Retroviral Coexpression of Two Different Types of Drug Resistance Genes to Protect Normal Cells from Combination Chemotherapy

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ABSTRACT

Drug resistance genes can protect normal hematopoietic cells from the toxicity of anticancer agents. Because chemotherapeutic agents are often used in combination in current clinical protocols, coexpression of two different drug resistance genes should be useful in protecting normal bone marrow cells from the hematotoxicities caused by combination chemotherapy. In this study, we have combined the human multidrug resistance gene (MDR1) and human O6-methylguanine DNA methyltransferase (MGMT) gene as drug resistance genes. For the coexpression of two drug resistance genes, we have constructed two bicistronic retrovirus vectors. One vector is Ha-MDR-ires-MGMT, in which translation of the MDR1 cDNA is cap-dependent and MGMT translation is dependent on an internal ribosome entry site (IRES). The other is Ha-MGMT-ires-MDR, which has cap-dependent MGMT translation and IRES-dependent MDR1 translation. MGMT-negative HeLa derivative (MR) cells transduced with these retroviruses showed resistance to vincristine (from MDR1) and 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea (ACNU; from MGMT). Cells transduced with Ha-MDR-ires-MGMT showed higher resistance to vincristine and lower resistance to ACNU than those transduced with Ha-MGMT-ires-MDR. In any case, the resistance levels of cells transduced with either vector were high enough to select transduced cells with vincristine or ACNU. The expression levels of P-glycoprotein or MGMT in the transduced cells determined by FACS and Western blot analysis correlated well with the extent of resistance to vincristine and ACNU, respectively. All of the MGMT-transduced cells expressed higher amounts of MGMT than the MGMT-expressing parental cell line HeLa S3. Murine bone marrow cells transduced with Ha-MDR-ires-MGMT and selected with vincristine also showed simultaneous resistance to vincristine and ACNU. These results suggest that bicistronic retroviral vectors allow the functional coexpression of two different types of drug resistance genes. This strategy could be applicable to any combination of drug resistance genes.

INTRODUCTION

In cancer chemotherapy, myelosuppression induced by anticancer agents is one of the major side effects that limits the effectiveness of the treatment. Introduction of genes into hematopoietic precursors that confer protection against the hematotoxicity of anticancer agents has been proposed to reduce myelosuppression.

The human MDR3 gene, MDR1, has been studied most extensively for this purpose (1-3). The MDR1 gene encodes the plasma membrane P-glycoprotein with a molecular weight of 170,000. P-glycoprotein acts as an ATP-dependent efflux pump for various structurally unrelated natural product anticancer agents, such as anthracyclines, vinca alkaloids, and taxanes (1). Retrovirus-mediated expression of the MDR1 cDNA has been shown to confer MDR in vivo when the MDR1-carrying vector is introduced into bone marrow cells of mice (4-6). Transgenic mice expressing the MDR1 mRNA at high levels in the bone marrow cells are resistant to daunomycin-induced leukopenia (7-9).

The human DNA repair protein MGMT (EC 2.1.1.63) has also been studied as a means to prevent myelosuppression caused by alkylating agents in cancer chemotherapy (10-14). Alkylating agents add a methyl group to DNA and cause point mutations, mismatch repair, or DNA interstrand cross-links. MGMT transfers a methyl group from O6-alkylguanine and other methylated moieties of DNA to itself. The expression of MGMT has been shown to protect cells from mutagenic and cytotoxic effects of alkylating agents. Transduction of murine bone marrow cells with an MGMT retrovirus increased the

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The abbreviations used are: MDR, multidrug resistance; MGMT, O6-methylguanine DNA methyltransferase; IRES, internal ribosome entry site; ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; FBS, fetal bovine serum; CS, calf serum; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; FACS, fluorescence-activated cell sorting; LTR, long terminal repeat; HSV-TK, herpes simplex virus thymidine kinase.

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survival of murine bone marrow cells following in vitro exposure to 1,3-bis(2-chloroethyl)-1-nitrosourea (13, 14).

