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Characterization of an etoposide-resistant human ovarian cancer cell line

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Abstract. Etoposide (VP-16) is one of the most important anticancer agents available and is used in many chemotherapeutic regimens. To characterize resistance to this drug, we established a VP-16-resistant human ovarian cancer cell line, SKOV3/VP, by continuous stepwise exposure of SKOV3 cells to VP-16. The degree of resistance to VP-16 of SKOV3/VP was about 25 times that of the parent cell line (SKOV3), and SKOV3/VP showed cross-resistance to teniposide, adriamycin, CPT-11, and vincristine. The accumulation of [^{3}H]-VP-16 observed in SKOV3/VP cells was about half that seen in SKOV3 cells, and the accumulation of Adriamycin by this resistant cell line was also lower than that of its parent. Overexpression of neither the multidrug resistance gene *mdr-1*, the multidrug-resistance-associated protein (*mrp*) gene, nor P-glycoprotein was detected using reverse transcriptase-polymerase chain reaction analysis and flow cytometry with MRK-16, a monoclonal antibody against P-glycoprotein. The topoisomerase II activity of nuclear extracts from SKOV3/VP cells was lower than that from the parental cells, as was the amount of DNA topoisomerase II, demonstrated by immunoblotting. These results suggest that the mechanism responsible for the multidrug resistance of this cell line may be attributable to changes on its DNA topoisomerase II and to its reduced accumulation of the drugs as compared with the parental line SKOV3.

Introduction

Etoposide (VP-16) is an effective drug against human ovarian cancer. Although the rate of response to regimens including VP-16 is high, the majority of patients eventually die due to tumor recurrence. One of the main reasons for chemotherapy failure is the development of drug resistance. Therefore, it is important to elucidate the mechanisms of drug resistance and to overcome them. Possible mechanisms involved in VP-16 resistance include decreased drug accumulation, detoxification by intracellular peptides and changes in DNA damage or repair [10]. The target of VP-16 is believed to be DNA topoisomerase II (topo II), which cleaves the tangled double-stranded DNA and rejoins it [2, 17, 22]. Changes in this activity and/or in intracellular topo II levels may also be associated with VP-16 resistance [17].

The aim of this study was to establish a VP-16-resistant human ovarian cancer cell line, SKOV3/VP, using the method of stepwise exposure of SKOV3 cells to increasing concentrations of the drug and to investigate the mechanisms responsible for its resistance to VP-16.

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Materials and methods

Chemicals. We obtained [^{3}H]-VP-16 (radiochemical purity, 90% as measured by high-performance liquid chromatography) from Monavek Biochemicals (Brea, Calif.), VP-16 and cisplatin were donated by Bristol-Myers Squibb Ltd. (Tokyo, Japan) and (4S)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino)carbonyloxy]-1H-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-3,14(4H,12H)-dionehydrochloridetrihydrate (CPT-11) was obtained from Yakult Co., Ltd. (Tokyo, Japan). Bleomycin was obtained from Nihon Kayaku Co., Ltd. (Tokyo, Japan); vincristine (VCR), from Shionogi Co., Ltd. (Osaka, Japan); 3′-diamino-3′-morpholino-13-deoxo-10-hydroxycarminomycin (MX2), from Kirin Brewery Co. Ltd. (Tokyo, Japan); and Adriamycin (ADM), from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Plasmid DNA pBR322 and kinetoplast DNA (kDNA) were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan), and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless stated otherwise.

Cell lines. The SKOV3 cell line, which was isolated in 1973 from the ascitic fluid of a patient with an ovarian cancer, was obtained from the American Type Culture Collection (Rockville, Md.). The human small-cell lung-cancer cell line H69, established at the National Cancer Institute (USA), was obtained from Dr. Y. Shimosato (National Cancer Center Research Institute, Tokyo, Japan), and H69/VP, which is a VP-16-resistant subline of the H69 cell line, was prepared as reported previously [18]. The topo II antiserum was a gift from Dr. L.F. Liu (Johns Hopkins Medical School, Baltimore, Md.).

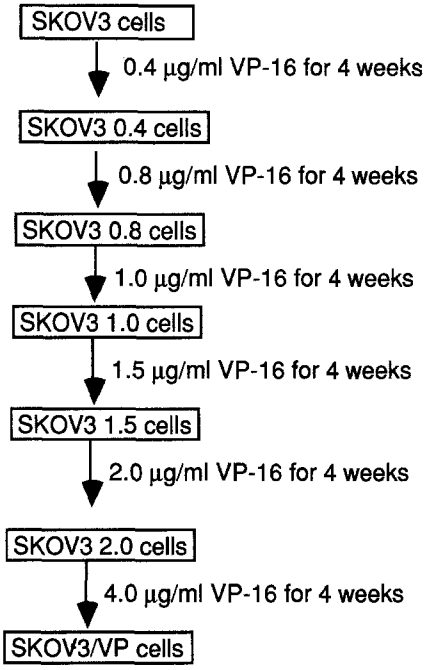

Fig. 1. Illustration of the method used to establish a VP-16-resistant SKOV3 cell line

