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Overexpression of Multidrug Resistance Protein Gene in Human Cancer Cell Lines Selected for Drug Resistance to Epipodophyllotoxins

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Overexpression of either the multidrug resistance 1 (MDR1) gene or multidrug resistance protein (MRP) gene is involved in acquisition of multidrug-resistant phenotypes in human cancer cells. In this study we examined whether selection for resistance to the epipodophyllotoxins, etoposide/teniposide (VP16/VM26), could induce overexpression of MDR1 or MRP. We have previously isolated two VP16/VM26-resistant KB cell lines. Two VP16/VM26-resistant KB cell lines, KB/VM-1 and KB/VM-4, which were selected by stepwise exposure to VM26 had decreased accumulation of [^{3}H]VP16 and increased levels of MRP, but no apparent expression of MDR1 gene was observed. Another VP16/VM26-resistant KB cell line, KB/VP-4, which was further isolated from a VP16-resistant KB cell line, KB/VP-2, had decreased accumulation of [^{3}H]VP16 and showed overexpression of MRP gene, but not that of MDR1 gene. We also isolated a VP16-resistant cell line, IN157/VP-1, from a human glioma cell line IN157. IN157/VP-1 cells showed decreased accumulation of [^{3}H]VP16 and overexpression of MRP gene, but not of MDR1. These findings suggest that selection for resistance to VP16/VM26, preferentially induces overexpression of MRP gene.

Key words: Multidrug resistance — MRP — Etoposide/Teniposide — ABC superfamily

The acquisition of multidrug resistance is often associated with the overexpression of the 170 kDa P-glycoprotein (P-gp) encoded by the multidrug resistance 1 (MDR1) gene,[1–5] and also with that of the multidrug resistance protein (MRP) gene, which encodes a 190-kDa transport protein of the membrane ATP-binding cassette (ABC) superfamily.[6] Cells transfected with an MRP expression vector display altered drug sensitivity to doxorubicin, vincristine and etoposide.[7–10] MRP, like P-gp, is a member of the ABC transporter superfamily, but the two have only minor sequence homology.[6] Several groups recently have reported that MRP is a glutathione-conjugate pump.[11–14]

Earlier studies have shown that overexpression of MDR1 gene or its product, P-gp, is often observed in cell lines selected for resistance to vincristine, colchicine or actinomycin D.[15–18] In contrast, overexpression of MRP was first reported in a non-P-gp-mediated multidrug-resistant human small cell lung cancer cell line that was selected for resistance to doxorubicin.[6] Overexpression of MRP gene was subsequently observed in various human cancer cell lines selected for resistance to doxorubicin[19–24] or epipodophyllotoxins, etoposide/teniposide (VP16/VM26).[25,26] Both MDR1 and MRP genes are overexpressed in doxorubicin-resistant human myeloid leukemia cells,[27] VP16-resistant human small cell lung cancer cells[28] and doxorubicin-resistant human bladder cancer cells.[23]

Thus, MDR1 gene appears to be often overexpressed when cell lines are selected for resistance to alkaloids such as colchicine, vincristine and vinblastine or anthracyclines such as doxorubicin and daunomycin. By contrast, MRP gene is often overexpressed when cells are selected for resistance to doxorubicin or epipodophyllotoxins. However, it remains unclear if the anticancer drug used for the selection determines whether MDR1 or MRP gene is overexpressed. In this study, we examined whether or not selection for resistance to VP16/VM26 preferentially induced overexpression of MRP gene.

MATERIALS AND METHODS

Cell lines and establishment of drug-resistant cells KB/VJ-300 cells isolated after a stepwise exposure to vincristine,[15] and VP16/VM26-resistant KB cell lines (KB/VP-2, KB/VM-1, KB/VM-4) isolated after sequential selection in the presence of increasing doses of VP16 or VM26 were as described previously.[29–31] KB/VP-4 was further selected after continuous exposure of KB/VP-2 cells to increasing concentrations of VP16. KB/VP-2 cells growing in 100-mm plastic dishes were first exposed to 1000 ng/ml VP16, a dose known to reduce the surviving fraction to 10% of the initial fraction, for 1 month. The cells were then exposed to 2000, 4000, and 6000 ng/ml of VP16, each for 1 month, as described previously.[30] Colonies appearing in the presence of VP16 at 6000 ng/ml were isolated and one cell line, KB/VP-4 was cloned.

