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Multidrug Resistance-associated Protein-mediated Multidrug Resistance Modulated by Cyclosporin A in a Human Bladder Cancer Cell Line

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A doxorubicin-resistant subline (5637/DR5.5) from human bladder cancer cells (5637) was induced by stepwise increase in the doxorubicin concentration. 5637/DR5.5 cells were cross-resistant to vinblastine and etoposide but not to mitomycin C and cisplatin. We analyzed the *mdr1*, MRP (multidrug resistance-associated protein), and DNA topoisomerase II gene expression using the reverse transcription polymerase chain reaction assay (RT-PCR) and investigated possible differences in the accumulation and efflux of radiolabeled daunorubicin. 5637/DR5.5 cells do not express the *mdr1* gene, but the expression levels of MRP are markedly higher than in drug-sensitive 5637 cells. The intracellular accumulation of radiolabeled daunorubicin was markedly decreased in the 5637/DR5.5 cells in comparison with the parent cells. This reduced drug accumulation was associated with an enhanced drug efflux, but was reversed when cells were incubated with cyclosporin A. Cyclosporin A at the concentration of 5 μM caused 3.4-fold enhancement of daunorubicin-sensitivity in the 5637/DR5.5 cells. On the other hand, there was no difference in DNA-topoisomerase II activity between the parent and resistant cells. The resistance of the 5637/DR5.5 cells is therefore associated with an enhanced drug efflux mediated by the MRP gene overexpression, as distinct from P-glycoprotein, and is modulated by cyclosporin A.

Key words: Multidrug resistance-associated protein — Cyclosporin A — Doxorubicin — Efflux — Bladder cancer

Effective therapy for various tumors is hampered by the development of drug resistance. Resistance to diverse drugs has been well studied. The best characterized mechanism of MDR[6] involves overexpression of the *mdr1* gene which encodes the 170-kDa membrane P-gp that is thought to function as a drug efflux pump. The presence of P-gp in the cell membrane has been linked to what has been referred to as "classical" MDR, characterized by resistance to a number of diverse natural product drugs that include Vinca alkaloids, as well as to a wide range of topoisomerase II-targeted drugs that include doxorubicin and its derivatives, amsacrine, mitoxantrone, etoposide, and teniposide.[1-3]

In contrast to the P-gp-mediated MDR, cytosolic and nuclear proteins have been identified *in vitro* in cells selected for resistance to diverse drugs.[4] For most of these proteins evidence of their involvement in MDR remains circumstantial and, for the most part, their biochemical functions have not been identified. Some of these proteins share sequence homology with ATP-binding cassette-type transport proteins, which suggests a possible role in drug transport. Recently, Cole and coworkers[5] isolated and cloned the cDNA for a putative transport protein (MRP) with some sequence homology to *mdr1* and the other ATP-binding cassette-type genes. This cDNA was isolated from MRP-overexpressing H69AR cells that had been derived from a small cell lung cancer cell line (H69) by selection for doxorubicin resistance. The H69AR cells did not have increased levels of P-gp or *mdr1* mRNA, and drug uptake studies showed that there were few differences in drug accumulation between the sensitive H69 and resistant H69AR cells.[6-8] As another mechanism, alterations in DNA topoisomerase II have been identified in a cell line selected for its resistance to various natural products, especially etoposide, mitoxantrone, doxorubicin, and teniposide.[9-13] In some cell lines, reduced sensitivity to topoisomerase II drugs is correlated with a reduction in DNA topoisomerase II levels and/or activity.[11-17]

Bladder cancer is one of the model diseases for the investigation of MDR. Since the introduction of cisplatin-based, combination chemotherapy regimens, chemotherapy has become the main treatment option for ad-

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[6] The abbreviations used are: MDR, multidrug resistance; P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein; kDNA, kinetoplast deoxyribonucleic acid; cDNA, complementary deoxyribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction; β_2m, β_2-microglobulin; PBS, phosphate buffer solution; SDS, sodium dodecyl sulfate; BPB, bromophenol blue.

vanced bladder cancer. Even though cisplatin itself is not a P-gp-related drug, current cisplatin-based chemotherapy regimens usually are combined with P-gp- and DNA topoisomerase II-targeted drugs.

