multipotent stem cells and the difficulty in obtaining efficient retroviral transduction. To assess potential means by which gene transfer into human hematopoietic stem cells might be enhanced, the retroviral transduction efficiency of human bone marrow cells (BM) or peripheral blood progenitor cells (PBPC) was compared at multiple time points after in vivo administration of granulocyte colony-stimulating factor (G-CSF). This was further compared with the transduction efficiency of cells mobilized with G-CSF plus stem cell factor (SCF) in a cohort of patients randomized to receive either one or two growth factors and with normal BM function. Using the LNL6 retrovirus, retroviral transduction efficiencies of up to 19% were observed for both PBPC and BM (n = 26 patients). There was at least a 100-fold increase in PBPC with G-CSF alone and a further 30-fold increase in the total number of progenitor cells available for retroviral transduction using the combination of SCF plus G-CSF. However, pretreatment of patients with G-CSF with or without SCF did not enhance the retroviral infectability of growth factor-mobilized progenitor cells. The effect of the growth factor, Flk-2/Flt3 ligand (FL), was also examined with respect to retroviral transduction efficiency of human progenitor cells. FL plus IL-3 in vitro increased the retroviral transduction efficiency up to eightfold compared with results observed using other combinations of cytokines tested (P < .001). These findings have clinical implications both for increasing the number of target cells for in vivo gene-marking/gene-therapy studies and improving the efficiency of gene transfer.

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Retroviral transduction of human progenitor cells: use of granulocyte colony-stimulating factor plus stem cell factor to mobilize progenitor cells in vivo and stimulation by Flt3/Flk-2 ligand in vitro

By Ngaire J. Elwood, Helen Zogos, Tracy Willson, and C. Glenn Begley

The clinical application of gene transfer is hindered by the availability of the multipotent stem cells and the difficulty in obtaining efficient retroviral transduction. To assess potential means by which gene transfer into human hematopoietic stem cells might be enhanced, the retroviral transduction efficiency of human bone marrow cells (BM) or peripheral blood progenitor cells (PBPC) was compared at multiple time points after in vivo administration of granulocyte colony-stimulating factor (G-CSF). This was further compared with the transduction efficiency of cells mobilized with G-CSF plus stem cell factor (SCF) in a cohort of patients randomized to receive either one or two growth factors and with normal BM function. Using the LNL6 retrovirus, retroviral transduction efficiencies of up to 19% were observed for both PBPC and BM (n = 26 patients). There was at least a 100-fold increase in PBPC with G-CSF alone and a further 30-fold increase in the total number of progenitor cells available for retroviral transduction using the combination of SCF plus G-CSF. However, pretreatment of patients with G-CSF with or without SCF did not enhance the retroviral infectability of growth factor-mobilized progenitor cells. The effect of the growth factor, Flk-2/Flt3 ligand (FL), was also examined with respect to retroviral transduction efficiency of human progenitor cells. FL plus IL-3 in vitro increased the retroviral transduction efficiency up to eightfold compared with results observed using other combinations of cytokines tested (P < .001). These findings have clinical implications both for increasing the number of target cells for in vivo gene-marking/gene-therapy studies and improving the efficiency of gene transfer.

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MATERIALS AND METHODS

Cells. Clinical samples were obtained from patients (n = 26) enrolled in a clinical trial to optimize PBPC mobilization in patients with untreated breast cancer, with normal BM function, and without breast cancer involvement. The design of this study will be de-
scribed elsewhere and was an extension of our related clinical study. Briefly, patients were randomized to receive either G-CSF (12 μg/kg/d) alone or in combination with SCF (5, 10, or 15 μg/kg/d) for 7 days. Only patients receiving 10 or 15 μg/kg/d of SCF were included in this analysis. BM was harvested (and blood samples were collected) from the patients before commencement of growth factors. Blood samples were collected for this study on days 1, 5, 8, and 12 after commencement of treatment with growth factor(s); on day 1, the blood was collected before the administration of growth factor. Subsequently, patients received high-dose chemotherapy, and the PBPC collected during the period of growth factor administration were reimplanted. This phase of the clinical study was not included in the analysis presented here. All aspects of this study were approved and monitored by the Institutional Ethics Committee.

Retroviral vector. The LN6.6 retrovirus was produced using the PA317 amphotropic retrovirus packaging cells containing the LN6.6 vector,9 which carries the neomycin phosphotransferase gene and confers resistance to G418. The retroviral titers of this packaging cell line were consistently between 5 X 10^6 and 1 X 10^7 infectious units (IU)/mL as assessed using an NIH3T3 assay.10 To ensure a comparable titer over the time of these studies, stocks from a single high-titer clone were thawed every 2 months and viral titer confirmed by NIH3T3 assay at approximately 1 X 10^7 IU/mL. The producer cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) high glucose (0.45% glucose) supplemented with 10% bovine calf serum (BCS) and, where required, vector-containing supernatants were collected from confluent cells, filtered, and frozen at -70°C. Titer of viral supernatants was repeatedly confirmed.

