MDR1 Up-Regulated by Apoptotic Stimuli Suppresses Apoptotic Signaling

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Purpose. Recently, MDR1 (P-glycoprotein) and related transporters have been suggested to play a fundamental role in regulating apoptosis, but little information is available concerning the role of MDR1. Here, the effect of apoptotic stimuli on the MDR1 mRNA and apoptotic signaling was examined in MDR1-overexpressing cells.
Methods. The expression levels of mRNA for MDR1, MRP1, MRP2, p53, p21, Bax, and Bcl-2 were measured by real time quantitative polymerase chain reaction in HeLa and its MDR1-overexpressing sublines. The effects of apoptotic stimuli by cisplatin (CDDP) on their levels were also assessed as well as on caspase 3, 8, and 9 activities.
Results. MDR1 was rapidly upregulated when the cells were exposed to apoptotic stimuli by CDDP. The increase in Bax mRNA to Bcl-2 mRNA ratio after treatment with CDDP was suppressed in *MDR1*-overexpressing cells. The increases in caspase 3 and 9 activities after treatment with CDDP were suppressed in MDR1-overexpression cells.
Conclusion. MDR1 is upregulated by apoptotic stimuli suppressed apoptotic signaling presumably via the mitochondrial pathway.

KEY WORDS: *MDR1* (P-glycoprotein); Apoptosis; Caspases; Mitochondrial pathway

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ABBREVIATIONS: MDR, multidrug resistance; MDR1, multidrug resistance transporter 1; MRP1 or 2, multidrug resistance-associated protein 1 or 2; PCR, polymerase chain reaction; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VLB, vinblastine sulfate; VCR, vincristine sulfate; DXR, doxorubicin hydrochloride; TXL, paclitaxel; 5-FU, 5-fluorouracil; CPA, cyclophosphamide monohydrate; CDDP, cisplatin.

INTRODUCTION

Multidrug resistance (MDR) is one of the most serious problems responsible for the failure of chemotherapy and cellular factors in MDR include: 1) down regulation of uptake or induction of the efflux system (e.g., MDR1 (P-glycoprotein)); 2) induction of inactivation enzymes including GSH-S-tranferase; 3) alteration of targeted enzymes including topoisomerase; 4) changes in DNA repair processes; and 5) alteration of apoptotic mechanisms (1). MDR1 was isolated from resistant tumor cells in 1976 (2). MDR1 is a glycosylated membrane protein of 1280 amino acids (170 kDa) consisting of two similar regions containing six putative transmembrane segments and intracellular binding sites for ATP. MDR1 has been understood to act as an efflux pump to remove antitumor agents from cells, and the magnitude of resistance depends on the MDR1 expression level (3,4). A gene, which was overexpressed in multidrug-resistant KB carcinoma cells, was isolated in 1986 and demonstrated to encode human MDR1 (5,6). Human MDR1 has been shown to be expressed in the apical membranes of normal tissues including the liver, kidneys, small and large intestines, and brain (7–10), and MDR1 is known to confer intrinsic resistance by exporting unnecessary or toxic exogeneous substances or metabolites out of the body.

Over the last decade, it has been demonstrated that MDR1 is also expressed on hematopoietic stem cells (11), natural killer cells (12), leukocytes (13), antigen-presenting dendritic cells (14), and T and B lymphocytes (14–16). MDR1 is expressed in the developing embryo (17) and placenta in a stage-specific manner (18). Transduction with the human MDR1 gene enables dramatic cell expansion of hematopoietic stem cells (19). MDR1 expression in hematopoietic progenitor cells is dependent on the level of differentiation (11). These observations have challenged the notion that MDR1 has evolved merely to facilitate efflux of xenobiotics and raised the possibility that MDR1 and related transporters might play a fundamental role in regulating immunology and apoptosis (20,21). Apoptosis is a type of cell death prevalent under many physiologic and pathologic conditions and consists of initiating stimuli, transduction pathways, effector mechanisms, nuclear fragmentation, and phagocytosis (22–24). Research in apoptosis has established a central role of cysteine-aspartate proteases, i.e., the caspase family, as activators or effectors (22–24). Recently, MDR1 has been suggested to protect cells against caspase-dependent apoptosis induced by cytotoxic drugs, Fas ligation, tumor necrosis factor, and ultraviolet irradiation (25,26). Although the relationship between MDR1 expression and caspase-dependent apoptosis has been elucidated, little information is available concerning the role of MDR1 in the system. In this study, the expression levels of mRNAs for MDR1, multidrug resistance-associated protein 1 or 2 (MRP1, MRP2), p53, a tumor suppressor, and p21, the product of a downstream target gene for wt p53 protein, and Bax and Bcl-2, well-known key proteins in the mitochondrial pathway, were quantitatively compared by real time quantitative polymerase chain reaction (PCR) in HeLa and its MDR1-overexpressing sublines, which were established by stepwise exposure to vinblastine sulfate (VLB) (27). The effects of cisplatin (CDDP) on the mRNA levels of MDR1, Bax, and Bcl-2 as well as on the activities of the

activator caspases 8 and 9 and effector caspase 3 were also evaluated. Chemotherapeutic agents including CDDP have been shown to induce the cell death by apoptosis (28). CDDP is not a substrate of MDR1 showing similar intracellular concentrations in HeLa and its MDR1-overexpressing sublines, and thus CDDP was a suitable agent to investigate the effects of MDR1 on apoptosis.