P-gp-mediated MDR can be circumvented by the addition of resistance modifiers such as cyclosporin A and verapamil.[18, 19] Cyclosporin A is one of the most widely studied *in vitro* modifiers of P-gp-related MDR. Although how it acts remains unclear, in many cases, restoration of sensitivity is associated with increased cytotoxic drug accumulation through its competitive binding to P-gp. On the other hand, there have been few reports about modifiers which affect chemosensitivity in MRP-overexpressing cells. We describe here a new variant of a human bladder cancer cell line, 5637, which was selected for resistance to doxorubicin. The subline (5637/DR5.5) possesses a pleiotropic cross-resistance phenotype resembling that of P-gp-mediated MDR cells. However, it does not express *mdr1* but expresses MRP gene at a significantly high level. Resistance in the 5637/DR5.5 cells appears to be associated with enhanced drug efflux and MRP overexpression, and it was reversed by cyclosporin A through a reduction of drug efflux.

MATERIALS AND METHODS

Isolation of the drug-resistant subline Human bladder cancer cell line 5637 cells were obtained from the American Type Culture Collection. A doxorubicin-resistant subline (5637/DR5.5) was established from 5637 cells by continuous exposure to gradually increasing concentrations of doxorubicin. The 5637/DR5.5 cells can be maintained in 55 ng/ml of doxorubicin in RPMI1640 medium supplemented with 10% fetal calf serum. The cells were grown in medium without doxorubicin for one passage prior to their use in the drug accumulation, drug efflux and decatenation assays.

Crystal violet assay for drug-sensitivity testing Exponentially growing cells were harvested, washed, and seeded into individual wells of a 24-well plate at 1×10^4 cells/well. The cytotoxic drugs and cyclosporin A were sequentially diluted with culture medium and added to the plated cells at a final volume of 0.1 ml per well, after which the treated cells were incubated at 37°C and 5% CO_2. After 4–5 days of culture, the cells were stained with 0.5% crystal violet in 20% methanol, and the amount of dye in the cells was determined by measuring the absorbance at 540 nm after elution with 0.1 *M* sodium citrate in 50% ethanol. Cell numbers were estimated from a calibration curve that related the cell number to the absorbance at 540 nm. Thus, we obtained survival curves against each cytotoxic drug by plotting the mean survival fraction of triplicate experiments. The relative resistance value was determined from the ratio of the IC_{50} of the doxorubicin-resistant subline to that of the parent cell line.

Determination of gene expression Total RNAs from the 5637 and 5637/DR5.5 cells were isolated by the method of Chomczynski and Sacchi.[20] The RT-PCR to determine the expressions of the *mdr1*, MRP and DNA topoisomerase II genes was done according to the method of Noonan *et al.*[21] with some modifications. Briefly, cDNA was prepared from 500 ng of total RNA by random priming using a First-Strand cDNA Synthesis kit (Pharmacia-LKB, Uppsala, Sweden). The PCR was carried out with a mixture consisting of the cDNA derived from 10 ng of the RNA, 10 pmol each of the sense and antisense primers for the sequences of the target gene (*mdr1*, MRP or DNA topoisomerase II) and the β_2m gene. The reaction mixture contained 200 μmol of deoxynucleotide triphosphate, 37 kBq of [α-^{32}P]dCTP, and Taq DNA polymerase in the reaction buffer (Wako Chemicals, Osaka) of final volume 12.5 μl. The sequences of the primers and the lengths of the products are given in Table I. The PCR conditions were denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. To determine the appropriate number of PCR cycles for quantification, the PCR was run from

Table I. Sequences of Amplification Primers Shown in the 5′→3′ Orientation

Gene	Primer[a]	PCR product (bp)[b]
MRP	S ATCAAGACCGCTGTCATTGG A GAGCAAGGATGACTTGCAGG	181
mdr1	S CGCCATTGCACGTGCCCTGG A CCTTTCTCTTTCATGAGTTC	243
Topoisomerase II	S GCTGTGGATGACAACCTCCT A GCCATCTAGCATTCGTCTGAC	189
β_2m	S ACCCCCACTGAAAAAGATGA A ATCTTCAAACCTCCATGATG	120

a) S and A indicate the sense and antisense primers.
b) Size of the amplified fragments obtained with each pair of primers.

20 to 50 cycles in steps of 5 cycles. The ratio of PCR products of the target gene to those of the β_2m gene was reasonably constant between 25 and 45 cycles (data not shown). The values at 30 PCR cycles therefore were defined as the expression of the target genes. Thirty cycles of PCR were done for each sample, after which the products were separated on 6% polyacrylamide gels. Radioactivity was measured with a Bioimaging Analysis System, BAS2000 (Fujix, Tokyo). Gene expression was represented by the relative yield of the target gene to the β_2m gene.

