ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10): 949~955
http://www.chinaphar.com

Genetic modification of hematopoietic progenitor cells for combined resistance to 4-hydroperoxycyclophosphamide, vincristine, and daunorubicin[1]

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KEY WORDS gene therapy; MDR genes; genetic vectors; gene expression; hematopoietic stem cells

ABSTRACT

AIM: To investigate whether human peripheral blood hematopoietic progenitor cells (PBPC) modified with human aldehyde dehydrogenase class-3 gene (ALDH-3) and multidrug resistance gene 1 (MDR1) would increase chemotherapy resistance to 4-hydroperoxycyclophosphamide (4-HC) and P-glycoprotein effluxed drugs. **METHODS**: A bicistronic retroviral vector G1Na-ALDH3-IRES-MDR1 cDNA was constructed and used to transfect the packaging cell lines PA317 by electroporation. $CD34^+$ PBPC were isolated with a high-gradient magnetic cell sorting system (MACS), and then were transfectced with supernatant of retrovirus containing human ALDH-3 and MDR1 cDNA. PCR, RT-PCR, Southern blot, Northern blot, FACS, and MTT assay were used to evaluate the transfection and expression of the transgene in target cells. **RESULTS**: The bicistronic retroviral vector construction was verified by PCR and restriction endonuclease analysis. Dual drug resistance genes were integrated into the genomic DNA of $CD34^+$ PBPC and expressed efficiently. The efficiency of gene transfection in $CD34^+$ PBPC was tested to be 18 % on colonies. Nested PCR and Neo^r rescue assay indicated that no helper virus was present in this system. Compared with the untransduced cells, transgene recipient cells confered 4.5-fold resistance to 4-HC, 6.6-fold and 7.8-fold resistance to P-glycoprotein effluxed drug, vincristine and daunorubicin, respectively. **CONCLUSION**: Efficient transduction of two different types of drug resistance genes into human peripheral blood hematopoietic progenitor cells and the co-expression may decrease cumulative myelosuppression of combination chemotherapy.

INTRODUCTION

Transduction of drug resistance gene into hematopoietic progenitor cells is a proposed strategy to overcome the dose-limiting myelosuppression of chemotherapeutic agents[1-4]. Hematopoietic progenitors are the most suitable target cells for the drug resistance gene therapy. Retrovirus is the first considered vector in gene transduction of hematopoietic progenitors. An increasing evidence suggests that the cytosolic enzyme gene, human aldehyde dehydrogenase class-3 gene (ALDH-3) may be involved in the mechanisms of cellular resistance to 4-hydroperoxycyclophosphamide (4-HC)[5]. ALDH-3 transduction into human peripheral blood hematopoietic progenitor cells also led to significant increases in cyclophosphamide resistance *in vitro*[6,7]. Multiple chemotherapeutic agents are used in current clinical protocols and co-expression of two different types of drug resistance gene would be useful to protect normal bone marrow cells from the hematotoxicity caused by combination chemotherapy[8-11]. This study was aimed to test whether the overexpression of the ALDH-3 gene and MDR1 gene in human peripheral blood hematopoietic progenitor cells induced 4-HC and P-glycoprotein effluxed

[1] Project partly supported by the Natural Science Foundation of Guizhou Province (№ E-99-2) and the Public Health Bureau of Guizhou Province (№ G-99-7).
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Received 2001-04-16 Accepted 2001-08-15

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
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drug resistance.