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We also established VP16-resistant and doxorubicin-resistant cell lines from the human glioma cell line, IN157,[32-34] after sequential treatment with increasing doses of VP16 or doxorubicin. IN157 cells growing in 100-mm plastic dishes were first exposed to 14 ng/ml of VP16, a dose known to reduce the surviving fraction to 10% of the initial fraction. They were subsequently exposed to 25, 50, 100 ng/ml of VP16, each for 1 month. After exposure to VP16 at 100 ng/ml for 1 month, colonies appearing in the presence of the drug were isolated and one cell line IN157/VP-1 was cloned. The doxorubicin-resistant cell line (IN157/DOX-1) was also isolated and cloned after exposure to doxorubicin at 1.0, 2.0, 5.0 and 10 ng/ml, each for 1 month. These cell lines were cultured as described previously.[29, 30, 32-34]

Drugs and chemicals Doxorubicin was a generous gift from Kyowa Pharmaceutical Co., Tokyo; vincristine was from Shionogi Pharmaceutical Co., Tokyo; cisplatin and VP16 were from Nihon Kayaku Co., Tokyo; VM26 was from Bristol Myers Co., Kanagawa. [^{3}H]VP16 was obtained from Moravek Biochemicals (Brea, CA).

Cell survival by colony formation Cell survival was determined by plating 300 cells of KB and its variants and 10^3 cells of IN157 and its variants in 35-mm dishes in the absence of drugs.[29, 30] Various drugs were added 24 h later. After incubation for 7 days at 37°C, the number of colonies was counted after Giemsa staining. All drugs were freshly prepared in physiological saline or dimethyl sulfoxide. All control experiments were done by adding equivalent volumes of saline or dimethyl sulfoxide. The 90% lethal dose (IC_{90}) for each cell line was determined from the dose-response curve.

Northern blot analysis Northern blot analysis was performed as described previously.[34, 35] The cDNA probes for MDR1, MRP, topoisomerase (topo) IIα and glyceraldehyde-3-phosphate dehydrogenase were described previously.[23, 34] The mRNA levels were quantified by densitometric analysis with a Fujix BAS 2000 bio-imaging analyzer (Fuji Photo Film Co., Tokyo).

Western blot analysis of MRP KB and IN157 cells in 100-mm plastic dishes were washed twice with ice-cold phosphate-buffered saline (PBS), and solubilized with lysis buffer (50 m*M* Tris-HCl, pH 7.5, containing 300 m*M* NaCl, 0.5% Triton X-100, 2 m*M* EDTA, 0.2 m*M* sodium orthovanadate, 10 m*M* aprotinin, 10 m*M* leupeptin and 1 m*M* PMSF). The lysate was centrifuged for 15 min at 12,000*g* at 4°C. Whole cell lysate (150 μg protein) was mixed with 4×sample buffer (250 m*M* Tris-HCl, pH 6.8, containing 20% β-mercaptoethanol and 9% SDS). Samples were then separated by 7.5% SDS-PAGE. Protein fractions from the gel were electrophoretically transferred onto nitrocellulose filters. The filters were blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween-20. They were further incubated for 1 h at room temperature with 1 : 5000 diluted MRP-specific monoclonal antibody (mAb) (QCRL-1).[23, 36] The second antibody, horseradish peroxidase-linked anti-mouse IgG, diluted 1 : 10000, was added, followed by incubation for 45 min at room temperature. The filters were then washed twice with 1% skim milk in TBS containing 0.1% Tween-20 and once with TBS containing 0.1% Tween-20. Antibody binding was detected by the enhanced chemoluminescence (ECL) Western blotting method (Amersham, Buckinghamshire, England) by fluorography on Hyperpaper-ECL Western (Amersham) for 30 min at room temperature.