MATERIALS AND METHODS

Materials

VLB, vincristine sulfate (VCR) sulfate, doxorubicin hydrochloride (DXR), paclitaxel (TXL), 5-fluorouracil (5-FU), and cyclophosphamide monohydrate (CPA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CDDP was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Other chemicals were of the highest purity available.

HeLa and Its MDR1-Overexpressing Sublines and Cell Culture

The human cervical carcinoma cell line HeLa-Ohio (HeLa) was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). HeLa cells (passage 396–401) were maintained in the culture medium consisting of Dulbecco's modified Eagle's medium (D-MEM with glucose [4.5 g/L], L-glutamine [4 mM], and sodium pyruvate [1 mM]; Cat. No. 12800-017, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Lot. No. AGM7413, HyClone, UT, USA) and 100 mg/l kanamycin sulfate (Invitrogen Corp.). Three MDR1-overexpressing HeLa sublines, Hvr1-1 (passage 34–38), Hvr10-6 (passage 107–113), and Hvr100-6 (passage 82–87) were established by stepwise exposure to VLB in 60 mm dishes (27) and maintained in the culture medium additionally supplemented with 1, 10, and 100 nM VLB, respectively. HeLa, Hvr1-1, Hvr10-6, and Hvr100-6 cells (4, 8, 8, 12×10^4 cells/cm^2, respectively) were seeded into culture flasks (Nunclon™ flasks; Nalge Nunc International, USA), grown in a humidified atmosphere of 5% CO_2-95% air at 37°C and subcultured every 3 or 4 days with 0.05% trypsin-0.02% EDTA (Invitrogen Corp.).

FACS Analysis of HeLa and Its MDR1-Overexpressing Sublines

Serial MDR1 expression in HeLa and its MDR1-overexpressing sublines, Hvr1-1, Hvr10-6, and Hvr100-6 was confirmed by fluorescence-activated cell sorter (FACS) analysis. They were treated with EDTA and harvested in Dulbecco's modified phosphate-buffered saline without magnesium and calcium (PBS; 137.9 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4). Each cell line (1×10^6 cells) was incubated with the MDR1-specific monoclonal antibodies MRK-16 (1:5 dilution; Funakoshi Co., Ltd.; Tokyo, Japan) and JSB-1 (1:10 dilution; Sanbio BV; Netherlands), or the multidrug resistance-associated protein (MRP)-specific monoclonal antibody MRPm6 (1:20 dilution, Sanbio BV) for 60 min on ice. MRK-16 and JSB-1 antibodies recognize the extracellular and intracellular epitopes of MDR1, respectively. As a secondary antibody, FITC-conjugated goat anti-mouse IgG (Becton Dickinson & Co.; NJ, USA) was used, and the cells were incubated for 30 min on ice. The cells were washed twice with PBS, and the fluorescence intensity of aliquots of 1×10^5 cells was determined (FACScan™, Becton Dickinson & Co.).

Antiproliferative Effects of Anticancer Drugs in HeLa and Its MDR1-Overexpressing Sublines

Antiproliferative effects of anticancer drugs were assessed in HeLa and its MDR1-overexpressing sublines by the WST-1 (tetrazolium salts) colorimetric assay using a Cell Counting Kit (Dojindo Laboratories; Kumamoto, Japan) as described (27). Briefly, cells (1,000 cells/well) were seeded on 96-well plates (Nalge Nunc International) in 100 μl of culture medium without any anticancer drug on day 0, and the culture medium was exchanged to that containing test anticancer drug at various concentrations on day 1. The anticancer drugs were added in ethanol, dimethyl sulfoxide, or double-distilled water. The maximum concentration of organic solvents or double-distilled water was 1%, which had no effect on cell viability or growth. After incubation for 3 days at 37°C (day 4), the culture medium was exchanged to 110 μl of that containing WST-1 reagent solution (10 μl WST-1 solution and 100 μl the culture medium), and 3 h later, the absorbance was determined at 450 nm with a reference wave length of 630 nm using a microplate reader (Sjeia Auto Reader II; Sanko Junyaku Co. Ltd.; Tokyo, Japan) according to the manufacturer's directions. Preliminary experiments demonstrated a good proportional relationship between the absorbance and cell number with acceptable precision and accuracy and very small day-to-day or within-day variations. The 50% growth inhibitory concentration (IC_{50}) of the anticancer drugs was calculated according to the sigmoid inhibitory effect model, $E = E_{max} \times [\ 1 - C^{\gamma}/ (C^{\gamma} + IC_{50}{}^{\gamma})\ ]$, using the nonlinear least-squares fitting method (WinNonlin®, ver. 2.1, Pharsight Corp.; CA, USA). E and E_{max} represent the surviving fraction (% of control) and its maximum, respectively, and C and γ represent the drug concentration and the sigmoidicity factor, respectively. Each series of experiments was conducted in quadruplicate, and mean values were presented with the SD.