Establishment of a VP-16-resistant human ovarian cancer cell line. We established a VP-16-resistant cell line by stepwise exposure of SKOV3 cells to increasing concentrations of the drug. The cells were propagated in RPMI 1640 medium supplemented with 10% (w/v) heat-inactivated fetal bovine serum (RPMI/FBS; Immuno-Biological Laboratories, Fujioka, Japan), penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37° C in an incubator with a highly humidified atmosphere containing 5% (v/v) CO_2. The VP-16-resistant cell line was selected as a result of stepwise and continuous exposure of SKOV3 cells to the drug, the starting and finishing concentrations of which were 0.1 and 4.0 μg/ml, respectively (Fig. 1). Then we cloned a VP-resistant subline by using a limiting dilution method three times. The VP-16-resistant cell line thus established was capable of growing in the medium containing VP-16 at 4.0 μg/ml and was designated SKOV3/VP. The cells were used in the experiments outlined below after being cultured in drug-free RPMI 1640 medium for 7 days as described above. The VP-16 resistance of SKOV3/VP was confirmed to be maintained at a constant level in VP-16-free RPMI medium for at least 6 months.

Drug sensitivity test. We determined the sensitivity of the parent and VP-16-resistant cell lines to anticancer agents using a growth-inhibition assay [20]. In brief, single-cell suspensions of SKOV3 and SKOV3/VP cells were harvested during the exponential growth phase, seeded (4,000 cells/well) into 96-well tissue-culture plates (Microtest III, Falcon 3072), and incubated at 37° C for 4 days, after which 20 μl 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 5 mg/ml) in phosphate-buffered saline (PBS) was added to each well and the plates were incubated at 37° C for 4 h. Then the medium was aspirated as completely as possible without disturbing the formazan crystals and cells on the plastic surface. Dimethylsulfoxide (DMSO, 200 μl) was added to each well, the plates were agitated on a plate shaker for 5 min to dissolve the formazan crystals, and the optical density of the solution in each well was measured at 562 nm using an automated microplate reader (model EL340, Bio-Tek Instruments Inc., Winooski, Vt.). The IC_{50} value for each drug, defined as the concentration that reduced the optical density by 50% as compared with the appropriate control, was determined graphically from the relevant concentration-response curve.

Accumulation study. The accumulation, net uptake, and efflux of [^{3}H]-VP-16 by the cell lines were determined using the method described by Ferguson and Cheng [8]. The parent and VP-16-resistant cells were harvested and 5×10^6 cells/ml were incubated with 30 μ*M* [^{3}H]-VP-16 at 37° C for up to 120 min. For the net-uptake study, 0.5 ml cell suspension was removed at each required time point, transferred to a fresh tube, and washed three times with ice-cold PBS; then, 0.5 ml 1 *N* NaOH was added to dissolve the cell pellet, followed by 0.5 ml 1 *N* HCl to neutralize the NaOH, and the protein content of the resulting solution was measured using the bicinchoninic acid-protein assay (Pierce Chemical Co., Rockford, Ill.). The suspension that remained after removal of the 0.5-ml sample was transferred to an equal volume of ACS II solution (Amersham Japan Co., Tokyo, Japan), and the radioactivity was measured using a Beckman liquid scintillation counter (LS 3801, Beckman Instruments Inc., Irvine, Calif.). For the efflux study, the cells were incubated with RPMI-FBS containing 30 μ*M* [^{3}H]-VP-16 at 37° C for 120 min and washed twice with drug-free RPMI-FBS, and the radioactivity was measured as described above.

The intracellular accumulation of ADM and MX2 by the cell lines was determined using the method of Tsuruo et al. [25] as modified by Horichi et al. [12, 24]. In brief, exponentially growing cells were collected; adjusted to 1×10^6 cells/ml; incubated with ADM or MX2 at 10, 30, 50, and 100 μg/ml for 120 min; and then washed with cold PBS. The absorbed anthracyclines were extracted with 200 μl DMSO, the cellular protein was precipitated by the addition of 1.8 ml absolute methanol, and the fluorescence intensities of the extracts were measured with a fluorescence spectrophotometer (Spectrophotometer FP-777; Japan Spectroscopic Co., Tokyo) at excitation and emission wavelengths of 470 and 550 nm, respectively.

Preparation of nuclear extracts. Crude nuclear extracts were prepared as described by Deffie et al. [7]. Cells were collected by centrifugation, washed twice with cold NB [2 m*M* K_2HP0_4, 5 m*M* $MgCl_2$, 150 m*M* NaCl, 1 m*M* ethylene glycol-bis(β-aminoethyl ether), *N*,*N*,*N*′,*N*′-tetraacetic acid, and 0.1 m*M* dithiothreitol, adjusted to pH 6.5], and resuspended in 1 ml cold NB, after which 9 ml cold NB containing 0.35% (w/v) Triton X-100 and 1 m*M* phenylmethylsulfonyl fluoride was added. The cell suspension was kept on ice for 10 min and then washed with cold NB containing 0.35% (w/v) Triton X-100. The nuclear protein was eluted for 1 h at 4° C with cold NB containing 0.35 *M* NaCl. A solution of nuclear protein was obtained by centrifugation of the resulting elute at 17,000 *g* for 10 min.

