Construction and expression of retroviruses encoding dual drug resistance genes in human umbilical cord blood CD34$^+$ cells

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Abstract A series of retroviral vectors encoding human *mdr1* gene alone as well as in combination with either human *mgmt* gene or human mutant Ser^{31}-*dhfr* gene are engineered. The resultant retroviruses are used to transduce human umbilical cord blood CD34$^+$ cells. It has been shown that expression of dual drug resistance genes in transduced cells confers a broad range of resistance to both kinds of corresponding drugs. These data suggest a rationale for the use of such double chemoresistance gene constructs in an *in vivo* model in which transduced hematopoietic cells will acquire multiple protection against the cytotoxic side effects of combination chemotherapy and may have future application in chemoprotection of normal tissues, thus killing tumor cells more effectively.

Keywords: tumor chemotherapy, retrovirus, drug resistance gene, cord blood CD34$^+$ cells.

The main problem of human cancer chemotherapy is how to ameliorate the cytotoxicity side effects of anticancer therapeutants so as to protect normal tissues, especially the hematopoietic system effectively. It has been demonstrated experimentally and clinically that introduction of one single drug resistance gene may result in increasing cellular resistance to one or one class of corresponding drugs. For instance, the multiple drug resistance gene (*mdr1*) renders cells resistance to various natural lipophilic drugs including colchicine, taxol, adriamycine, vincristine, etc. In contrast, the dihydrofolic acid reductase gene (*dhfr*) confers resistance to antifolates like methotrexate (MTX) and synthetic derivatives thereof, whereas O^6-methylguanine DNA methyltransferase gene (*mgmt*) confers resistance to nitrosoureas such as ACNU and BCNU[1]. Since combinations of anticancer agents are commonly used in cancer therapy to increase clinical effectiveness, it is of interest to investigate the potential of co-expression of several distinct drug resistance genes in hematopoietic stem and progenitor cells that can confer multiple chemoprotection against different classes of non-cross-resistant myelotoxic agents. For this purpose, a series of retroviral vectors containing human *mdr1* gene as well as in combination with either human *mgmt* gene or human *dhfr* gene were constructed and transduced into human umbilical cord blood CD34$^+$ cells. It was observed that co-expression of dual chemoresistance genes in transduced cells increased significant resistance to a wide spectrum of antitumor drugs. This may

provide theoretic and experimental basis for tumor chemotherapy and gene therapy.

1 Materials and methods

(i) Cell cultures. The retrovirus packaging cell lines, GP+E-86 and GP+envAM12 line (a gift from Dr. Arthur Bank of Columbia University, New York), an ecotropic and an amphotropic line respectively, were grown in DMEM supplemented with 10% fetal calf serum (FCS) as well as either HXM (15 μg/mL hypoxanthine, 250 μg/mL xanthine, 25 μg/mL mycophenolic acid, Sigma) or HXM added with hygromycin B (200 μg/mL, Sigma), respectively.

(ii) Retroviral vectors construction and retrovirus production. Human *mgmt* cDNA[2] (a gift from Petter Karran of Imperial Cancer Research Fund, UK) and human mutant Ser^{31} *dhfr* cDNA[3] (a gift from Joseph R. Bertino of Memorial Sloan-Kettering Institute for Cancer Research, US) were used with the 5′ *Sal* I site and 3′ Xho I site being introduced by PCR amplification. Following a *Sal* I / *Xho* I digestion, the *mgmt* and *dhfr* cDNA were inserted into the downstream from SV40 promoter in pHaMASV[4], the retrovirus vector containing human *mdr1* cDNA under control of the Harvey murine sarcoma virus LTR. The resultant retroviral vectors were then introduced into packaging cell lines GP+E-86 and GP+envAM12 using LipofectAMINE (Gibco) mediated method. The ecotropic and amphotropic producer colonies were selected with 60 ng/mL colchicine (Serva). To increase viral titer, a modified supernatant "ping-pong" method was used[5]. Helper virus production was monitored with vector rescue assays.

