RETROVIRAL MEDIATED EFFICIENT TRANSFER ANDEXPRESSION OF MULTIPLE DRUG RESISTANCE GENE TO HUMAN LEUKEMIC CELLS

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ABSTRACT

Objective: To investigate retroviral-mediated transfer and expression of human multidrug resistance (MDR) gene MDR1 in leukemic cells. Methods: Human myeloid cells, K562 and NB4, were infected by MDR retrovirus from the producer PA317/HaMDR, and the resistant cells were selected with cytotoxic drug. The transfer and expression of MDR1 gene was analyzed by using polymerase chain reaction (PCR), flow cytometry (FCM) and semisolid colonies cultivation. Results: The resistant cells, K562/MDR and NB4/MDR, in which integration of the exogenous MDR1 gene was confirmed by PCR analysis, displayed a typical MDR phenotype. The expression of MDR1 transgene was detected on truncated as well as full-length transcripts. Moreover, the resistant cells were P-glycoprotein postiive at 78.0% to 98.7% analyzed with FCM. The transduction efficieny in K562 cells was studied on suspension cultures and single-cell colonies. The transduction was more efficient in coculture system (67.9%~72.5%) than in supernatant system (33.1%~46.8%), while growth factors may improve the efficiency. Conclusion: Retrovirus could allow a functional transfer and expression of MDR1 gene in human leukemia cells, and MDR1 might act as a dominant selectable gene for coexpression with the genes of interest in gene therapy.

Key words: Gene transfer, Retrovirus, MDR genes, Gene expression, Leukemia, Cell lines

Multidrug resistance (MDR) is a well-defined phenomenon of cross-resistance of cells to a number of anticancer agents. In many instances, this is mediated by overexpression at the cell surface of the MDR1 gene product, P-glycoprotein (P-gp), that functions as an energy-dependent efflux pump.[1] Both transfection and transgenic mice studies have clearly shown that P-gp is responsible for MDR.[1,2] MDRmediated by P-gp is thought to be primarily due to reduced intracellular concentration of drug and failure of the drug to reach its target, which was explained by a "flippase" model lately.[3] Therefore, transfer of MDR1 gene to hematopoietic cells offers an attractive strategy to overcome the dose-limiting side effect, suppression of hematopoiesis, in cancer treatment, and may be useful to enrich or expand genetically modified cells *in vivo* as a dominant selectable marker.[2,4] Herein, we report the transfer and expression of the MDR1 gene into human leukemic cells mediated by safe retroviral vector efficiently, which resulted the recipients in an MDR phenotype.

MATERIALS AND METHODS

Cell Culture

The ecotropic packaging cell line GP+E86, the amphotropic packaging cell line PA317 (ATCC CRL9078), and NIH3T3 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 12% newborn calf serum (NCS). Human myeloid leukemic cells K562 and NB4 were grown in RPMI 1640 medium (Gibco-BRL) with 10% NCS. All cells were cultured in 5%CO_2 humidified atmosphere at 37°C.

MDR Retrovirus Preparation

The retrovirus producer lines were prepared as

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previously described.[5] Briefly, GP+E86 cells were transfected with a Harvey murine sarcoma virus (HaMSV)-based retroviral vector HaMDR, using DOSPER Transfection Reagent (Boehringer mannheim). Supernatants from these resistant cells were used to transduce PA317 cells repeatedly. Clones expressing high levels of P-gp were selected in 100μg/L of colchicine (COL). Viral supernatant was collected from subconfluent PA317/HaMDR cell monolayer 24hr after medium change, and filtered through a 0.45 μm filter. The titer of virus in the supernatant was about 8.5×10^5 particles per milliliter assayed on NIH3T3 cells.

Transduction of Human Leukemia Cells

For infection, K562 and NB4 myeloid leukemia cells were cultured in medium containing cell-free supernatant from PA317/HaMDR cells supplemented with 4mg/L of Polybrene (Sigma) for 2 hours. Cells were selected with COL (50~100μg/L) for 2 weeks. After cultivation in drug-free medium for 2 weeks, the IC_{50}s were determined by an MTT [3–(4,5-dimethylthiazol z-yl 2,5- diphenyl -tetrazolium bromide, Sigma] colorimetric method.

To define optimal conditions for retroviral-mediated transduction of hematopoietic cells, K562 cells were transduced as a model by the MDR virus in parallel using three transduction conditions. The first was cocultivation K562 cells with subconfluent producer cells. The second was supernatant only. The third was supernatant in addition with 20% 5637 cells conditioned medium as growth factors. K562 cells were plated at a density of 10^5/ml in RPMI 1640 medium with 20% NCS and Polybrene was added to 4 mg/L. At 24-hour intervals for 2 days, transduced cells were collected and analyzed after an additional 48 hours by flow cytometry (FCM) or colony-forming assay, respectively.