Real Time Quantitative PCR in HeLa and Its MDR1-Overexpressing Sublines

MDR1, MRP1, MRP2, p53, p21, Bax, and Bcl-2 mRNA levels were measured in HeLa and its MDR1-overexpressing sublines by real time quantitative PCR. HeLa, Hvr1-1, Hvr10-6, and Hvr100-6 cells (4, 8, 8, 12×10^4 cells/cm^2, respectively) were seeded into culture flasks (Nunclon™ flasks; Nalge Nunc International) and cultured in the culture medium with 0, 1, 10, and 100 nM VLB, respectively, for 3 or 4 days. Cell suspensions were prepared by 0.05% trypsin-0.02% EDTA (Invitrogen Corp.). Total RNA was extracted from cell suspensions using an RNeasy Mini Kit (Quiagen, Hilden; Germany) according to the manufacturer's protocol. To prevent contamination of genomic DNA, an RNase-Free DNase Set (Quiagen; Hilden, Germany) was also used. RT reaction was conducted using 4 μl of the extracted total RNA (2 μg/mL) in 20 μl of TaqMan® Reverse Transcription Reagents (Applied Biosystems; Foster City, CA), containing 1×TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM dATP, 500 μM dGTP, 500 μM dCTP, 500 μM dUTP, 2.5 μM Random Hexamer, 0.4 U/μl of

RNase Inhibitor, and 1.25 U/μl MultiScribe™ Reverse Transcriptase. The mixture was incubated at 25°C for 10 min and subsequently at 48°C for 30 min. Reverse transcription was terminated by heating at 95°C for 5 min followed by cooling at 4°C for 5 min giving the RT product.

Table I shows the primer pairs and TaqMan probes for *MDR1, MRP1, MRP2*, p53, p21, Bax, and Bcl-2 mRNA, which were designed using the Primer Express 1.0 program (Applied Biosystems). Primers and the TaqMan probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems (TaqMan® GAPDH Control Reagent Kit). All TaqMan probes were labeled with a reporter fluorescent dye (6-carboxy-fluorescein [FAM] or 2,7-dimethoxy-4,5-dichloro-6-carboxy-fluorescein [JOE])] at the 5′ end and a quencher fluorescent dye (6-carboxy-tetramethyl-rhodamine [TAMRA]) at the 3′ end, by which the reporter dye emission was quenched when the probe was intact.

Real time PCR was conducted using 1 μl of RT product in 25 μl of TaqMan® PCR Core Reagents Kit with AmpliTaq Gold (Applied Biosystems) containing 1×TaqMan buffer A, 5.5 mM $MgCl_2$, 400 μM dUTP, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 0.01 U/μL AmpErase UNG and 0.025 U/μL AmpliTaq Gold DNA Polymerase with 200 nM of each forward and reverse primer, and 100 nM TaqMan probe. The reaction was performed in triplicate for each RT product after activation of AmpErase UNG at 50°C for 2 min. During the extension phase of PCR consisting of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, the nucleolytic DNA polymerase cleaved the hybridization probe and the resulting relative increase in the reporter fluorescent dye emission was monitored in real time using a sequence detector (ABI prism 7700 Sequence Detector; Applied Biosystems). The fluorescent dye emission was a function of cycle number and was determined using the Sequence Detector™ software (ver.1.6.3, Applied Biosystems), giving the threshold cycle number (C_T) at which PCR amplification reached a significant threshold. The value of C_T was linearly correlated with logarithmic value of genomic DNA quantity.

The PCR products obtained by this real time quantitative PCR procedure were confirmed to be the expected products by electrophoresis through 3.0% agarose gels in the presence of ethidium bromide with visualization under UV (data not shown).

A representative sample obtained from HeLa cells was used as the authentic standard and in each run of the assay mRNAs of GAPDH and target protein (i.e., MDR1, MRP1, MRP2, p53, p21, Bax, and Bcl-2) were analyzed in five-fold serially diluted authentic samples from HeLa cells (0.32, 1.6, 8, 40, 200 ng as total RNA determined spectrophotometrically). The standard lines were constructed by plotting mean C_T values against quantity (the relative copy number). The mRNA levels of GAPDH and target protein in each sample were calculated from the mean C_T values and that of target protein was expressed as concentration relative to GAPDH mRNA.

Effects of CDDP on MDR1 mRNA and Apoptotic Signaling in HeLa and Its MDR1-Overexpressing Subline

The effects of CDDP on the mRNA levels of MDR1, Bax, and Bcl-2 as well as on the caspase 3, 8, and 9 activities

Table I. Sequences of Oligonucleotide Primers and Probes Used for Real Time Quantitative PCR

Target protein	Sequence	Accession no.
MDR1		
Forward primer	5′-AGG AAG CCA ATG CCT ATG ACT TTA-3′	M14758
Reverse primer	5′-CAA CTG GGC CCC TCT CTC TC-3′	
TaqMan probe	5′-ATG AAA CTG CCT CAT AAA TTT GAC ACC CTG G-3′	
MRP1		
Forward primer	5′-CTT GGC CAC GTA CAT TAA CAT GAT-3′	L05628
Reverse primer	5′-CCG ATT GTC TTT GCT CTT CAT G-3′	
TaqMan probe	5′-ATG GTC CTC ATG GTG CCC GTC AAT-3′	
MRP2		
Forward primer	5′-TGC AGC CTC CAT AAC CAT GA-3′	U63970
Reverse primer	5′-GGA CTT CAG ATG CCT GCC A-3′	
TaqMan probe	5′-TCG AAC ACT TAG CCG CAG TTC TAG GTC CA-3′	
p53		
Forward primer	5′-TGC GTG TGG AGT ATT TGG ATG-3′	AF136270
Reverse primer	5′-TGG TAC AGT CAG AGC CAA CC-3′	
TaqMan probe	5′-AAA CAC TTT TCG ACA TAG TGT GGT GGT GCC-3′	
p21		
Forward primer	5′-AGC AGA GGA AGA CCA TGT GGA C-3′	L25610
Reverse primer	5′-TTT CGA CCC TGA GAG TCT CCA G-3′	
TaqMan probe	5′-TGT CAC TGT CTT GTA CCC TTG TGC CTC G-3′	
Bax		
Forward primer	5′-AAG CTG AGC GAG TGT CTC AAG C-3′	L22474
Reverse primer	5′-ACT CGG AAA AAG ACC TCT CGG-3′	
TaqMan probe	5′-CTG GAC AGT AAC ATG GAG CTG CAG AGG A-3′	
Bcl-2		
Forward primer	5′-TGC CTT TGT GGA ACT GTA CGG-3′	M14745
Reverse primer	5′-CCA AAC TGA GCA GAG TCT TCA GAG-3′	
TaqMan probe	5′-CCC AGC ATG CGG CCT CTG TTT GAT TT-3′	