CD34+ cell selection. CD34+ cells were selected using the Miltenyi MiniMacs Immunomagnetic Separation system and the CD34+ progenitor cell isolation kit (Becton Dickinson, Knoxfield, Victoria, Australia) with slight modification of the manufacturer's instructions. Briefly, mononuclear cells (MNC) from BM samples or PBPC were isolated by centrifugation on Ficoll density gradient and suspended in Iscove's modified Dulbecco’s medium (IMDM) and 20% fetal calf serum (FCS). A total of 10^3 to 3 X 10^5 MNC were incubated with 150 μL of degased buffer/10^6 cells (phosphate-buffered saline supplemented with 0.5% bovine serum albumin and 5 mmol/L EDTA, pH 7.2). Fifty microliters of reagent A1 (human IgG) was mixed with 50 μL of reagent A2 (modified CD34 antibody, QBEND/10, mouse IgG1) per 10^6 cells and incubated for 15 minutes at 4°C. Cells were washed once in 5 mL buffer/10^6 cells and then resuspended in 200 μL/10^6 cells. Reagent B (50 μL/10^6 cells; colloidal superparamagnetic MACS microbeads recognizing reagent A2) was added and incubated for 15 minutes at 6°C. Cells were washed once and resuspended in 400 μL buffer/10^6 cells. Cells were then applied to the MiniMacs magnetic column (type MS, without flow resistor), allowed to pass through the column, and washed with 4 X 500 μL buffer. The column was removed from the magnet, 1 mL of buffer was added to the column, and the cells were eluted. The purity of the isolated cells was assessed by staining cells with the CD34 antibody (HPCA-2; Becton Dickinson) before analysis with a fluorescence activated cell analyzer (Coulter Electronics Profile II, Hialeah, FL). The purity of the CD34+ fraction was consistently between 90% and 96%.

Retroviral transduction. MNCs from BM or blood (2.5 X 10^6 cells) or CD34+-purified cells (2.5 X 10^5 cells) were cocultivated for 3 days (37°C, 5% CO_2) in 60-mm dishes (Falcon; Becton Dickinson, Lincoln Park, NJ) with 10^5 irradiated (3,000 rad) PA317/LN6.6 retrovirus producer cells. For all experiments, control or mock-cocultivations were performed, using the packaging cell line PA317. For retroviral transductions performed using viral supernatant (titer at approximately 10^7 IU/mL), supernatant was added at a final dilution of 1:10 and supplemented daily. Control transductions were performed using media in place of supernatant-containing virus. All retroviral transductions were performed in 5 mL IMDM/10% FCS in the presence of the following concentrations of IL-3 (100 ng/mL), IL-6 (100 ng/mL), GM-CSF (100 ng/mL), SCF (100 ng/mL), G-CSF (500 U/mL), and 4 μg/mL polybrene (Sigma, New South Wales, Australia). These cytokines were recombinant human proteins provided by AMGEN (Thousand Oaks, CA). In an attempt to improve retroviral transduction efficiency, the cytokines listed above were added in various combinations with or without a supermaximal concentration of purified recombinant murine FL (FL; 100 ng/mL)23 during the cocultivation period. Recombinant FL was produced as previously described.23 Both BM and PBPC from all of the above-mentioned mobilization regimens were used as the source of cells for the FL experiments. After retroviral transduction, nonadherent cells were collected and the efficiency of infection was determined by progenitor cell assay in the presence of G418.

Progenitor cell assay. Progenitor cells were grown in agar culture prepared by adding 1 vol of double-strength asparagine IMDM (A1MDM) and 1 vol of FCS to 2 vol of 0.6% agar (Difco Laboratories, Detroit, MI) in a final volume of 1 mL.2 Cultures were stimulated by purified, recombinant human GM-CSF (100 ng/mL), G-CSF (500 U/mL), and SCF (100 ng/mL) for granulocyte-macrophage progenitor cells (GM-CFC) and GM-CSF (100 ng/mL), IL-3 (100 ng/mL), IL-6 (100 ng/mL), SCF (100 ng/mL), and erythropoietin (EPO; 2 U/mL) for erythroid progenitors (BFU-E). Cells from BM were cultured at concentrations of both 10^6 and 10^7 peripheral blood (PB) at both 2 X 10^6 and 1 X 10^6 cells/dish. CD34+ cells were cultured at both 1 X 10^6 and 3 X 10^6 cells/dish. To determine the efficiency of retroviral infection, cultures were grown in the presence or absence of G418 (active concentration, 2 mg/mL; Life Technologies, Melbourne, Australia). To accurately quantitate progenitor cells, multiple replicate cultures were examined; results shown for colonies grown in G418 are the mean of 9-replicate cultures. All other results are the mean of triplicate cultures. The percentage of infection efficiency was defined as the number of G418-resistant (G418R) CFC as a function of the total number of CFC, multiplied by 100. The retroviral infection efficiencies reported throughout this study were assessed by ability to confer resistance to G418 and, as discussed below, probably underestimated the true retroviral transduction efficiency.