Flow cytometry. A murine anti-human P-glycoprotein monoclonal antibody, MRK-16, was provided by Dr. T. Tsuruo of Tokyo University (Tokyo, Japan) and a fluorescein isothiocyanate (FITC)-conjugated F(ab′)2 fragment of rabbit anti-mouse immunoglobulin preparation was purchased from DACO Japan Co. (Kyoto, Japan). The SKOV3/VP and SKOV3 cells (1×10^6) were washed with PBS and incubated for 30 min with 50 μl MRK-16 (10 ng/ml) at 4° C. They were also incubated under identical conditions with nonspecific murine immunoglobulins (10 ng/ml) at 4° C and used as isotype controls. The cells were washed with cold PBS and incubated for a further 30 min with 50 μl FITC at 4° C. The fluorescence intensity of the FITC for each cell line preparation was measured by flow cytometry (Hewlett-Packard model 9000–340 computer interfaced with FACScan).

Quantitative analysis of mdr-1 and multidrug-resistance-associated protein gene expression using the reverse transcriptase-polymerase chain reaction. The total cellular RNA was obtained using the acid guanidinium thiocyanate-phenol-chloroform extraction method [3]. The total cellular RNAs (1 mg) of the SKOV3, SKOV3/VP, and H69/VP cells were annealed with a random hexadeoxynucleotide primer (Takara Shuzo), and each mixture (14 μl) was transcribed with M-MLV reverse transcriptase (RT; Batheda Research Laboratories, Gaithersburg, Md.) containing a ribonuclease inhibitor, RNasein.

The names and nucleotide sequences of the primers used for cDNA amplification are listed in Table 1. Aliquots (5 μl) of each RT mixture were subjected directly to the polymerase chain reaction (PCR) to amplify the *mdr-1*, multidrug-resistance-associated protein (*mrp*), β_2-microglobulin (β2MG), and β-actin cDNA regions in each mixture (50 μl). For quantitation, 0.5 μCi (1 Ci = 37 GBq) [α-^{32}P]-deoxycytidine triphosphate (Amersham) was added to each reaction mixture. The resulting PCR products were diluted with 20 vols. of loading solution

Table 1. Oligonucleotides used for amplification of cDNA

mRNA species	Sequence	Size of PCR product (bp)	Reference
MDR-1:			
(upstream)	5′ CCCATCATTGCAATAGCAGG 3′	167	Noonan et al. [21]
(downstream)	5′ GTTCAAACTTCTGCTCCTGA 3′		
MRP:			
(upstream)	5′ GGGAATTCTGGGACTGGAATGTCACG 3′	245	Zaman GJR et al. [26]
(downstream)	5′ CGGGATCCAGGAATATGCCCCGACTTC 3′		
Beta$_2$-microglobulin:			
(upstream)	5′ ACCCCCACTGAAAAAGATGA 3′	120	Kinoshita et al. [15]
(downstream)	5′ ATCTTCAAACCTCCATGATG 3′		
Beta-actin:			
(upstream)	5′ TACATGGCTGGGGTGTTGAA 3′	218	Noonan et al. [21]
(downstream)	5′ AAGAGAGGCATCCTCACCCT 3′		

Table 2. Morphological characteristics of the cell lines

	SKOV3	SKOV3/VP
Diameter (μ*M*)	13.5	14.2
Cell Volume (pl)	1.3	1.49
Doubling time (h)	41	40.9
Protein content (μg/10^6 cells)	267	251

Diameter and volume were measured using a Coulter channelizer 256. There was no significant difference between SKOV3 and SKOV3/VP

Table 3. Responses of SKOV3 and SKOV3/VP cell lines to various anticancer agents

Drugs	IC_{50} (μg/ml)		RR[a]
	SKOV3	SKOV3/VP	
VP-16	0.76	19	25
VM-26	0.21	2.5	12
ADM	0.13	0.74	5.7
MX2	0.12	0.19	1.6
mAMSA	9.4	8.7	0.93
CPT-11	1.8	10	6.3
VCR	0.0008	0.0027	3.3
paclitaxel	0.0072	0.0061	0.85
Cisplatin	1.5	1.6	1.1
Bleomycin	5.3	4.6	0.82
Mitomycin	0.36	0.72	2

IC_{50}, Drug concentration that inhibits cell growth by 50%
[a] Relative resistance (RR) equals the IC_{50} value for the resistant cell line divided by the IC_{50} value for the parental cell line

containing 20 m*M* ethylenediaminetetraacetic acid (EDTA), 0.05% (w/v) bromophenol blue and xylene cyanol, 0.1% (w/v) sodium dodecyl sulfate (SDS), and 5% (w/v) glycerol, applied (1 μl/lane) to a 6% (w/v) polyacrylamide gel, and electrophoresed at 15 W for 35 min. The gel was dried on filter paper and the radioactivity of each band was determined using a BAS2000 image analyzer (Fuji Film Co. Ltd., Tokyo).

