High-Efficiency Gene Transfer and Selection of Human Hematopoietic Progenitor Cells with a Hybrid EBV/Retroviral Vector Expressing the Green Fluorescence Protein

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Abstract

We report a retroviral expression vector (PINCO) that allows high-efficiency gene transfer and selection of hematopoietic progenitor cells (HPCs). The main characteristics of this vector are the presence outside the two long terminal repeats of the EBV origin of replication and the EBNA-1 gene and the presence in the retrovirus of the cDNA that encodes for the enhanced green fluorescence protein (GFP), controlled by a cytomegalovirus promoter. Transient transfection of PINCO in Phoenix packaging cells results in episomal propagation of the plasmid and generates viral titers as high as $10^7$ colony-forming units/ml. Infection of established cell lines with the PINCO retrovirus yields more than 95% GFP-expressing cells. GFP expression remains stable for months in infected cell cultures and can easily be monitored by fluorescent microscopy or fluorescence-activated cell-sorting (FACS) analysis of living cells. The PINCO vector allows efficient expression of a second gene (thymidine kinase, Shc, and PML), and there is strict correlation between GFP and second gene expression levels in the infected cells. PINCO was used to infect human HPCs; infection efficiency was about 50%. GFP-positive cells can be FACS sorted to yield a homogeneous population of infected cells. FACS-sorted GFP-positive HPC cells have, with respect to uninfected packaging cell lines, the same frequency of long-term culture initiating cells and an identical capacity to undergo multilineage and unilineage differentiation. The entire gene transfer procedure, from the transfection of the packaging cell line to the infection of target cells, requires less than a week. The high viral titer and the easy obtainment of homogeneously infected cell populations without drug selection procedures make PINCO an ideal vector for gene transfer of human primary hematopoietic cells.

Introduction

Genetic manipulation of hematopoietic stem cells is both a great opportunity and a challenge to gene therapy strategies. Insertion of exogenous genes into hematopoietic stem cells would enable adoptive transfer of genetic information into progenitors and progeny of blood cell lineages. This approach would allow diseases associated with genetic defects, such as immunodeficiencies and hemoglobinopathies, to be treated with genetically engineered cells. Moreover, stem cells and their progeny could be engineered to ubiquitously express enzymes missing from other tissues or could be endowed with higher resistance to chemotherapeutic damage or oncogenic processes (1, 2).

Crucial aspects of the genetic manipulation of HPCs are the efficiency of the transduction system, the choice of the appropriate target cell, and the type of selection of the transduced cells. Among the available gene transfer systems, retroviral vectors still seem to be the best, because they provide stable integration of exogenous DNA into genomic DNA and gene transmission to progeny (1, 2). Classic approaches involve the infection of bone marrow cells with recombinant retroviruses that express the gene of interest and a selectable marker. The most used selectable markers are genes that confer resistance to specific drugs. However, there are severe limitations to all of these approaches: (a) the low and variable efficiency of the transduction system (the titer of the commonly used recombinant retroviruses rarely exceeds $10^6$ CFU/ml); (b) the use of mixed bone marrow populations, which further reduces the percentage of transduced HPCs; (c) the need for drug selection of infected cells, which requires 2–3 weeks of in vitro bone marrow culture, thereby provoking partial loss of multipotentiality of HPCs; and (d) the competition between expression of the selectable marker and the transduced gene.

We combined three different procedures to improve current approaches of gene transfer into hematopoietic cells: (a) the use of a new selectable marker, GFP (3); (b) the generation of high-titer hybrid EBV/retroviral vectors (4); and (c) the use of purified HPCs as the target cell population (5, 6).

Expression of the GFP yields a strong spontaneous fluorescent signal in heterologous cell types (3). It has been observed that GFP can be fused to resident proteins or to specific targeting signals without altering its fluorescence properties (7). GFP therefore seems to be a unique tool in cell biology, in that it allows specific proteins to be visualized in living cells or GFP-transfected cells to be identified.

The nuclear replication and retention functions of EBV have been used to maintain retroviral vectors episomally within packaging cell lines. These hybrid EBV/retroviral vectors are capable of producing helper-free recombinant retrovirus in as little as 48 h and for at least 30 days after transfection into ectopic and/or amphotropic packaging cells. Viral titers greater than $10^7$ CFU/ml can be obtained after selection of transfected packaging cells (4).