MATERIALS AND METHODS

Construction of retroviral expression vector The human ALDH-3 cDNA (1.5 kb) was synthesized by reverse transcriptase polymerase chain reaction (RT-PCR) as described previously[5,6]. This ALDH-3 cDNA was subcloned into the pLXSN retroviral vector at *Eco*R Ⅰ cloning site in the sense orientation, and the resulting vector was pLXSN-ALDH-3 (a gift kindly provided by Dr Cowan KH, NIH, USA). A vector p-SXLC-IRES-MDR1 (generously provided by Professor Pastan I, NCI, USA) contains an internal ribosome entry site (IRES) fragment isolated from encephalomyocarditis (EMC) virus and the human MDR1 cDNA (4 kb) that is translated under control of the IRES. To construct the bicistronic retroviral vector expressing ALDH-3 and MDR1 genes, the ALDH-3 cDNA was removed from the plasmid pLXSN-ALDH-3 with *Eco*R Ⅰ-*Xho* Ⅰ and was subsequently ligated to the *Eco*R Ⅰ-*Xho* Ⅰ site of the pBS cDNA to form the shuttle vector, pBS-ALDH-3. The ALDH-3 cDNA was released from the pBS-ALDH-3 by *Not* Ⅰ-*Xho* Ⅰ restriction and ligated to the equally restricted G1Na vector producing G1Na-ALDH-3. The IRES-MDR1 fragment was released from the plasmid p-SXLC-IRES-MDR1 by digestion with *Sal* Ⅰ-*Xho* Ⅰ, the IRES-MDR1 insert was gel-purified and then ligated into the G1Na-ALDH-3 vector to give the final bicistronic retroviral vector G1Na-ALDH3-IRES-MDR1 (GL3M). The construction was verified by both restriction and polymerase chain reaction (PCR) analysis.

Cell culture The ecotropic packaging cells GP + E86 (kindly supplied by Professor MAO Ning, Institute of Basic Medical Sciences, Academy of Sciences of Military Medicine). The amphotropic retrovirus packaging cells PA317 and the mouse fibroblast NIH3T3 cells were grown on monolayer cultures in 37 ℃, 5 % CO_2 in DMEM medium (Dulbecco's modified Eagle's medium, Gibco/BRL) supplemented with 15 % heat-inactivated new-born calf serum and passaged at intervals of 2-3 d.

Establishment of amphotropic viral producer cells To generate ALDH-3 and MDR1 retrovirus, the PA317 and GP + E86 packaging cells were transfected with G1Na-ALDH3-IRES-MDR1 using electroporation, followed by selection with vincristine (VCR, 50 μg/L). To increase viral titer, a modified supernatant "ping-pong" method[12] was used and the packaging cell GP + E86 or PA317 was selected by VCR (20 - 200 μg/L) and/or 4-HC (10 - 50 μmol/L, a gift kindly provided by Dr Colvin OM, Duke University, NCI, USA). After transfected with this successfully constructed vector that carried pairs of drug resistance genes, the titer was calculated by transducing NIH3T3 cells and the replication-competent retrovirus (RCR) was detected by nested PCR analysis of *env* gene.

Detection of helper virus *env* gene by nested PCR and determination of transduction efficiency using clone assay To establish the safety of gene transfer system mediated by retrovirus vector, the replication-competent retrovirus was detected by nested PCR analysis of *env* gene. The GL3M and G1Na plasmid (as control) were transferred into amphotropic packaging cell line GP + E86 respectively, retroviral supernatant with higher titer was used to infect human leukemia cell lines U937, K562, and HL60. The *env* gene of the helper virus was detected by PCR using the primers *env*1: sense 5′-GGG AGT CCT GTT AGG AGT AGG G-3′, antisence 5′-ATG ATG TGG GCT TCC AGT AAG C-3′; *env*2: sense 5′-ACC AAC CTG ATG ACT GGG CG-3′, antisence 5′-CCC GAC TTT ACG GTA TGC CC-3′. The efficiency of transduction of $CD34^+$ PBPC was determined by mixing a cell suspension (2×10^8/L) with an equal volume of the same viral supernatant used to titer the 3T3 cells and then an aliquot of the infected cells was then plated (2×10^4 in 35-mm dishes) in 0.8 % methylcellulose in RPMI-1640 supplemented with 30 % fetal bovine serum (FBS) and in the presence of hematopoietic growth factors, duplicate culture also contained G418 (0.6 g/L active material). Colonies were counted after 14 d and the percent transduction efficiency was calculated as 100 × (number of colonies in the presence of G418)/(number of colonies in the absence of G418).