DNA topo II activity. The reaction mixture comprised 50 m*M* TRIS-HCl (pH 8.0), 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 0.5 m*M* EDTA, 10 μg bovine serum albumin/ml, and 1 m*M* adenosine 5′-triphosphate (pH 7.7). The catalytic activity of topo II was determined by decatenation of kDNA as described elsewhere [14, 19]. Crude nuclear extracts from the parent and VP-16-resistant cells, 1 μg kDNA, and the reaction mixture were incubated at 30° C for 30 min, after which 5 μl dye solution containing 5% (v/v) SDS, 0.025% (v/v) bromophenol blue and 50% (v/v) glycerol was added and the samples were applied to 1% (w/v) agarose gel and electrophoresed using 40 m*M* TRIS-2 m*M* acetate-1 m*M* EDTA (pH 8.0) at 60 V for 3 h. The gels were stained with ethidium bromide and photographed under UV light.

Immunoblotting. The nuclear protein was extracted from each cell line as described above. Each sample was mixed with an equal volume of 2 × sample buffer [0.25 *M* TRIS-HCl, 2% (w/v) SDS, 30% (v/v) glycerol, 10% (w/v) 2-mercaptoethanol, and 0.01% (w/v) bromophenol blue, pH 6.8], heated for 3 min at 95° C, and subjected to electrophoresis on a 7.5% (w/v) SDS-polyacrylamide gel (SDS-PAG PLATE 10, Daiichi Pure Chemicals, Tokyo, Japan). The gels were then equilibrated for 15 min with transfer buffer [25 m*M* tris, 192 m*M* glycine, 0.02% (w/v) SDS, and 20% (v/v) methanol, pH 8.3] and transferred electrophoretically from the polyacrylamide gel to a polyvinylidene difluoride membrane (0.45 μm; Nihon Millipore Kogyo, Tokyo, Japan). The membranes were blocked by incubation for 2 h at room temperature with 5% (v/v) bovine serum albumin in PBS, incubated at 4° C for 2 h with anti-topo II antibody, diluted with PBS (1:500), and then rinsed with PBS containing 0.1% (v/v) Triton X-100 (PBS-T). Next, the membranes were incubated with anti-rabbit immunoglobulin G (Fc)-horseradish peroxidase conjugate diluted with PBS-T containing 1% (w/v) bovine serum albumin for 1 h at room temperature and finally washed with PBS-T. The membranes were amplified with BLAST, a blotting amplification system (Dupont NEN Research Product, Boston, Mass.), developed with Western blotting-enhanced chemiluminescence-detection reagents (Amersham).

Results

General characteristics

The SKOV3 cell line that was resistant to VP-16, termed SKOV3/VP, was subcultured in RPMI 1640 medium containing VP-16 at 4.0 μg/ml, in which it showed predominantly attached growth, as did the parental SKOV3. The mean cell diameter and volume, protein content, and doubling time of SKOV3/VP were almost identical to those of the parent cell line (Table 2).

Drug sensitivity

Table 3 shows the cytotoxic effects of various anticancer agents on the parent and VP-16-resistant cells. The IC_{50} value of VP-16 was 0.76 μg/ml for the parent line and 19 μg/ml for the resistant line. Therefore, SKOV3/VP was 27

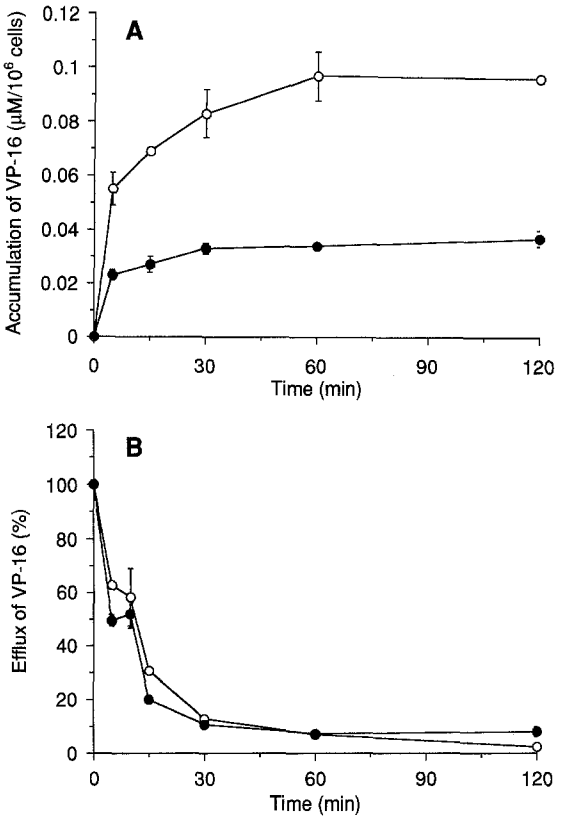

Fig. 2A, B. Time-course study of the accumulation (**A**) and efflux (**B**) of VP-16 by SKOV3 (*open circles*) and SKOV3/VP (*filled circles*) cells after the addition of 30 μ*M* [^{3}H]-VP-16 to the medium. After incubation with the drug for 120 min, the cells were cultured in VP-16-free medium for the efflux study (**B**)