There have been two recent advances in the culturing of human bone marrow cells: (a) methods for isolating HPCs from donor peripheral blood that give high yields and purification levels; and (b) unilineage suspension cultures of erythroid, granulopoietic, eosinophilic, megakaryopoietic, and monocytic progenitors (6, 8, 9).

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3 The abbreviations used are: HPC, hematopoietic progenitor cell; CFU, colony-forming unit; GFP, green fluorescence protein; FACS, fluorescence-activated cell sorting; IL, interleukin; LTR, long terminal repeat; CMV, cytomegalovirus; TK, thymidine kinase; LT-IC, long-term culture initiating cell.
We report the generation of a hybrid EBV/retroviral vector expressing the GFP as a selectable marker (PINCO vector). Infection of purified HPCs with PINCO allowed transduction of approximately 40–50% of the population and FACS sorting selection of a virtually homogeneous population of transduced cells without interfering with their proliferative and differentiative properties.

Materials and Methods

Culture of Cell Lines and HPCs and Differentiation of HPCs. U2OS osteosarcoma and NIH-3T3 mouse fibroblasts were cultured in DMEM supplemented with 10% FCS or 10% calf serum, respectively. The human myeloid leukemia cell line TF-1 and the mouse myeloid cell line 32D were cultured in RPMI 1640/10% FCS supplemented with 10 ng/ml recombinant human granulocyte macrophage colony-stimulating factor and 10 units of IL-3, respectively. HPCs were purified according to published procedures (5,6) and stimulated to enter the cell cycle by adding IL-3, IL-6, KL, and Flt3 ligand (cycling mixture) in a serum-free medium, as reported previously (10). Infection cycles were started 48 h after the addition of growth factors. HPCs were studied for their proliferative and differentiative potential by clonogenic assays, liquid phase cultures, and LTC-IC cultures, as described previously (5,6,8–10).

Construction of PINCO Retroviral Plasmid, Transient Production of Retroviral Particles, and Infection of Target Cells. The reported LZRSpBMN-Z plasmid (4) was digested with HindIII and NotI to substitute the lacZ gene with the EGFP cDNA (pEGFP-N1; Clontech, Palo Alto, CA). A CMV promoter enhancer derived from the pRC/CMV plasmid (Invitrogen Corp., San Diego, CA) was inserted upstream to the EGFP cDNA in the EcoRI-HindIII sites. The resulting plasmid was named PINCO. The amphotropic packaging cell line Phoenix was transfected by the calcium-phosphate/paraformaldehyde, and incubated for indirect immunofluorescence with monoclonal antibodies against shc (17) or PML (14). A rhodamine-conjugated antibody to the p53 protein was used (Becton Dickinson, San Jose, CA). The production of the fluorescent protein was evaluated in Phoenix cells with a fluorescence microscope equipped with standard FITC filters. As shown in Fig. 2A, about 80% of the cells became highly fluorescent 48 h after transfection of the PINCO plasmid. Although the percentage of detectable positive cells remained constant over time, the expression of the fluorescent protein slightly tended to decrease in epidosmal plasmid copy number (data not shown).

Viral titers were quantified by cloning the neomycin resistance gene (Neo) in the PINCO plasmid under the control of the 5' LTR. Serial dilutions of the Neo-PINCO viral supernatant were collected 2 or 5 days after transfection of Phoenix cells and used to infect the human osteosarcoma U2OS cell line. In parallel experiments, supernatants were collected from Phoenix cells after 5 days of puromycin selection. The number of G418-resistant colonies was evaluated after 14 days of drug selection. The Neo-PINCO virus titer was approximately 7–8 × 10^6 CFU/ml in supernatants collected at both 2 and 5 days in the absence of puromycin and was 0.9–1.1 × 10^7 CFU/ml in the puromycin-selected ones. Note that these titers were obtained with a retrovirus expressing two genes, Neo and the EGFP cDNA. Therefore, these viral titers should be representative of those obtainable with PINCO retroviruses that express the GFP selectable marker and a gene of interest (see below).

We next investigated the efficiency of infection and GFP expression in target cells by infecting different cell lines: the NIH-3T3

![Fig. 1. Limited restriction enzyme map of the PINCO vector. The indicated components of the PINCO plasmids are described in the text.](image-url)
GFP-EXPRESSING RETROVIRUS

Fig. 2. Evaluation of GFP expression in cells transfected (A) or infected (B–D) with PINCO. Phoenix cells were transiently transfected with the PINCO plasmid using the calcium phosphate precipitation procedure and analyzed after 48 h (A). NIH-3T3 (B), U2OS (C), and TF-1 (D) cells were infected with PINCO retrovirus and analyzed after 48 h. GFP expression was analyzed in intact cells with a fluorescence microscope equipped with a standard filter for FITC. Exposure times were adjusted to obtain the optimal view of GFP-positive cells. The untransfected cells were detected by 4',6-diamidino-2-phenylindole staining and analyzed with a standard UV filter.