Double immunocytochemical staining of MRP and β-COP KB and IN157 cells were incubated on cover slips and then fixed with 3% paraformaldehyde in PBS at room temperature for 15 min. Cells were permeabilized with 0.1% saponin in PBS at room temperature for 10 min and then stained with both mouse anti-MRP-specific mAb (QCRL-3) (diluted 1 : 100)[36] and rabbit anti-β-COP peptide antibody (diluted 1 : 100)[37] for 1 h at room temperature. The cells were washed with PBS five times with 0.1% saponin in PBS, then incubated with 1 : 100 diluted secondary antibodies, FITC-conjugated goat anti-mouse IgG (Organon Teknika N.V., Tuonhout, Bergium) and TRITC-conjugated swine anti-rabbit IgG (DAKA A/S, Denmark), for 45 min at room temperature. The cells were washed five times and mounted on coverslips with 50% glycerol in PBS. The samples were observed by fluorescence microscopy (Nikon) with a BIO-RAD MRC600 laser scanning confocal imaging system.[35]

VP16 accumulation The cells ($1-2\times10^5$ per 24-well plate) were plated and incubated for 48 h at 37°C. After they had reached subconfluence, the plates were placed on ice in water at 4°C for 15 min and the cells were washed twice with ice-cold PBS. The medium was then replaced with 200 μl of buffer (serum-free MEM and 20 m*M* Hepes, pH 7.5) containing [^{3}H]VP16 (1 μCi/ml), and the cells were incubated at 37°C for 60 min as described previously.[29, 30, 33] They were then washed with ice-cold PBS three times, treated with 400 μl of 0.25 *N* sodium hydroxide, and kept at 37°C for 30 min. The cell lysates were mixed thoroughly with 4 ml of Scintisol EX-H (Wako Chemicals, Osaka) and the radioactivity was determined.

Intracellular distribution of doxorubicin using confocal microscopy KB and glioma cells in the exponential growth phase were centrifuged and suspended in DMEM supplemented with 10% fetal bovine serum at 1×10^5/ml. The cells were seeded on a glass slide and incubated at 37°C for 24 h. They were further incubated with doxorubicin (1 μg/ml) for 60 min at 37°C, followed by washing with ice-cold PBS twice, and mounted on coverslips with 50% glycerol in PBS. Doxorubicin fluorescence in the cells was examined by fluorescence microscopy (Nikon)

with a BIO-RAD laser scanning confocal imaging system (MRC-600).[24, 33, 34]

RESULTS

In this study, we examined the expression of the MRP gene in VP16/VM26-resistant cell lines from human epidermoid cancer KB cells and human glioma IN157 cells. We have previously isolated a P-gp-mediated MDR cell line, KB/VJ-300,[15] and also a VP16/VM26-resistant cell line, KB/VM-4, which had decreased levels of VP16 accumulation and topo IIα.[29, 31] We further isolated a VP16-resistant cell line, KB/VP-4, after exposure of KB/VP-2 cells to increasing doses of VP16. KB/VP-2 is a VP16/VM26-resistant KB cell line containing reduced levels of a mutant topo IIα.[30, 31] The four VP16/VM26-resistant cell lines, KB/VP-2, KB/VP-4, KB/VM-1 and KB/VM-4, showed 51-, 200-, 20- and 112-fold higher resistance to VP16 than their parental KB cells, respectively, and they are all cross-resistant to VM26, doxorubicin and vincristine, but not to cisplatin (Table I). Both VP16- and doxorubicin-resistant cell lines, IN157/VP-1 and IN157/DOX-1, derived from human glioma IN157 cells were also cross-resistant to doxorubicin and vincristine (Table I).