were also assessed in HeLa and MDR1-overexpressing Hvr100-6 cells. They were seeded on dishes at a density of 2 $\times 10^5$ cells/100 mm dish and 6 $\times 10^5$ cells/100 mm dish, respectively and cultured in VLB-free culture medium for 4 days. Subconfluent monolayers of HeLa and Hvr100-6 cells were treated for 4 h with VLB-free culture medium containing 0, 0.3, 1, 3, and 10 μM CDDP. Then, each cell line was incubated in VLB-free and CDDP-free culture medium for 1 day. Cells were scraped off from subconfluent monolayers of HeLa and Hvr100-6 cells, and the cell pellets were washed twice with ice-cold PBS(-). Aliquots of each cell pellet were subjected to real time quantitative PCR assay for Bax and Bcl-2 mRNA levels as described above, and remaining cell pellet was used to assay of caspase 3, 8, and 9 activities. Caspase 3 activities were measured using an assay kit (EnzChek™ caspase 3 assay kit#2, E-13184; Molecular Probes Inc.; Engene, OR, USA). Cell pellets were suspended in cell lysate buffer supplied with the kit, then subjected to freeze-thaw cycling. In accordance with the instruction manual, the lysates were centrifuged at 5,000 rpm for 5 min in a microcentrifuge, and the supernatants were assayed for caspase 3 activity. Caspase 9 activities were measured by alteration of the substrate according to the manufacture's instruction. Caspase 8 activities were also measured using an assay kit (caspase 8 fluorometric assay, BF2100; R&D systems; Minneapolis, MN, USA). The fluorescence was measured using a Fluoromark plate reader (Bio-Rad Laboratories; Hercules, CA, USA) with excitation at 355 nm and emission at 460 nm. The protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Bovine γ-globulin was used as the standard.

Data and Statistical Analysis

Data are shown as the means ± SE, except for the IC_{50} values, where the data are as the means ± SD. Multiple comparisons were performed for the data of HeLa and its MDR1-overexpressing sublines by analysis of variance (ANOVA) followed by Sheffé's test, provided the variance was similar. If this was not the case, a Sheffé-type test followed by Kruskal-Wallis test was performed. For comparisons between HeLa and Hvr100-6 cells, unpaired *t* test or Mann-Whitney's *U* test was performed. The p values of less than .05 were considered significant.

RESULTS AND DISCUSSION

Figure 1 shows the fluorescence histograms for HeLa and its MDR1-overexpressing sublines, Hvr1-1, Hvr10-6, and Hvr100-6 on which the results obtained with MDR1-specific monoclonal antibody MRK-16 were superimposed, suggesting serial induction of MDR1 by stepwise exposure to VLB. No shifts in signal were found with JSB-1 or MRPm6 antibodies suggesting that there was no abnormal orientation of the expression of MDR1 and no induction of MRP (data was not shown). The estimated IC_{50} values of VLB were 0.135 ± 0.036, 1.62 ± 0.245, 22.3 ± 6.45, and 67.0 ± 30.1 nM, respectively. Serial increases in estimated IC_{50} value was also found for VCR (0.597 ± 0.335, 11.5 ± 2.43, 107 ± 16.4, and 196 ± 118 nM, respectively), DXR (2.94 ± 0.535, 4.06 ± 1.05, 35.8 ± 11.5, and 153 ± 56.8 nM, respectively) and TXL (0.136 ± 0.238, 0.422 ± 0.0783, 131 ± 22.3, and 562 ± 255 nM, respectively),

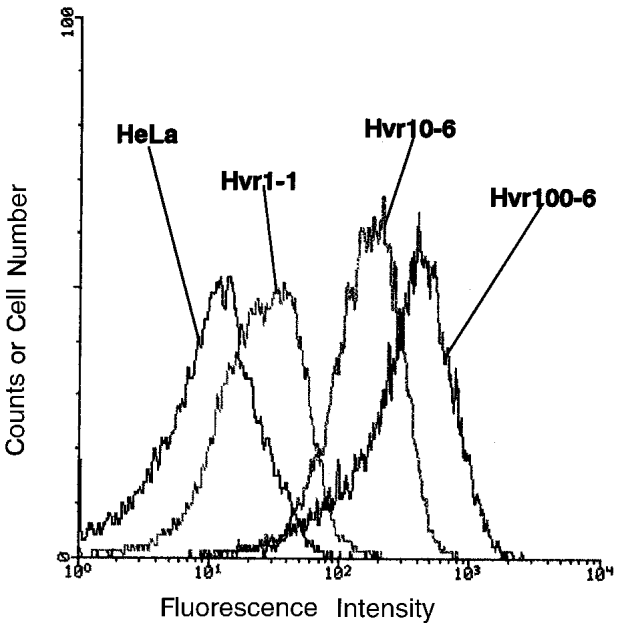

Fig. 1. Fluorescence histograms for HeLa and its MDR1-overexpressing sublines, Hvr1-1, Hvr10-6, and Hvr100-6 by flow cytometric analysis, in which the results obtained with the MDR1-specific monoclonal antibody MRK-16 are superimposed. No signal shift was found with JSB-1 and MRPm6 suggesting there was no abnormal orientation of the expression of MDR1 and no induction of MRP.

