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Acute Induction of Adriamycin-resistance in Human Colon Carcinoma HT-29 Cells Exposed to a Sublethal Dose of Adriamycin

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To study the mechanisms of the acute induction of drug resistance in cancer cells, we have established a model system in which adriamycin (ADM) induces immediate drug resistance. In this system, human colon carcinoma HT-29 cells were pretreated for 1 h with a subtoxic dose of ADM (0.3 μg/ml) and incubated for 24 h in drug-free medium. Then the cells were treated for 1 h with ADM, and the cell survival was determined in terms of colony-forming ability. The survival of the pretreated cells was increased up to 100-fold, as compared with that of untreated cells. Such increased survival, however, was observed only after high doses of ADM (2 to 8 μg/ml); more than 99% of the cells were killed. These results indicate that only a small fraction of ADM-pretreated cells acquire the ADM-resistant phenotype. Similar induced resistance was observed in five of seven subclones isolated from HT-29 cells by limiting dilution, suggesting that the majority of cells in the parental HT-29 population could acquire the ADM-resistant phenotype. In the subclone HT-29T9, the ADM pretreatment induced concomitant resistance to daunomycin, VP-16, and VM-26 but not to agents other than topoisomerase II inhibitors. The ADM-induced drug resistance did not accompany MDR1 gene expression and could not be overcome by verapamil, a P-glycoprotein inhibitor. The present system could be useful to study the acute induction mechanism(s) of ADM-resistance, which could be relevant to clinical resistance in patients.

Key words: Adriamycin — Drug resistance — P-glycoprotein — Stress response

The failure of cancer chemotherapy is mainly due to the development of cellular drug resistance. ADM[4] is one of the most active anticancer agents and is used in a wide variety of malignancies. The development of cellular resistance to ADM is attributed to two processes: genetic (mutational) and epigenetic (non-mutational). These processes frequently lead to the same mechanisms of ADM resistance, such as alteration in the level of target enzymes.

ADM-resistant mutants have been established by many research groups. According to a mutation-selection hypothesis for drug-resistance in cancer, the ADM resistance is mainly due to the selection of resistant cells.[1] Most of these ADM-resistant mutants show cross-resistance to various structurally unrelated antitumor drugs. The mechanism of multidrug resistance was first explained in terms of the increased expression of the MDR1 gene, which encodes P-glycoprotein, an energy-dependent efflux pump for lipophilic compounds.[2–4] An alternative mechanism of multidrug resistance is the alteration of DNA topoisomerase II. Topoisomerase II poisons, such as ADM and VP-16, stabilize the cleavable enzyme-DNA complexes and DNA breaks result.[5–7] Decreases in the quantity of topoisomerase II or alterations in the enzyme's ability to bind DNA and drug could confer drug resistance by decreasing the number of cleavable complexes.[8,9] Other mechanisms involved in multidrug resistance have been described in recent reviews.[4,10]

Epigenetic alterations in cells can also cause ADM resistance. Cells exposed to a variety of nonphysiological stimuli, such as elevated temperature and nutrient or oxygen deprivation, can induce stress proteins, such as HSPs and GRPs.[11–13] Many studies have demonstrated that the cellular stress responses could be associated with ADM resistance.[14–16] The development of ADM resistance correlates with the induction and repression of GRPs.[14] The glucose-regulated stress can induce resistance to VP-16 in Chinese hamster cells.[17] The mechanism of this induction involves a reduction of the nuclear level of topoisomerase II.[18] On the other hand, the induction of HSPs, which generally correlates with thermotolerance, also correlates with a transient emergence

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[4] The abbreviations used are: ADM, adriamycin; ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea; VP-16, etoposide; VM-26, teniposide; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; cDNA, complementary DNA; PBS(−), phosphate-buffered saline without calcium or magnesium; HSPs, heat shock proteins; GRPs, glucose-regulated proteins.

of cross-tolerance to several cytotoxic agents, including ADM.[15, 16, 19, 20] Some of the HSP inducers were found to induce resistance to anticancer drugs such as ADM and VM-26,[15, 21] and conversely, drugs such as VM-26 and bleomycin induced transcription of a certain heat shock gene.[21, 22] Heat shock and arsenite can induce increased expression of MDR1 mRNA and P-glycoprotein in a human renal carcinoma cell line.[23] Some antitumor agents were reported to stimulate transcription of a reporter gene from the human MDR1 promoter.[24] Furthermore, it was recently reported that MDR1 expression in several human cell lines could be induced by transient exposure to different cytotoxic drugs.[25] These findings raise the possibility that drug resistance can be induced directly by antitumor drugs through cellular stress responses.

In the present study, we found that pretreatment of human colorectal carcinoma HT-29 cells with sublethal doses of ADM led to the emergence of ADM-resistant subpopulations. The implication of the emergence of this resistance *in vitro* and *in vivo* is discussed.