Transfection of G1Na-ALDH3-IRES-MDR1 retroviral vector into $CD34^+$ cells Peripheral blood mononuclear cells (MNC) were collected from apheresis samples by density centrifugation using Ficoll (Biochrom KG, Berlin, Germany). $CD34^+$ PBPC were recovered by positive immunoselection using the MACS $CD34^+$ multisort cell isolation kit (Miltenyi Biltec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The selected fractions contained a median of 92 % $CD34^+$ cells (range, 46 % - 98 %) and then transfected with supernatant of

retrovirus containing human ALDH-3 and MDR1 cDNA, $CD34^+$ cells were prestimulated for 48 h in presence of Flt-3 ligand (100 μg/L), stem cell factor (100 μg/L), IL-3 (50 kU/L), and IL-6 (100 kU/L). Retrovirus-containing supernatant was changed twice a 24-h interval. After five days, the cells were selected in the medium containing VCR 50 μg/L and/or 4-HC 10 μmol/L.

Analysis of ALDH-3 and MDR1 transgenes in $CD34^+$ cells by PCR assay The integration of ALDH-3 and MDR1 genes in transduced $CD34^+$ cell genomic DNA was analyzed by PCR as described[8]. The ALDH-3 gene specific primers used for this reaction were: 5′-GAG AGA TCA TAC ATC CGA ATT TGT C-3′ and 5′-GCT GAG CCA GTC ACC TGT GTT CCA G-3′; MDR1 gene was analyzed using primers 5′-CCC ATC ATT GCA ATA GCA GG-3′ and 5′-GTT CAA ACT TCT GCT CCT CA-3′. The reaction parameter of PCR is 95 ℃ for 45 s, 55 ℃ for 1 min, 72 ℃ for 90 s, 30 circles in all. PCR was performed on a DNA thermal cycler (Gene Amp9600, Perkin Elmer). Ten microliters of each PCR products was separated by electrophoresis on a 1.5 % agarose gel and visualized under UV light after ethidium bromide staining.

Southern blot analysis of genomic DNA Southern blot analysis was performed according to the standard procedures using ALDH-3 cDNA or MDR1 cDNA released from plasmid GL3M as probes. The probes were labelled using Random Primed DNA Labelling kit (Boehringer Mannheim, GmbH, Germany) with [α-^{32}P]-dATP. The PCR products were seperated on a 1.5 % agarose gel. After denaturing and neutralization steps, the DNA was transferred by capillary blotting onto a Hybond-N nylon membrane and covalently crosslinked to the membrane by ultraviolet light and backed at 80 ℃ for 2 h. The blots were hybridized overnight at 65 ℃ with constant agitation using ^{32}P-labelled ALDH-3 cDNA or MDR1 cDNA and washed three times at room temperature before the exposure to X-ray film.

Northern blot and RT-PCR analysis of ALDH-3 and MDR1 mRNA expression Total RNA was extracted using TRIzol isolation reagent (Life Technologies) from human $CD34^+$ PBPC. Total RNA (20 μg per lane) was separated on 1.5 % agarose formaldehyde gels, blotted on to Hybond-N nylon filters (Amersham, USA), and hybridized overnight at 42 ℃ with random-primer ^{32}P-dATP-labelled ALDH-3 cDNA or MDR1 cDNA (the primer using in the reaction was ALDH-3: 5′-AGC AGG ATC AGC GAG GCC GTG AAG-3′, 5′-ACT CCG ATG GGA CAC AGT ATG GCC-3′and MDR1: 5′-CCC ATC ATT GCA ATA GCA GG-3′, 5′-GTT CAA ACT TCT GCT CCT CA-3′), the blots were washed with 0.2 × SSC and 0.1 % SDS first at room temperature and afterwards at 56 ℃. Blots were exposed to X-ray film with two intensifying screens at −70 ℃. Total RNA was isolated using TRIzol isolation reagent, and the cDNA synthesis was carried out with Moloney mouse leukemia virus-reverse transcriptase (Life Technologies) 200 U at 37 ℃ for 1 h. After cDNA synthesis, the transcripts were amplified with ALDH-3 and MDR1 gene specific primers as described above.

Analysis of P-glycoprotein (P-gp) expression by flow cytometry (FCM) Human P-gp expression on the cell surface was examined by direct immunofluorescence staining and FCM analysis. Briefly, cells (1×10^6) harvested after trypsinization were washed and incubated with phycoeruthrin (PE)-anti-P-glycoprotein (BECTON-DICKINSON, BD), reacted specifically with human P-gp, at 4 ℃ in the dark for 1 h. Flow cytometric analysis was performed with a Becton Dickinson FACScan cytometer. PE-conjugated mouse gammar-1 antibody were used as negative control.