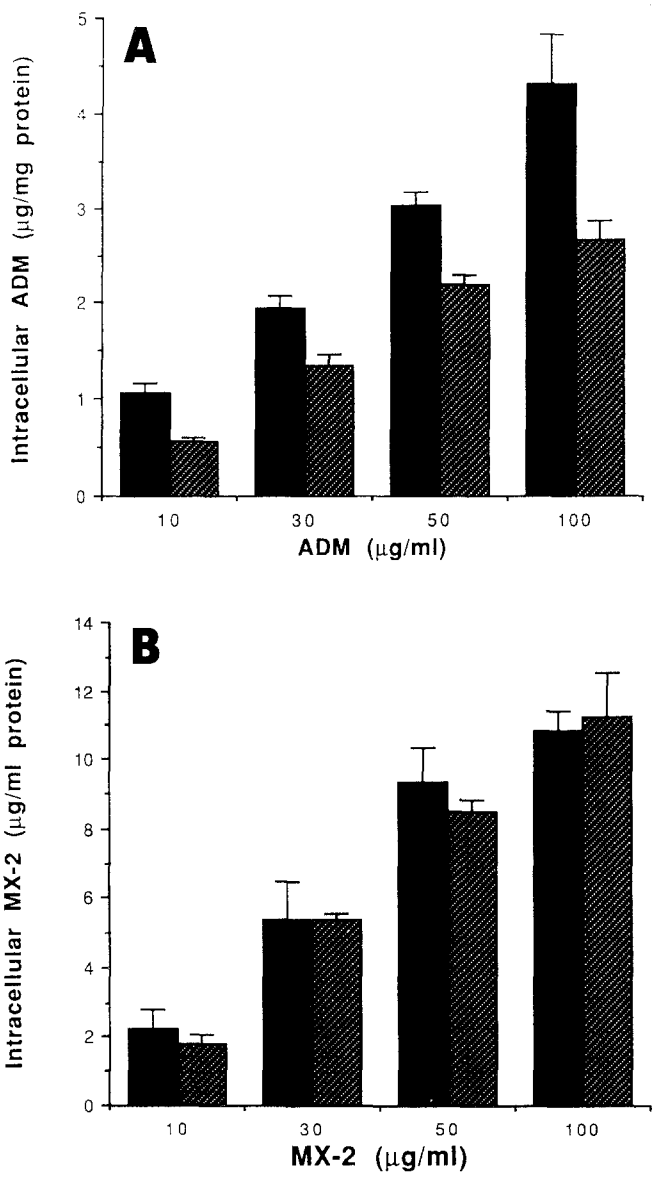

Fig. 3A, B. Concentration-dependent accumulation of drug after incubation for 2 h with ADM (**A**) and MX2 (**B**) by SKOV3 (*filled bars*) and SKOV3/VP (*hatched bars*) cells. Data represent mean values ± SD for 3 independent experiments

times more resistant than SKOV3 to the drug. The resistant cell line demonstrated cross-resistance to teniposide (VM-26), ADM, CPT-11, and VCR but not to the anthracycline derivative MX2 [12] or paclitaxel. The growth-inhibition assay results showed that SKOV3/VP had MDR phenotype characteristics different from those of H69/VP.

Accumulation study

Figure 2 shows the drug accumulation for SKOV3 and SKOV3/VP. The accumulation of VP-16 by both cell lines reached a plateau within 60 min (Fig. 2A), when the intracellular VP-16 concentration of SKOV3 was 5 times that of SKOV3/VP. In the efflux study, the intracellular VP-16 levels of both cell lines decreased steeply, but the efflux of VP-16 from SKOV3/VP was not increased as compared with that from SKOV3 (Fig. 2B).

The SKOV3/VP cell line showed cross-resistance to ADM. The intracellular ADM concentrations measured after the addition of 10–100 μ*M* to the culture medium are shown in Fig. 3A. At steady state, the intracellular ADM concentrations determined in SKOV3 and SKOV3/VP were 1.03 and 0.52 μg/mg protein, respectively, after incubation with ADM at 10 μg/ml. When ADM was used at 30, 50, or 100 μg/ml, the intracellular drug concentrations of SKOV3/VP were also significantly lower than those of SKOV3 ($P < 0.01$, Students *t*-test). Accumulation of ADM by SKOV3/VP was about 50%–70% of that by SKOV3. Although MX2 is a derivative of ADM, SKOV3/VP cells showed no cross-resistance to it. The intracellular MX2 concentrations determined after the addition of 10–100 μ*M* to the culture medium are shown in Fig. 3B. At each concentration of MX2, the intracellular concentrations measured in SKOV3 and SKOV3/VP did not differ significantly.