To determine whether acquisition of VP16/VM26 resistance in KB and IN157 cells was due to altered expression of MDR-associated genes, Northern blot analyses of total cellular RNA were performed with MDR1, MRP and topo IIα cDNA probes (Fig. 1A). MDR1 mRNA was overexpressed only in KB/VJ-300 cells, in accordance with our previous study.[15] Cellular topo IIα mRNA levels are often much lower in VP16/VM26-resistant cell lines than their parental counterparts.[38, 39] Fig. 1A also demonstrated decreased mRNA levels of topo IIα in KB/VP-2 and KB/VM-4 compared to parental KB cells, and also in IN157/VP-1 and IN157/DOX-1 compared to parental IN157 cells. However, no further reduction of topo IIα mRNA levels was observed in KB/VP-4 derived from KB/VP-2 (Fig. 1A). Relative mRNA levels of topo IIα were calculated from densitometric scanning of the bands and normalization with respect to GAPDH mRNA levels. The quantitative data on topo IIα mRNA levels and the qualitative data on both P-gp and MRP are presented in Table II. Cellular mRNA levels of MRP were found to be 4- and 10-fold higher in KB/VM-4 and KB/VP-4, respectively, compared to KB/VM-1 cells. Both KB and KB/VP-2 lines had no detectable MRP mRNA in Northern blot analysis (Fig. 1A and Table II). IN157/VP-1 and IN157/DOX-1 also showed overexpression of MRP mRNA, but their parental IN157 cells did not.

Western blot analysis with an MRP-specific monoclonal antibody also demonstrated the presence of MRP in KB/VM-1, KB/VM-4 and KB/VP-4 cells, but not in KB or KB/VP-2 cells (Fig. 1B). Both Northern and Western blot analysis demonstrated a dramatic increase in the cellular levels of both MRP mRNA and protein in KB/VP-4 cells. MRP was also detected in IN157/VP-1 and IN157/DOX-1 cells, but not in their parental IN157 cells (Fig. 1B).

Immunocytochemical staining with an MRP-specific mAb demonstrated localization of MRP in both the plasma membrane and cytoplasmic vesicles of KB/VP-4 cells (Fig. 2). There appeared to be no co-localization of MRP and β-COP, a marker protein of Golgi and related vesicles, in KB/VP-4 cells. We also observed no co-localization of MRP and a lysosome-specific protein (data not

Table I. Relative Drug Resistance in MDR Cancer Cell Lines Derived from Human Epidermoid Carcinoma KB and Human Glioma IN157 Cells

Cell lines	Relative resistance[a]				
	VP16	VM26	Doxorubicin	Vincristine	Cisplatin
KB	1.0	1.0	1.0	1.0	1.0
KB/VP-2	51.0	30.0	9.0	1.7	0.5
KB/VP-4	200.0	nt[b]	20.0	3.8	0.5
KB/VM-1	20.0	12.0	5.9	2.1	1.5
KB/VM-4	112.0	95.0	11.0	1.8	0.5
KB/VJ-300	4.0	5.0	10.0	400.0	1.0
IN157	1.0	nt[b]	1.0	1.0	1.0
IN157/VP-1	16.0	nt[b]	12.0	3.3	0.9
IN157/DOX-1	14.0	nt[b]	19.0	3.3	0.9

a) Relative resistance was obtained by dividing the IC_{90} of resistant cell lines by that of parental cell lines (KB cells, 45±3.1 ng/ml for VP16; 25±2.1 ng/ml for VM26; 5.1±0.23 ng/ml for doxorubicin; 5.2±0.47 ng/ml for vincristine; 130±11 ng/ml for cisplatin. IN157 cells, 14±2.4 ng/ml for VP16; 1.0±0.15 ng/ml for doxorubicin; 0.28±0.053 ng/ml for vincristine; 110±8 ng/ml for cisplatin).
b) nt: not tested.