We have developed a retroviral vector system, pSXLC/pHa, that uses an IRES isolated from encephalomyocarditis virus (15–18). In this construct, a single mRNA is transcribed under control of an upstream promoter, and two gene products are translated independently from a bicistronic mRNA. This vector system allows transfer and expression of two independent genes with very high efficiency. Because multiple chemotherapeutic agents are used in current clinical protocols, coexpression of two different types of drug resistance genes could be useful to protect normal bone marrow cells from the hematotoxicity caused by combination chemotherapy. To examine the feasibility of this strategy, we have chosen the MDR1 gene and the MGMT gene as examples of two distinct drug resistance genes.

We constructed two bicistronic retroviral vectors to coexpress the human MGMT cDNA and the MDR1 cDNA. One vector is Ha-MDR-ires-MGMT, in which translation of the MDR1 open reading frame is cap-dependent and translation of MGMT is dependent on an IRES. The other is Ha-MGMT-ires-MDR, which has cap-dependent translation of MGMT cDNA and IRES-dependent translation of MDR1. Cells transduced with these vectors acquired simultaneous resistance to vincristine and ACNU.

**MATERIALS AND METHODS**

**Cell Culture.** The amphotropic retrovirus packaging cells PA317 (19) were grown in DMEM supplemented with 10% FBS. The mouse fibroblast NIH 3T3 cells were cultured in DMEM supplemented with 10% CS. Two HeLa-derived cell lines, wild-type S3 and MGMT-nonexpressing MR (12, 20), were cultured in DMEM supplemented with 6.7% CS and 3.3% FBS.

**Construction of Vectors.** The construction of the pSXLC/pHa retrovirus system was described previously (15). The human MGMT cDNA was generated from mRNA of human ovarian carcinoma cells A2780 by reverse transcription-PCR according to the published sequence of human MGMT (11). The bicistronic vector plasmids made in this study were pHa-MDR-MGMT, which has the MDR1 cDNA upstream from IRES and the MGMT cDNA downstream from the IRES, and pHa-MGMT-ires-MDR, which has the MGMT cDNA upstream from the IRES and the MDR1 cDNA downstream from the IRES. To construct pHa-MDR-ires-MGMT, we first inserted the MGMT cDNA between the NcoI and XhoI sites of pSXLC (pSXLC-MGMT). Next we subcloned the SacII/Xbal-digested MDR1 cDNA between the SacII and XhoI sites of pSXLC-MGMT (pSXLC-MDR-MGMT). The MDR-ires-MGMT insert of this plasmid was isolated after SacII digestion and transferred into the pHa retroviral vector (pHa-MDR-ires-MGMT). To construct pHa-MGMT-ires-MDR, we subcloned the BamHI/SalI insert of the MGMT cDNA into the BamHI/SalI-digested pSXLC-MDR (pSXLC-MDR-MGMT). The MGMT-ires-MDR insert of pSXLC-MDR-MGMT was transferred into the pHa retrovirus vector (pHa-MGMT-ires-MDR). As control single-gene vectors, we used pHaMDR (2) and pHaMGMT, which carry the MDR1 cDNA and the MGMT cDNA, respectively. The schematic structures of the retroviral vectors are presented in Fig. 1.

**Retrovirus Producer Cells.** To generate MDR1 retrovirus producers, the PA317 packaging cells were transfected with either pHa-MDR-ires-MGMT or pHa-MGMT-ires-MDR using the calcium phosphate coprecipitaion method (21) and selected with 25 ng/ml vincristine. The supernatants from drug-selected transfectant clones were tested for the production of MDR1 retroviruses using NIH 3T3 as recipient cells. A HaMDR-retrovirus-producing cell line 3P26 and a HaMGMT-retrovirus-producing cell line l35P1 were used as controls.

