HIGH EFFICIENCY RETROVIRUS-MEDIATED GENE TRANSFER TO LEUKEMIA CELLS

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Abstract

Objective: To establish an efficient and safe gene transfer system mediated by retrovirus for gene marking and gene therapy of human leukemia. Method: The retroviral vector LXSN, containing the neomycin resistance (Neo^R) gene, was transferred into amphotropic packaging cells GP+envAm12 by liposome transfection or by ecotropic retrovirus transduction. Amphotropic retrovirus in supernatants with higher titer was used to infect human leukemic cell lines NB4, U937, and THP-1. The efficiency of gene transfer was assayed on colonies formed by transduced K562 cells. Results: The titer of DOSPER directly transfected GP+envAm12 cells determined on NIH3T3 cells was 8.0×10^5 CFU/ml, while that of producer infected with retrovirus was 1.6×10^7 CFU/ml. Integration of Neo^R gene into all leukemia cells was confirmed by polymerase chain reaction (PCR). Absence of replication-competent virus was proved by both nested PCR for *env* gene and marker gene rescue assay. Gene transfer with the efficiency as high as 93.3 to 100% in K562 cells was verified by seminested PCR for integrated Neo^R gene on colonies after 7 days' culture. Conclusion: The efficiency and safety of retrovirus mediated gene transfer system might provide an optimal system in gene therapy for leukemia or genetic diseases.

Key words: Retrovirus, Leukemia, Gene transfer, Transfection, Gene therapy, Polymerase chain reaction.

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Gene therapy with gene transfer as its molecular basis promises an effective strategy for the treatment of cancer and inherited diseases. Retroviral vectors derived from the Moloney murine leukemia virus (MoMLV) backbone are widely studied as vehicles for gene transfer based on their potential for highly efficient transduction and integration in the host genomic DNA.[1,2] Several clinical trails have been ongoing using MoMLV derived vectors. Retroviral vectors encoding selectable reporter molecules, such as the neomycin phosphotransferase (Neo^R) and the bacterial β-galactosidase (lacZ), have been used to study the efficiency of transduction and gene marking in a variety of cell types. Here we demonstrate the transfer of Neo^R gene in human myeloid leukemia cells mediated by replication-incompetent retroviral vector LXSN to develop an efficient gene transfer protocol for gene therapy or gene marking.

MATERIALS AND METHODS

Cell Culture and Retroviral Vector

Human leukemic cell lines NB4, U937, THP-1, and K562 were cultured in RPMI 1640 (Gibco-BRL) supplemented with 15% newborn calf serum (NCS). The GP+E86/Neo^R cells previously established in this laboratory, which produced ecotropic retrovirus at titer of 8.1×10^6 CFU/ml, the amphotropic packaging cell line GP+envAm12, which was kindly provided by Dr. Bank (Columbia University, New York), and NIH3T3 fibroblasts were cultured in high glucose Dulbecco's modified Eagle's medium (glucose 4.5 g/L, DMEM, Gibco-BRL) with 15% NCS. The culture were maintained at 37°C with 5% CO_2 in a humidified atmosphere. The vector LXSN containing the selectable Neo^R gene was a gift of Dr. Cowan (NCI, NIH, Maryland).

DNA Transfection Mediated by Liposome

The GP+envAm12 cells were transfected with LXSN vector using liposome transfection reagent DOSPER according to the manufacturer's protocols (Boehringer Mannheim), and subsequently selected in the presence of 0.8 g/L G418 (Gibco-BRL) to generate a polyclonal population of retroviral producer cells. Retroviral titers were assessed by transducing NIH3T3 cells with different volumes of supernatant.

Production of High-titer Amphotropic Retrovirus Carrying Neo^R Gene

Supernatants containing ecotropic retrovirus were collected by culturing 60–80% confluent GP+E86/Neo^R cells overnight. 0.45 μm filtered supernatants were added, with Polybrene (Sigma) to a final concentration of 8 mg/L, to GP+envAm12 cells seeded 24 hours before. Infection was performed for 2 hours at 37°C and repeated twice, then the concentration of Polybrene was reduced to 2 mg/L. The transduced packaging cells were selected in medium containing 0.8 g/L G418 following an additional culture of 24 h, the amphotropic retroviral producer line with higher titer was thus obtained.

Leukemia Cells Transduction

Human leukemia cells NB4, U937, THP-1, and K562 were transduced by replacing 50% of their medium with filtered amphotropic retrovirus-containing supernatants supplemented with Polybrene (8 mg/L) for 2 hours. After infection the medium was replaced with fresh RPMI 1640. Twenty-four hours after infection, the transduced leukemia cells were selected with G418.