Cultures were incubated in a fully humidified atmosphere of 5% CO_2 in air at 37°C for 14 days. Replica cultures were scored for colonies (>40 cells) after 14 days using a dissection microscope at 35 X magnification.

Polymerase chain reaction (PCR). In 3 experiments, PCR to detect the NeoR gene was performed on DNA extracted from 90 individual CFC colonies. Individual colonies were plucked from agar culture into 50 μL water. Samples were heated to 99°C for 10 minutes, digested with proteinase K (0.4 mg/mL; Worthington Biochemical Corp, Freehold, NJ) at 55°C for 60 minutes, heated again to 99°C for 10 minutes, and then kept on ice. DNA (6 μL) was amplified in a 50 μL reaction containing 200 μmol/L dNTPs, 1 μmol/L oligonucleotide primers, 1.25 U Taq polymerase (Boehringer Mannheim, Germany), and the supplied reaction buffer. PCR was performed using a Hybaid Omniogene Thermal Cycler (Hybaid, Teddington, UK). Cycle conditions were 94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 3 minutes, with 7 minutes of extension time for the last cycle. Reactions were performed for 35 cycles. The sequence of PCR primers to detect the NeoR gene (353-bp fragment) was sense 5'-GAGTGAATTGCAAGACGAGG-3' and antisense 5'-AGGCCTGCGCAACAGTTC-3'. PCR products were analyzed by electrophoresis through 1.2% agarose gels (40 μmol/L Tris-acetate (pH 8.2), 1 mmol/L EDTA), transferred to nitrocellulose filters, and prehybridized/hybridized with a 20-mer oligonucleotide (GTATCC-
ATCATGGCTGATGC) internal to the primers used for PCR. Oligonucleotides were labeled by addition of a radioactive in the samples used as negative controls (viz water alone, autoradiographed. In all experiments, NeoR DNA was not detected test.

PA3 17).

were washed to a final stringency of

were not observed in mock-transduced patient samples.

Abbreviation: NT, sample not tested.

RESULTS

Growth factor-mobilized cells are not more efficiently transduced by retrovirus. Initial experiments examined the retroviral transduction efficiency for normal BM-derived GM-CFC and BFU-E. Transduction efficiency was determined using colony growth in cultures containing 2 mg/mL G418. This concentration of G418 was higher than that usually used by others (usually 0.8 to 1.2 mg/mL).\textsuperscript{14,15,17,34-36} This concentration was selected based on titrations of G418; occasionally, spontaneously G418R CFCs were observed at concentrations up to 1.6 mg/mL (data not shown). An active concentration of 2 mg/mL was therefore deliberately used throughout these studies as a stringent selection for transduced cells expressing high levels of neomycin phosphotransferase. PCR was performed on DNA extracted from individual GM-CFC and BFU-E colonies. This was performed as an independent indicator of transduction efficiency. In three experiments, the NeoR gene was detected by PCR in 39%, 68%, and 79% of colonies that were grown in cultures without G418 (total of 90 colonies examined). The extent of gene transfer in the same three experiments, as assessed by colony formation in the presence of G418, was much lower (ie, 0.1%, 7%, and 3%, respectively). These findings are consistent with results of similar studies\textsuperscript{14,34-36} and may reflect the loss of neomycin phosphotransferase expression as cells in the hematopoietic colonies differentiate.\textsuperscript{17}

As expected from earlier studies,\textsuperscript{37} there was a broad inter-individual variation in number of GM-CFC (from 1.6% to 17.5% BM GM-CFC transduced). A similar variation was seen with BM-derived BFU-E, ranging from 0.1% to 6.5% (n = 17 examined, representative examples shown in Table 1). Retroviral transduction efficiency was similar for resting blood samples (day 1) when compared with bone marrow samples from that individual. For all cell samples, gene transfer into BFU-E was consistently less than observed with GM-CFC (P < .05).