Accumulation of daunorubicin Cells were harvested with 1.0 ml of trypsin/EDTA then plated as a monolayer in 35 mm 6-well plates at the concentration of 5×10^5 cells/well. After 24 h of culture at 37°C, the medium was discarded, then 100 n*M* [^{3}H]daunorubicin in 1.5 ml of medium was added to each well. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. At 0, 10, 30, 60, and 120 min after the addition of the drug, the medium was removed, and the plates were washed ($\times3$) with 2 ml of PBS (4°C). Finally, 0.5 ml of 0.3 *N* NaOH was used to remove the cells from the well. After 3 h of incubation at 37°C under 5% CO_2, the cell suspensions were transferred to a microcentrifuge tube. A 50 μl sample of this cell suspension was transferred to a scintillation vial, then 500 μl of 0.5 *N* alkali solution (NCS-II) was added and the mixture was lysed. Then 3 ml of RI cocktail was added, and the radioactivity of the lysate determined by liquid scintillation.

Efflux of daunorubicin Cells were incubated at 37°C for 2 h with 100 n*M* [^{3}H]daunorubicin in the culture medium. They were washed with 1 ml of daunorubicin-free medium, and 1 ml of fresh medium was added. At various times during incubation (0, 5, 10, 20, 30, 60, and 120 min), 50 μl of medium was transferred to a scintillation vial, and 50 μl of fresh medium was added to the well. After 500 μl of 0.5 *N* alkali solution and 3 ml of RI cocktail had been added to the scintillation vial, the radioactivity was checked. The medium was discarded 120 min later, and the cells were treated as described above for the accumulation of daunorubicin in order to measure the amount of radioactivity remaining in the cells.

Cyclosporin A modulation of the accumulation and efflux of daunorubicin To study the modulatory effect of cyclosporin A on daunorubicin accumulation, cells were treated with 100 n*M* [^{3}H]daunorubicin and 10 μ*M* cyclosporin A or 100 n*M* [^{3}H]daunorubicin alone in the accumulation study described above. To examine the modulatory effect of cyclosporin A on the efflux of daunorubicin, cells were incubated at 37°C for 2 h with 100 n*M* [^{3}H]daunorubicin in the culture medium. After 2 h, the medium was changed to fresh daunorubicin-free medium containing cyclosporin A at one of five concentrations (10, 5, 2, 1, and 0 μ*M*). Subsequent procedures were the same as those described above for the drug-efflux study.

Protein assay The protein concentrations in the accumulation and efflux studies of daunorubicin and in the decatenation assay were determined with Bio-Rad Protein Assay Reagent (Bio-Rad, Herculus, CA).

Nuclear extraction and the decatenation assay All the nuclear extraction procedures were done in a cold room (4°C). Cells were collected with a rubber scraper in 1 ml of RPMI1640 containing 10% fetal calf serum cells (5×10^6) were pelleted by centrifugation at 2,000 rpm for 5 min. The pellets were resuspended in 10 ml of washing buffer [10 m*M* tris-HCl (pH 7.5), 130 m*M* NaCl, 5 m*M* KCl, 8 m*M* $MgCl_2$], and the suspension was centrifuged at 2,000 rpm for 5 min. The pellets obtained were resuspended in 5 ml of hypotonic buffer [20 m*M* HEPES-KOH (pH 7.9), 5 m*M* KCl, 500 μ*M* $MgCl_2$] and kept on ice for 10 min. Homogenization was achieved by gently pipetting the suspension 40 times then centrifuging it at 2,000 rpm for 10 min. The pellets were resuspended in 5 ml of extraction buffer [20 m*M* HEPES-KOH (pH 7.9), 25% glycerol, 500 m*M* NaCl, 1.5 m*M* $MgCl_2$, 200 μ*M* EDTA-NaOH (pH 8.0)], and the whole was mixed gently by mechanical rotation for 1 h, after which it was centrifuged at 15,000 rpm for 30 min. The supernatant was collected, and the protein concentration measured. Topoisomerase II catalytic activity was assayed by means of the decatenation assay. The reaction mixture was 100 m*M* tris-HCl (pH 7.5), 170 m*M* KCl, 20 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 1 m*M* EDTA, 2 m*M* ATP, and bovine serum albumin (0.06 mg/ml). Decatenation of the kDNA was done by incubating 5 μl of the crude nuclear extract with 0.1 μg of kDNA for 30 min at 30°C in a final reaction mixture volume of 20 μl. The reaction was terminated by the addition of a stop buffer (2% SDS, 0.05% BPB, 50% glycerol), after which samples were electrophoresed in a 1% agarose gel in 89 m*M* tris-borate, and 2 m*M* EDTA (pH 8.3) at 34 V for 4 h. The gel was stained with ethidium bromide (1.0 μg/ml) for 45 min then destained for 2 h in distilled water. DNA bands were made visible by transillumination with UV and photographed.

