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Non-P-glycoprotein-mediated Atypical Multidrug Resistance in a Human Bladder Cancer Cell Line

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A human bladder cancer cell line resistant to adriamycin (ADM), T24/ADM9 has been established *in vitro* by exposing T24 parent cells to progressively higher concentrations of the drug over a period of 12 months. The T24/ADM9 cells were found to be 9 times more resistant to ADM than the T24 parent, and showed various degrees of cross-resistance to an ADM derivative, vinca alkaloids and a DNA topoisomerase II (Topo II)-targeting agent, etoposide. No significant difference was observed in the cellular accumulation of ADM between the T24/ADM9 and T24 parent cells. A Northern blot analysis showed an overexpression of multidrug resistance-associated protein (MRP) mRNA, but no overexpression of multidrug resistance-1 (MDR1) mRNA was observed in the T24/ADM9 cells. A flow cytometric analysis showed that the MDR1 gene product, P-glycoprotein (Pgp), is not expressed on the T24/ADM9 cells. T24/ADM9 showed approximately the parental level of DNA Topo II catalytic activity. In Western blot and Northern blot analyses, however, the cellular level of DNA topo II was apparently much lower in T24/ADM9 than in the T24 parent. Thus, these results suggest that a decreased cellular level of DNA Topo II and an overexpression of MRP gene may be responsible for the expression of an MDR phenotype in the T24/ADM9 cells and that such non-Pgp-mediated, atypical MDR may develop in bladder cancer treated with chemotherapy including ADM.

Key words: Bladder cancer — Multidrug resistance — DNA topoisomerase II — P-glycoprotein — Adriamycin

ADM[4] and vinblastine, which are MDR1-related agents, are frequently used to treat advanced bladder cancer. Sternberg *et al.*[1] reported that M-VAC chemotherapy including these agents showed about a 70% response rate in advanced transitional cell carcinoma of the urothelium. However, it is important to note that the remaining 30% of the patients do not respond, and even patients who achieve a partial response still have a residual tumor which is resistant to chemotherapy. In order to increase the therapeutic effects of such chemotherapy, it seems to be essential to clarify the mechanisms of MDR and eventually to develop a means to overcome such resistance.

We previously demonstrated that about one-third of untreated human bladder cancers expressed Pgp and there was a good correlation between Pgp expression and MDR phenotype.[2] However, the mechanism of MDR is complicated, and many other factors such as an increased detoxifying capacity,[3,4] altered and/or mutated DNA Topo II level[5,6] and an overexpression of MRP[7] are also considered to be involved.

To date, several MDR human bladder cancer cell lines have been established *in vitro* as models for investigating drug resistance.[8–10] However, most of the cell lines are Pgp-mediated classical MDR lines, and to date little has been reported on the mechanism of non-Pgp-mediated atypical MDR in human bladder cancer. We herein report the establishment and characterization of an atypical MDR human bladder cancer cell line, T24/ADM9 which does not express MDR1-mediated-Pgp.

MATERIALS AND METHODS

Cell culture and the establishment of T24/ADM9 T24, which was established from a transitional cell carcinoma of the bladder, grade 3,[11] was used as a parent line. The cells were grown as a monolayer culture in complete MEM (Eagle's MEM supplemented with 10% FBS, sodium pyruvate, non-essential amino acids, L-glutamine and two-fold vitamin solution) (Gibco, Grand Island, New York). The T24 cells were expanded, frozen and stored in liquid nitrogen. The resistant subline, T24/ADM9 was established by the continuous exposure of the T24 parent cells to culture media containing ADM at concentrations from 6.0×10^{-2} to 2×10^{-1} μg/ml over a

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[4] Abbreviations: ADM, adriamycin; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; Pgp, P-glycoprotein; MEM, minimum essential medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide; PBS(−), phosphate-buffered saline free of calcium and magnesium; cDNA, complementary DNA; DNA Topo, DNA topoisomerase; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

12-month period. Next, the cells were expanded in an ADM-free medium, and stored in liquid nitrogen. All experiments were performed within 6 passages after thawing the cells.

A K562 human myelogenous leukemia cell line and its ADM-resistant subline, K562/ADM, which was established by Tsuruo *et al.*[12] and is known to express Pgp on the cell surface,[8,12] were maintained in a suspension culture in RPMI 1640 (Gibco) supplemented with 10% FBS. These two lines were used as a control in flow cytometric analysis.