Chemosensitivity assay According to methods described previously[2,13], a stock solution of MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 5 g/L in PBS 0.01 mol/L, NaCl 0.14 mol/L, pH 7.2] was prepared, and 20 μL of this solution was added to each well (1×10^3 cells) of a 96-well plate, and the tested drugs (VCR and 4-HC) were added at varying concentrations. After 3 − 4 d, the number of viable cells was determined by colorimetric assay, and the IC_{50} and index of resistance were calculated.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and the statistical significance was evaluated using paired t-test.

RESULTS

Retroviral vector construction and generation of amphotropic producer cell lines The structure of the bicistronic retrovirus vector G1Na-ALDH3-IRES-MDR1 was summarized in Fig 1. The purified expression vector was transfected into ecotropic GP + E86 cells mediated using electroporation. The resistant colonies were selected with VCR (50 μg/L) and 4-HC (10 μmol/L), and the supernants were harvested with

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
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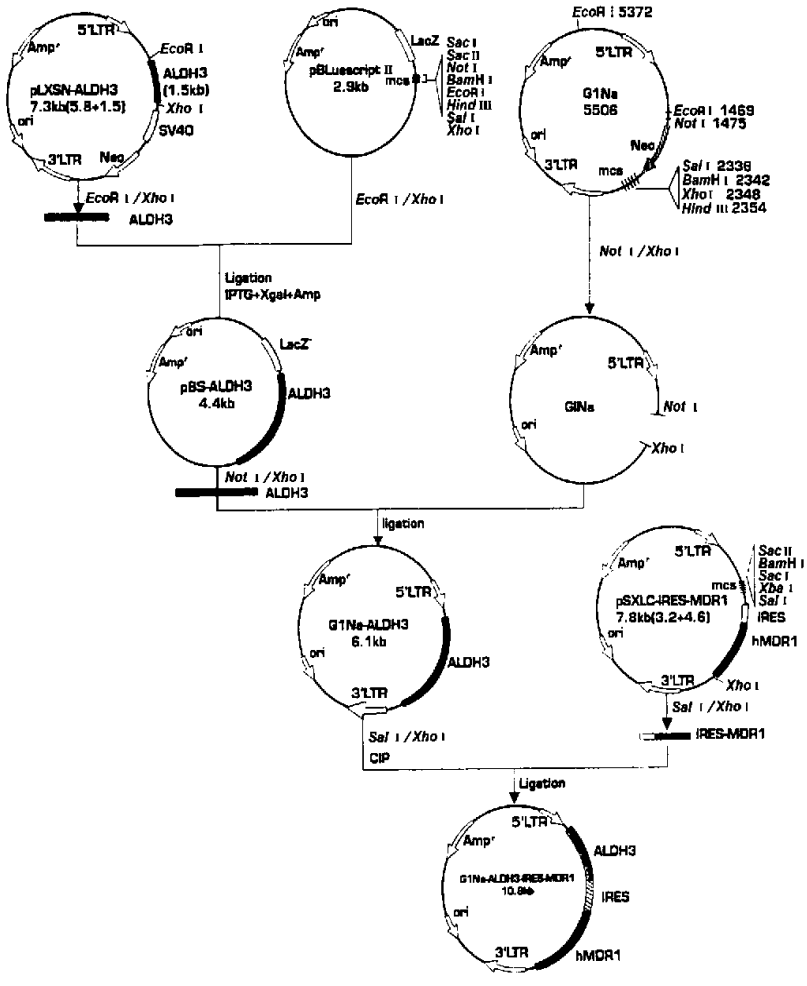

Fig 1. The schematic structures of the retroviral vector G1Na-ALDH3 -IRES-MDR1.

the titer of 1.2×10^8 CFU/L for transduction. To generate high titer amphotropic ALDH-3 and MDR1 retrovirus, PA-317 cells were infected with six rounds of exposure to supernatants from GP + E86 cells. After continuous selecting with 4-HC (20 μmol/L) and VCR (50 – 200 μg/L), we obtained PA-317/GL3M producer cells with a titer of 3.5×10^8 CFU/L assayed on NIH3T3 cells.