P-glycoprotein and mdr-1 gene expression

The flow-cytometric data obtained for P-glycoprotein (P-gp) detection and presented as overlaid histograms are shown in Fig. 4. For H69/VP cells, which were used as a positive control, there were distinct differences between the

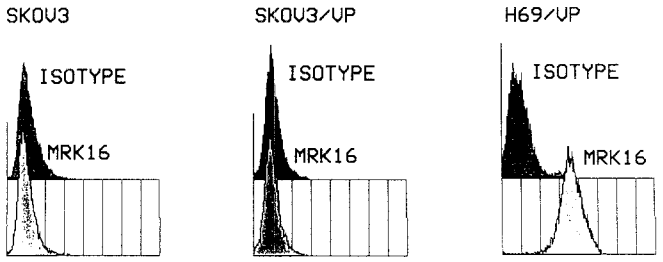

Fig. 4. P-gp expression by MRK-16-treated and isotype control SKOV3 and SKOV3/VP cells. In each histogram, the horizontal axis represents the fluorescence of FITC (a single scale corresponds to 0.5 log) and the vertical axis represents the number of cells. *Left*, SKOV3; *center*, SKOV3/VP; *right*, H69/VP. The H69/VP cell line was used as a positive control comprising typical *mdr-1*-expressing cells

fluorescence of the MRK-16-treated cells and that of the isotype controls, and this cell line was adjudged positive for P-gp expression. For the SKOV3/VP cells, virtually no difference was observed between the MRK-16-treated and the isotype control cells in any experiment and, therefore, SKOV3/VP was considered negative for P-gp expression.

To confirm that SKOV3/VP did not possess the P-gp-mediated multidrug resistance phenotype, we investigated *mdr-1* mRNA expression in SKOV3/VP using RT-PCR analysis. VP-16-resistant H69/VP cells were used as a positive control, as they are typical *mdr-1*-expressing cells. The cDNAs were synthesized from the total cellular RNAs isolated from the SKOV3, SKOV3/VP, and H69/VP cell lines, and the amplified *mdr-1*, β2MG, and β-actin fragments were separated by electrophoresis. Figure 5A shows an autoradiogram of the PCR products as analyzed using the sequential cycling method. The amplified β2MG and β-actin mRNAs were used as internal controls. Increased amounts of β2MGand β-actin mRNA with increasing cycle numbers were observed in the SKOV3, SKOV3/VP, and H69/VP cell lines. In the H69/VP cell line, increased expression of *mdr-1* mRNA was detected, whereas no *mdr-1*