An MDR cell line, VJ300, which was derived from human epidermoid cancer KB cells and is known to overexpress MDR1 mRNA,[13] was maintained as a monolayer culture in complete MEM and used as a positive control in the Northern blot analysis.

Drugs All the anticancer agents used were obtained from commercial sources, and were either dissolved or diluted immediately before use. Verapamil and [^{14}C]ADM (95 μCi/mg) were purchased from Eisai Co., Ltd., Tokyo and Amersham Japan Ltd., Tokyo, respectively.

Drug sensitivity determined by MTT assay Experiments on the effects of the drugs were performed with the MTT assay as described by Mosmann[14] with some modification. The tumor cell suspensions were adjusted to a concentration of 2.5×10^4/ml of complete MEM for T24 and 3×10^4/ml of complete MEM for T24/ADM9. A portion (100 μl) of each suspension was then seeded into the wells of 96-well microtiter plates. In preliminary experiments, these seeding densities were determined to be such that the cultures did not become confluent before the assay was conducted. The cell number per well was proportional to the absorbance of the solubilized formazan. The plates were incubated at 37°C for 24 h, and then 100 μl of various concentrations of anticancer agents was added to each well, with complete MEM added to control wells. To examine the effect of verapamil on ADM sensitivity, 100 μl of ADM at various concentrations in the presence or absence of verapamil was added. Quadruplicate wells were used for each drug concentration. After a further 3 days of incubation, the culture supernatant was discarded, and a colorimetric reaction was initiated by adding 10 μl of MTT at a concentration of 0.4 μg/ml and 10 μl of 10 m*M* sodium succinate to each well. The formazan crystals formed from MTT were dissolved in 150 μl of dimethyl sulfoxide, and the absorbance at 540 nm was quantitated with a microtiter plate spectrophotometer (Immunoreader NJ-2001, Nippon Intermed K.K., Tokyo). The results were expressed as a percentage of the controls. The IC_{50}s of the drug were determined by plotting the logarithm of the drug concentration versus the growth rate (percentage of control) of the treated cells. The assays were repeated three times for each drug.

Drug accumulation The cellular accumulation of ADM was examined according to the method described previously.[8] In brief, 5×10^4 cells were plated into the wells of 96-well microtiter plates and incubated overnight at 37°C. The medium was then replaced with 100 μl of complete MEM containing [^{14}C]ADM (2 μg/ml), and the plates were incubated at 37°C. At 30, 60 or 120 min after replacing the medium, the drug solution was aspirated off, and the cells were washed twice with PBS(−). The adherent cells were lysed with 100 μl of 0.2% sodium dodecyl sulfate. The lysates were harvested and added to 5 ml aliquots of scintillation fluid in glass vials, and the cell-associated radioactivity was determined with a Beckman β-scintillation counter. The radioactivity was expressed as cpm/10^6 cells, and cellular accumulation of the drug was normalized by comparison with the accumulation in T24 parental cells at 30 min.

Northern blot analysis A Northern blot analysis was performed as described previously.[5,6] A human MDR1 cDNA, which was a kind gift from M. M. Gottesman (National Cancer Institute, Bethesda, Md.), human MRP cDNA from S. P. C. Cole (Queen's University, Kingston, Canada), human DNA Topo I cDNA from O. Koiwai and T. Andoh (Aichi Cancer Center, Nagoya) and human DNA Topo II cDNA from J. C. Wang (Harvard University, Cambridge, Mass.) were used as probes. The harvested tumor cells were suspended in 4 *M* guanidinum isothiocyanate, 25 m*M* sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1 *M* β-mercaptoethanol. Then, 2 *M* sodium acetate (pH 4.0), water-saturated phenol and chloroform were added successively to samples. After having been vigorously mixed, the samples were left on ice for 20 min, and then centrifuged at 10,000*g* for 20 min. The aqueous phase was removed, mixed with isopropanol and kept at −20°C for 60 min. The samples were then centrifuged at 10,000*g* for 20 min, and the RNA pellet was washed with 75% ethanol and dissolved in sterile, RNase-free water. The RNA (20 μg) was fractionated through a 1% agarose gel containing 2.2 *M* formaldehyde and then transferred onto a Hybond N^+ filter (Amersham Japan, Ltd., Tokyo). The filter was hybridized with each ^{32}P-labeled cDNA probe in Hybrisol for 24 h at 40°C, and then washed in 2× SSC and 0.1 % SDS at room temperature, followed by further washing in 0.2× SSC and 0.1% SDS. The mRNA levels were quantified by densitometric analysis with a Fujix BAS 2,000 bio-imaging analyzer (Fuji Photo Film Co., Tokyo).