Analysis of MDR1 Gene by Polymerase Chain Reaction (PCR)

Genomic DNA was analyzed by PCR for the integrity of the MDR provirus. The primers, specifically amplifying a 283-bp fragment from full-length MDR1 cDNA as well as a fragment at 830bp from genomic DNA, were hMDR1 and hMDR2.[5] PCR was performed for 32 cycles on an DNA thermalcycler (GeneAmp9600, Perkin Elmer). After amplification, reaction mixtures were run on a 1.8% agarose gel and the specific amplified fragments were detected under an UV light.

To investigate the expression level of exogenous MDR1 gene, total RNAs were extracted from resistant cells using the TRIzol Reagent (Gibco-BRL). The cDNA synthesis was performed in a total volume of 40μl, containing 2μg of total RNA, 100ng of random hexamer (Promega), 40U RNAsin (Promega), 200U MoMLV-reverse transcriptase (Gibco-BRL), and 0.5mmol/L dNTPs (Sangon) at 37°C for 1hr. After cDNA synthesis, the full-length MDR1 gene transcript was amplified as described above, and the aberrantly spliced cDNA of MDR1 gene transcript was amplified as described above, and the aberrantly spliced cDNA of MDR1 gene was specifically amplified by PCR using the primers hMDR5 (5'-CATGA ATCTG GAGGA AGACA-3') and hMDR4 (5'-GTTCA AACTT CTGCT CCTGA-3') with products at 460bp.[6] Additionally, the primer hMDR3(5'-CCCAT CATTG CAATA GCAGG-3') was combined with the primer hMDR4 to amplify a 157-bp fragment from both full-length and spliced form cDNA. The RNA integrity was evaluated by c-abl gene.

FCM Analysis of P-gp

Transduced or untransduced leukemia cells were incubated with R-phycoerythrin conjugated P-gp monoclonal antibody UIC2 (Coulter-Immunotech), which bind to an extracellular domain on P-gp, for 30 minutes at 4°C. Control studies were performed with nonspecific murine IgG2a isotype. After washing, cells were analyzed on a flow cytometer (Epics XL, Coulter) as previously described.[5]

Methylcellulose Assays

After infection, cells (5×10^2) were added to 1 ml 1%(w/v) methylcellulose (Fisher Scientific) medium containing 0, or 25 μg of COL per liter. Cell mixtures were plated in 35-mm culture dishes (NUNC), and incubated at 37°C. Colony formation was scored after 7~10 days, and the extent of transduction was analyzed by COL-resistant colony-forming assay. The assay was performed in duplicate.

RESULTS

Characteristics of MDR Transduced Leukemia Cells

To establish a model for MDR1-mediated drug resistance with human hematopoietic cells, the HaMDR vector was introduced into human leukemia cells, e.g., K562 and NB4, mediated by MDR retrovirus. After selecting with COL, the resistant cells were pooled and named as K562/MDR and

NB4/MDR, respectively. K562/MDR and NB4/MDR cells were 9.6- to 77.5-fold higher resistance to cytotoxic drugs (COL, vincristine/VCR, or daunorubicin) as compared with the parental cells. Resistance remained stable when cells were grown in drug-free medium for more than 40 weeks. Absence of the replication-competent retrovirus was confirmed for *env* gene of retrovirus by a PCR assay. Additionally, contrary to irregularity of human resistant HL-60(VCR) myeloid leukemic cells obtained by growing HL-60 cells in progresively increasing concentrations of VCR in our laboratory, the MDR1 gene-transduced resistant cells did not change in morphology in comparison with the sensitive cells, indicated the overexpression of P-gp was not the true reason for the morphological alteration of HL-60(VCR) cells.

Integration and Expression of MDR1 Transgene

Transformation of K562 or NB4 cells with MDR virus resulted in integration of full-length human MDR1 cDNA in resistant cells. This was verified by amplification of an MDR1 cDNA specific fragment at 283bp with an internal control product at 830bp from human genomic DNAs of the resistant cells. Moreover, an aberrant proviral MDR1 form, that could be detected with primers hMDR5 and hMDR4 (460bp), was also present in genomic DNA from K562/MDR or NB4/MDR cells. This demonstrates the resistant cells contain more than one proviral copy per cell genome.

To determine the level of transcription from integrated proviral sequences, semi-quantitative RT-PCR was performed with sequence specific primers. Fugure 1 shows the results of RT-PCR analysis for full-length and spliced MDR1 transcripts. HaMDR vector DNA shows amplification product only with the full-length primers as expected. However, cDNA prepared from resistant leukemia cells showed almost equivalent amounts of both full-length and spliced transcripts, as seen in PA317/HaMDR cell. This result indicate the aberrant spliced subgenomic viral RNA within the producer cells results in passage of the truncated proviral form to target cells.