Table 1. Retroviral Infection Efficiency Into BM or PBPC After Treatment With G-CSF Alone or SCF Plus G-CSF

<table>
<thead>
<tr>
<th>Mobilization Protocol</th>
<th>Patient No.</th>
<th>Percentage of G418-Resistant GM-CFC (total GM-CFC/10^5 cells) (days after treatment)</th>
<th>Percentage of G418-Resistant BFU-E (total BFU-E/10^5 cells) (days after treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (BM)</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>G-CSF</td>
<td>1</td>
<td>5.7 (975)</td>
<td>2.9 (35)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.0 (575)</td>
<td>4.0 (25)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.3 (790)</td>
<td>7.2 (8)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>17.5 (948)</td>
<td>=0.1 (2)</td>
</tr>
<tr>
<td>G-CSF + SCF</td>
<td>5</td>
<td>7.8 (795)</td>
<td>2.7 (77)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5.9 (605)</td>
<td>6.0 (138)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5.8 (608)</td>
<td>7.2 (32)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>3.9 (255)</td>
<td>2.6 (170)</td>
</tr>
</tbody>
</table>

Four representative samples of each of the two treatment protocols are shown. Patient samples were analyzed at the time points shown. Samples were BM at day 0 and PBPC at days 1, 5, 8, and 12 after the commencement of treatment. The percentage of G418R GM-CFC (top) or BFU-E (bottom) represents the number of G418R CFC as a function of the total number of CFC, multiplied by 100. Brackets show the total number of CFC per 10^5 cells observed in the absence of G418. G418R CFC were not observed in mock-transduced patient samples.

Statistical analyses. Statistical analyses of the SCF/G-CSF patient data were performed using either an unpaired Student's t-test or the Mann-Whitney test, as indicated within the text. Statistical analysis of the FL data was performed using a paired Student's t-test.

RESULTS

Growth factor-mobilized cells are not more efficiently transduced by retrovirus. Initial experiments examined the retroviral transduction efficiency for normal BM-derived GM-CFC and BFU-E. Transduction efficiency was determined using colony growth in cultures containing 2 mg/mL G418. This concentration of G418 was higher than that usually used by others (usually 0.8 to 1.2 mg/mL).\textsuperscript{14,15,17,34-36} This concentration was selected based on titrations of G418; occasionally, spontaneously G418R CFCs were observed at concentrations up to 1.6 mg/mL (data not shown). An active concentration of 2 mg/mL was therefore deliberately used throughout these studies as a stringent selection for transduced cells expressing high levels of neomycin phosphotransferase. PCR was performed on DNA extracted from individual GM-CFC and BFU-E colonies. This was performed as an independent indicator of transduction efficiency. In three experiments, the NeoR gene was detected by PCR in 39%, 68%, and 79% of colonies that were grown in cultures without G418 (total of 90 colonies examined). The extent of gene transfer in the same three experiments, as assessed by colony formation in the presence of G418, was much lower (ie, 0.1%, 7%, and 3%, respectively). These findings are consistent with results of similar studies\textsuperscript{14,34-36} and may reflect the loss of neomycin phosphotransferase expression as cells in the hematopoietic colonies differentiate.\textsuperscript{17}

As expected from earlier studies,\textsuperscript{37} there was a broad inter-individual variation in number of GM-CFC (from 1.6% to 17.5% BM GM-CFC transduced). A similar variation was seen with BM-derived BFU-E, ranging from 0.1% to 6.5% (n = 17 examined, representative examples shown in Table 1). Retroviral transduction efficiency was similar for resting blood samples (day 1) when compared with bone marrow samples from that individual. For all cell samples, gene transfer into BFU-E was consistently less than observed with GM-CFC (P < .05).

Treatment of patients with G-CSF alone did increase the number of retrovirally infected PBPC (GM-CFC and BFU-E/10^5 MNCs compared with baseline blood samples (day 1). The median number of G418R GM-CFC/10^5 cells at days 1, 5, and 8 was 1, 13, and 18, respectively, for patients receiving G-CSF alone (Fig 1A). By day 12, the total number of retrovirally infected cells returned to near-baseline levels.
(median, 4 G418R GM-CFC/10^5 cells). A similar pattern was observed for G418R BFU-E/10^5 cells, although the median values for BFU-E were usually at least twofold lower than for GM-CFC (Fig 1B). This increase in gene transfer paralleled the overall increase in progenitor cells observed in this study and previously noted with G-CSF and thus reflected an increase in the total number of PBPC/10^5 MNCs in the blood.

However, despite the increase in the total number of transduced cells, there was no consistent change in efficiency of gene transfer after in vivo administration of G-CSF (Table 1). For example, at the peak level of PBPC after 5 days of treatment with G-CSF, the infection efficiency of GM-CFC was 0.7% to 18.6% (n = 10), compared with 0.1% to 12.4% (n = 17) for baseline PB (P = .20).