Western blot analysis A Western blot analysis of DNA Topo II was performed as described previously.[5,6] In brief, nuclear protein fractions extracted from 1×10^7 cells were run in 7.5% SDS-PAGE. Protein fractions from the gel were electrophoretically transferred onto nitrocellulose filters in 25 m*M* Tris-HCl (pH 8.3)-92 m*M*

glycine-20% methanol for 2 h at 20 V. Nitrocellulose membranes were further incubated with antibody against human DNA Topo II (1:2,000) for 1 h at room temperature. The membranes were rinsed with PBS, treated with biotinylated secondary antibody, and developed according to the manufacturer's specification (Vectastain ABC-Go kit; Vector Laboratories, Burlingame, Calif.). Antibodies to the human DNA Topo II were a gift from Dr. L. F. Liu (Johns Hopkins University Medical School, Baltimore, Md.)

DNA Topo II assays DNA Topo II assay was performed as described previously.[5,6] In brief, the standard reaction for the DNA Topo II assay contained 50 m*M* Tris-HCl (pH 7.5), 85 m*M* KCl, 10 m*M* $MgCl_2$, 5 m*M* dithiothreitol, 0.5 m*M* EDTA, BSA (0.03 mg/ml), and 1 m*M* ATP. Decatenation reaction of catenated DNA was carried out with serial dilutions of extract and 0.4 μg of kDNA in a final volume of 15 μl at 30°C for 10 min. The kDNA was prepared from *Crithidia fasciculata*, which was obtained from Dr. P. Englund (Johns Hopkins Medical School). kDNA was purified from a Sarkosyl extract of the trypanosomes by cesium chloride-ethidium bromide density centrifugation as described by Englund.[15] The reaction was terminated with 2 μl of 1.2 mg/ml proteinase K and 22 m*M* EDTA, followed by incubation for 15 min at 37°C. Then, 5 μl of 0.05% bromophenol blue in 50% glycerol was added to the reaction mixture. Samples were electrophoresed through a 1% agarose gel. After staining with ethidium bromide, gels were photographed under UV illumination.

Flow cytometric analysis of Pgp expression The expression of Pgp on tumor cells was examined by flow cytometry using monoclonal antibody MRK 16, which was prepared and characterized by Hamada and Tsuruo,[16] and kindly provided by Dr. T. Tsuruo, Institute of Applied Microbiology, University of Tokyo, Tokyo. Tumor cell suspensions were adjusted to 1×10^5 cells/ml of PBS(−) containing 0.3% BSA and 0.05% NaN_3 (designated as washing buffer). The cells were blocked with 20-fold-diluted normal mouse serum at 4°C for 15 min, and incubated for 30 min at 4°C with either mouse IgG (Coulter Co., Hialeah, Flor.) or MRK 16 at a final concentration of 10 μg/ml. They were washed three times with washing buffer, and stained with fluorescein isothiocyanate-conjugated goat anti mouse IgG (Fc specific antibody) (Organon Teknika Co., Durham, N.C.) diluted 1:100 in PBS(−) containing 0.3% BSA. The stained cells were then analyzed by an EPICS ELITE (Coulter Co.).

RESULTS

Characteristics of T24/ADM9 *in vitro* The morphology of T24/ADM9 cells was different from that of the T24 parent cells. The resistant cells were bizarre in shape and showed weak cell-to-cell attachment as compared to the T24 parent cells. The doubling time for the T24/ADM9 cells was 29 h, compared to 21 h for the T24 parent cells.

Drug resistance to anticancer agents The IC_{50} values along with the relative resistance, expressed as the ratio of the IC_{50} concentration for the T24/ADM9 versus T24 cells, are presented in Table I. The IC_{50} of T24/ADM9 was 5.51×10^{-1} μg/ml and the relative resistance was 9.2. The ADM-resistance of T24/ADM9 cells was maintained for at least 4 weeks in the drug-free medium. The T24/ADM9 cells showed various degrees of cross-resistance to a closely related analogue (epirubicin), vinca alkaloids (vinblastine and vincristine) and a DNA Topo II-targeting agent, etoposide, but mild collateral sensitivity to mitomycin C. The sensitivity to cisplatin and 5-fluorouracil remained unchanged.