Materials Doxorubicin, daunorubicin, vinblastine, cisplatin and mitomycin C were purchased from Sigma (St. Louis, MO) and etoposide was obtained from Bristol-Myers Squibb K.K. (Tokyo). Cyclosporin A was supplied from Sandoz (Basel, Switzerland). [^{3}H]Daunorubicin (62.9 GBq/mmol) was purchased from Du Pont/NEN Research Products (Boston, MA).

RESULTS

Drug sensitivities The relative resistance to doxorubicin and daunorubicin of 5637/DR5.5 cells was 7.6- and

5.4-fold (Table II), respectively. In addition, the 5637/DR5.5 cell line was 4.2- and 16.7-fold cross-resistant to vinblastine and etoposide, respectively, whereas they remained relatively sensitive to cisplatin. Interestingly, the 5637/DR5.5 cells were 1.7-fold more sensitive to mitomycin C than the parent 5637 cells.

Accumulation and efflux of [^{3}H]daunorubicin To examine whether altered membrane permeability is involved in the mechanism of drug resistance in 5637/DR5.5 cells, we compared the uptake and efflux of radiolabeled daunorubicin by the 5637 and 5637/DR5.5 cells. There was a 2.7-fold reduction in the accumulation of [^{3}H]daunorubicin in 5637/DR5.5 cells relative to that in 5637 cells after 120 min of exposure to the radiolabeled drug (Fig. 1). The efflux of the drug from 5637 and 5637/DR5.5 cells that had been incubated for 2 h with [^{3}H]daunorubicin was also compared (Fig. 2). After removal of the radiolabeled drug from the medium, the decrease in intracellular radioactivity was more rapid from 5637/DR5.5 than from 5637 cells, and more than 65% of the radioactive daunorubicin was ejected from the 5637/DR5.5 cells within 120 min. In contrast, approximately 70% of the radiolabeled drug remained in 5637 cells incubated under the same conditions. The resistance of the 5637/DR5.5 cells was therefore mainly associated with enhanced drug efflux and/or decreased intracellular drug accumulation.

Effect of cyclosporin A on drug accumulation and efflux Cyclosporin A, a potent immunosuppressive agent, is an effective modifier that can reverse the MDR of human and rodent tumor cells,[18, 19] although its mechanism of circumvention of MDR has not been fully clarified. We therefore examined the effect of cyclosporin A on daunorubicin accumulation and efflux in 5637/DR5.5 and 5637 cells. The cellular accumulation of [^{3}H]daunorubicin was measured after scheduled exposure to the radiolabeled drug with or without cyclosporin A. In the 5637/DR5.5 cell line, the uptake of [^{3}H]daunorubicin alone reached the steady-state level of accumulation within 60 min, but the presence of 10 μM cyclosporin A produced a large increase in the amount of intracellular [^{3}H]dau-

Table II. IC_{50} Values and Relative Resistance to Drugs

Drug	IC_{50} (μg/ml)[a]		Relative resistance[b]
	5637	5637/DR5.5	
Doxorubicin	3.7×10^{-2}	2.8×10^{-1}	7.6
Daunorubicin	4.6×10^{-3}	2.5×10^{-2}	5.4
Vinblastine	5.2×10^{-4}	2.2×10^{-3}	4.2
Etoposide	1.2×10^{-1}	2.0×10^{-0}	16.7
Mitomycin C	1.7×10^{-2}	9.8×10^{-3}	0.6
Cisplatin	1.4×10^{-1}	1.8×10^{-1}	1.3

a) IC_{50} concentrations were determined by the crystal violet assay as described in "Materials and Methods."
b) Relative resistance is the ratio of the IC_{50} (the drug concentration causing 50% inhibition of cell growth) for 5637/DR5.5 to the IC_{50} for 5637 cells.

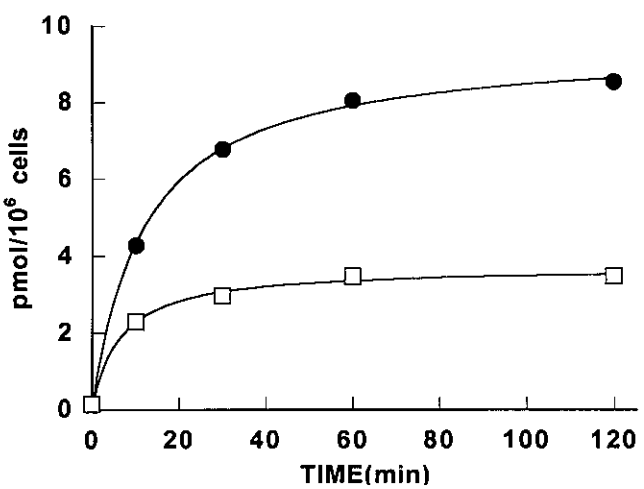

Fig. 1. Time course of the uptake of radiolabeled daunorubicin (100 nM) into parent and resistant cells, calculated from triplicate determinations. Cells were the parent 5637 drug-sensitive strain (●) or the drug-resistant subline 5637/DR5.5 selected with doxorubicin (□).