suggesting serial acquisition of MDR, whereas no alterations were detected for 5-FU (2.85 ± 0.747, 2.82 ± 0.927, 4.09 ± 0.778, 11.5 ± 2.33 μM, respectively), CDDP (0.574 ± 0.112, 0.445 ± 0.125, 0.626 ± 0.355, 0.223 ± 0.0458 μM, respectively) or CPA (1689 ± 247, 1744 ± 315, 1318 ± 971, 1388 ± 378 μM, respectively).

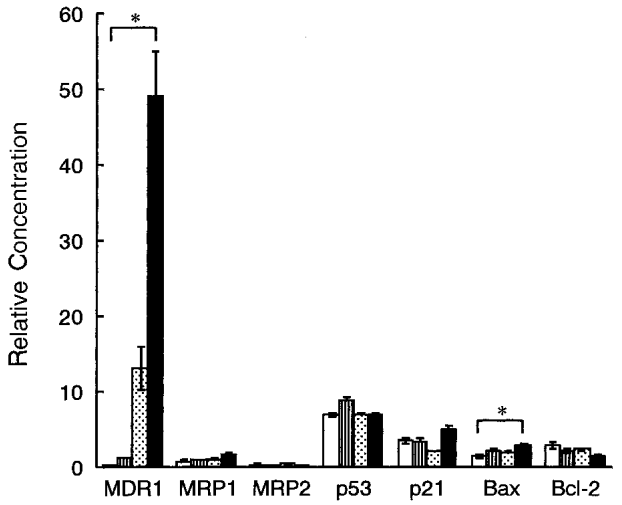

Fig. 2. Concentrations of mRNAs for MDR1, MRP1, MRP2, p53, p21, Bax, and Bcl-2 relative to GAPDH mRNA in HeLa and its *MDR1*-overexpressing sublines, Hvr1-1, Hvr10-6, and Hvr100-6 cells determined by real time quantitative PCR. The cells were exposed to 0, 1, 10, and 100 nM VLB, respectively. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Each column represents the mean ± SE of 3–4 independent experiments.

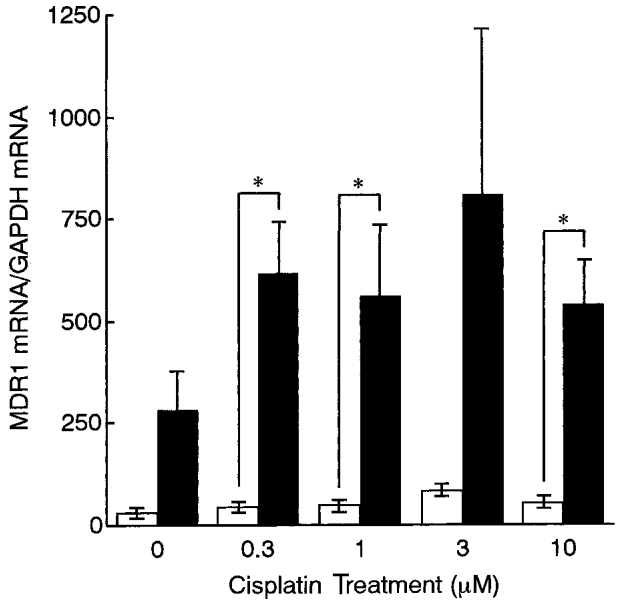

Fig. 3. Effects of cisplatin (CDDP) on the relative concentrations of MDR1 mRNA in HeLa and MDR1-overexpressing Hvr100-6 cells. The expression levels of mRNA were determined by real time quantitative PCR. The cells were cultured with VLB-free culture medium for 4 days, treated with 0–10 μM of CDDP for 4 h, and VLB-free and CDDP-free culture medium for 1 day. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Open and closed columns show the data for HeLa and Hvr100-6 cells, respectively. Each column represents the mean ± SE of 3–4 independent experiments.

Figure 2 shows the relative concentrations of MDR1, MRP1, MRP2, p53, p21, Bax, Bcl-2 mRNA to GAPDH mRNA level in HeLa and its MDR1-overexpressing sublines. These observations were made under continuous exposure to 0, 1, 10 and 100 nM VLB, respectively. The relative concentrations of MDR1 mRNA were serially increased, whereas those of MRP1 and MRP2 mRNA were not altered. There were no significant alterations in p53, p21, or Bcl-2 mRNA levels, suggesting that continuous stimuli causing MDR1 overexpression had no effect on the apoptotic cascade. Bax mRNA level was serially increased (Fig. 2), and the ratio of Bax mRNA to Bcl-2 mRNA was increased in Hvr100-6 cells (0.54 ± 0.13, 0.97 ± 0.08, 0.87 ± 0.07, and 2.00 ± 0.40, respectively ($p < 0.05$)). Various type of biologic events have been discussed by p53 activation and inactivation including cell growth arrest, apoptosis, DNA repair, angiogenesis, and stress responses (29,30). To date, it has been accepted that Bax is activated and Bcl-2 is repressed by p53 activation (29). A large amount of experimental evidences indicate that both p53 and MDR1 play important roles in chemoresistance (30). Transcriptional dependence of the MDR1 gene promoter by p53 has been strongly suggested, however, the relationship between MDR1 and p53 is conditional, that is, dependent on the cellular environments and the drug used (30). Taken together, it was suggested that increases in the Bax mRNA level or the ratio of Bax mRNA to Bcl-2 mRNA in MDR1-overexpression cells (Fig. 2) were due to transcriptional regulation by p53 activation. Since p53 mRNA was not altered in MDR1-overexpression cells (Fig. 2), this might be explained by p53 activation through the suppression of p53 inhibitor, mdm2 (29). In addition to p53, various types of transcription factors have been suggested, which are involved in MDR1 expression, such as NF-Y, C/EBP β, EGR1, NF-IL6, NF-R2, and NF-κB (10). There are substantial reports on these fac-