Effect of verapamil on ADM sensitivity The IC_{50} values of ADM in the presence or absence of verapamil for T24 and T24/ADM9 are presented in Table II. As with T24, verapamil, even at a concentration of 10 μg/ml, enhanced the ADM sensitivity of T24/ADM9 by only

Table I. IC_{50} and the Relative Resistance of T24 and T24/ADM9 Cells to Different Chemotherapeutic Agents

Chemotherapeutic agent	IC_{50} (μg/ml)[a]		Relative resistance
	T24	T24/ADM9	
Adriamycin	$5.97\ (\pm0.55)\times10^{-2}$	$5.51\ (\pm0.40)\times10^{-1}$	9.2
Epirubicin	$1.38\ (\pm0.63)\times10^{-1}$	$5.24\ (\pm1.24)\times10^{-1}$	3.8
Vinblastine	$1.43\ (\pm0.31)\times10^{-3}$	$9.37\ (\pm2.00)\times10^{-3}$	6.6
Vincristine	$5.67\ (\pm1.41)\times10^{-3}$	$8.86\ (\pm0.48)\times10^{-2}$	15.6
Etoposide	$2.40\ (\pm0.63)\times10^{0}$	$8.98\ (\pm0.40)\times10^{0}$	3.7
Cisplatin	$1.76\ (\pm0.47)\times10^{0}$	$1.83\ (\pm0.61)\times10^{0}$	1.0
5-Fluorouracil	$4.11\ (\pm0.42)\times10^{0}$	$4.92\ (\pm0.51)\times10^{0}$	1.2
Mitomycin C	$2.90\ (\pm0.37)\times10^{-1}$	$1.12\ (\pm0.32)\times10^{-1}$	0.4

a) The mean values (±SD) for each cell line were derived from three separate experiments.

Table II. IC_{50} of ADM in the Presence or Absence of Verapamil for T24 and T24/ADM9

Verapamil concentration (μg/ml)	IC_{50} of ADM (μg/ml)[a]	
	T24	T24/ADM9
0	$6.72\ (\pm 0.62) \times 10^{-2}$	$5.89\ (\pm 0.39) \times 10^{-1}$
1	$4.45\ (\pm 0.53) \times 10^{-2}$ (1.5)	$3.03\ (\pm 0.43) \times 10^{-1}$ (1.9)
10	$2.21\ (\pm 0.34) \times 10^{-2}$ (3.0)	$2.09\ (\pm 0.33) \times 10^{-1}$ (2.8)

a) The mean values (±SD) for each cell line were derived from three separate experiments. The number in parentheses is the ratio of IC_{50} (ADM alone) to IC_{50} (ADM plus verapamil).

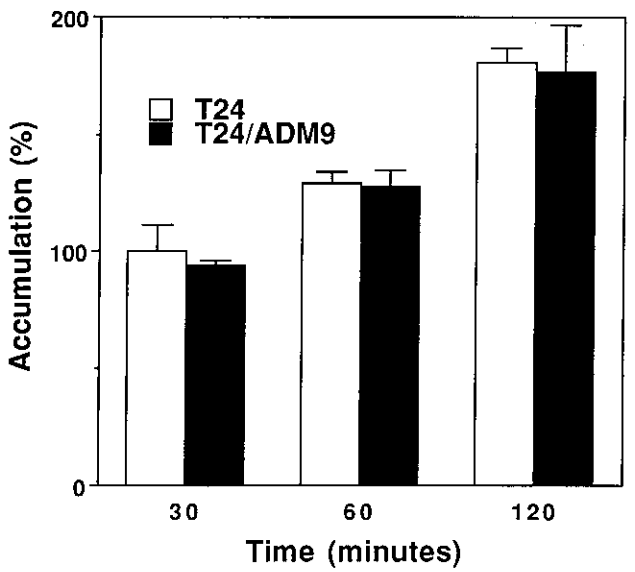

Fig. 1. Cellular accumulation of [^{14}C]ADM. Each value represents the average of triplicate wells. Bars, standard deviation.