**Assay of Drug Sensitivity.** A cell growth inhibition assay was used to determine the sensitivity of cells to vincristine, Adriamycin, Taxol, ACNU, MNNG, and VP-16 (etoposide). To evaluate the sensitivity of cells to vincristine, Adriamycin, Taxol, and VP-16, cells were cultured for 5–6 days in serum-containing growth medium, with continuous exposure to various concentrations of the drug. To evaluate the sensitivity of cells to vincristine, Adriamycin, Taxol, and VP-16, cells were cultured for 5–6 days in serum-containing growth medium, with continuous exposure to various concentrations of the drug.

**Table 1.** Drug sensitivity of isolated clones of HeLa MR cells transduced with HaMDR, Ha-MDR-ires-MGMT, HaMGMT, or Ha-MGMT-ires-MDR

<table>
<thead>
<tr>
<th></th>
<th>Vincristine (ng/ml)</th>
<th>ACNU (µg/ml)</th>
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<tbody>
<tr>
<td>MR</td>
<td>0.3 ± 0.2 (1)</td>
<td>0.9 ± 0.1 (1)</td>
</tr>
<tr>
<td>MR/HaMDR</td>
<td>31.7 ± 0.3 (105)</td>
<td>ND</td>
</tr>
<tr>
<td>26M1</td>
<td>9.4 ± 0.5 (31)</td>
<td>ND</td>
</tr>
<tr>
<td>26M2</td>
<td>19.7 ± 2.3 (65)</td>
<td>7.8 ± 1.0 (8)</td>
</tr>
<tr>
<td>27M1</td>
<td>32.3 ± 0.2 (107)</td>
<td>8.3 ± 0.2 (9)</td>
</tr>
<tr>
<td>27M2</td>
<td>48.0 ± 2.8 (160)</td>
<td>13.8 ± 2.2 (15)</td>
</tr>
<tr>
<td>MR/HaMGMT</td>
<td>ND</td>
<td>22.0 ± 0.2 (24)</td>
</tr>
<tr>
<td>135M1</td>
<td>ND</td>
<td>20.7 ± 0.1 (23)</td>
</tr>
<tr>
<td>135M2</td>
<td>1.5 ± 0.1 (5)</td>
<td>22.2 ± 0.5 (24)</td>
</tr>
<tr>
<td>46M1</td>
<td>6.4 ± 0.6 (21)</td>
<td>24.0 ± 1.3 (26)</td>
</tr>
<tr>
<td>46M2</td>
<td>1.2 ± 0.1 (4)</td>
<td>21.0 ± 1.3 (23)</td>
</tr>
</tbody>
</table>

**ND, not determined.**
ACNU or MNNG, cells were first exposed for 1 h to various concentrations of the drug in serum-free DMEM at 37°C, washed, and subsequently cultured for 5–6 days in serum-containing, drug-free growth medium. Cell numbers were determined in triplicate with a Coulter counter (cultured cell lines) or hemocytometer (bone marrow cells), and the IC50s and degrees of resistance were calculated.

**FACS Analysis.** To examine the expression of human P-glycoprotein on the cell surface, FACS analysis was carried out using human P-glycoprotein-specific monoclonal antibody, MRK16 (22). For the detection of P-glycoprotein expressed in HeLa variants, cells (10^5–10^6) harvested after trypsinization were incubated with the F(ab')2 fragment of MRK16 (100 μg/ml), washed, and incubated with a fluorescein-conjugated F(ab')2 fragment of goat anti-mouse IgG F(ab')2 (1:10 diluted; Cappel, Durham, NC). For the detection of P-glycoprotein expressed in murine hematopoietic cells, cells were incubated with the biotinylated F(ab')2 fragment of MRK16 (100 pg/ml; Ref. 25), washed, and incubated with peroxidase-conjugated streptavidin (25 pg/ml; Life Technologies, Inc., Grand Island, NY). The fluorescence staining level was analyzed using FACSort (Becton Dickinson, San Jose, CA).