MATERIALS AND METHODS

Chemicals Antitumor agents were generous gifts from the following companies: ADM and mitomycin C from Kyowa Hakko Kogyo Co., Ltd., Tokyo; cisplatin, VP-16, and VM-26 from Bristol Meyers Squibb Co., Ltd., Tokyo; bleomycin and methotrexate from Nippon Kayaku Co., Ltd., Tokyo; daunomycin from Meiji Seika Kaisha, Ltd., Tokyo; camptothecin from Yakult Co., Ltd., Tokyo; ACNU from Sankyo Co., Ltd., Tokyo; vincristine from Eli Lilly Japan Co., Ltd., Kobe; verapamil from Eisai Co., Ltd., Tokyo. 5-Fluorouracil was purchased from Wako Pure Chemical Industries, Osaka. All other reagents were of analytical grade.

Cell culture conditions Human colon carcinoma cell line HT-29 was obtained from Dr. R. Shoemaker of the National Cancer Institute (NIH, Bethesda, MD). Sublines of HT-29 were cloned by the limiting dilution technique in our laboratory. Human ovarian cancer line A2780 and its ADM-resistant subline (2780^{AD}) were provided by Drs. R. Ozols and T. Hamilton, Medicine Branch, NCI, NIH.[26] All cell lines were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum and 100 μg/ml kanamycin (complete medium), and were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Treatment with cytotoxic agents Cells were harvested from logarithmically proliferating cultures by brief trypsinization and seeded into Corning 12-well plates at a density of 2×10^4 cells/well, each well containing 2 ml of complete medium. After incubation for 2 days to allow the cells to overcome the lag period, they were pretreated for 1 h with ADM at concentrations between 0.1 and 0.3 μg/ml. Then, the cells were washed with PBS(−) and incubated for a further 24 h in the ADM-free complete medium. Likewise, the cells were pretreated for 1 h with daunomycin, VP-16, and VM-26 at indicated concentrations (Table I) and were incubated as above.

Control cells and cells pretreated as above with ADM were then treated for 1 h with various concentrations of ADM (0.2–8 μg/ml), daunomycin (0.25–1.5 μg/ml), VP-16 (10–150 μg/ml), VM-26 (1–15 μg/ml), 5-fluorouracil (25–400 μg/ml), cisplatin (5–30 μg/ml), mitomycin C (0.25–2 μg/ml), and ACNU (50–125 μg/ml), or for 24 h with bleomycin (5–30 μg/ml), camptothecin (1–100 ng/ml), methotrexate (0.05–0.5 μg/ml), and vincristine (5–100 ng/ml). The 24-h exposure was necessary to induce measurable cytotoxicity. To examine the effect of verapamil, an inhibitor of P-glycoprotein,[27, 28] on ADM-cytotoxicity, control cells and ADM-pretreated cells were preincubated for 1 h with 10 μg/ml verapamil before the ADM treatment. Verapamil at 10 μg/ml slightly reduced (less than 5%) the colony-forming ability of the cells. After the treatment with the drugs, cells were tested for colony-forming ability.

Colony-forming ability assay Immediately after drug treatment, the cells were washed with PBS(−), trypsinized, and plated at appropriate dilutions in 100-mm dishes in drug-free complete medium. After 7–12 days of incubation at 37°C, the colonies were fixed with formaldehyde and stained with crystal violet for enumeration. The relative survival was corrected for the plating efficiency of the appropriate control. The typical plating efficiency of untreated control cells was 0.6 to 0.8 for HT-29 and its subclones.

Cellular uptake of ADM Cells were incubated in 1 ml of complete medium containing 1 μg/ml of [^{14}C]ADM (1.92 GBq/mmol; Amersham Japan Ltd., Tokyo) for 2 h at 37°C. The cells were then washed with ice-cold PBS, and the intracellular drug accumulation was determined as described previously.[29]

RNA extraction and cDNA-PCR analysis Total cellular RNA was extracted by a small-scale SDS extraction procedure.[30] The cDNA synthesis and PCR amplification of MDR1 and β_2-microglobulin (internal control) cDNA sequences were carried out as described by Noonan *et al.*[31] After 40 cycles of PCR, the products were separated on 15–25% polyacrylamide gradient gels and silver stained.

Antibodies and immunoblot analysis Mouse monoclonal antibody T14C against human topoisomerase I was established in our laboratory by using purified topoisomerase I from human myelogenous leukemia K562 as the antigen.[32] Mouse monoclonal antibody 2H5 against the recombinant Mr 75,000 N terminus of human topoisomerase IIα was provided by Dr. T. Andoh. Another anti-

body, KF4, to human topoisomerase II was obtained from Cambridge Research Biochemicals Inc., Wilmington, DE.

Cells were solubilized with 2% SDS, 1% 2-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride, and 50 m*M* Tris-HCl (pH 7.5). The protein content was determined by the method of Bradford.[33] Solubilized whole cell lysates (50 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis, and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Dasse, Germany). For detection of topoisomerases, blots were incubated for 1 h with the above antibodies to topoisomerases and visualized with horseradish peroxidase-conjugated sheep anti-mouse IgG under chemiluminescence detection (Amersham Japan, Ltd.).