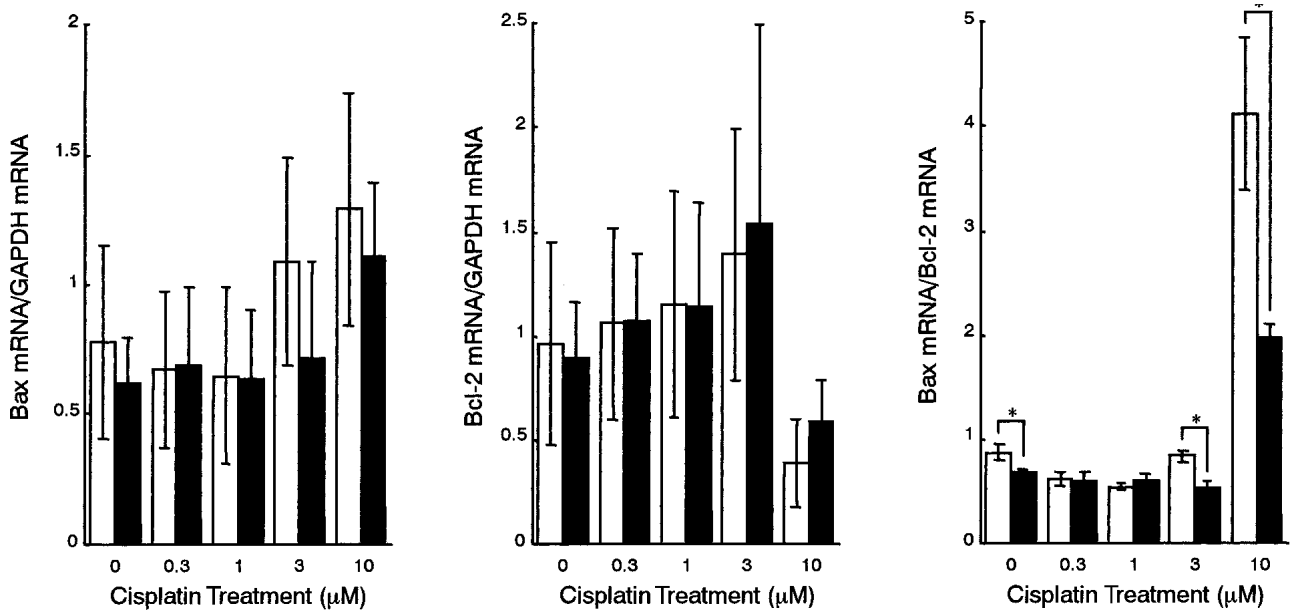

Fig. 4. Effects of cisplatin (CDDP) on the relative concentrations of mRNA for Bax (left panel), Bcl-2 (central panel) and the ratio of Bax mRNA to Bcl-2 mRNA (right panel) in HeLa and MDR1-overexpressing Hvr100-6 cells. The expression levels of mRNA were determined by real time quantitative PCR. The cells were cultured with VLB-free culture medium for 4 days, treated with 0–10 μM of CDDP for 4 h, and VLB-free and CDDP-free culture medium for 1 day. GAPDH was selected as an endogenous RNA control to normalize for differences in the amount of total RNA. Open and closed columns show the data for HeLa and Hvr100-6 cells, respectively. Each column represents the mean ± SE of 3–4 independent experiments.

Table II. Effects of Cisplatin (CDDP) on Caspase 3, 8, and 9 Activities (FL/μg Protein) in HeLa and MDR1-Overexpressing Hvr100-6 Cells

	Caspase 3		Caspase 8		Caspase 9	
	HeLa	Hvr100-6	HeLa	Hvr100-6	HeLa	Hvr100-6
CDDP 0 μM	1.08 ± 0.33	1.46 ± 0.35	10.91 ± 3.61	12.79 ± 3.32	5.27 ± 0.23	5.98 ± 0.20
CDDP 0.3 μM	2.02 ± 0.92	1.42 ± 0.29	8.64 ± 2.76	10.09 ± 3.45	4.74 ± 0.20	4.70 ± 0.04
CDDP 1.0 μM	1.94 ± 0.77	1.20 ± 0.08	8.48 ± 2.78	10.70 ± 3.85	4.61 ± 0.09	5.12 ± 0.19
CDDP 3.0 μM	3.00 ± 1.11	1.33 ± 0.18	7.06 ± 2.74	9.50 ± 3.30	4.54 ± 0.07	4.36 ± 0.33
CDDP 10.0 μM	26.31 ± 2.93	14.92 ± 1.86*	14.56 ± 3.41	23.77 ± 1.21*	21.82 ± 0.21	12.19 ± 0.21*

Note: The cells were cultured with VLB-free culture medium for 4 days, treated with 0–10 μM of CDDP for 4 h and VLB-free and CDDP-free culture medium for 1 day. Values are the mean ± SE of 3–6 independent experiments.
*Statistically significant when compared with HeLa cells ($p < .05$).

tors, and further investigations should be addressed on the relationship between the MDR1 expression and apoptotic mechanisms.