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fibroblast cell line; the U2OS osteosarcoma cell line; and the TF-1 human myeloid leukemia cell line. Target cells were submitted to one or two infection cycles (see “Materials and Methods”) using supernatants of Phoenix cells collected 2 days after transfection of the PINCO plasmid. Infected cells were analyzed for GFP expression 2 days after infection. After a single cycle of infection, more than 95% of NIH-3T3 (Fig. 2B) and U2OS (Fig. 2C) cells and 40–50% of the TF-1 myeloid cells expressed GFP (Fig. 2D). With two infection cycles, the percentage of positive TF-1 cells rose to 95% (data not shown). To determine whether the GFP expression was sufficiently intense for fluorometric analysis, a population of TF-1 cells that was 40% GFP positive at fluorescence microscopy was analyzed by FACS. FACS analysis revealed a clear quantitative separation between GFP-positive and GFP-negative cells (data not shown). We finally kept a PINCO-infected NIH-3T3 population in continuous culture and found that GFP expression remains stable for at least 3 months (data not shown).

These results provide evidence that transient production of PINCO retroviruses in the Phoenix cell line allows high-efficiency transduction of the GFP marker in target cells, and that GFP expression is stable in the transduced cells and is suitable for microscopic evaluation and FACS analysis.

Evaluation of the Efficiency of Expression of a Second Gene Cloned within the PINCO Vector. To study the expression efficiency of genes of interest by the PINCO vector, cDNAs encoding the Shc adaptor protein (13), the TK (15), or the PML protein (16) were cloned under the control of the 5' LTR (Shc-PINCO, TK-PINCO, and PML-PINCO). TF-1, U2OS, and NIH-3T3 cells were infected with Phoenix cell supernatants collected 48 h after transfection with each plasmid. The three cell lines yielded more than 95% GFP-positive cells as determined by direct fluorescence microscope evaluation 48 h after infection (data not shown). The bulk population of TK-PINCO-infected U2OS cells was treated with ganciclovir, which induced death in virtually all GFP-positive cells (data not shown). Shc-PINCO-infected cells were evaluated simultaneously for GFP and Shc expression, respectively, by microscopic evaluation after fixation.
(Fig. 3A) and by indirect immunofluorescence with anti-Shc polyclonal antibodies (Fig. 3B). Because the antibody used hardly recognizes endogenous Shc polypeptides, the staining derives mainly from exogenous Shc proteins (17). All GFP-positive cells were also Shc positive. In addition, there was a strict correlation between the expression intensities of GFP and Shc (compare Fig. 3A with the anti-Shc antibody staining in Fig. 3B). This correlation was also documented in cells infected by PML-PINCO. In this case, PML-PINCO-infected 32D myeloid cells were separated by FACS sorting, based on the levels of GFP expression in two populations that were evaluated for PML expression by Western blotting and immunofluorescence (data not shown). The strict parallel between GFP and associated gene expression could be explained by the common integration site of the two genes or the presence of multiple integrated copies of the provirus. Southern blotting analysis of integration sites was carried out on eight clones obtained by limiting dilution from a TK-PINCO-infected U2OS bulk population, four with high and four with low GFP expression. A single copy of the virus was integrated in the four low fluorescence intensity clones, but more than one copy was integrated (up to four) in the high-fluorescence clones (data not shown). It is therefore likely that when infection is performed on cell lines at high viral multiplicity, multiple viral copies can enter the cell and contribute to high expression of both GFP and the associated gene.

In summary, GFP can be considered a true selectable marker for rapid selection of homogeneously transduced cells. The entire procedure from transfection of the packaging cells to collection of infected cells can be accomplished in just 1 week. Moreover, because the brightness of fluorescence of the PINCO-infected cells correlates with the expression levels of the associated gene, bulk cell populations with differing expression levels of the GFP-associated gene can be identified or FACS purified without biases of clonal selection and variability. This provides an experimental approach for a quantitative analysis of gene expression effects in mammalian cells.