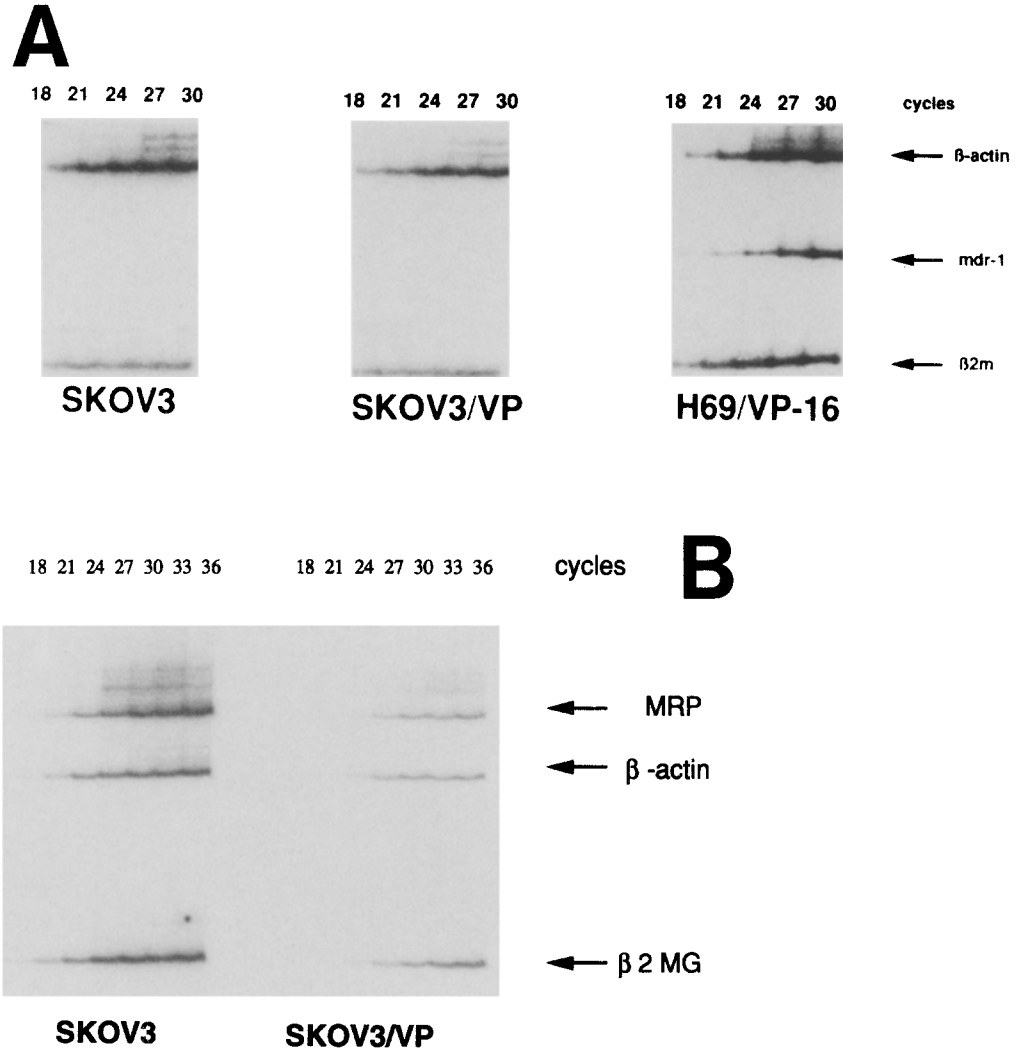

Fig. 5. A Expression of *mdr-1* mRNA by SKOV3/VP as analyzed using RT-PCR. An autoradiogram of the PCR products, analyzed using the sequential cycling method, is shown. The H69/VP cells were used as a positive control, and amplified β-actin and β2-MG mRNA were used as internal controls. Increased amounts of β-actin and β2-MG mRNA with increasing cycle number were observed in SKOV3, SKOV3/VP, and H69/VP cells. In the H69/VP cells, marked expression of *mdr-1* mRNA was detected, whereas no *mdr-1* mRNA was detected in either the SKOV3 parental cells or the SKOV3/VP cells. **B** Expression of *mrp* mRNA by SKOV3/VP as analyzed using RT-PCR. Amplified β-actin and β2-MG mRNA were used as internal controls and increased amounts of both were observed with increasing cycle number in the SKOV3, SKOV3/VP, and H69/VP cells. The magnitude of the increase in *mrp* mRNA expression was the same as that observed in the internal controls

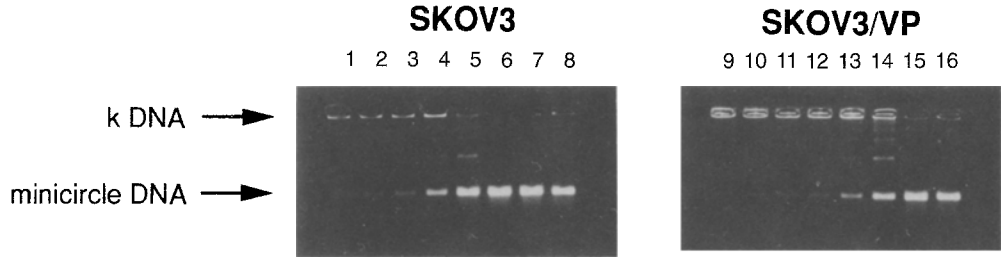

Fig. 6. Decatenation assay of DNA topo II in SKOV3 and SKOV3/VP cells. The reaction mixture, kDNA, and the nuclear extract were incubated at 30° C for 30 min. *Lanes 1, 9,* no nuclear extract; *lanes 2, 10,* $0.5\times(1/2)^6$ μg; *lanes 3, 11,* $0.5\times(1/2)^5$ μg; *lanes 4, 12,* $0.5\times(1/2)^4$ μg; *lanes 5, 13,* $0.5\times(1/2)^3$ μg; *lanes 6, 14,* $0.5\times(1/2)^2$ μg; *lanes 7, 15,* $0.5\times(1/2)$ μg; *lanes 8, 16,* 0.5 μg

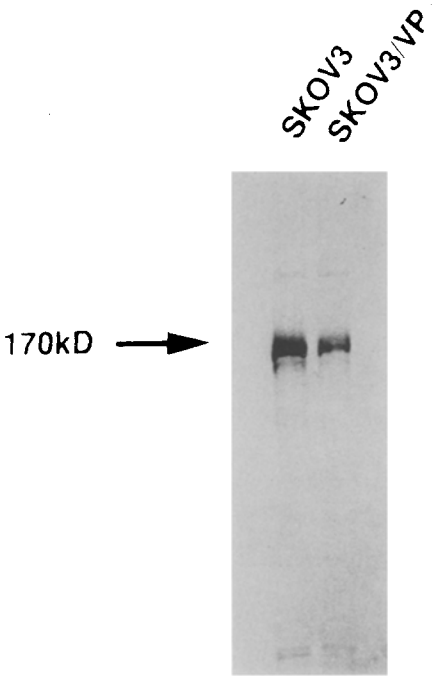

Fig. 7. Immunoblotting of DNA topo II from SKOV3 and SKOV3/VP cells

mRNA was detected in the parental SKOV3 line or the SKOV3/VP cell line. Therefore, the SKOV3/VP cell line did not appear to possess a typical multidrug resistance phenotype.

mrp Gene expression

The multidrug-resistant cell line SKOV3/VP did not overexpress P-gp. A multidrug-resistance-associated protein (MRP) has been cloned in a multidrug-resistant cell line, H69AR, by Cole et al. [5, 23], and amplification of the *mrp* gene has been suggested to be associated with the expression of drug resistance in solid-tumor cell lines. We compared *mrp* gene expression by SKOV3 and SKOV3/VP using RT-PCR (Fig. 5B) and found that *mrp* mRNA was expressed by both the parental SKOV3 line and the SKOV3/VP cell line, but the latter did not appear to overexpress *mrp* mRNA.