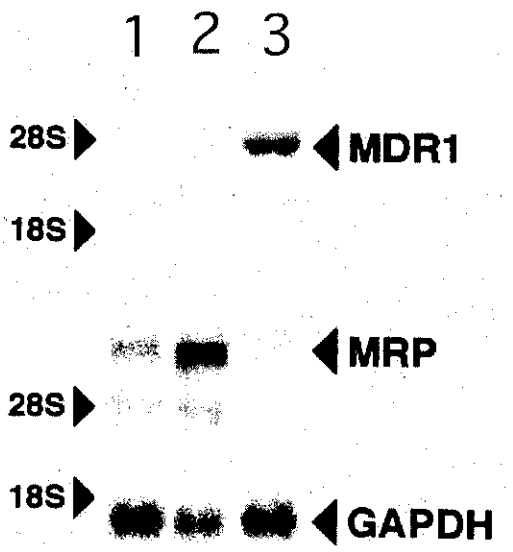

Fig. 2. Northern blot analysis of MDR1 and MRP mRNAs. Lane 1, T24; Lane 2, T24/ADM9; Lane 3, VJ300. The equivalent loading of total RNA is confirmed by the GAPDH blot.

about three times and complete reversal of ADM resistance was never obtained.

Cellular accumulation of ADM No significant difference was observed in the cellular accumulation of [^{14}C]ADM between the T24 parent and the T24/ADM9 at 3 time points after its addition (Fig.1). The experiments were repeated twice and the reproducibility of the results was confirmed.

Expression of MDR 1 and MRP genes In contrast to the classical MDR cell line, VJ300, no expression of MDR1 mRNA was evident in either T24/ADM9 or the T24 parent. However, the expression of MRP mRNA was found to be much higher in T24/ADM9 than the T24 parent (Fig. 2).

Flow cytometric analysis of Pgp expression The negative control, K562 did not show any expression of Pgp, whereas the positive control, K562/ADM showed a strong expression of Pgp (Fig. 3A). In contrast to K562/ADM, the T24/ADM9 cells did not show any expression of Pgp, like the T24 parent (Fig. 3B).

Cellular levels of DNA Topo I and II The expression of DNA Topo I mRNA was similar in both T24 and T24/ADM9, whereas the cellular level of DNA Topo II mRNA expression was much lower in T24/ADM9 than in the T24 parent (Fig. 4). The decrease of DNA Topo II gene product in T24/ADM9 was confirmed by Western blot analysis (Fig. 5). As shown in Fig. 6, however, decatenation of kDNA by serial dilutions of the nuclear extracts from T24/ADM9 cells showed activities similar to those of the T24 parent cells.

DISCUSSION

We have established a 9 times more ADM-resistant subline of T24 human bladder cancer (T24/ADM9) *in*