(iii) Separation and *ex vivo* culture of human umbilical cord blood $CD34^+$ cells. Human cord blood samples were obtained from naturally puerperal infants in Peking Union Hospital. Mononuclear cells were collected by Ficoll-Hypaque (d=1.077 g/mL) gradient centrifugation and enriched for CD34-positive cells using the Mini MACS magnetic cell sorting system (Miltenyi Biotec GmbH, Germany). The quality of $CD34^+$ cells was determined by fluorescence-activated cell sorting (FACS) analysis. A total of 1×10^4 cells/mL were plated in IMDM containing 15% horse serum, 1% bovine serum albumin, 1×10^{-4} mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 10 ng/mL rhIL-3, 10 ng/mL rhIL-6, 10 ng/mL rhTPO (all purchased from Peprotech), 50 ng/mL rhSCF (a generous gift of Amgen) and 10 ng/mL rhFLT3L (Genzyme). After incubation at 37℃ and 5% CO_2, half of the medium was changed weekly.

(iv) Retroviral transduction. After 24 h *ex vivo* expansion, cell-free retroviral supernatants transduction is performed in the same medium added with 4 μg/mL protamine (Sigma). This procedure was repeated for additional 3 times at 24 h intervals. One week later, cells were harvested and stained with MoAb MRK-16 (ImmunoTech) to P-glycoprotein, the *mdr1* gene product on cell surface. The transduction efficiency was evaluated by FACS analysis.

(v) Proviral integration and expression. Genomic DNA was prepared from retroviral transduced hematopoietic cells and tested by PCR for the presence of the human *mgmt*, *dhfr* or *mdr1* cDNA. Meanwhile, purified total RNA was detected by RT-PCR for the expression of transduced genes. The integrity of RNA samples was confirmed using specific primers of human *actin* gene (~120 bp). Genomic DNA contamination was excluded by controls in the absence of reverse transcriptase.

(vi) Progenitor CFC assays. Retroviral transduced or nontransduced $CD34^+$ cells (1×10^4 / mL) were cultured in IMDM containing 0.8% methyl cellulose (Sigma), 30% horse serum, 1% BSA, 1×10^{-4} mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 10 ng/mL rhIL-3, 1 U/mL hEPO (Toyobo), 10 ng/mL rhG-CSF (Amgen), 50 ng/mL rhSCF and 10 ng/mL rhGM-CSF (Glaxo) in the presence or absence of colchicine (10 ng/mL), taxol (10 ng/mL, paclitaxel, generous gift from Faulding), ACNU (5 μg/mL, Sankyo) or MTX (5×10^{-8} mol/L, Sigma). Cultures were conducted in triplicate in 0.5 mL aliquots in 24-well plates at 37℃, with 5% CO_2. After 14 d, colonies greater than 50 cells were enumerated as colony forming cells (CFC).

2 Results

(i) Retrovirus production. Three kinds of retroviruses, containing *mdr1* cDNA (HaMASV), *mdr1* and *mgmt* cDNA (HaMASV*mgmt*), *mdr1* and *dhfr* cDNA (HaMASV*dhfr*) respectively, were

packaged with titers of 5×10^4 colchicine-resistance CFU per milliliter of cell-free supernatants. Virus preparations were found to be free of replication competent retrovirus. Proviral forms of vector genomes as present after retroviral replication are shown in fig. 1.

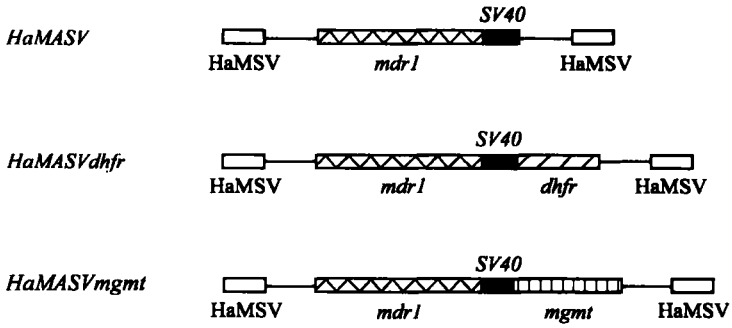
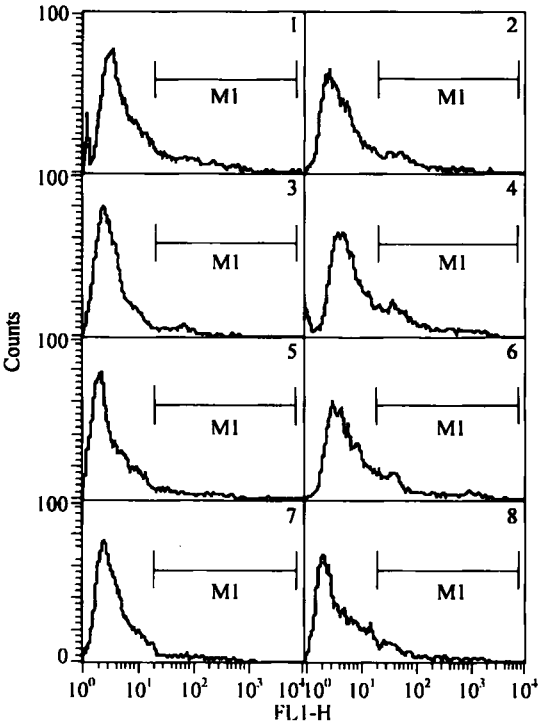