P-gp Expression on Virus-transduced Leukemic Cells

The expression of P-gp on the surface of transduced cells was analyzed by FCM using human P-gp-specific monoclonal antibody UIC2. Parental K562 and NB4 cells did not express endogenous P-gp. Figure 2A shows that approximately 99% of K562/MDR cells were P-gp positive. After transduction and selection, more than 78% of NB4/MDR cells express human P-gp on their surface when probed with UIC2 antibody (Figure 2B). These suggest that functional high-level expression of P-gp could be mediated by MDR virus in hematopoietic cells.

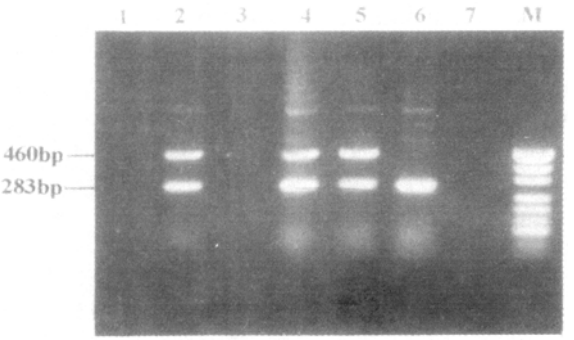

Fig. 1. .MDR1 gene expresion analyzed by semi quantitative RT-PCR.
1:K562 cells; 2: K562/MDR cells; 3: NB4 cells; 4: NB4/MDR cells; 5: PA317/HaMDR cells; 6: Vector HaMDR control; 7: Negative control; M: pUC19/MspI DNA Marker (MBI).

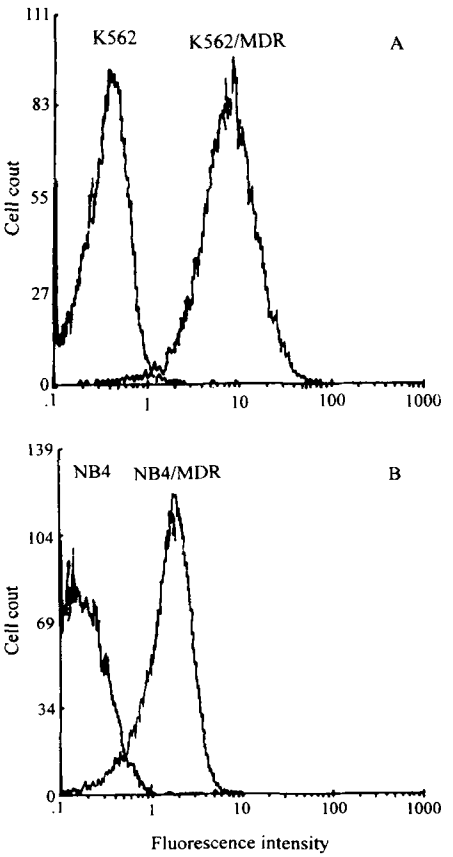

Fig. 2. Detection of P-gp expression on the cell surface analyzed by FCM. A: K562 cells and K562/MDR cells; B: NB4 cells and NB4/MDR cells.

Comparison of Gene Transduction Efficiency

To optimize the conditions for retroviral-mediated transduction of human early hematopoietic cells, K562 cells were employed as a model to compare MDR vector transduction efficiency in parallel using three conditions. As shown in Figure 3, when assessed 48 hours later by FCM analysis, the transduction efficiencies obtained with three procedures ranged from 38.1% to 72.5%. (It was showed the cocultivation 1) markedly improved transduction of K562 by 90% (supernatant only, 2) or 55% (supernatant with growth factor; and 3, respectively.) When determined by semisolid culture assay, the transduction efficiencies, defined as the percentage of COL-resistant K562 colonies, ranged from 33.1% to 67.9%, confirmed the results from FCM analysis of P-gp expression above.

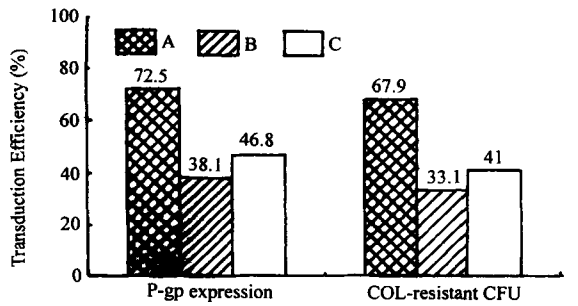

Fig. 3. Comparison of transduction efficiency of K562 cells: effect of different infection conditions. The efficiency was calculated as the percentage of P-gp expressing cells or COL-resistant colonies.