In Vitro CFU-L Assay

K562 cells harvested after transduction were plated into tissue culture dishes (35 mm, Nunc) in semisolid methylcellulose medium containing 1% (w/v) methylcellulose (Fisher Scientific) in RPMI 1640 with 20% fetal bovine serum at concentration of 10^2 cells per ml. Seven to ten days later, single, well-isolated colonies were plucked from the dish using a micro-pipette for detection of Neo^R gene transfer.

Amplification of Neo^R Gene by Polymerase Chain Reaction (PCR)

For amplification of LXSN sequence from transduced leukemia cells, the cells were treated with a lysis buffer with proteinase K (Merck) and the DNA lysate was used directly in PCR as described by else.[3] Thirty cycles of PCR (95°C for 30s, 63°C for 45s) with primers specific for Neo^R gene sequence, Neo1: 5'-CGTTG TCACT GAAGC GGGAA GG-3' (sense), Neo2: 5'-CCATG ATATT CGGCA AGCAGGC-3' (antisense), and Neo3: 5'-CAAGA TGGAT TGCAC GCAGG-3' (sense), all from Sangon (Shanghai), were performed in DNA thermal cycler (GeneAmp System 9600, Perkin Elmer) using primer pair 1–2 for amplification of NB4, U937, and THP-1 cells and primer pair 3–2 then 1–2 for single colonies of K562 cells (seminested PCR). Ten microliters of the PCR product was examined on a 2% agarose gel with ethidium bromide.

Nested PCR Analysis for *Env* Gene of Helper Virus

Env gene of mouse leukemia virus 4070A integrated in genomic DNA of packaging cells GP+envAm12 without packaging signal, was amplified by nested PCR from genetic transduced cells for detection of *env* gene transfer, indicating the presence of helper virus. The primers specific for the *env* gene were kindly provided by Dr. Daru Lu (Institute of Genetics, Fudan University).[4] The structure of helper virus, the location of the *env* primers and the sizes of PCR products were summarized in Figure 1.

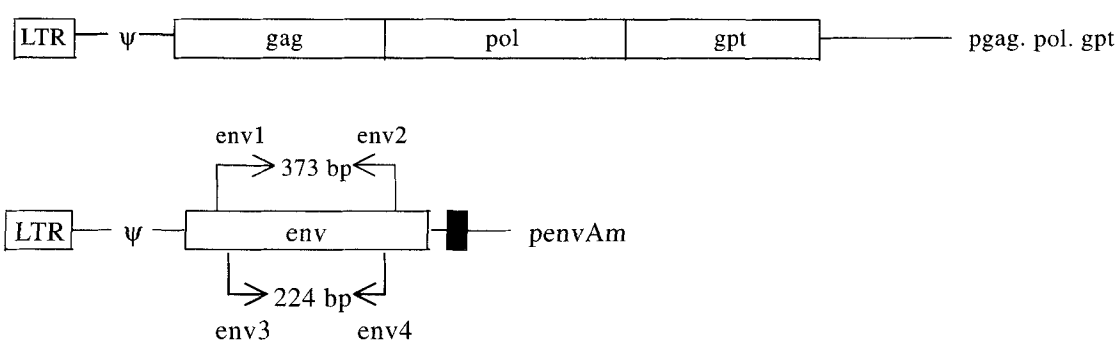

Fig. 1. Schema of helper viral sequences (*gag*, *pol*, and *env*) in GP+envAm12 packaging cells.
MoMLV LTR and the ψ deletion (ψ$^-$) are indicated. The location of the *env* primers and the sizes of PCR products are shown also.

Marker Rescue Assay for Helper Virus

Functional helper virus (replication-competent retrovirus, RCR) was analyzed by Neo^R gene marker rescue assay with 3T3 cell amplification.[4]

RESULTS

Generation and Characterization of Virus Producer Lines

The retroviral vector LXSN was transferred into GP+envAm12, an NIH3T3-derived amphotropic retroviral packaging line, mediated by DOSPER or ecotropic virus. A number of G418-resistant colonies appeared on day 10–14 after selection. The titer produced by a mixed population of GP+envAm12/Neo^R cells transfected by DOSPER was 8.0×10^5 CFU/ml when NIH3T3 cells were used as recipients, whereas the titer of a producer line transduced with different host-range retrovirus from ecotropic GP+E86/Neo^R cells was 1.6×10^7 CFU/ml. This result confirmed that the titer of retrovirus could be markedly elevated by cross-infection between ecotropic and amphotropic packaging lines, known as the so-called ping-pong transduction. In addition, the integration of Neo^R gene carried by LXSN vector into GP+envAm12 cells was corroborated by PCR analysis.