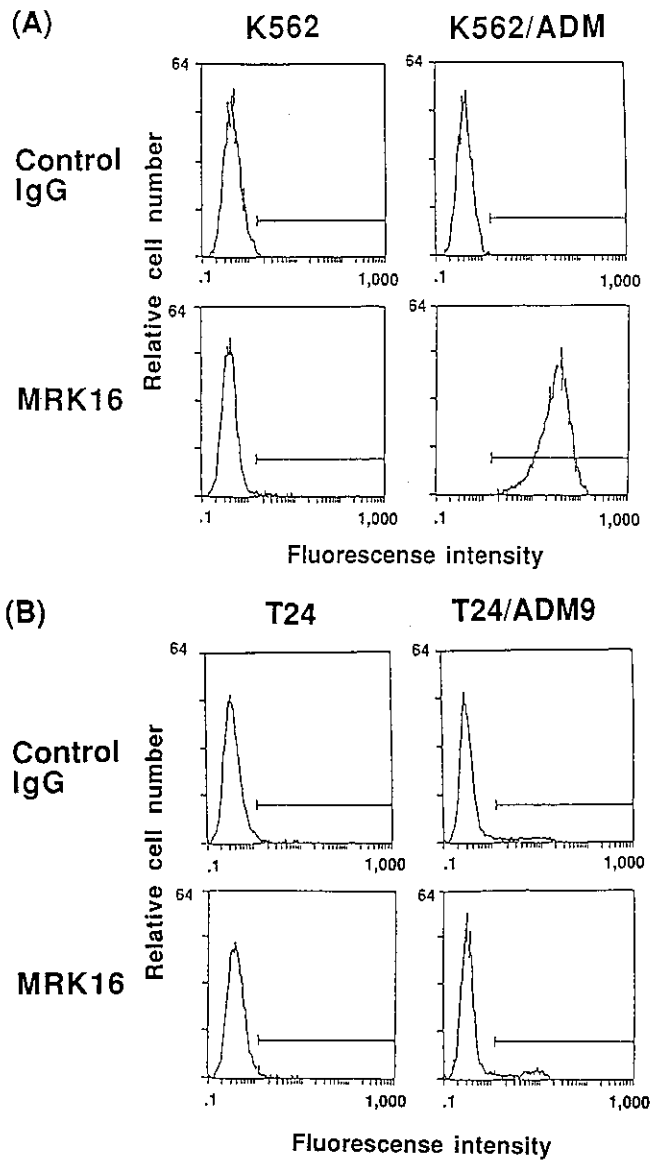

Fig. 3. A flow cytometric analysis of the P-glycoprotein expression in K562 and K562/ADM as the control (A), and T24 and T24/ADM9 (B). The vertical axis is a linear scale indicating the relative cell number. The horizontal axis is a logarithmic scale indicating the relative fluorescence intensity.

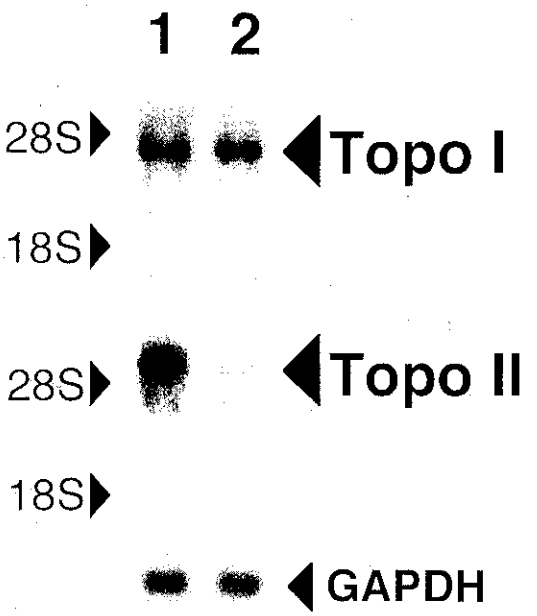

Fig. 4. Northern blot analysis of DNA Topo I and II mRNAs. Lane 1, T24; Lane 2, T24/ADM9. The equivalent loading of total RNA is confirmed by the GAPDH blot.

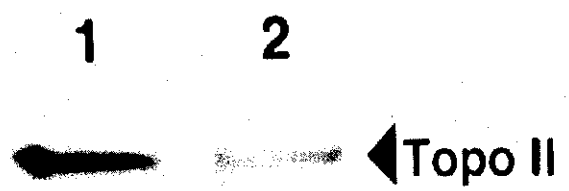

Fig. 5. Western blot analysis of DNA Topo II.

vitro by continuous exposure of the parental cells to gradually increased levels of ADM. The T24/ADM9 cells showed alterations in morphology and growth rate, as observed in KK47/ADM cells.[8] However, the frequent appearance of giant cells that was observed in MGH-U1R,[10] was not evident in T24/ADM9.

It has been demonstrated that when tumor cells acquire resistance to ADM, they generally show a cross-resistance to a wide range of structurally and functionally unrelated drugs.[8, 12, 17] T24/ADM9 also showed various degrees of cross-resistance not only to ADM derivatives but also to vinca alkaloids and etoposide. Many studies, including those on KK47/ADM, have shown that MDR is accompanied with a decrease in drug accumulation mediated by MDR1-encoded Pgp, which plays a significant role in the active outward transport of the drug.[8, 12, 17] However, no overexpression of the MDR1 gene was observed in T24/ADM9. A flow cytometric analysis also demonstrated that T24/ADM9 did not show any overexpression of Pgp, in contrast to the clas-

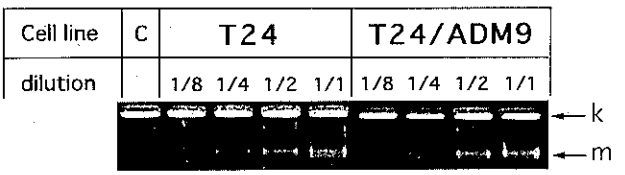

Fig. 6. A comparison of the decatenation activities using nuclear extracts from T24 and T24/ADM9 cells. The reaction mixture was incubated in the absence of nuclear extract (control, C) or in the presence of various concentrations of nuclear extracts. The protein concentration diluted at 1/1 corresponded to 25 ng of nuclear extract protein in each cell line, and the protein concentrations in the extracts from the two lines were equivalent. k, kDNA; m, free minicircles.