**Western Blot Analysis.** For Western blot analysis, whole cell extracts were prepared by solubilizing intact cells with SDS sample buffer (24, 25). Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA). The cell extracts were separated on SDS-polyacrylamide gels (24) and transferred to nitrocellulose membranes. The blots were incubated with 2.5 μg/ml of rabbit anti-MGMT polyclonal antibody (24) and transferred to nitrocellulose membranes. The blots were incubated with 2.5 μg/ml of rabbit anti-MGMT polyclonal antibody (25), washed, and incubated with peroxidase-conjugated goat antirabbit IgG (1:500 diluted; Amersham, Arlington Heights, IL). The peroxidase on the blots was visualized using the ECL chemiluminescence detection kit (Amersham).

**Transduction of Mouse Bone Marrow Cells.** Seven-week-old male CDF1 mice were treated i.v. with 150 mg/kg 5-fluorouracil. After 6 days, the bone marrow was harvested from both femurs, and mononuclear cells were isolated. The resulting cells were cultured in Iscove's modified Dulbecco's medium supplemented with 20% FBS, 50 μM 2-mercaptoethanol, 100 ng/ml rat stem cell factor (gift from Amgen, Thousand Oaks, CA), 20 ng/ml mouse interleukin 3 (gift from KIRIN Brewery Co. Ltd., Tokyo, Japan), and 100 ng/ml human interleukin 6 (R&D Systems, Minneapolis, MN). On the next day, retrovirus-containing supernatant was added to the bone marrow cells with 6 μg/ml polybrene. Retrovirus-containing supernatant was changed twice with a 24-h interval. Two days after the transduction, the cells were selected in the medium containing 25 ng/ml vincristine.

### RESULTS

**Drug Sensitivity of MR Clones Transduced with Retroviruses.** The PA317 retrovirus packaging cells transfected with either pHa-MDR-RES-MGMT or pHa-MGMT-RES-MDR showed vincristine resistance. The culture supernatants of the transfected, vincristine-resistant PA317 clones were tested for the production of MDR1 retroviruses. Titers of these retrovirus-producing cell lines ranged 10^2 to 10^5 colony-forming units/ml, which were 2–20-fold less than that of our control HaMDR-producer, 3P26. Among them, two clones, Ha-MDR-RES-MGMT-producer 27P26 and Ha-MGMT-RES-MDR-producer 48P28, were selected for further studies. The MR cells were transduced with HaMDR, Ha-MDR-RES-MGMT or Ha-MGMT-RES-MDR retroviruses and selected with 2 ng/ml vincristine for 7 days. The MR cells transduced with HaMGMT were selected with 20 μg/ml ACNU. As shown in Table 1, the MR clones transduced with Ha-MDR-RES-MGMT showed 50–120-fold higher resistance to vincristine than the parental cells. The drug resistance of the Ha-MDR-RES-MGMT-transduced clones was similar to that observed in HaMDR-transduced clones. The Ha-MGMT-RES-MDR-transduced clones showed 4–21-fold higher resistance to vincristine than the parental cells (Table 1).

To test if the MGMT gene was functionally expressed in transduced cells, these clones were tested for sensitivity to ACNU (Table 1). The Ha-MGMT-RES-MDR-transduced clones showed 23–26-fold higher resistance to ACNU than the parental cells. The drug resistance of the Ha-MGMT-RES-MDR-transduced clones was similar to that observed in HaMGMT-transduced clones. The MR clones transduced with Ha-MDR-RES-MGMT showed 7–12-fold higher resistance to ACNU than the parental cells (Table 1).

Cross-resistance of these vincristine- or ACNU-selected transduced cells to various cytotoxic agents were examined using one each of the transduced clones. As shown in Table 2, cells transduced with the MDR1-expressing retroviruses showed resistance to vincristine, Adriamycin, and Taxol, but they showed drug sensitivity to ACNU, MNNG, and VP-16 similar to that shown by the parental cells. Taxol seemed to be the best MDR substrate among these three MDR drugs. On the other hand, cells transduced with the MGMT-carrying vectors showed resistance to ACNU, but the sensitivity of these cells to vincristine, Adriamycin, Taxol, and VP-16 was not changed (Table 2).