Retroviral transduction efficiency of PBPC mobilized with the combination of SCF plus G-CSF was therefore compared with transduction of G-CSF-mobilized PBPC. As noted above, there was a wide variation between individuals in the number of cells transduced. However, the baseline values were comparable between the two groups: equally low levels of blood GM-CFC and BFU-E were retrovirally marked at this time (median of <1/10^5 progenitor cells and a median of 36 BM GM-CFC per 10^5 cells were G418R). This finding suggested that the two patient groups were similarly matched (Fig 1A).

Treatment of patients with SCF plus G-CSF did not alter the proportion of retrovirally infected progenitor cells when compared with G-CSF alone. After treatment with SCF plus G-CSF, the median number of G418R GM-CFC at days 1, 5, and 8 was 0.6, 13, and 14, respectively. This was similar to that observed for G-CSF alone (Fig 1A). At day 12, there was the suggestion of a difference between the two cohorts (median, 7; range, 2 to 93 G418R GM-CFC/10^5 cells for SCF plus G-CSF; v median, 4; range, 0.3 to 8 G418R GM-CFC/10^5 cells for the G-CSF group; P = .1). A similar pattern was observed for G418R BFU-E/10^5 cells (Fig 1B). The difference at day 12 between the two groups reflected a difference in the total number of GM-CFC/10^5 cells (median, 287; range, 130 to 750 GM-CFC/10^5 cells for SCF plus G-CSF compared with median, 81; range, 33 to 232 GM-CFC/10^5 cells for G-CSF alone; P < .01).

Thus, somewhat surprisingly, there was no consistent change in retroviral transduction efficiency of PBPC after treatment with G-CSF alone. Similarly, there was no increase in transduction efficiency after SCF plus G-CSF. However, in some individuals (eg, patient no. 8 in Table 1), this may have occurred. The variability between individuals in terms of percentage of cells retrovirally transduced was also unaltered by the administration of growth factor. Therefore, the administration of G-CSF or a stem cell-active molecule, SCF, did not enhance the ability to retrovirally infect PBPC populations.

Treatment of patients with SCF plus G-CSF increased the number of retrovirally transduced cells compared with G-CSF treatment alone. Treatment of patients with SCF plus G-CSF increased the absolute number of retrovirally transduced GM-CFC and BFU-E per milliliter of blood by up to 30-fold when compared with G-CSF alone. Baseline values (both G418R BM cells and day 1 G418R PBPC) were the same for the two groups (Fig 1). The difference between the SCF plus G-CSF cohort versus G-CSF alone cohort was most evident at day 12 (median, 230 G418R GM-CFC/mL v 19 G418R GM-CFC/mL, respectively; P < .05, Mann Whitney test). SCF plus G-CSF was also significantly superior at day 8 (1,048 G418R GM-CFC/mL v 533 G418R GM-
CFC/mL; \( P < .05 \), unpaired \( t \)-test). This difference was also evident, although not statistically significant at day 5 (median, 1,451 G418R GM-CFC/mL v 559 G418R GM-CFC/mL; Fig 2A). Thus, despite the wide patient to patient variation, this represented an overall increase in the number of retrovirally marked GM-CFC of about twofold at days 5 and 8 and about 12-fold at day 12. Similar results were observed for the number of G418R BFU-E per milliliter (Fig 2B), with a twofold increase in BFU-E at day 5 and 8 (\( P < .05 \), unpaired \( t \)-test) and a 30-fold increase in favor of SCF plus G-CSF at day 12 (\( P < .01 \), Mann-Whitney test).

As with the increase in absolute numbers of retrovirally transduced progenitor cells noted with G-CSF above, the increase in absolute numbers of retrovirally transduced GM-CFC and BFU-E was a consequence of the increase in the total number of progenitor cells observed in patients treated with the growth factor combination. The total number of GM-CFC recovered after the 3-day coculture period was a median of 48,315 (day 5), 39,054 (day 8), and 3,936 per milliliter of blood (day 12) with SCF plus G-CSF. In contrast, the median number of GM-CFC recovered per milliliter of blood for patients treated with G-CSF alone was 30,989 (day 5), 11,231 (day 8; \( P < .01 \), unpaired \( t \)-test), and 370 per milliliter of blood (day 12; \( P < .05 \), unpaired \( t \)-test). Similar differences were also observed in the total number of BFU-E recovered (data not shown). Together, these results indicated that treatment of patients with SCF plus G-CSF did not alter the retroviral infectability of progenitor cells but rather increased the absolute number of progenitor cells available for retroviral marking when compared with mobilization of PBPC using G-CSF alone.