Fig. 1. Schematic representative of pro-retroviruses encoding dual drug resistance genes.

(ii) Separation, *ex vivo* expansion and retroviral transduction of $CD34^+$ cells. The purity of magnetic bead-enriched $CD34^+$ cells analyzed by FACS reached up to 90%, with an average yield of 0.4%—0.6% of total mononuclear cells. Within one week of liquid culture with rhIL-3, rhIL-6, rhSCF, rhTPO and rhFLT3L, $CD34^+$ cells expanded from 1×10^4 to $(5.6\pm0.2)\times10^5$ cells (n=3). The number of progenitors derived from 1×10^4 $CD34^+$ cells increased from 353.3 ± 30.6 to 473.3 ± 40.4 (n=3, P<0.05). After four consecutive exposures to cell-free supernatants, 3 retroviral transduced cells, termed *mdr1*, *mdr1/mgmt* and *mdr1/dhfr* respectively, were obtained. Mock-transduced cells with the supernatant from the parental GP+envAM12 cells were used as negative control.

(iii) Transduction efficiency. FACS analysis of the transduced cells showed that 7%—20% of the cells analyzed increased the amount of *mdr1* gene expression on their surface over identically treated mock-transduced aliquots of cells (fig. 2).



	Marker	% Gated	Geo Mean
1	All	100.00	5.58
	M1	10.81	64.21
2	All	100.00	5.04
	M1	11.45	46.86
3	All	100.00	3.14
	M1	2.85	49.17
4	All	100.00	9.27
	M1	22.74	63.59
5	All	100.00	3.21
	M1	3.89	67.86
6	All	100.00	7.04
	M1	16.08	55.33
7	All	100.00	3.71
	M1	3.38	69.28
8	All	100.00	4.55
	M1	10.78	52.21

Fig. 2. FACS analysis of P-glycoprotein in transduced cord blood cells. 1, 2, Controls; 3, 4, *mdr1*; 5, 6, *mdr1/mgmt*; 7, 8, *mdr1/dhfr*. 1, 3, 5 and 7 represent negative controls in each group without primary antibodies.

(iv) Transduced gene expression. The presence of transduced cDNAs was confirmed by PCR amplification from genomic DNA of transduced cells (figure not shown). To determine the expression of transduced drug resistance genes, RT-PCR was further performed. As shown in fig. 3, *mdr1* mRNA (~415 bp) was detected in all retroviral transduced cells, whereas *mgmt* mRNA (~670 bp) and *dhfr* mRNA (~600 bp) were detected in *mdr1/mgmt* and *mdr1/dhfr* transduced cells respectively. These results suggest that the dual drug resistance genes have been successfully introduced and expressed in human cord blood cells through retroviral mediated transduction.

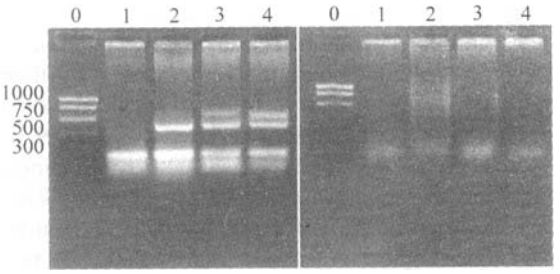


Fig. 3. RT-PCR analysis of transduced gene expression in human cord blood cells. 0, DNA marker; 1, control; 2, *mdr1*; 3, *mdr1/mgmt*; 4, *mdr1/dhfr*. The right graph shows negative controls in the absence of reverse transcriptase.