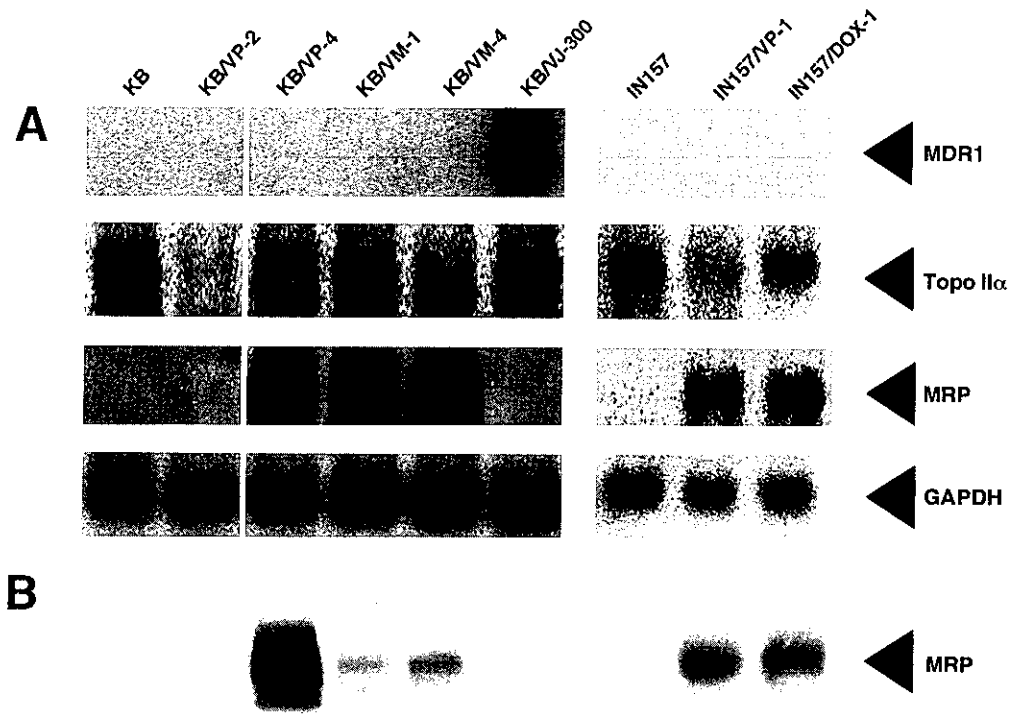

Fig. 1. Northern blot analysis for MDR1, Topo IIα and MRP mRNA (A), and Western blot analysis of MRP (B). In (A), 20 μg of total RNA from each cell line was loaded in each lane. The equivalent loading of total RNA is shown by the GAPDH blot. In (B), 150 μg of the whole cell lysates was loaded in each lane. Cellular mRNA levels of Topo IIα were quantified by densitometric analysis normalized with respect to the GAPDH mRNA level (see Table II).

Table II. Cellular Level of MDR1, MRP and Topoisomerase IIα mRNA, and [^{3}H]VP16 Accumulation of MDR Human Epidermoid Carcinoma KB and Human Glioma IN157 Cell Lines

Cell lines	mRNA levels[a]			[^{3}H]VP16[b] accumulation
	MDR1	MRP	Topo IIα	
KB	−	−	100	100
KB/VP-2	−	−	12	100
KB/VP-4	−	+	92	29
KB/VM-1	−	+	98	63
KB/VM-4	−	+	44	44
KB/VJ-300	+	−	99	nt[c]
IN157	−	−	100	100
IN157/VP-1	−	+	36	46
IN157/DOX-1	−	+	71	nt[c]

a) Cellular mRNA levels of Topo IIα were expressed as a percent of levels in their parental cell line when normalized by GAPDH mRNA level. (+) and (−) indicate enhancement of MDR1 or MRP mRNA levels in comparison with each parental counterpart.
b) Cellular accumulation of [^{3}H]VP16 in MDR cell lines is expressed as a percent of levels in their parental cell line (KB: 5.2±0.3 dpm × $10^3/10^6$ cells).
c) nt: not tested.

shown). These findings suggest that the cytoplasmic localization of MRP in KB/VP-4 cells is not in the Golgi or lysosomes. By contrast, MRP was mostly localized in the plasma membranes of IN157/VP-1 cells, but not in cytoplasmic vesicles (Fig. 2).