Helper virus assay and the determination of transduction efficiency After retroviral supernatant infection of human leukemia cell lines, the wildtype retrovirus *env* gene transfer was not found in GINa-ALDH3-IRES-MDR1-containing retrovirus transduced cells using nested PCR. Supernatants were collected and cultured with fresh NIH3T3 cells. Replication-competent virus was not detected by either assay, indicating no helper virus present in this system (Fig 2). The efficiency of gene transduction in $CD34^+$ PBPC was tested on colonies formed with an efficiency of 18 %. The nested PCR results suggested that bicitronic retroviral vectors were effective and safe.

Integration and overexpression of ALDH-3 and MDR1 transgenes in $CD34^+$ PBPC Integration of inact ALDH-3 and MDR1 provirus was verified by PCR with specific primers respectively in transgenes $CD34^+$ PBPC, which produced 1.5 kb and 157 bp amplification bands (data not shown). The products of PCR amplification were seperated by electrophoresis on 2 % agarose gel and visualized on Southern blots using ^{32}P-labelled ALDH-3 and MDR1 cDNA-specific sequences (Fig 3). The double gene mRNA expression was detected by RT-PCR using ALDH-

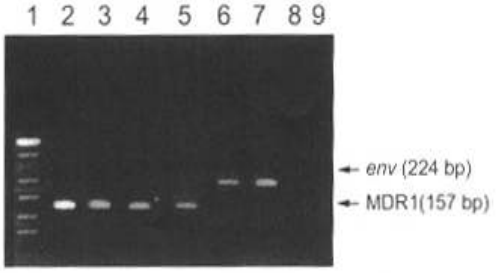

Fig 2. Detetion of helper virus *env* gene by nested PCR. Lane 1: pUC19DNA/Msp marker; Lane 2, 3: K562/GL3M; Lane 4, 5: HL60/GL3M; Lane 6, 7: PA317/GL3M; Lane 8, 9: Negative control.

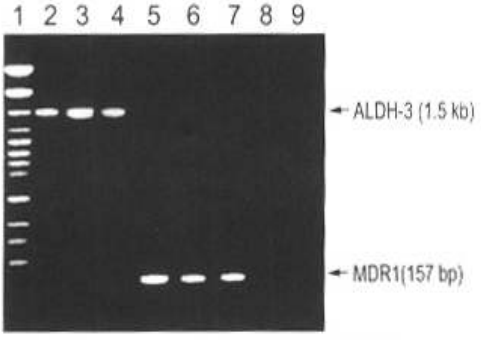

Fig 3. Detection of ALDH-3 and MDR1 mRNA expression in PA 317, K562, and CD34$^+$ PBPC transduced with GL3M by RT-PCR. Lane 1: DNA ladder marker; Lane 2, 3, 4: RT-PCR amplified ALDH-3 product (1.5 kb) from transduced PA317, K562, and CD34$^+$ PBPC; Lane 5, 6, 7: RT-PCR amplified MDR1 product (157 bp) from transduced PA317, K562, and CD34$^+$ PBPC with GL3M; Lane 8, 9: Negative control, RT-PCR amplified CD34$^+$ PBPC/untransduced ALDH-3 and MDR1 products.

3 (1.5 kb) and MDR1 (157 bp) specific primer pairs respectively (Fig 4). Northern blot analysis showed that the transcriptional fusion of two genes resulted in only one transcription in transduced CD34$^+$ PBPC (Fig 5).

P-gp expression on the CD34$^+$ PBPC surface by FACS analysis Flow cytometric analysis showed that 22.6 % CD34$^+$ PBPC/GL3M were P-gp positive, while the untransduced CD34$^+$ cells were only 5.8 % positive, transgenes CD34$^+$ cells expressed higher levels of gp 170 than that in nontransfected cells (3.6 *vs* 1.5 in mean fluorescence intensity). These results demonstrated that MDR1 transgene could be translated to P-gp under control of the IRES.