(v) Drug resistance of retroviral transduced hematopoietic progenitor cells. To clarify wheth-er these dual drug resistance gene vectors would confer multiple chemoprotection in hematopoietic progenitor cells, several relevant drugs, including colchicine and taxol for *mdr1* gene, ACNU for *mgmt* gene and MTX for *dhfr* gene, were chosen in clonogenic assays. Compared with mock-transduced controls, dual drug resistance gene transduced CFCs show 2.4—4.7, 7.9—13.2, 7.2 and 10.2-fold ($P<0.05$) increase in percentage of drug resistance colonies, to colchicine, taxol, ACNU and MTX respectively (fig. 4). This finding indicates that simultaneous expression of dual drug resistance genes provides multiple protection of hematopoietic cells against a broad range of distinct cytotoxic agents.

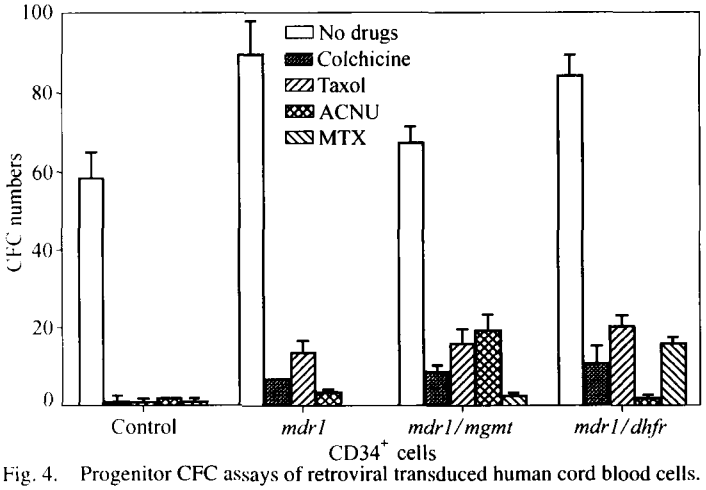


Fig. 4. Progenitor CFC assays of retroviral transduced human cord blood cells.

3 Discussion

It is well known that almost all of human hematopoietic precursor cells exist in mononuclear cells expressing the $CD34^+$ antigen. Considering undetectable $CD34^+$ expression in solid tumor cells, $CD34^+$-selected blood cells may represent the safest target population for gene transfer and transplantation of human hematopoietic cells. Currently, umbilical cord blood has been shown to have a high content of lineage-restricted as well as pluripotent progenitor cells. The percentage of early hematopoietic progeny is comparable to or even larger than that in adult bone marrow or peripheral blood cells harvested after mobilization, which means the excellent *in vitro* expansion potential. These characteristics have made cord blood cells an optimal target for gene transfer trials.

To date, retroviral transfer with single drug resistance gene has been performed using human $CD34^+$ cells derived from cord blood[6], peripheral blood[7] and bone marrow[8], resulting in protection from one or one class of relevant drugs. Phase I clinical trial has already proven the feasibility and safety of this approach[9]. The study presented here demonstrates for the first time that retroviral transduction of human umbilical cord blood $CD34^+$ cells with dual drug resistance gene may protect

hematopoietic progenitor cells from the toxic effects of two classes of chemotherapeutic drugs *in vitro*. Such multiple chemoprotection has the potential to decrease or eliminate much of the host toxicity associated with combination chemotherapy. Furthermore, it is noticed that the transduction efficiency in this experiment is relatively low. This limitation may be due to the apparent low titer of retrovirus containing the large size of inserted *mdr1* cDNA (~4.1 kb), as well as the interference between two separate promoters in dual gene vectors. By choosing different configurations of retroviral vector and optimized transduction protocol, the improved transduction efficiency can be expected. Indeed, low transduction efficiency is the main obstacle in current gene therapy for human hematopoietic progenitor cells. The highest efficiency reported so far in progenitor cells was only 5% after reinfusion of transduced autologous bone marrow. Although a variety of reasons contribute to this issue, retroviral construct carrying drug resistance gene, especially dual drug resistance genes, might permit enrichment of the transduced hematopoietic cells population by *in vivo* selection. This strategy may have significant implications for future cancer gene therapy protocols.

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