**FL in vitro increased retroviral transduction of primary hematopoietic cells.** Given that FL supports proliferation of hematopoietic progenitor cells and the requirement of cell cycling for retroviral integration, we examined whether addition of FL during the cocultivation period enhanced retroviral transduction efficiency. Nine different cytokine combinations were examined during the cocultivation period in an attempt to optimize retroviral transduction. The combination of FL plus IL-3 was always superior and consistently produced a twofold to eightfold increase in transduction efficiency above our standard five-factor combination used (\( n = 28 \), \( P < .001 \)). A typical experiment is shown in Fig 3, in which the cocultivation was performed in the presence of the usual combination of five cytokines (IL-3, IL-6, GM-CSF, SCF, and G-CSF), IL-3 alone, FL alone, or FL plus IL-3. The absolute number of GM-CFC or BFU-E detected in the culture system was similar regardless of the cytokine combination. However, FL plus IL-3 produced about a sevenfold increase in the number of G418R GM-CFC compared with the five-factor combination or FL alone. A significant increase in retrovirally transduced BFU-E was also observed with FL plus IL-3. In the experiment shown in Fig 3, this was about twofold greater than the five-factor combination and was always slightly more than the effects seen with IL-3 alone. Although subtle, this erythroid effect was a consistent finding with all patient samples.

A summary of the retroviral infection efficiency of 10

![Fig 2](https://example.com/fig2.png)
To exclude the possibility that the effect of FL plus IL-3 was due to an interaction with the fibroblast packaging cell line, we performed four experiments in which retroviral transductions were undertaken using viral supernatant alone. Two examples are represented by patients no. 9 and 10 in Fig 4. Retroviral infection using viral supernatant in the presence of FL plus IL-3 produced a similar increase in transduction efficiency as observed for transductions per-

Fig 3. Typical experiment showing number of progenitor cells after 3 days of cocultivation with PA317/LNL6 in the presence of the usual combination of five cytokines (IL-3, IL-6, GM-CSF, SCF, and G-CSF), IL-3 alone, FL, or FL plus IL-3. Agar cultures were established using 1,000 CD34+ cells in the presence (III) or absence (II) of G418 (2 mg/mL). Data points are the mean ± SD of triplicate cultures (III) or 9-replicate cultures (II). (A) GM-CFC. (B) BFU-E. G418R CFC were not observed in mock-transduced samples for all cytokine conditions tested.

Patient samples transduced in the presence of the usual five-factor combination or FL plus IL-3 is shown in Fig 4. The presence of FL plus IL-3 gave on average a twofold to eightfold increase in infection efficiency of GM-CFC above that observed using the standard five-factor combination ($P < .001$). Similarly, an increase in transduction efficiency of BFU-E was also consistently observed (twofold to fivefold; $P < .001$). The effects of FL occurred for both BM and PBPC. There were no apparent differences in the degree of FL effect for the different sample sources.

Fig 4. Efficiency of retroviral infection of 10 patient samples transduced in the presence of the standard five-factor combination (IL-3, IL-6, GM-CSF, SCF, and G-CSF; III) or FL plus IL-3 (II). The percentage of infection efficiency represents the number of G418R CFC as a function of the total number of CFC, multiplied by 100. Patients no. 1 through 5 were retrovirally infected by the cocultivation with PA317/LNL6, whereas patients no. 9 and 10 were retrovirally transduced using viral supernatant. (A) GM-CFC. (B) BFU-E.
Fig 5. Four representative experiments (A through D) showing the number of GM-CFC after 3 days of cocultivation with PA317/LNL6 in the presence of the usual combination of five cytokines (IL-3, IL-6, GM-CSF, SCF, and G-CSF), IL-3 + FL, IL-3 + SCF, or IL-3 + SCF + FL. Agar cultures were established using $10^5$ PB MNC (A through C) or $10^3$ CD34$^+$ cells (D) in the presence (■) or absence (●) of G418 (2 mg/mL). Data points are the mean ± SD of triplicate cultures (■) or 9-replicate cultures (●).

formed by cocultivation. Again, the increase was observed for both GM-CFC and BFU-E. Thus, it appeared that FL plus IL-3 appeared to exert a direct action on CD34$^+$ cells in improving retroviral transduction.

Given that FL shares a number of overlapping properties with SCF, it was interesting to note that the combination of SCF plus IL-3 did not have the same effect in enhancing retroviral transduction efficiency as did FL plus IL-3 (Fig 5). In fact, the addition of SCF to the FL plus IL-3 combination appeared to abrogate this effect; representative examples are shown in Fig 5. For each patient sample, the absolute number of GM-CFC was similar regardless of the cytokines present during the cocultivation. The combination of FL plus IL-3 during the transduction enhanced the number of retrovirally marked progenitor cells for the 4 patients approximately fivefold. In contrast, the combination of IL-3 plus SCF produced a similar result to the five-factor combination of cytokines. Moreover, the addition of SCF to the FL plus IL-3 combination showed no enhancement of the retroviral transduction efficiency.