Intracellular accumulation of [^{3}H]VP16 was compared among the various drug-resistant cell lines. Cellular levels of [^{3}H]VP16 in KB/VP-4, KB/VM-1, and KB/VM-4 cells were 29, 63 and 44%, respectively, of that of KB or KB/VP-2 cells. Both KB and KB/VP-2 lines accumulated similar levels of VP16 (Table II). The cellular level of [^{3}H]VP16 in IN157/VP-1 cells was 46% of that of IN157 cells (Table II). Nuclear accumulation of doxorubicin was observed when cells were exposed to the drug at 1 μg/m for 60 min (0 min) followed by further incubation for 60 min in the absence of drug (60 min) (Fig. 3). A dramatic reduction of doxorubicin was observed in both KB/VP-4 and KB/VJ-300 cells at 60 min, when their parental KB cells still retained a similar amount of the drug to that at 0 min (Fig. 3). Intranuclear content of doxorubicin was also markedly decreased in IN157/VP-1 cells at 60 min, when the drug still remained in the parental IN157 cells (Fig. 3). Dox-

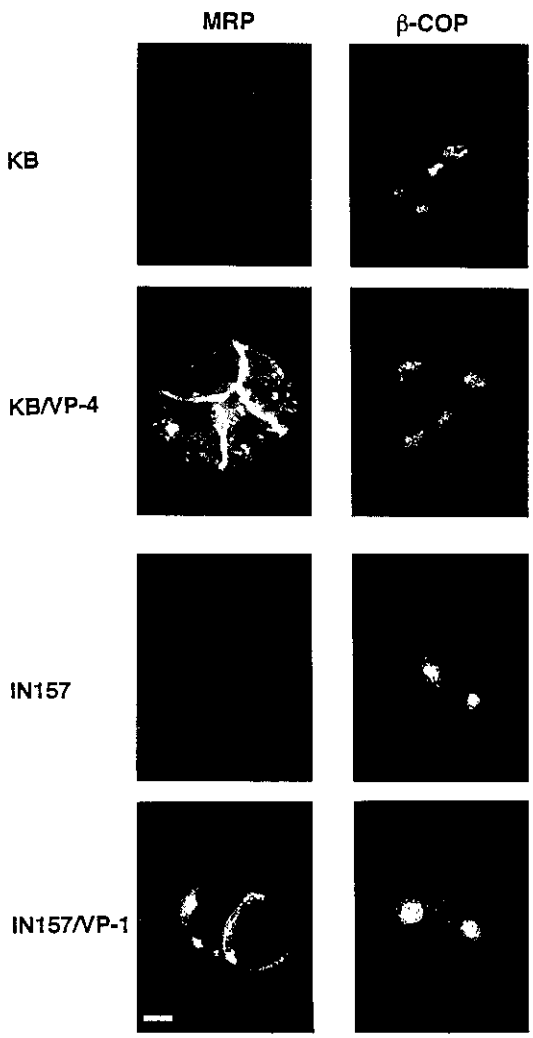

Fig. 2. The distribution of MRP and β-COP in KB, KB/VP-4, IN157, and IN157/VP-1 cells. Exponentially growing cells were stained with monoclonal antibody QCRL-3, which reacts with MRP, and also with anti-β-COP antibody to detect the Golgi apparatus. The staining was observed under a Nikon fluorescence microscope with a BIO-RAD MAC-600 laser scanning confocal imaging system. Bar=20 μm.