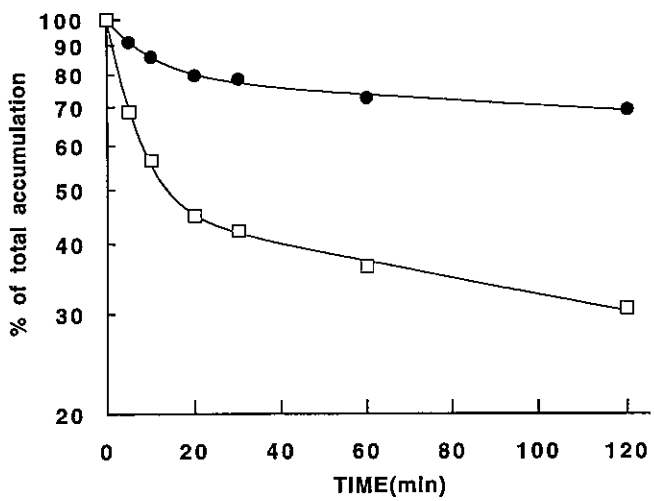

Fig. 2. Time course of the efflux of daunorubicin from 5637 cells (●) and 5637/DR5.5 cells (□). Each value is the mean of triplicate experiments. The cells were incubated with 100 nM [^{3}H]daunorubicin for 2 h at 37°C, after which the medium was changed to daunorubicin-free medium. The intracellular concentration of daunorubicin at 0 min (100%) was 68.3 pmol/10^6 cells in 5637 and 36.5 pmol/10^6 cells in DR5.5.

norubicin (Fig. 3A), while such an increase in intracellular accumulation of daunorubicin was not observed in 5637 cells (Fig. 3B). To investigate the action of cyclosporin A on drug efflux, 5637/DR5.5 cells first were loaded with [^{3}H]daunorubicin, then the efflux of the radiolabeled drug from the loaded cells was examined during subsequent incubation in daunorubicin-free medium containing cyclosporin A at five different concentrations. Approximately 30% of the drug remained in the 5637/DR5.5 cells after incubation for 2 h in cyclosporin A-free medium. In contrast, up to 50% of the radioactive drug remained in the cyclosporin A-loaded cells in a dose-dependent manner (Fig. 4A). Incubation of 5637 cells with 10 μM cyclosporin A had no effect on [^{3}H]daunorubicin efflux (Fig. 4B). Simultaneous addition of 5 μM cyclosporin A enhanced daunorubicin sensitivity 3.4-fold (Fig. 5), while it did not show any sensitizing effect on the parent cells (data not shown).

MRP expression Because our results suggested the association of a drug transport protein with MDR in 5637/DR5.5 cells, we examined the expression of MRP gene in 5637/DR5.5 cells. MRP mRNA was readily detectable in both 5637 and 5637/DR5.5 cells on RT-PCR analysis, but the amount of MRP mRNA in the 5637/DR5.5 cells was 5.6 times that in the 5637 cells (Fig. 6A).