DISCUSSION

The data presented in this study have shown two potential means by which gene transfer into human hematopoietic cells may be improved. Firstly, in vivo administration of SCF plus G-CSF mediated an increase in the number of progenitor cells in the blood available for gene transfer. These cells could be retrovirally transduced as efficiently as
BM cells. Secondly, the retroviral transduction efficiency of hematopoietic cells was enhanced by the addition of FL plus IL-3 during the transduction period.

**Retroviral transduction of mobilized PBPC.** Retroviral transduction of mobilized human PBPC has been previously examined by others. However, this is the first comprehensive analysis of gene transfer into human progenitor cells mobilized by cytokines alone and from individuals with no abnormality of bone marrow function. Retroviral transduction of PBPC mobilized by G-CSF alone has been reported to be more efficient than infection of cells mobilized by high-dose chemotherapy plus G-CSF. However, only 6 patient samples were examined in this study and retroviral transduction efficiency was assessed by specific glucocerebrosidase enzyme activity in transduced PB cells. The infection efficiencies we obtained for PBPC mobilized by either G-CSF alone or SCF plus G-CSF were comparable to those reported by groups for whom cells were mobilized with high-dose chemotherapy plus cytokines, but lower than that reported by others; however, the active concentrations of G418 used make it difficult to compare results. In this study, a concentration of 2 mg/mL G418 was used to provide a stringent selection for transduced cells.

Mobilization of PBPC with G-CSF alone increased the number of retrovirally transduced progenitor cells compared with baseline blood samples. The number of genetically marked PBPC at days 5 and 8 was comparable to that observed in normal BM. After withdrawal of G-CSF in vivo, the proportion of retrovirally transduced PBPC had returned towards baseline levels by day 12. These changes observed were not due to increases in the transduction efficiency of the cells per se but probably reflected the increase in total PBPC observed after G-CSF alone.

Mobilization of PBPC with SCF plus G-CSF did not further enhance the proportion of retrovirally marked cells above that observed for G-CSF alone but did increase the total number of PBPC per milliliter of blood available for gene transfer. The difference in the number of G418R PBPC per milliliter of blood at day 5 after G-CSF alone or SCF plus G-CSF was not statistically different, although a trend in favor of the combination was apparent. At days 8 and 12, a statistically significant difference was evident. This was shown by using an unpaired t-test (day 8) and Mann-Whitney test (day 12). The requirement for the Mann-Whitney test reflects the wide interpatient variation and the fewer number of samples analyzed from patients receiving G-CSF alone. However, support is given to the validity of these findings because we conclude that the increase in G418R PBPC per milliliter of blood after SCF plus G-CSF was a reflection of an increase in the total number of PBPC per milliliter of blood (which was also significantly different at day 8 and 12). Furthermore, the patient samples presented here are a subset of the patient samples analyzed for progenitor cell mobilization in a more extensive study in which the differences between SCF plus G-CSF and G-CSF alone have been further validated (Begley et al, manuscript in preparation).

The effect of SCF plus G-CSF to increase the number of target cells for gene transfer is consistent with animal data. In mice, it has been shown that SCF plus G-CSF mobilized at least twofold more long-term repopulating cells and short-term repopulating cells than did G-CSF alone and subsequently led to more sustained engraftment. However, it was surprising that administration of SCF in vivo did not enhance retroviral transduction efficiency. This was particularly so given that SCF enhances the in vitro proliferation of progenitor cells from primates marrow in response to various hematopoietic growth factors and may play a role in the maintenance of cell cycle. In addition, SCF in combination with IL-3 and IL-6 has been shown to increase expression of the amphotropic receptor. Furthermore, Bodine et al showed that treatment of splenectomized mice treated with SCF plus G-CSF mobilized large numbers of stem cells into the PB that were more efficiently marked by retroviruses than equivalent cells from the BM of 5-fluorouracil-treated mice. Although there was no control group that received G-CSF alone, a role for SCF in enhanced gene transfer in human gene studies was predicted. Our data suggest that the combination of SCF plus G-CSF in vivo is not superior to G-CSF alone in terms of enhancing efficiency of retroviral transduction of human progenitor cells. For any hematopoietic gene therapy approach it is desirable to show that retrovirally transduced cells are able to provide long-term reconstitution of the patients’ hematopoietic system. Although a number of assays have been proposed as alternatives to GM-CFC assays, in the context of gene marking, they are all surrogate assays for cells that provide long-term hematopoietic reconstitution. Moreover, the time-consuming and cumbersome nature of these assays substantially limits their application to clinical studies. For these reasons, we examined gene transfer into progenitor cells. Based on these results, we concluded that in vivo administration of SCF plus G-CSF did not offer any further advantages over G-CSF alone in terms of retroviral infectability of progenitor cells, although it did provide an increase in the number of progenitor cells available for gene marking.