Neo^R Gene Transfer Mediated by Retrovirus

Human leukemia cells NB4, U937, and THP-1 were infected with virus-containing supernatants from GP+envAm12/Neo^R cells with higher titer. After selection with G418, the resistant cells, labeled NB4/Neo^R, U937/Neo^R, and THP-1/Neo^R respectively, were obtained. The presence and integration of Neo^R gene in all transduced cells were verified by DNA PCR with Neo^R gene specific primers yielding a 347 bp product. This indicated the leukemic cells have been marked by Neo^R gene.

Analysis of Gene Transduction Efficiency

The K562 cell line was used as a model to tested the transduction efficiency mediated by retrovirus system in leukemia cells by means of single colony forming and seminested PCR technique without cytokines. In three independent experiments, we found that 34 of 35 (97.1%) individual colonies (93.3–100%) contained provirus, which suggested that the target genes can be efficiently transferred into leukemia cells by using this system.

Detection of Helper Virus

It is well known that the recipients infected by replication-incompetent virus should not be integrated by trans-structural gene of virus including *gag*, *pol*, or *env*. In this study, the presence of helper virus was tested by nested PCR for *env* gene. The results show that the 224 bp band representing *env* gene could only be detected in the positive control cells, but not in the recipients (Figure 2). In all leukemia cells, a 830 bp product specific for human MDR1 genomic DNA were found confirming the successful DNA preparation and PCR processes. By marker rescue assay, we have approved that the recombinant virus can only mediate Neo^R gene transfer into NIH3T3 cell, but the transduced 3T3 cells can not passage the marker to parental 3T3 cell again, which was confirmed by the fact that no colony alive in selection with G418. These denoted the absence of RCR from both genetic and functional analysis in this transfer system.

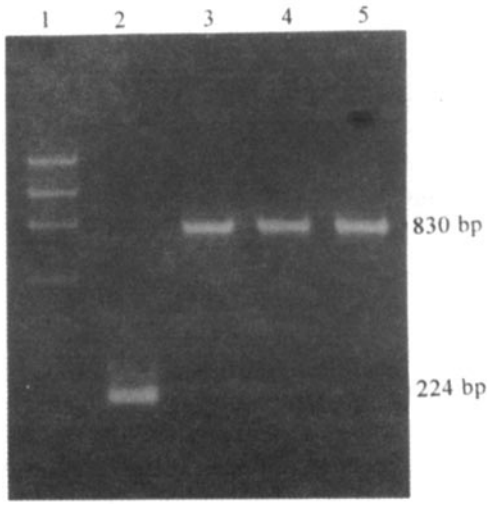

Fig. 2. PCR detection of *env* gene in genetically modified leukemia cells.
1. ϕ×174 RF DNA/HaeIII Marker (Gibco-BRL)
2. GP+envAm12 cells
3. NB4/Neo^R cells
4. U937/Neo^R cells
5. THP-1/Neo^R cells

DISCUSSION

In this study the transduction mediated by retroviral vector expressing the Neo^R gene was documented in myeloid leukemia cells. The leukemic cells were highly susceptible to retroviral gene transfer with 93.3% to 100% of CFU-L demonstrating the integration of provirus in K562 model. Similarly, human peripheral blood stem cells or progenitors can be transduced by retrovirus with efficiency as high as 93% in the presence of growth factors. This provides a useful tool to study on gene marking and gene therapy by using hematopoietic cells as recipient

cells.

The retroviral-mediated transduction of $CD34^+$ hematopoietic stem cells and progenitors, or of leukemia cells has been carried out in clinical trails.[1,2,5] This strategy provided an efficient, budding protocol for the treatment of a variety of diseases. However, a major consideration for the safety of retroviral vectors is the presence of RCR in vector releasing packaging cell lines. Several immuno-suppressed monkeys developed T cell lymphomas when RCR were administered along with a vector although other people have demonstrated that high level of RCR have no detrimental effects when injected into non-human primates.[6] The safety of retroviral-mediated gene transfer can be improved on both vectors and packaging cells. In this paper, the MoMLV-based vector, LXSN, was used as a vehicle for gene marking. The homology of sequence between the vector and the virus genome in packaging line was minimized. The *gag* start codon in vector was replaced with a stop codon TAG and the *env* gene was removed from the vector resulting in a lower rate of recombination. In the same way, GP+envAm12 cell line was constructed in which the *gag*, *pol*, and *evn* genes of the helper virus are separated on two different plasmids and in which the ψ packaging signals and 3' long terminal repeats are deleted (Figure 1). We have examined the safety issue of GP+envAm12 showing that RCR cannot be detected by sensitive nested PCR analysis and rescue assay, perhaps because at least the three-recombination events are necessary to form an intact genome. The GP+envAm12 cell may be safer than another amphotropic packaging cells PA317 in which the trans-acting helper viral gene presented on same plasmid. In GP+envAm12 cell transfer system, detection of the *env* gene by PCR was not sufficient to confirm the presence of helper virus or RCR developing since the presence of *env* protein only cannot package the vector into virions.