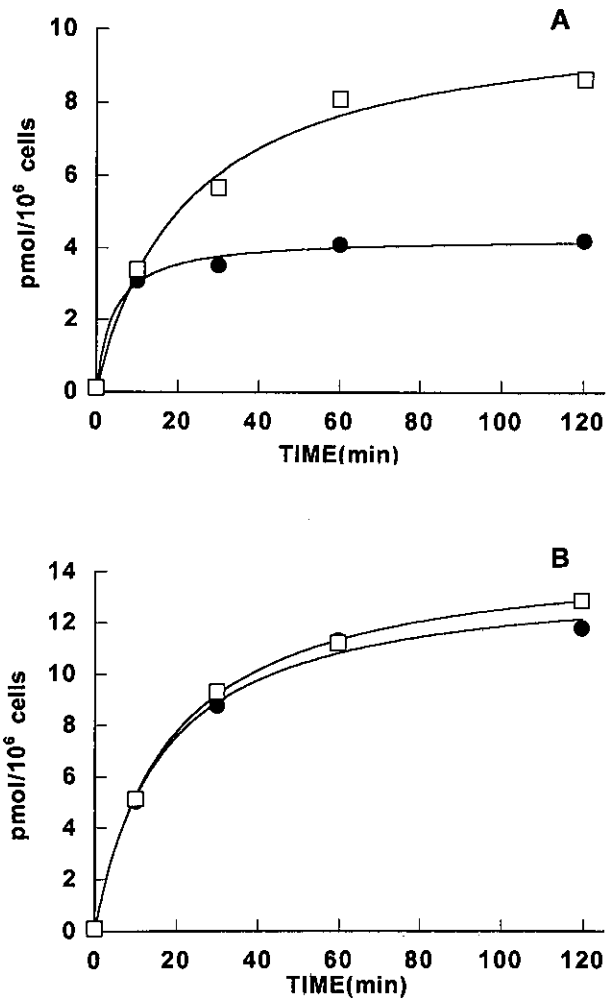

Fig. 3. Effect of 10 μM cyclosporin A on the uptake of daunorubicin by the drug-resistant 5637/DR5.5 (panel A) and the drug-sensitive 5637 (panel B) cells, determined by triplicate experiments. Closed symbols show the time course of daunorubicin uptake without cyclosporin A, open symbols that with 10 μM cyclosporin A.

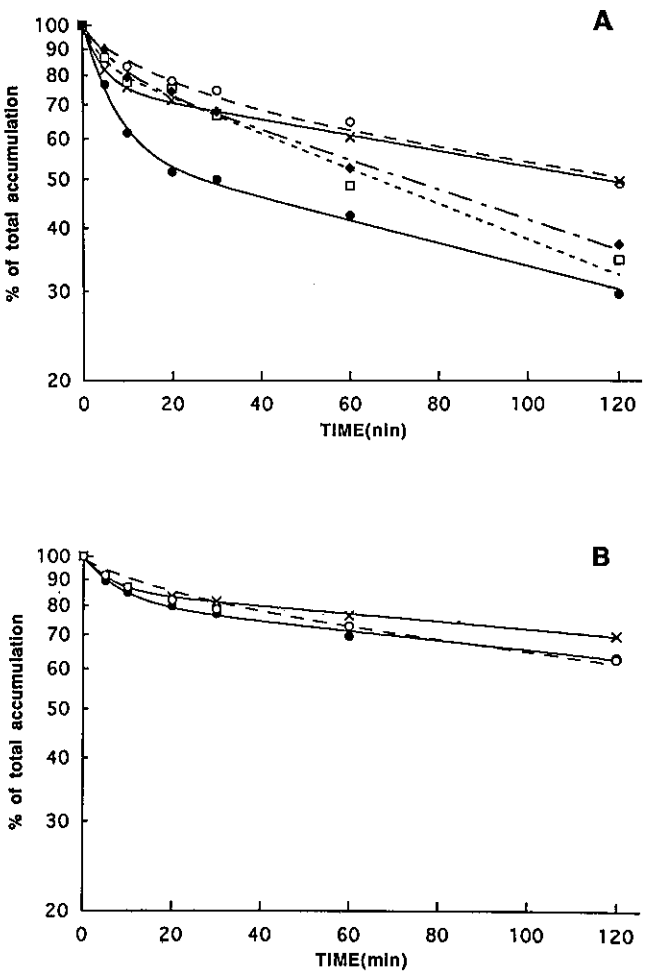

Fig. 4. Effect of cyclosporin A on the daunorubicin efflux from 5637/DR5.5 (panel A) and 5637 (panel B) cells, determined by triplicate experiments.. Incubation was done under the conditions given in Fig. 2. After 2 h, the medium was changed to fresh daunorubicin-free medium containing 10 μM (×), 5 μM (○), 2 μM (□), 1 μM (◆), or 0 μM (●) cyclosporin A.

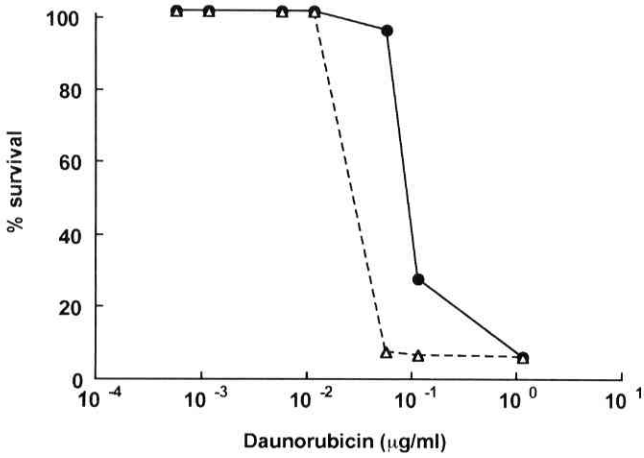

Fig. 5. Sensitizing effect of 5 μM cyclosporin A on daunorubicin sensitivity of 5637/DR5.5 cells. Survival fraction of the cells exposed to different concentrations of daunorubicin with (△) or without (●) 5 μM cyclosporin A was calculated from 3 replicates of the crystal violet assay.