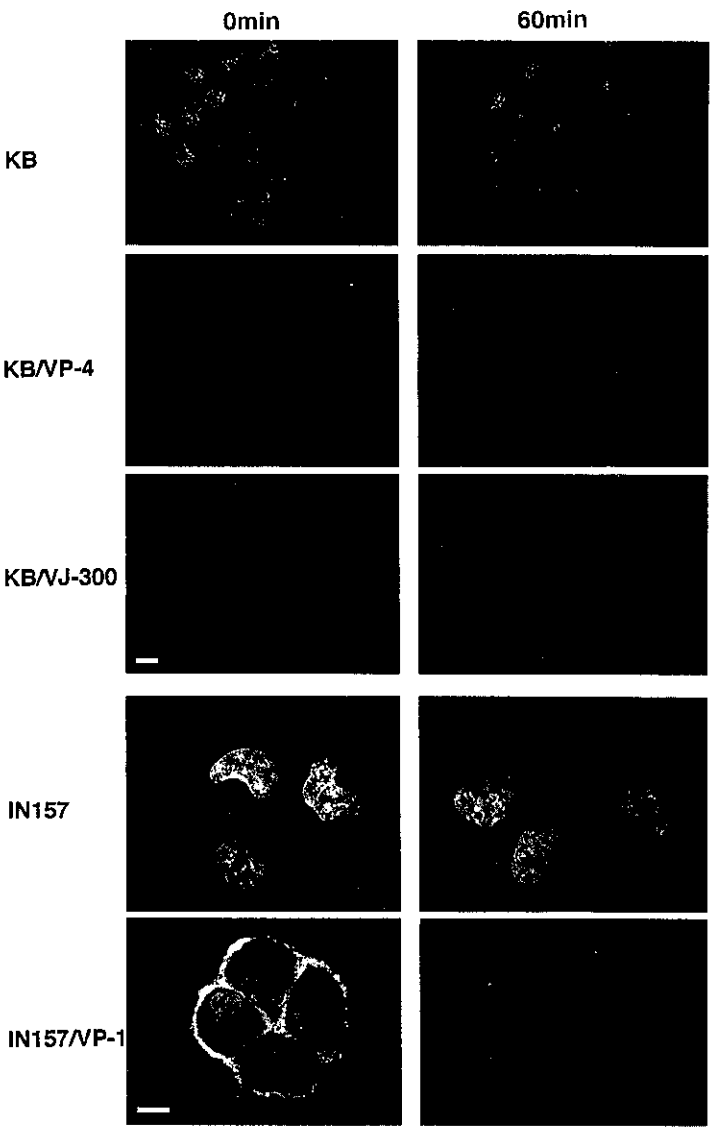

Fig. 3. Comparison of doxorubicin accumulation in KB, KB/VP-4, KB/VJ-300, IN157 and IN157/VP-1 cells using fluorescence microscopy. The cells were incubated for 60 min in the presence of doxorubicin (1.0 μg/ml) (0 min), followed by incubation in drug-free medium for 60 min (60 min). We conducted three independent assays, and obtained essentially the same data as in this analysis. Bar=20 μm.

orubicin was mainly localized in nuclei of all cell lines, but was also associated with plasma membranes of IN157/VP-1 cells. MRP-overexpressing cell lines thus exhibited a much more rapid decrease of intracellular accumulation of VP16 and doxorubicin compared to their parental cell lines.

DISCUSSION

Most multidrug-resistant cell lines which are selected for resistance to colchicine or vinca alkaloids and overexpress P-gp show a weak cross-resistance to VP16/VM26 and doxorubicin, such as is observed in KB/VJ-300 cells (Table I). Selection for resistance to doxorubicin leads to overexpression of MRP or P-gp, resulting in acquisition of a multidrug-resistant phenotype.[1-5, 40] Selection for resistance to VP16/VM26 in human breast cancer MCF7 cells[25] and human prostatic cancer PC-3 cells[26] also leads to overexpression of MRP. The overex-

pression of both MRP and P-gp was observed in human myeloid leukemia U-937 cells selected in doxorubicin,[27] in human small cell lung cancer H69 cells selected in VP16[28] and also in human bladder cancer KK47 cells selected in doxorubicin.[23]