Sensitivity to cytotoxic drugs To examine

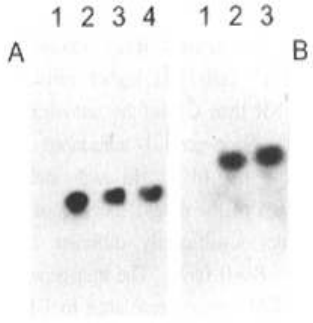

Fig 4. Southern blot analysis of double drug resistance gene integration in transduced CD34$^+$ PBPC. (A) Lane 1: Untransduced CD34$^+$ PBPC (none) serves as negative control; Lane 2, 3, and 4: Transduced CD34$^+$ PBPC genomic DNA PCR product hybridize with ALDH-3 probe; (B) Lane 1: Untransduced CD34$^+$ PBPC (none) serves as negative control; Lane 2, 3: Transduced CD34$^+$ PBPC genomic DNA PCR product hybridized with MDR1 probe.

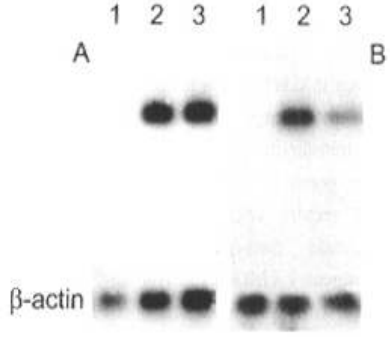

Fig 5. Northern blot analysis of ALDH-3 and MDR1 mRNA expression in CD34$^+$ PBPC transduced with retroviral vector G1Na-ALDH3-IRES-MDR1. (A) Lane 1: Untransduced CD34$^+$ cells (none) serves as negative control; Lane 2, 3: The results of Northern blot analysis which indiates retrovirally transcribed ALDH3-MDR1 mRNA expression using ^{32}P-labelled ALDH-3 cDNA; (B) Lane 1: Untransduced CD34$^+$ cells (none) serves as negative control; Lane 2, 3: The results of Northern blot analysis which indiates retrovirally transcribed ALDH3-MDR1 mRNA expression using ^{32}P-labelled MDR1 cDNA. The transcriptional fusion of two genes resulted in only one transcript band in transduced CD34$^+$ PBPC.

the effectiveness of GL3M vector, the extent of drug resistance in ALDH-3 and MDR1 gene transduced CD34$^+$ PBPC were determined by MTT. The bicistronic retroviral vector transduction resulted in significant

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Oct; 22 (10)
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increases in VCR, daunorubicin (DNR), and 4-HC resistances. The survival data indicated that GL3M modified $CD34^+$ cells had higher resistant to 4-HC, VCR, and DNR than G1Na (the retroviral vector which contained only Neor gene) transduced $CD34^+$ cells, respectively ($P < 0.01$). In each individual experiment, with each of the drugs, the IC_{50} of $CD34^+$ cells/G1Na was not significantly different compared with parental cells ($P > 0.05$). The transgene recipient cells confered 4.5-fold stronger resistance to 4-HC and 6.6 to 7.8-fold to P-glycoprotein effluxed drugs (VCR and DNR) than untransduced cells (Tab 1).

DISCUSSION

The genetic transduction of drug resistance genes into hematopoietic cells is an attractive approach to overcoming myelosuppression caused by high-dose chemotherapy. Mobilized peripheral blood progenitor cells (PBPC) are an attractive target for the retrovirus-mediated transfer of cytostatic drug resistance genes[14,15]. However, chemotherapeutic agents are often given in combination to increase their effectiveness. Consequently, there is an advantage in designing vectors for gene transfer that are capable of expressing two drug resistance genes. We have constructed a bicistronic retroviral vector, which contains the human aldehyde dehydrogenase class-3 (ALDH-3) and multidrug resistance gene 1 (MDR1). Human $CD34^+$ PBPC were transduced with this vector and their resistance to 4-HC and P-glycoprotein effluxed drugs was evaluated. PCR, RT-PCR, Southern blot, and FACS analysis showed that dual drug resistance genes have been integrated into the genomic DNA of PBPC and expressed efficiently. The efficiency of gene transduction in $CD34^+$ PBPC was tested on colonies formed with an efficiency of 18 %. The transduced target cells showed high levels of resistance to 4-HC (4.5 folds), VCR (6.6 folds), and DNR (7.8 folds) compared with untransduced cells. Using enzymatic assays, we observed a coordinate increase in resistance to 4-HC and DNR agents following an VCR selection. In addition, K562 cells transduced with the bicistronic vector also showed drug resistance to both 4-HC and P-glycoprotein effluxed drugs. Interestingly, the double-gene construct conferred an equivalent level of drug resistance compared with single-gene vectors bearing only ALDH-3 or MDR-1 genes in the hematopoietic cells (data not shown). These results demonstrate the potential of the GL3M vector to confer dual drug resistances and may have future application in chemoprotection of normal hematopoietic cells in patients with cancer.