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### Table 2  Cross-resistance patterns of isolated clones of HeLa MR cells transduced with HaMDR, Ha-MDR-RES-MGMT, HaMGMT, or Ha-MGMT-RES-MDR

<table>
<thead>
<tr>
<th>Vincristine (μg/ml)</th>
<th>Adriamycin (μg/ml)</th>
<th>Taxol (μg/ml)</th>
<th>ACNU (μg/ml)</th>
<th>MNNG (μg/ml)</th>
<th>VP-16 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR/HaMDR, 26M1</td>
<td>160</td>
<td>84</td>
<td>2400</td>
<td>0.91</td>
<td>1.1</td>
</tr>
<tr>
<td>MR/HaMDR-RES-MGMT, 27M2</td>
<td>68</td>
<td>21</td>
<td>480</td>
<td>8.3</td>
<td>84</td>
</tr>
<tr>
<td>MR/HaMGMT, 135M1</td>
<td>0.83</td>
<td>0.88</td>
<td>0.85</td>
<td>23</td>
<td>140</td>
</tr>
<tr>
<td>MR/Ha-MGMT-RES-MDR, 46M1</td>
<td>4.1</td>
<td>2.0</td>
<td>53</td>
<td>22</td>
<td>130</td>
</tr>
</tbody>
</table>

*SD for each value was less than 20% of the value.
Because experiments of Tables 1 and 2 were done separately, degrees of resistance in each clone is sometimes not the same between the two experiments.

Expression of P-Glycoprotein in the Transduced Cells. The expression of P-glycoprotein on the surface of transduced cells was analyzed by FACS using the Fab'2 fragment of human P-glycoprotein-specific monoclonal antibody MRK16. Parental MR cells did not express endogenous P-glycoprotein (Fig. 2). The vincristine-selected, Ha-MDR-IRES-MGMT-transduced clones showed similar levels of P-glycoprotein expression as did HaMDR-transduced clones (Fig. 2). The expression of P-glycoprotein in the vincristine-selected, Ha-MGMT-IRES-MDR-transduced clones was slightly lower than that of Ha-MDR-IRES-MGMT-transduced clones (Fig. 2). Among the Ha-MGMT-IRES-MDR-transduced clones, 46M2, which showed the highest (21-fold) resistance to vincristine, expressed higher amounts of P-glycoprotein than the other two Ha-MGMT-IRES-MDR-transduced clones (Table 1 and Fig. 2).

Western Blot Analysis. To determine whether the MGMT cDNA was efficiently translated to yield detectable amounts of protein, we performed Western blot analysis of cell extracts from MR clones described above. As shown in Fig. 3, high-level expression of MGMT was observed in all three clones transduced with Ha-MGMT-IRES-MDR. Three clones transduced with Ha-MDR-IRES-MGMT also showed significant levels of MGMT expression that were higher than that of HeLa S3, which expresses endogenous MGMT. Among the Ha-MDR-IRES-MGMT-transduced clones, 27M3, which showed the highest (15-fold) resistance to ACNU, expressed higher amounts of MGMT than the other two Ha-MDR-IRES-MGMT-transduced clones (Table 1 and Fig. 3).

Transduction of Murine Bone Marrow Cells with Ha-MDR-IRES-MGMT Retrovirus. 5-Fluorouracil-treated murine bone marrow cells were transduced with HaMDR or Ha-MDR-IRES-MGMT and selected with vincristine (25 ng/ml). Five days after the vincristine-selection, the bone marrow cells were assayed for resistance to ACNU. Both HaMDR-transduced cells and Ha-MDR-IRES-MGMT-transduced cells showed vincristine resistance (Fig. 4A). The Ha-MDR-IRES-MGMT-transduced cells showed 7.5-fold higher resistance to...
coexpressed with HSV-TK (16), a-galactosidase A (17), or a gene of interest.