A: cocultivation; B: Supernatant;

C: Suprnatant with growth factor

DISCUSSION

HaMSV-based vector, that have safety, packaging, and gene expression features, has been widely used to transfer the MDR1 gene into mammalian cells, resulted in a selectable phenotype of recipient cells both *in vitro* and *in vivo*.[6-8] Therefore, MDR1 transduction not only makes bone marrow cells more resistant to chemotherapy, but also exploit drug-resistance gene to help deliver and expand cells cotransduced with other therapeutic genes that could be used in the treatment of both cancer and nonmalignant disorders, especially by using bicistronic vectors.[2-4,6,8-10] Moreover, leukemia cells modified by MDR1 gene, for instance K562/MDR and NB4/MDR cells established in this study, can be used to clarify the effects of P-gp overexpression on cell differentiation and apoptosis.[11-13] In the present works, we have developed a retroviral-mediated MDR1 gene transfer system for efficient expression, and obtained two human resistant leukemia cells for further studies in the future.

Several groups have conducted clinical MDR1 gene transfer to human hematopoietic stem/progenitor cells, although the level of gene marking detected at the time of reconstitution has been low, thus limiting any potential benefit from the myelosuppressive effects of MDR drugs[4,9,14] Recently, Bunting et al.[8] found that hematopoietic cells transduced with the HaMDR vector and then cultured for an additional 12 days in the presence of cytokines produced a myeloproliferative disorder in recipient mice. This report raises important safety concerns, though it is not clear how to produce this effect. In contrary to it, when act as a dominant selectable marker, MDR1 gene has several advantages in human gene therapy. First, P-gp, an ATP-dependent flippase expressed at low level in hematopoietic stem cells (HSC), is not antigenic. Secondly, the substrates of P-gp are well-characterized drugs that have already been applied *in vivo*. Thirdly, it is possible to limit the selection to hematopoietic cells without causing extensive damages to the other tissues. Moreover, expression of P-gp on cell surface makes it easily to isolate the genetically modified cells by magnetic cell sorting or fluorescence-activated cell sorting, as described in other cell-surface molecules.[4] Among them, only P-gp can function as a dominant selectable marker both *in vitro* and *in vivo*.

Maximizing vector transduction will be important for clinical application of gene therapy. We initially optimized the conditions for transduction on human CD34+early hematopoietic cell K562. In the current study, it was confirmed that cocultivation with virus-producing cells (1) was superior to supernatant transduction with (2) or without growth factor (3).[4] And the transduction was increased by the addition of cytokines from conditioned media of 5637 bladder carcinoma cells. This result also showed the potential use of P-gp as an efficient reporter molecule in genetic modification and selection of hematopoietic cells. However, cocultivation of HSC with producer cells is not suitable in human gene therapy for the safety notion. Further studies should address the gene transduction system based on repeated addition of virus-containing supernatant to the stem cells supplemented with cytokines and/or irradiated stromal cells.

Although both transgenic as well as knockout mice of MDR1 gene were generated with the lack of an observable biolgoical phenotype, some groups has demonstrated that P-gp expression could prevent cells

from differentiation and apoptosis in a number of systems.[8,11-13] Despite earlier reports of Kizaki et al,[11,12] we found that differentiation of NB4/MDR cells induced by all-trans retinoic acid did not differ significantly in comparison with parental NB4 cells (unpublished data). This result directly suggests no role of P-gp on differentiation of NB4 cells. Although the mechanism of anti-apoptotic effect of p-gp remains to be defined, some hypotheses have been proposed, that somewhat are related to changes in the intracellular concentration of ceramide or that of ATP, or alteration the membrane distribution of phosphatidylserine.[13] Therefore, K562/MDR cells could be used as models for studying the action of P-gp on apoptosis in hematopoietic cells.

Inefficient retroviral-mediated gene transfer to human HSC and insufficient gene expression due to transcriptional silencing in progeny cells derived from transduced HSC are two major problems associated with HSC-based gene therapy. Thus, it is necessary to develop novel vectors for efficient expression of transgenes in HSC.[7,15] Unexpectedly, we also found that all MDR virus-transduced cells contained a shortened provirus and expressed a truncated MDR1 mRNA (deletion~2kb), that was mediated by cryptic splice donor and acceptor sites within the MDR1 cDNA.[6] So, engineering MDR1 vectors by removing cryptic sites to test if functional expresion can be augmented or by replacing wild-type MDR1 gene with its cyclosporin-resistant mutant to examine if the level of drug resistance in bone marrow can be increased in resistant caner patients, should be performed to develop the second-generation MDR1 vectors for clinical use of human gene therapy.[5,6]

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