The multidrug transporter (MDR1) has been suggested as a useful selective marker for gene therapy. In this work, we tried to co-express ALDH-3 and MDR1 cDNA in a retroviral vector using an internal ribosome entry site (IRES) from encephalomyocarditis virus expressing a single mRNA on which translation of the ALDH-3 gene is cap-dependent and translation of the MDR1 gene is IRES-dependent. This system produced a bicistronic mRNA containing both ALDH-3 and MDR1, which enables co-expression of the dual genes and also allows the two proteins to be translated separately[8]. However, MDR1 expression was proportional to the stringency of the vincristine selection. Hence the retroviral system described in this work may serve as a useful tool to evaluate the strategies involving *in vivo* dominant selection for gene therapy of tumor patients.

In addition, the transfer safety was performed and replication-competent virus did not be found in the supernatant of the transferred NIH3T3 by nested PCR. This safety-modified vector might be useful for introducing the dual genes into bone marrow cells to protect normal cells from the toxic side effects of cancer chemotherapy.

Tab 1. Drug sensitivity of umbilical cord blood $CD34^+$ cells transduced with double gene retroviral vectors. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$ *vs* $CD34^+$ cells group. $^fP < 0.01$ *vs* $CD34^+$ cells/G1Na group.

Group	IC_{50} (Degree of resistance/folds) 4-HC/$\mu mol \cdot L^{-1}$	VCR/$\mu g \cdot L^{-1}$	DNR/$\mu g \cdot L^{-1}$
$CD34^+$ Cells	$3.0 \pm 0.5(1)$	$12 \pm 3(1)$	$6.0 \pm 1.6(1)$
$CD34^+$ Cells/G1Na	$3.2 \pm 0.6(1.4)^a$	$10.0 \pm 2.8(0.8)^a$	$7.8 \pm 2.2(1.3)^a$
$CD34^+$ Cells/GL3M	$13.5 \pm 2.8(4.5)^f$	$79 \pm 10(6.6)^f$	$47 \pm 6(7.8)^f$

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造血干/祖细胞遗传学修饰对4-氢过氧化环磷酰胺、长春新碱和柔红霉素的联合抗性[1]

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关键词 基因疗法; MDR 基因; 遗传载体; 基因表达; 造血干细胞

目的: 探讨经醛脱氢酶基因(ALDH-3)和多药耐药基因(MDR1)遗传学修饰的人外周血造血干/祖细胞能否同时增强活性环磷酰胺(4-HC)和 MDR1 基因靶药的抗性. **方法**: 构建同时含 ALDH-3 和 MDR1 双耐药基因的逆转录病毒表达质粒 G1Na-ALDH3-IRES-MDR1, 经电穿孔导入 PA317 包装细胞, 将免疫磁珠分离系统(MACS)分离纯化后的人外周血 CD34$^+$ 细胞用含有 ALDH-3 和 MDR1 双耐药基因重组病毒的上清感染, 用 PCR、RT-PCR、Southern blot、Northern blot、ACS 和 MTT 等方法检测外源 ALDH-3 与 MDR1 基因在 CD34$^+$ 细胞中的转移和表达. **结果**: 用 PCR 与酶切分析法鉴定了双顺反子逆转录病毒载体构建的正确性, 双耐药基因已整合入转染靶细胞中基因组, 并获得有效表达, 应用集落计数测定基因转导效率为 18 %. 巢式 PCR 及补救分析均未检测到辅助病毒存在. 经双耐药基因修饰的 CD34$^+$ 造血干/祖细胞对 4-HC 的 IC_{50} 较对照组提高 4.5 倍, 对多药耐药基因靶药(长春新碱和柔红霉素)的 IC_{50} 较未转染细胞分别高 6.6 和 7.8 倍. **结论**: 逆转录病毒载体介导双耐药基因转导外周血造血干/祖细胞高效共表达可降低联合化疗骨髓毒性.

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