**Infection of Hematopoietic Progenitors.** A purification procedure for human early HPCs based on the depletion of all mature cells has recently been designed (5, 6). The final cell population is 95% CD34+, has a high cloning efficiency, can be differentiated along all hemopoietic lineages, and contains 0.3–1.5% LT-TCs representative of human stem cells (5, 6, 9).

To establish the efficiency of gene transfer of human HPCs and stem cells using our GFP-based retroviral system, HPCs were transduced with the PINCO and TK-PINCO vectors. HPCs were stimulated to enter the cell cycle by adding growth factors, and four infection cycles were performed, starting on day 2 after growth factor addition (see "Materials and Methods"). The percentage of transduced cells was evaluated 48 h after the last infection by FACS analysis in repeated experiments. The GFP-positive cell population was 40 ± 5% in TK-PINCO-infected cells (data not shown) and 50 ± 5% in PINCO-infected cells (Fig. 4, A–C), as evaluated by FACS analysis and microscopic evaluation. GFP-positive cells disappeared from TK-PINCO-infected HPC populations within 10 days of culture in the presence of ganciclovir (data not shown).

We next analyzed the biological properties of GFP-positive HPCs in homogenous populations of PINCO-infected HPCs obtained by FACS sorting. The positive and negative sorted populations were reevaluated for GFP expression and were found, respectively, to be 99% positive and 100% negative (see Fig. 4, D–F, for the GFP-positive cells). Sorted HPCs were then studied for their clonogenic and differentiative properties and compared with the unfractionated HPC population. To this purpose, cells were plated in methylcellulose or liquid culture with diverse combinations of growth factors to induce multilineage and unilineage differentiation. Additional cultures were established to study the presence of LTC-ICs. As shown in Table 1, GFP-positive cells were essentially indistinguishable from untransduced cells in their ability to differentiate toward the individual hemopoietic lineages, both in liquid culture and in the methylcellulose colony formation assays. In addition, the number of LTC-ICs that are regarded as putative stem cells was similar in the control and GFP-positive populations. Overall, transduction with GFP did not alter the biological properties of highly purified human hematopoietic progenitors.

In conclusion, the PINCO-based gene delivery system described in this paper allows efficient and rapid gene transfer into human HPCs. The infection efficiency of HPC using PINCO-based retroviruses is about 50%, and the transduced cells can be easily sorted from the
nontransduced cells to provide a 100% pure population of cells that express the gene of interest. This selection procedure does not require long-term culture in the presence of potentially toxic drugs like G418 or puromycin and does not seem to alter the growth or differentiative potential of HPCs. As in the case of established cell lines, the procedure of infection/selection of HPCs can be undertaken within 1 week of transfection of the packaging cell line. Similar results can be achieved by other previously reported techniques, such as the one that uses the expression of nerve growth factor receptor or CD24 as a selection marker (18, 19). However, these techniques suffer from serious limitations that are eliminated when GFP is used, for example, the necessity of using antibodies that may perturb the cell surface or

Table 1  Methylcellulose hemopoietic colonies obtained from noninfected HPCs or from GFP-expressing HPCs obtained after PINCO infection and FACS sorting

<table>
<thead>
<tr>
<th></th>
<th>CFU-GEMM</th>
<th>BFU-E</th>
<th>CFU-G</th>
<th>CFU-M</th>
<th>CFU-GM</th>
<th>CFU-MK</th>
<th>LTC-IC</th>
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<tr>
<td>Noninfected cells</td>
<td>5.5 ± 1.5</td>
<td>47.5 ± 1</td>
<td>19 ± 2</td>
<td>10.5 ± 2.5</td>
<td>11.5 ± 3.5</td>
<td>3.5 ± 0.5</td>
<td>0.13</td>
</tr>
<tr>
<td>PINCO-infected cells</td>
<td>6 ± 1</td>
<td>61 ± 3</td>
<td>28.5 ± 5</td>
<td>7 ± 2</td>
<td>13 ± 3</td>
<td>4 ± 1</td>
<td>0.11</td>
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Fig. 4. Infection of HPCs with the PINCO retroviral vector and sorting of GFP-positive cells. HPCs were infected with the PINCO retrovirus and analyzed before (left panels) and after (right panels) GFP expression-based FACS sorting. A and D, GFP expression by FACS analysis; B and E, 4',6-diamidino-2-phenylindole stainings; C and F, GFP expression by fluorescence microscope analysis.
the introduction in the cells of molecules with a potential biological activity. The validation of the P1NCO vector system for human gene therapy experimentation is the natural next step.

References

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