In our present study, we examined whether selection for resistance to VP16/VM26 could preferentially induce overexpression of MRP gene in human cancer cells. We found that: 1) KB/VM-1 and KB/VM-4 cells showed decreased cellular accumulation of VP16 and increased expression of MRP gene; 2) KB/VP-4 cells showed decreased accumulation of VP16 and increased expression of MRP; 3) IN157/VP-1 cells had decreased accumulation of VP16 and increased MRP mRNA levels. Our results might support the hypothesis that selection for resistance to epipodophyllotoxins such as VP16 and VM26 preferentially induces overexpression of MRP rather than MDR1.

KB/VM-1 cells show a low level of VP16/VM26 resistance and decreased accumulation of VP16, while KB/VM-4 cells show a high level of VP16/VM26 resistance and decreased accumulation of VP16 (Tables I and II), consistent with our previous report.[29] Unlike KB/VM-4 cells, KB/VM-1 cells show similar levels of topo IIα mRNA to their parental KB cells (Fig. 1 and Table II). The reduced intracellular accumulation of VP16 in KB/VM-1 and KB/VM-4 cells appears to be due to overexpression of MRP. During exposure of KB cells to increasing doses of VP16/VM26, overexpression of MRP appears to precede a reduction in topo IIα gene expression during establishment of KB/VM-4 (Fig. 1). Takano *et al.*[30] established the KB/VP-2 cell line, which shows 30- to 50-fold higher resistance to VP16/VM26. A dramatic overexpression of MRP was observed in KB/VP-4, which was further selected after exposure to increasing doses of VP16 (Fig. 1). A reduced accumulation of VP16 in KB/VP-4 cells thus appears to be due to the increased expression of MRP gene. In addition, the relative decrease in VP16 accumulation in KB/VM-1 and KB/VM-4 appeared not appreciably different from that in KB/VP-4, which had higher MRP levels than KB/VM-1 or KB/VM-4. Overexpression of MRP may affect drug accumulation, but other unknown process(es) which might be associated with acquisition of multidrug resistance in KB/VP-4 cells or additional mutation(s), may also contribute to drug resistance in KB/VP-4 cells.

During selection of KB/VP-2 and KB/VP-4 cells, a reduction of topo IIα gene expression precedes the overexpression of MRP gene. To our surprise, the topo IIα level of KB/VP-4 cells was found to be restored to that of the wild-type KB cells. It is not clear whether or not the overexpression of MRP gene is directly associated with the restoration of topo IIα gene to the normal level. There might be no preferred sequence for altered expression of either topo IIα gene or MRP gene during the appearance of various levels of resistance in cell lines exposed to various doses of VP16/VM26. During establishment of the VP16/VM26-resistant line, IN157/VP-1 human glioma cell line, decreased expression of topo IIα and increased expression of MRP were both observed. The ordering of the altered expression of the two genes is not clear in IN157/VP-1 cells, but expression of MRP appears to be correlated with expression of topo IIα gene in IN157/VP-1 cells, as well as in other VP16/VM26-resistant cell lines.

There was a difference in the subcellular distribution of MRP between KB/VP-4 and IN157/VP-1 cells. In KB/VP-4 cells, MRP was found on the plasma membrane and cytoplasmic vesicles. By contrast, MRP was mostly located on the plasma membrane in IN157/VP-1 cells. MRP has been reported to be present primarily in endoplasmic reticulum of HL60/Adr cells[22] and in the plasma membrane of others,[9, 41, 42] including MRP-transfected HeLa cells. The reason for these differences in the subcellular distribution of MRP is unknown. It may depend on the cell type.

In conclusion, our present study shows that selection for resistance to epipodophillotoxins preferentially induces overexpression of MRP gene.[40] Preferential expression of such multidrug resistance-related genes by selecting agents may be of importance in the clinical application of anticancer agents.

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