MDR1 construction of bicistronic vectors in which the express drug-selectable genes such as the gene into mammalian cells (3, 26). In a previous study, transduced bone marrow cells into live mice (4, 5) also suggest MDR1 large subunit of cytochrome b55 (18) and demonstrated that all already started (28-31).

**DISCUSSION**

we reported the generation of a flexible bicistronic retroviral vector system pSXLC/pHa that uses an IRES sequence to co-express drug-selectable genes such as the Harvey murine sarcoma virus LTR has been used to transfer the function was observed. Studies of the transplantation of normal hematopoietic precursors have demonstrated that all already started (28-31).

**DISCUSSION**

A HaMDR retrovirus vector that uses the promoter of the bone marrow cells examined by FACs with a human P-glycoprotein-specific monoclonal antibody, MRK16. Vincristine-resistant murine bone marrow cells transduced with HaMDR-ires-MGMT or HaMDR as described above showed high-level expression of human P-glycoprotein (Fig. 4). The shifts in the transduced cells suggest that almost all of the bone marrow cells surviving selection with vincristine express human P-glycoprotein. The expression of MGMT was examined by Western blot analysis. The human MGMT protein was detected in Ha-MDR-ires-MGMT-transduced cells without vincristine (Fig. 4B). In contrast, as expected, vincristine-resistant bone marrow cells transduced with HaMDR did not show any resistance to ACNU.

The expression of P-glycoprotein on the cell surface of the bone marrow cells was examined by FACs with a human P-glycoprotein-specific monoclonal antibody, MRK16. Vincristine-resistant murine bone marrow cells transduced with HaMDR-ires-MGMT or HaMDR as described above showed high-level expression of human P-glycoprotein (Fig. 4). The shifts in the transduced cells suggest that almost all of the bone marrow cells surviving selection with vincristine express human P-glycoprotein. The expression of MGMT was examined by Western blot analysis. The human MGMT protein was detected in Ha-MDR-ires-MGMT-transduced cells without vincristine, and strong expression of MGMT was observed in Ha-MDR-ires-MGMT-transduced cells surviving selection with vincristine (Fig. 6).

**DISCUSSION**

A HaMDR retrovirus vector that uses the promoter of the Harvey murine sarcoma virus LTR has been used to transfer the MDR1 gene into mammalian cells (3, 26). In a previous study, we reported the generation of a flexible bicistronic retroviral vector system pSXLC/pHa that uses an IRES sequence to co-express drug-selectable genes such as the MDR1 gene with the gene of interest (15). Using this system, we have reported the construction of bicistronic vectors in which the MDR1 gene is coexpressed with HSV-TK (16), α-galactosidase A (17), or a large subunit of cytochrome b56a (18) and demonstrated that all of the drug-selected cells expressed the other nonselectable genes. A bicistronic vector with MDR1 and glucocerebrosidase has also been reported (27). In the present paper, we show that it is possible to create bicistronic vectors that express two different drug resistance genes.

Transfer of drug resistance genes for the chemoprotection of normal hematopoietic cells is a promising strategy for gene therapy of cancer. Characteristics required for candidate drug resistance genes are as follows: the drug is effective against the patient’s tumor; high-dose administration of the drug does not cause severe toxicity to other tissues; efficient introduction of the gene into hematopoietic precursors is possible; introduction of the drug resistance gene results in reasonably high degrees of drug resistance in hematopoietic cells; and expression of the gene in hematopoietic cells does not disturb normal function.