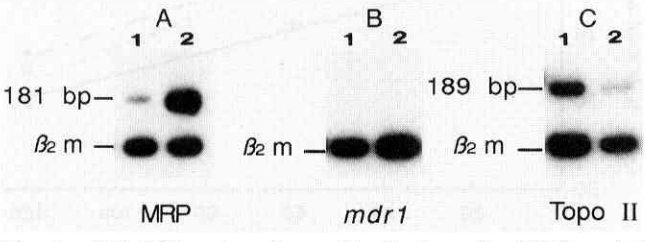

Fig. 6. RT-PCR autoradiographic findings for MRP, *mdr1* and DNA topoisomerase II. The amplified fragments (1, 5637; 2, 5637/DR5.5) were electrophoresed on a 6% polyacrylamide gel, and the autoradiographs were exposed for 24 h at room temperature.

***mdr1*/P-gp expression** We used RT-PCR and immunohistochemical staining to examine the levels of *mdr1*/P-gp expression in both cell lines. As shown in Fig. 6B, we did not detect *mdr1* mRNA in either 5637/DR5.5 or 5637 cells. Furthermore, immunocytochemical staining detected no P-gp in either cell line (data not shown). For both the RT-PCR and the immunocytochemical analyses, a multidrug-resistant cell line, KB-8-5 (a kind gift from Dr. Kazumitsu Ueda, Kyoto University), was used as a positive control, and *mdr1*/P-gp expression was consistently observed in it as described elsewhere.[22] The reduction in the daunorubicin accumulation and the development of doxorubicin resistance in the 5637/DR5.5 cells therefore was not associated with *mdr1*/P-gp overexpression.

DNA topoisomerase II expression and catalytic activity When we checked the mRNA levels of topoisomerase II by RT-PCR, the expression of the DNA topoisomerase II gene was 3.2-fold lower in the 5637/DR5.5 cells than in the 5637 cells (Fig. 6C). Topoisomerase II catalytic activity was analyzed in nuclear extracts by decatenation of the kDNA network.[23,24] This assay showed that the DNA topoisomerase II activities of these cell lines were almost identical (Fig. 7). This suggests that alteration of this enzyme activity is not involved in the resistant phenotype, in contrast to the results for mRNA expression.

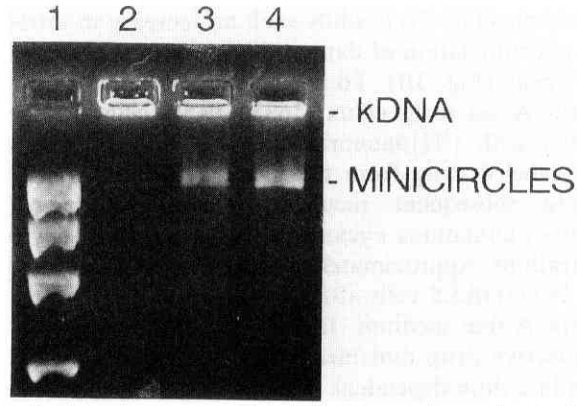

Fig. 7. Topoisomerase II activity in nuclear extracts from 5637 and 5637/DR5.5 cells. Topoisomerase II activities in the nuclear extracts from the 5637 (lane 3) and 5637/DR5.5 (lane 4) cells were evaluated by means of the decatenation assay as described in "Materials and Methods." The amounts of extract protein added were the control, no extract protein (lane 2), and 7 μg (lanes 3 and 4). Lane 1 shows the λ/*Hin*dIII size marker.

DISCUSSION

MDR is characterized by cross-resistance within a single class of unrelated drugs.[25] Drugs associated with MDR are generally amphipathic, lipophilic, natural substances and include the anthracyclines, epipodophyllotoxins, and Vinca alkaloids. Although the actual physiological function of P-gp is unknown, overproduction of P-gp pumps drugs out of cells, resulting in a decrease in the drug content in MDR cells.[26–30] In some MDR cells, changes in intracellular drug accumulation, binding, or sequestration have been noted in the absence of P-gp expression.[31,32] Evidence from several laboratories suggests that this form of MDR involves changes in DNA topoisomerase II activity.[33–36]

Recently, another class of protein, MRP, which is one of the candidates for non-P-gp MDR, has been found.[5] MRP is a 190-kDa protein and a member of the ATP-binding cassette superfamily of membrane transport proteins, as is P-gp, but it is markedly different from P-gp. Although MRP protein is distributed mainly in the endo-

plasmic reticulum and, to a lesser degree, in the plasma membrane,[37] its role in causing MDR remains unknown.