Here, the effects of apoptotic stimulation with CDDP on the mRNA levels of MDR1, Bax, and Bcl-2 as well as on caspase 3, 8, and 9 were also evaluated. It is noted that the cells were exposed to higher concentration of CDDP only for 4 h. Most antineoplastic drugs used in chemotherapy of leukemia and solid tumors including CDDP-induce apoptosis in drug-sensitive target cells (28). This exposure condition was supposed to cause the spontaneous cell death and was different from that, by which the antiproliferative effects were assessed. In this condition, CDDP displayed more cytotoxicity in HeLa than Hvr100-6 cells at 0.3–10 μM; for example, 34.8 ± 1.8% of HeLa cells and 57.6 ± 2.7% of Hvr100-6 cells remained alive at 5 μM of CDDP. Figure 3 shows the relative concentrations of MDR1 mRNA in HeLa and Hvr100-6 cells. As shown in Fig. 3, MDR1 mRNA was susceptible to upregulation by CDDP in both cell lines, suggesting that MDR1 mRNA was rapidly up-regulated to protect the cells against the apoptotic stimuli. The up-regulation was more marked in Hvr100-6 cells than in HeLa cells, with the difference between the cells being statistically significant at 0.3, 1, and 10 μM CDDP. Figure 4 shows the relative concentrations of Bax and Bcl-2 mRNA. Bax and Bcl-2 mRNA levels tended to increase by CDDP treatment for both cell lines (Fig. 4a and 4b). The ratios of Bax mRNA to Bcl-2 mRNA were also increased in both cell lines (Fig. 4c), but the increase was found at a higher CDDP concentration in Hvr100-6 cells than in HeLa cells. The remarkable increase in the ratio of Bax mRNA to Bcl-2 mRNA was found at 3–10 μM CDDP, which induced the cell death. Table II shows the activities of caspases 3, 8, and 9. Caspase 3, 8, and 9 activities were increased at 10 μM CDDP. The increases in caspase 3 and 9 activities were suppressed in Hvr100-6 cells as compared with HeLa cells whereas caspase 8 activity was higher in Hvr100-6 cells. In response to cellular damage and certain physiologic cues, cells enter the suicide program termed apoptosis, which is executed by caspases (22–24). Recent experimental observations have suggested that mitochondria contribute to many forms of apoptosis. A number of death stimuli target mitochondria and stimulate several proteins including cytochrome c, which binds to its adapter, Apaf-1, and activates activator caspase 9. Caspase 9 can signal downstream and finally activates effector caspase 3. The release of cytochrome c can be regulated by different Bcl-2 family proteins, such as Bax, Bid, Bcl-2, and Bcl-X(L. The first two potentiate cytochrome c release, whereas the latter two antagonize this event. Other death signaling pathways, such as those mediated by the CD95 death receptor, which directly activates activator caspase 8 and subsequently caspase 3, have been identified. The increases in Bax mRNA to Bcl-2 mRNA ratio and in levels of caspases 3 and 9 by CDDP treatment were suppressed in Hvr100-6 cells as compared with HeLa cells (Fig. 4 and Table II), which indicated that MDR1 suppresses apoptotic signaling, especially for that via the mitochondrial pathway. In contrast, MDR1 overexpression was accompanied by acceleration of the increase in caspase 8 activity caused by CDDP (Table II). This suggested that MDR1 was negatively related with the apoptotic signaling via the nonmitochondrial pathway, e.g., direct activation of caspases after death receptor binding, but further experiments should be performed to explain this finding.

In summary, it was demonstrated that: 1) MDR1 was rapidly upregulated when the cells were exposed to apoptotic stimuli by CDDP; 2) the increase in Bax mRNA to Bcl-2 mRNA ratio after treatment with CDDP was suppressed in MDR1-overexpressing cells; and 3) the increases in caspase 3 and 9 activities after treatment with CDDP were suppressed in MDR1-overexpression cells. Taken together, it was suggested that MDR1 up-regulated by apoptotic stimuli suppressed apoptotic signaling, presumably via the mitochondrial pathway.

REFERENCES

1. D. M. Bradshaw and R. J. Arceci. *J. Clin. Oncol.* **16**:2674–2690 (1998).
2. R. L. Juliano and V. Ling. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta.* **455**:152–162 (1976).
3. K. Ueda, A. Yoshida, and T. Amachi. Recent progress in P-glycoprotein research. *Anti-Cancer Drug Design* **14**:115–121 (1999).
4. C. Ramachandran and S. J. Melnick. Multidrug resistance in human tumors – molecular diagnosis and clinical significance. *Mol. Diagnosis* **4**:81–94 (1999).
5. K. Ueda, D. P. Clark, C. Chen, I. B. Roninson, M. M. Gottesman, and I. Pastant. The human multidrug resistance (mdr1) gene. *J. Biol. Chem.* **262**:505–508 (1987).
6. K. Ueda, C. Cardarelli, M. M. Gottesman, and I. Pastant. Expression of full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad. Sci. USA* **84**:3004–3008 (1987).
7. F. Thiebaut, T. Tsuruo, H. Hamada, M. M. Gottesman, I. Pastant, and M. C. Willingham. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc. Natl. Acad. Sci. USA* **84**:7735–7738 (1987).
8. H. Kusuhara, H. Suzuki, and Y. Sugiyama. The role of P-