Although a number of foreign genes could be efficiently transferred by retroviral vectors in many cell types, one of the major problems was the low vector titers in comparison with adenovirus and the study on improvement of this system was necessary. A study by Pages et al.[7] has shown that sodium butyrate alone or in combination with dexamethasone could significantly enhance viral production and increase the titer, through activation of MoMLV long terminal repeat. Other strategies to increase the titer were focused on growing producer cells at 32°C instead of 37°C and cross-infecting of packaging cells with different host ranges to amplify the copy of provirus. When PA317 cell and GP+E86 cell were cocultured, the titer was elevated generally up to 10^{10} CFU/ml.[8] We have successfully transduced GP+envAm12 cells by GP+E86/Neo^R producer cells with a simple supernatant protocol without cocultivation. This resulted in a 20-fold increase of vector titer in comparison with DOSPER transfection, and no RCR was found.

The Neo^R gene, which confers resistance to the antibiotic G418, is the most common marker gene used in gene transfer experimentation.[9,10] For example, using Neo^R gene as marker, evidence was provided that peripheral blood $CD34^+$ cells could engraft durably and contribute to multiple lineages and the optimal transduction condition of primitive progenitors was thus defined.[9] However, for the enrichment of transduced progenitors this gene is less suitable, mostly because a selective purpose is obtained only after time-consuming exposure to G418 under *in vitro* culture conditions. Several genes encoding marker molecule expressed at the cell surface, allowing for the direct identification of transduced cells with specific antibodies based on flow cytometric analysis, were developed. Examples of such genes are human nerve growth factor, CD24, mouse heat-stable protein, and human MDR1, also provided a potential selection *in vivo*.[9] Recently, the green fluorescent protein (GFP) has been introduced as a promising reporter for monitoring gene transfer and expression.[11] The application of GFP or enhanced GFP (EGFP) as a reporter has no need for secondary molecules for analysis of expression by a FACS or fluorescence microscopy. Together with all, the efficient, safe transfer system of Neo^R gene, generated in present study, would be used to understand the stem cell biology or to treat leukemia via transfer of suicide genes or antisense for oncogenes. Today, the retroviral vectors, in which the Neo^R gene or MDR1 gene was severed as selectable markers, were used to transduce human peripheral blood mobilized stem cells from cancer patients and $CD34^+$ stem cells from cord blood (unpublished data, 1998). We believe that it will offer unique insights into biology of transplantation, immunology, and hematopoietic disease in combination with other strategies.

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REFERENCES

[1] Anderson WF. Human gene marker/therapy clinical protocols. Hum Gene Ther 1995 4:129.

[2] Bagnis C, Chabannon C, Mannoni P. Gene transfer into haemopoietic cells: a challenge for gene therapy. Gene Ther 1996; 3:362.

[3] Tafuro S, Zentilin L, Falaschi A, et al. Rapid

retrovirus titration using competitive polymerase chain reaction. Gene Ther 1996; 3:679.

[4] Lu DR, Qiu XY, Zhen B, et al. Replication-competent retrovirus detection in gene therapy study. Chin J Virol 1996; 12:135.

[5] Hanania EG, Giles RE, Kavanagh J, et al. Results of MDR-1 vector modification trial indicate that granulocyte/macrophage colony-forming unit cells do not contribute to posttransplant hematopoietic recovery following intensive systemic therapy. Proc Natl Acad Sci USA 1996; 93:15346.

[6] Smith KT, Shepherd AJ, Boyd JE, et al. Gene delivery systems for use in gene therapy: an overview of quality assurance and safety issues. Gene Ther 1996; 3:190.

[7] Pages J-C, Loux N, Farge D, et al. Activation of Moloney murine leukemia virus LTR enhances the titer of recombinant retrovirus in ψ CRIP packaging cells. Gene Ther 1995; 2:547.

[8] Lynch CM, Miller AD. Production of high-titer helper virus-free retroviral vectors by cocultivation of packaging cells with different host ranges. J Virol 1991; 65:3887.

[9] Dunbar CE, Young NS. Gene marking and gene therapy directed at primary hematopoietic cells. Curr Opin Hematol 1996; 3:430.

[10] Wu T, Bloom ML, Yu J-M, et al. Murine bone marrow expressing the neomycin resistance gene has no competitive disadvantage assessed *in vivo*. Hum Gene Ther 1998; 9:1157.

[11] Limon A, Briones J, Puig T, et al. High-titer vectors containing the enhanced green fluorescent protein gene for efficient expression in hematopoietic cells. Blood 1997; 90:3316.