Our doxorubicin-resistant human bladder tumor cells (5637/DR5.5) were 7.6- and 5.4-fold resistant to doxorubicin and daunorubicin, respectively, but they also had a 4.2- and 16.7-fold cross-resistance to vinblastine and etoposide, respectively. The drug-resistance spectrum of 5637/DR5.5 was similar to that of P-gp-expressing MDR cells. In addition, the pharmacokinetic experiments of the present study demonstrated that the resistance in 5637/DR5.5 cells was brought about by decreased drug accumulation through increased drug efflux, as in the P-gp mediated MDR cells. However, we could not detect any *mdr1* gene expression by either the sensitive RT-PCR assay or immunohistochemistry in the resistant line, but, instead, we found overexpression of the MRP gene. Thus, we conclude that the reduction of drug accumulation in 5637/DR5.5 cells is not mediated by P-gp, but rather by MRP. Decreased drug accumulation has been reported for several non-P-gp MDR cell lines in which MRP gene is over-expressed. A study by Grant *et al.*[38] indicated that increased resistance to doxorubicin in the transfected cells is directly attributable to the overexpression of MRP. Overexpression of MRP in transfected cells did not alter the levels of mRNA specifying topoisomerase II isoform, annexin II or *mdr1*. A recent study with SW-1573 human lung cancer cell line transfected with MRP expression vector clarified the drug-resistance mechanism of MRP.[39] The transfected cells showed decreased drug accumulation and increased drug efflux. On the other hand, in H69AR small cell lung cancer cells, from which MRP gene was originally isolated, no reduction of drug accumulation was observed.[5] Further experiments are needed to establish the mechanisms of MRP action.

Non-P-gp MDR cells in which MRP is over-expressed sometimes simultaneously show an altered DNA topoisomerase II activity.[12, 40] We, therefore, investigated whether decreased expression or altered topoisomerase II activity also contributed to MDR phenotype of our resistant cell line. Although expression of the DNA topoisomerase II mRNA was 3.2-fold lower in 5637/DR5.5 cells than in 5637 cells, there was no detectable difference in the catalytic activity of DNA topoisomerase II between the parent and drug-resistant cells as analyzed in the decatenation assay. Based on these results, we consider that the altered topoisomerase II does not contribute the MDR phenotype in 5637/DR5.5 cells.

Because the circumvention of MDR is essential in current cancer chemotherapy, several clinical trials designed to circumvent MDR have been conducted. Cyclosporin A is one of the most widely studied *in vitro* modifiers of P-gp-mediated MDR. Its action mechanism is not yet clear, but it seems likely that there is not a single mechanism. It acts, at least in part, by binding to P-gp, thereby modifying drug accumulation, but also through a less specific interaction with the plasma membrane, and possibly by the inhibition of protein kinase C.[19, 41–43] On the other hand, it is still controversial whether cyclosporin A has potential to overcome drug-resistance in non-P-gp MDR, particularly in MRP-overexpressing cells. Barrand *et al.*[44] reported that there was no chemosensitization effect of cyclosporin A or its derivative, PSC-833, on MRP-overexpressing MDR large cell lung cancer cells. In contrast, Ross *et al.*[45] found some enhancing effect of cyclosporin A on drug accumulation in HL60/AR cells in which MRP was overexpressed. The present study showed that cyclosporin A brought about almost complete restoration of the drug accumulation to the parent cell level and inhibited the efflux of daunorubicin in a dose-dependent manner. Moreover, daunorubicin sensitivity was enhanced 3.4-fold by addition of 5 μM cyclosporin A in 5637/DR5.5 cells. We therefore believe that the resistance to doxorubicin in 5637/DR5.5 cells can be ascribed mainly to increased drug efflux mediated by MRP, which appears to have a role like that of P-gp. In addition, cyclosporin A modifies drug transport, especially the efflux. Through the modification of membrane transport, cyclosporin A restores the sensitivity associated with increased cytotoxic drug accumulation, especially in 5637/DR5.5 cells.

In conclusion, we have isolated a doxorubicin-resistant human bladder cancer subline which shows pleiotropic drug resistance. Overexpression of MRP gene was observed in it, though no *mdr1* gene expression was detected and DNA topoisomerase II enzyme activity was unchanged. Reduction of drug accumulation and enhancement of drug efflux were found, which suggests that the resistance in these cells is associated with the overexpression of MRP. Moreover, the present study is the first to report that cyclosporin A clearly modulates drug transport in MRP-overexpressing cancer cells with MDR phenotype. It is of particular interest to clarify whether cyclosporin A and a number of its less toxic analogues are clinically potent as sensitizers for chemotherapy in MRP-overexpressing neoplasms, including bladder cancer.

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