Use of the MDR1 gene for chemoprotection in the treatment of ovarian and breast tumors with Taxol satisfies all of the above criteria. To study the effect of expression of the exogenous human MDR1 gene in tissues that normally do not express MDR1 mRNA, MDR1-transgenic mice were engineered (7-9). The expression of the MDR1 gene in one line of the MDR1-transgenic mice was limited to several different cell types in the bone marrow, and the expression level was high enough to confer drug resistance in normal bone marrow cells in vivo. The transgenic animals were healthy, and no defect in bone marrow function was observed. Studies of the transplantation of MDR1-transduced bone marrow cells into live mice (4, 5) also suggest the safety of MDR1 gene expression in bone marrow cells that normally do not express the MDR1 gene. Therefore, in principle, MDR1 gene transfer into bone marrow cells appears to be safe, and we do not anticipate any side effects associated with expression of an exogenous MDR1 gene. Clinical trials of the MDR1 gene transfer into normal hematopoietic precursors have already started (28-31).
Retroviral Coexpression of MDRI. A. Biotinylated fragment of MRK16 (100 μg/ml; Ref. 23), washed, and incubated with R-phycocerythrin-conjugated streptavidin (25 μg/ml). The fluorescence staining level was analyzed using FACSort. As controls, the cells were stained with R-phycocerythrin-conjugated streptavidin (25 μg/ml) without MRK16 staining and analyzed (---). A. nontransduced cells; B. HaMDR-transduced cells selected with vincristine; C. Ha-MDR-RES-MGMT-transduced cells selected with vincristine.

One of the problems associated with MDRI gene therapy is the limited cross-resistance of MDRI-transduced cells to anticancer drugs. For instance, expression of the MDRI gene does not confer resistance to alkylating agents. To protect normal cells from the toxicity of antitumor agents that are not substrates for the MDRI-encoded efflux pump, other distinct drug resistance genes have been studied. Dihydrofolate reductases, especially mutant cDNAs isolated from drug-resistant cells, mediate resistance to methotrexate (32-34). Rat glutathione S-transferase Yc confers resistance to alkylating agents (35, 36). Transfer of MGMT into mammalian cells results in resistance to nitrosoureas (10-14). These drug resistance genes have been demonstrated to confer drug resistance when they are introduced into cells that do not overexpress the genes.

Because multiple chemotherapeutic agents are used in current clinical protocols, coexpression of two different types of drug resistance genes should be useful to protect normal bone marrow cells from the hematotoxicity of combination chemotherapy. Our bicistronic vector system could be used to coexpress two distinct drug resistance genes in each transduced cell. We have chosen the MDRI gene and the MGMT gene as models of two distinct drug resistance genes. Transfer of the MGMT gene also satisfies most of the above criteria. Nitrosoureas are one of the most effective anticancer agents against brain tumors. As shown in this study, retroviral transfer of the MGMT gene confers resistance to ACNU. Overexpression of the MGMT gene in transgenic mice does not affect the normal function of hematopoietic cells (37). We have constructed two types of bicistronic retroviral vectors, Ha-MDR-RES-MGMT and Ha-MGMT-RES-MDR, and demonstrated that cells transduced with either of these retroviral vectors acquired simultaneous resistance to vincristine and ACNU.

In a previous study, we compared the expression level of the MDRI gene and β-galactosidase A gene placed upstream or downstream from the IRES of bicistronic vectors and concluded that the cDNA whose translation was cap-dependent was expressed at higher levels than when the same cDNA was translated in an IRES-dependent manner in our bicistronic vectors (17). In this study, we have shown that cells transduced with Ha-MDR-RES-MGMT showed higher resistance to vincristine and lower resistance to ACNU than those transduced with Ha-MGMT-RES-MDR. Higher expression of P-glycoprotein and lower expression of MGMT were observed in cells transduced with Ha-MDR-RES-MGMT than in those with Ha-MGMT-RES-MDR. This supports our previous observation that the cap-dependent translation is more efficient than the IRES-dependent translation. This vector system should therefore make it possible to express different ratios of expression of two drug resistance genes, depending on which gene is upstream from the IRES and which is downstream.

Cells transduced with Ha-MDR-RES-MGMT were as resistant to vincristine as those transduced with HaMDR, and cells transduced with Ha-MGMT-RES-MDR were as resistant to ACNU as those transduced with HaMGMT. These results suggest that the introduction of second drug resistance genes controlled by the IRES did not affect the activity of drug resistance genes, the translation of which was cap-dependent. The activity of the second drug resistance gene downstream from the IRES was significantly reduced. However, the resistance levels of
transduced cells were still high enough to select transduced cells with vincristine or ACNU. Therefore, our bicistronic vectors that confer double resistance might be more useful than the original one-gene vectors under certain protocols involving combination chemotherapy.