Topo II activity

The DNA topo II activities of SKOV3 and SKOV3/VP were determined by decatenation of kDNA. In the decatenation assay (Fig. 6), no free minicircle was formed by the samples that contained nuclear extract (lanes 1, 9). Minicircle formation by both cell lines increased as the amount of crude nuclear extract increased. However, fewer free minicircles were formed by SKOV3/VP than by SKOV3 with the same amount of nuclear extract; twice the amount of nuclear extract was required for SKOV3/VP to produce the same number of minicircles as SKOV3. This suggests that the catalytic activity of SKOV3/VP topo II was approximately half that of the SKOV3 enzyme.

Topo II content

The topo II contents of the parent and VP-16-resistant cell lines were determined by immunoblotting analysis using topo II rabbit antiserum. The amount of the topo II homodimer subunit with a relative molecular mass of 170,000 Da detected in the SKOV3/VP nuclear extract was considerably lower than that found in SKOV3 extract (Fig. 7). When these bands were subjected to densitometry, the intensity of the SKOV3/VP topo II was approximately half that of the SKOV3 enzyme. Therefore, the lower topo II protein content was considered likely to be the reason for the lower SKOV3/VP topo II activity.

Discussion

We established a VP-16-resistant human ovarian cancer cell line, SKOV3/VP, by stepwise selection of SKOV3 cells with VP-16. Other VP-16-resistant cell lines that have been similarly selected include a human nasopharyngeal cancer (human KB cell) cell line established by Ferguson et al. [9], a human squamous carcinoma of the tongue (HN-l) cell line established by Hill et al. [11], and the H69/VP line established in our laboratory [18]. All ot these VP-16-resistant cell lines show cross-resistance to ADM and VCR. The SKOV3/VP cell line showed cross-resistance to VM-26, ADM, VCR, and CPT-11 and had a multidrug resistance (MDR) phenotype but overexpressed neither P-gp nor *mdr-1* genes. Therefore, SKOV3/VP was considered to possess

non-P-pg-mediated MDR. Overexpression of the *mrp* gene has been proposed to be one of the characteristic features of non-P-gp-mediated MDR in some solid-tumor cell lines [23]. However, this would not appear to be the mechanism responsible for the MDR of SKOV3/VP, as it did not overexpress the *mrp* gene.

SKOV3/VP showed cross-resistance to a topo I inhibitor, CPT-11. This phenomenon seems to be unique for MDR, because CPT-11 shows high sensitivity to typical MDR cells and CPT-11 resistance is due to the decreased topo I activity, which is sometimes caused by point mutation of the topo I gene [13, 16]. To elucidate the mechanism of cross-resistance to CPT-11, we measured the topo I activity and protein amount of topo I in SKOV3/VP. The topo I activity of SKOV3/VP was the same as that of SKOV3. No difference was found in the amount of topo I protein between SKOV3/VP and SKOV3 by immunoblotting (data not shown). In addition, we compared the growth-inhibitory effect of SN-38, which is an active metabolite of CPT-11, on SKOV3 and SKOV3/VP. SN-38 showed the same growth-inhibitory effect against SKOV3 and SKOV3/VP. At present, we cannot explain the mechanism of cross-resistance to CPT-11.

In this study, the accumulation of both VP-16 and ADM, but not MX2, observed in SKOV3/VP cells was lower than that seen in SKOV3 cells. These results are consistent with the cross-resistance to ADM but not to MX2 shown by the SKOV3/VP line and suggest that decreased drug accumulation is a possible cause of its MDR.

The VP-16 efflux rates demonstrated by SKOV3/VP and SKOV3 in the time-course study were the same (Fig. 2B). However, the initial VP-16 influx rate was lower for SKOV3/VP than for SKOV3 (Fig. 2A). This accumulation pattern appears to differ from that of cells with the typical MDR phenotype.

The mechanisms of topo II-mediated VP-16 resistance have been suggested to involve qualitative changes in the activity of topo II and quantitative changes in its content [10]. In our study, both the topo II content and the topo II catalytic activity of SKOV3 were about half those of SKOV3/VP. Furthermore, no point mutation of topo II was detected by single-strand conformation polymorphism analysis of the SKOV3/VP PCR products (data not shown). Topo II changes are believed to be involved in MDR; this phenomenon has been called "atypical MDR" by some researchers [1,6]. Therefore, the MDR of SKOV3/VP can be considered to be "atypical."

SKOV3/VP is a non-P-pg and non-*mrp*-expressing, VP-16 resistant ovarian cancer cell line with multifactorial mechanisms of drug resistance such as decreased accumulation and decreased topo II activity and content. It also shows cross-resistance to CPT-11, a typical topo I inhibitor, although its topo I activity and content did not differ from those of SKOV3. There is no VP-16-resistant line with these kinds of characteristics. VP-16 is an essential drug for the treatment of ovarian cancer. A clinical evaluation of samples from lung cancer patients demonstrated that overexpression of the P-gp and *mdr-1* genes did not always correlate with clinical resistance [4]. The VP-16-resistant cell line that we developed (SKOV3/VP) may be a useful tool for studying non-P-gp-mediated MDR.

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