glycoprotein and canalicular multispecific organic anion trasnporter in the hepatobiliary excretion of drugs. *J. Pharm. Sci.* **87**: 1025–1040 (1998).
9. Y. Tanigawara. Role of P-glycoprotein in drug disposition. *Ther. Drug Monit.* **22**:137–140 (2000).
10. C. J. Matheny, M. W. Lamb, K. L. R. Brouwer, and G. M. Pollack. Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy* **21**:778–796 (2001).
11. P. M. Chaudhary and I. B. Roninson. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell* **66**:85–94 (1991).
12. A. S.-F. Chong. P. N. Markham, H. M. Gebel, S. D. Bines, and J. S. Coon. Diverse multidrug-resistance-modification agents inhibit cytolytic activity of natural killer cells. *Cancer Immon. Immunother.* **36**:133–139 (1993).
13. W. T. Klimecki, B. W. Futscher, T. M. Grogan, and W. S. Dalton. P-Glycoprotein expression and function in circulating blood cells from normal volunteers. *Blood* **83**:2451–2458 (1994).
14. G. J. Randolph, S. Beaulieu, M. Pope, I. Sugawara, L. Hoffman, R. M. Steinman, and W. A. Muller. A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. *Proc. Natl. Acad. Sci. USA* **95**:6924–6929 (1998).
15. G. Raghu, S. W. Park, I. B. Roninson, and E. B. Mechetner. Monoclonal antibodies against P-glycoprotein, an MDR1 gene product, inhibit interleukin-2 release from PHA-activated lymphocytes. *Exp. Hematol.* **24**:1258–1264 (1996).
16. J. Drach, A. G. Gsur, G. Hamilton, S. Zhao, J. Angerler, M. Fiegl, N. Zojer, M. Raderer, I. Haberl, M. Andreeff, and H. Huber. Involvement of P-glycoprotein in the transmembrane transport of interleukin-2 (IL-2), IL-4, and interferon-γ in normal human T lymphocytes. *Blood* **88**:1747–1754 (1996).
17. C. K. van Kalken, G. Giaccone, P. van der Valk, C. M. Kuiper, M. M. N. Hadisaputro, S. A. A. Bosma, R. J. Scheper, C. J. L. M. Meijer, and H. M. Pinedo. Multidrug resistance gene (P-glycoprotein) expression in the human fetus. *Am. J. Pathol.* **141**: 1063–1072 (1992).
18. A. Macfarland, D. R. Abramovich, S. W. B. Ewen, and C. K. Pearson. Stage-specific distribution of P-glycoprotein in first-trimester and full-term human placenta. *Histochem. J.* **26**:417–423 (1994).
19. K. D. Bunting, J. Galipeau, D. Topham, E. Benaim, and B. P. Sorrentino. Transduction of murine bone marrow cells with an MDR1 vector enables ex vivo stem cell expansion, but these expanded grafts cause a myeloproliferative syndorome in transplated mice. *Blood* **92**:2269–2279 (1998).
20. R. W. Johnstone, A. A. Ruefli, and M. J. Smyth. Multiple physiological functions for multidrug transporter P-glycoprotein? *Trends Biochem. Sci.* **25**:1–6 (2000).
21. R. W. Johnstone, A. A. Ruefli, K. M. Tainton, and M. J. Smyth. A role for P-glycoprotein in regulating cell death. *Leukemia Lymphoma* **38**:1–11 (2000).
22. J. M. Adams and S. Cory. The Bcl-2 protein family: arbiters of cell survival. *Science* **281**:1322–1326 (1998).
23. M. Pollack and C. Leeuwenburgh. Apoptosis and aging: role of mitochondria. *J. Gerontology* **56A**:B475–482 (2001).
24. J. D. Robertson and S. Orrenius. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. *Crit. Rev. Toxicol.* **30**: 609–627 (2000).
25. M. J. Smyth, E. Krasovskis, V. R. Sutton, and R. W. Johnstone. The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. *Proc. Natl. Acad. Sci. USA* **95**:7024–7029 (1998).
26. R. W. Johnstone, E. Cretney, and M. J. Smyth. P-Glycoprotein protects leukemia cells against caspase-dependent, but not caspase-independent, cell death. *Blood* **93**:1075–1085 (1999).
27. N. Iida, K. Takara, N. Ohmoto, T. Nakamura, T. Kimura, A. Wada, M. Hirai, T. Sakaeda, and K. Okumura. Reversal effects of antifungal drugs on multidrug resistance in MDR1-overexpressing HeLa cells. *Biol.Pharm.Bull.* **24**:1032–1036 (2001).
28. Y. A. Hannun. Apoptosis and the dilemma of cancer chemotherapy. *Blood* **89**:1845–1853 (1997).
29. D. P. Guimaraes and P. Hainaut. TP53: a key gene in human cancer. *Biochimie* **84**:83–93 (2002).
30. J. A. Bush and G. Li. Cancer chemoresistance: the relationship between p53 and multidrug transporters. *Int. J. Cancer* **98**:323–330 (2002).