There still remains another potential problem associated with retroviral transfer of drug-resistant genes into normal bone marrow cells. In transducing bone marrow cells of cancer patients with the MDR1 retrovirus, for instance, if the bone marrow contains contaminating cancer cells, and the cancer cells receive the MDR1 retrovirus, then multidrug-resistant cancer cells will result. To eliminate such unintentionally transduced cells, coexpression of a suicide gene, HSV-TK, that confers hypersensitivity to a nucleoside analogue ganciclovir would be valuable. In a previous study, we constructed a bicistronic retrovirus Ha-MDR-IRES-TK, which carries the MDR1 gene and the HSV-TK gene and demonstrated that it is possible to eliminate MDR1-transduced cells with ganciclovir (16). This strategy could be applied to MDR-MGMT bicistronic retroviral vectors as well. The resulting polycistronic vectors Ha-MDR-IRES-MGMT-IRES-TK or Ha-MGMT-IRES-MDR-IRES-TK would solve this problem if the two drug resistance genes were used for chemoprotection.

Mouse bone marrow cells were transduced with Ha-MDR-IRES-MGMT. We used the retrovirus, which has a cap-dependent MDR1 cDNA, for this experiment because we have had good success in transducing human and mouse bone marrow cells with this type of vector. The transduction efficiency of mouse bone marrow cells with Ha-MDR-IRES-MGMT retrovirus were usually 5–10% (data not shown). Higher transduction rates could be obtained by repeated transduction. After 5 days of selection of transduced bone marrow cells with vincristine, almost all of the bone marrow cells were shown to express exogenous P-glycoprotein, indicating that the MDR1 gene expressed from the bicistronic vector confers resistance to vincristine in murine bone marrow cells. Expression of MGMT was dramatically increased after vincristine selection. According to our previous results, more than 95% of vincristine-resistant cells transduced with MDR1-bicistronic retroviruses express the second, nonselected gene (16). The difference in MGMT expression levels in bone marrow cells before and after vincristine selection suggests that 3–5% of bone marrow cells were initially transduced with Ha-MDR-IRES-MGMT. We have shown that the vincristine-selected populations of Ha-MDR-IRES-MGMT-transduced cells were 7.5-fold more resistant to ACNU than the HaMDR-transduced cells. These results clearly indicate that the expression of the IRES-dependent MGMT cDNA in Ha-MDR-IRES-MGMT retrovirus could confer a significant level of ACNU resistance in mouse bone marrow cells. This is the first report that two different drug resistance genes are functionally coexpressed in bone marrow cells from bicistronic retrovirus vectors. The results obtained in this study are indispensable to start the next experiments using animal models. Studies to examine the functional expression of the two drug resistance genes in murine bone marrow cells in vivo are currently ongoing in our laboratory.

Another serious side effect of chemotherapy using alkylating agents is drug-induced carcinogenesis and the emergence of secondary tumors. Formation of alkylated DNA could result in point mutations, mismatch repair, or DNA interstrand cross-links. Cells transfected with the MGMT cDNA were shown to be resistant to alkylating agent-induced mutagenesis in vitro (38). The MGMT-transgenic mice treated with alkylating agents did not develop thymic lymphomas (37). Therefore, MGMT gene transfer could be useful to protect cancer patients from secondary carcinogenesis. For this purpose, it is desirable that all of the hematopoietic cells express the MGMT gene. In this study, we have shown that it is possible to use the MDR1 gene as a drug-selectable marker to enrich MGMT-expressing murine bone marrow cells. The use of a dominant drug-selectable marker such as MDR1 is a powerful way to ensure that the transduced cells become the major population in vivo. Further preclinical studies in various animal model systems are needed to prove this hypothesis.

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Retroviral coexpression of two different types of drug resistance genes to protect normal cells from combination chemotherapy.


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