**Enhanced retroviral transduction by FL plus IL-3 in vitro.** Examination of the number of retrovirally transduced progenitor cells showed that the most efficacious combination of cytokines during retroviral infection was FL plus IL-3. It was of interest that addition of SCF to this combination appeared to abrogate this effect. The combination of IL-3, IL-6, and SCF has been documented as the optimum combination to achieve retroviral transduction of human CD34+ PB and CD34+ BM cells and has recently been used in a clinical study. The addition of IL-1α may also be of benefit. In these reported studies, transduction efficiencies ranging from 2% to 50% G418R CFCs were recorded with wide variability in infectivity of patient samples. The infection efficiencies obtained in the current study were comparable with those observed by others and also with wide variation between individuals.

FL plus IL-3 appeared to exert a direct action on CD34+ cells in improving retroviral transduction. This effect was seen both in coculture experiments using the packaging cell line and in experiments using the viral supernatant. The presence of FL plus IL-3 during transduction with retroviral
supernatant remained up to 10-fold better than the usual combination. In all experiments (n = 28), FL plus IL-3 also enhanced the transduction efficiency of BFU-E above the usual combination. This is not surprising given that CD34+ cells express receptors for FL and display erythroid potential.45 Furthermore, antiseed studies directed against the FL receptor inhibited BFU-E formation by CD34+ human BM cells.28 However, FL does not appear to have a direct proliferative effect on committed erythroid progenitors.18,19,22,46.49

The mechanism by which FL plus IL-3 enhanced retroviral transduction is unknown. It did not appear to be associated with enhanced clonogenicity. There was no consistent difference between the cytokine combinations in terms of the total number of progenitor cells after the transduction period (data not shown). However, enhanced clonogenicity has been shown with murine Lin- Sca-1 BM cells22 and Thy1 Sca-1 cells21 stimulated with FL plus IL-3. FL has been shown to increase the clonogenicity of the more primitive Lin-Kit Sca-1 progenitor cells, although leaving the high generative capacity of the more primitive Lin-Kit Sca-1 cells unaltered.22 Furthermore, FL plus IL-3 stimulated greater clonogenic of these cells than FL plus SCF. Thus, it is possible that, rather than enhancing self-generation of the most primitive stem cells, FL may act to generate more mature multipotential cells. However, an action of FL to stimulate self-generation has been shown using the M1 leukemic cell line.32 Perhaps with a more highly purified population of CD34+ cells, a difference in clonogenic potential may have been observed. Based on the above literature with highly purified murine cells, FL may act to maintain clonogenic potential and possibly facilitate self-renewal, thereby explaining the enhanced retroviral integration that we observed. It is also possible that the combination of FL plus IL-3 altered the cycling status of the progenitor cells, thus allowing more efficient retroviral integration. The FL receptor-positive fraction of primitive cells contains almost 10-fold more cycling cells than the FL receptor-negative subpopulation.37,46 Equally, the combination of FL plus IL-3 may increase amphotropic receptor binding to human stem cells to allow improved retroviral transduction efficiency. This has been observed after prestimulation of CD34+ cells in IL-3, IL-6, and SCF.43

FL plus IL-3 appeared to favor predominantly macrophage and some granulocyte differentiation in liquid culture.7 This preference of FL to generate cells of the myeloid lineage was also observed in morphologic analysis in the current study after the 3-day transduction period (data not shown). However, from both the colony morphology and cell morphology analysis, there was no suggestion that FL plus IL-3 was promoting differentiation beyond that observed using the other cytokine combinations, although the generation of dendritic cells has been noted in similar culture conditions.49

Whatever the mechanism for the enhanced retroviral transduction efficiency in the presence of FL plus IL-3, this is the first time such a function for FL has been described. It is now of interest to examine whether the long-term repopulating ability of such transduced cells is maintained. Furthermore, the combination of FL plus IL-3 may prove useful for retroviral supernatant infection of mobilized cells for clinical protocols. It may also be useful to include FL in systems involving allogeneic stromal cultures51 or BM extracellular matrix molecules.51 It would also be of interest to examine the action of FL in clinical PBPC mobilization strategies as an approach to obtaining cells potentially susceptible to enhanced retroviral gene transfer.

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RETROVIRAL TRANSDUCTION OF HUMAN PBPC

4481

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NJ Elwood, H Zogos, T Willson and CG Begley