sical MDR cell line, K562/ADM.[8, 12] Furthermore, there was no significant difference in the cellular accumulation of ADM between the two lines. These results indicate that the main mechanism of MDR of T24/ADM9 is not a decrease in drug accumulation mediated by MDR1-encoded Pgp.

It has been demonstrated that a calcium channel blocker, verapamil, can inhibit the outward transport of the ADM and enhance the cytotoxic effects on resistant cells with an overexpression of Pgp.[18, 19] However, the enhancing effect of verapamil on the ADM sensitivity was only slight (about 3 times) in the T24/ADM9 cells as well as in the T24 parent cells, and complete reversal of the resistance could not be obtained. The concentration of verapamil used in this study was sufficiently high to reverse the ADM resistance in other cell lines.[18, 19] Therefore, these results also suggest that the main mechanism of MDR of T24/ADM9 is not an overexpression of Pgp. Shinohara *et al.*[9] reported that one of the 3 human bladder cancer cell lines showed little change in ADM sensitivity in the presence of verapamil, in spite of a clear expression of Pgp, and they considered that more than one pathway of MDR may be present in human bladder cancer cell lines.

The expression of non-Pgp-mediated MDR phenotype is considered to involve reduction of DNA Topo II[5, 6] and/or an overexpression of MRP gene,[19] which was isolated from the MDR human small cell lung cancer cell line H69AR by Cole *et al.*[7] T24/ADM9 showed both reduction of DNA Topo II and an overexpression of MRP mRNA as compared with the T24 parent. It has been reported that H69AR, which showed an overexpression of MRP, but not Pgp, display a cross-resistance profile very similar to those of classical MDR cell lines with an overexpression of Pgp: H69AR was resistant to vinca alkaloids and VP16 as well as anthracycline analogues.[20] Grant *et al.*[21] reported that the transfected HeLa cells with MRP expression vectors became resistant to vinca alkaloid, VP16, and ADM, and mentioned that MRP overexpression confers an MDR phenotype similar to that associated with an elevated level of Pgp. T24/ADM9, as well as H69AR, also showed a quite similar cross-resistance profile to that of classical MDR cell lines with an overexpression of Pgp.[8, 12, 13] The overexpression of MRP may thus be responsible for the cross resistance to vinca alkaloids and VP16 in T24/ADM9.

DNA Topo II, which catalyzes DNA conformational changes such as winding-unwinding, catenation-decatenation and condensation-decondensation, is an important cellular target of a number of antitumor agents, such as etoposide and anthracyclines, including ADM.[22] Takano *et al.*[5] demonstrated that reduced expression of DNA Topo II mRNA is closely associated with acquired etoposide resistance in KB cells. Thus, cellular sensitivity to DNA Topo II-targeting agents is considered to be regulated by the cellular DNA Topo II levels.[22] T24/ADM9 showed a quantitative loss of the DNA Topo II but has approximately the parental level of DNA Topo II catalytic activity. Similar findings have been reported in the VP16-resistant cell lines, KB/VP-1 and KB/VP-2: the acquisition of VP16-resistant phenotype in these cell lines was more closely correlated with decreased levels of stable DNA-Topo II complex formation in the presence of VP16 than with the catalytic activity of DNA Topo II.[5] Decreased content but not catalytic activity of DNA Topo II may also be one of the factors responsible for the expression of resistance to the DNA Topo II-targeting agents, ADM or etoposide in T24/ADM9.

In conclusion, these results suggest that an increased expression of MRP mRNA and a reduced expression of DNA Topo II may be responsible for the expression of MDR in the T24/ADM9 cells and that such non-Pgp-mediated MDR may develop in human bladder cancer treated with chemotherapy including ADM. For the treatment of such atypical MDR bladder cancer, the application of modulators to neutralize Pgp may not be appropriate and other new chemotherapeutic agents or modulators may be required. Thus, T24/ADM9 may be a useful model for developing new chemotherapeutic strategies against atypical MDR bladder cancer.

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