RESULTS

Induction of ADM-resistance in HT-29 cells Pretreatment of human colorectal carcinoma HT-29 cells with sublethal doses of ADM induced resistance to ADM at high doses of the drug (Fig. 1). In this case, HT-29 cells were pretreated for 1 h with ADM and subsequently incubated for 24 h without ADM to allow for recovery. Under these conditions, the values of colony-forming efficiency of the cells pretreated with 0.1, 0.2 and 0.3 μg/ml of ADM were above 95%, 70–75% and 55–60%, respectively. To estimate the induction of ADM resistance, we treated the pretreated cells with between 0.2 and 8 μg/ml of ADM for 1 h. The cells pretreated with 0.2 μg/ml and 0.3 μg/ml, but not 0.1 μg/ml, showed significant resistance to ADM at 2–8 μg/ml (Fig. 1B). The survival of the ADM-pretreated cells at 0.3 μg/ml was increased up to 100-fold over control cells. At 0.2–0.8 μg/ml of ADM, however, the survival of the ADM-pretreated cells was lower than that of the control cells (Fig. 1A).

Induction of ADM-resistance in subclones of HT-29 cells To investigate what proportion of the HT-29 cells could acquire the ADM-resistant phenotype mentioned above, we isolated some subclones by limiting dilution of HT-29 cells. Pretreatment of the clones with 0.3 μg/ml of ADM produced ADM resistance in 5 of 7 clones examined, indicating that the majority of the clones in the parental HT-29 population could be transformed to the ADM-resistant phenotype. Fig. 2 shows the results with two representative clones; one (HT-29T9) acquired ADM-resistant phenotype on ADM pretreatment, but the other (HT-29T17) did not. HT-29T9 clone, which showed a similar sensitivity profile to the parental HT-29 cells (compare Fig. 1 and Fig. 2), was used for further studies.

We next studied the effect of a recovery period after the ADM pretreatment on the emergence of ADM-resistant cells (Fig. 3). The ADM resistance could be induced at 24, 36, and 48 h after the pretreatment, but not at 12 h. More than 24 h of recovery was required for the development of the DNA resistance.

The effects of more than 48 h of recovery could not be investigated, since the density of HT-29T9 cells at 48 h

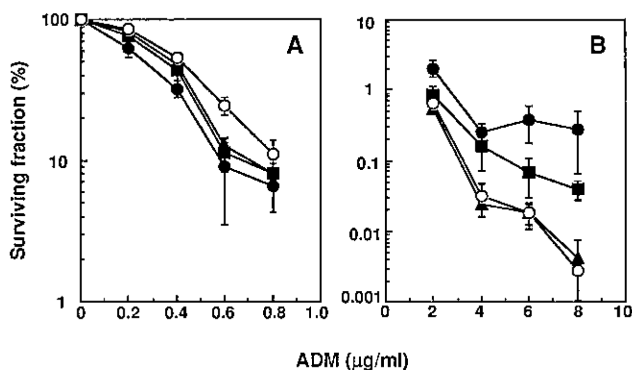

Fig. 1. Induction of ADM resistance by pretreating HT-29 cells with ADM. Exponentially growing HT-29 cells were pretreated for 1 h with ADM at 0.1 (▲), 0.2 (■) or 0.3 μg/ml (●) and incubated for 24 h in drug-free complete medium. Control (open symbols) and ADM-pretreated cells (closed symbols) were exposed for 1 h to ADM at various concentrations; A, 0.2 to 0.8 μg/ml; B, 2 to 8 μg/ml. The survival curves were determined by assaying colony-forming ability. Points represent mean values and bars indicate the SD of quadruplicate determinations in two independent experiments performed in duplicate.

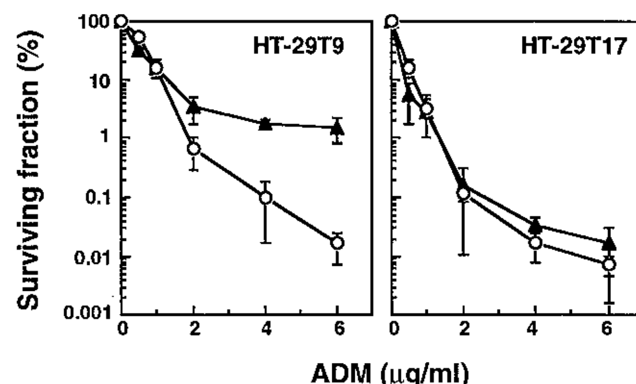

Fig. 2. Induction of ADM resistance by pretreating HT-29 subclones with ADM. Exponentially growing cells were pretreated for 1 h with 0.3 μg/ml of ADM and incubated for 24 h without ADM. Control (open symbols) and ADM-pretreated cells (closed symbols) were treated for 1 h with ADM at various concentrations. The survival curves were determined by assaying colony-forming ability. Points represent mean values and bars indicate the SD of quadruplicate determinations in two independent experiments performed in duplicate.

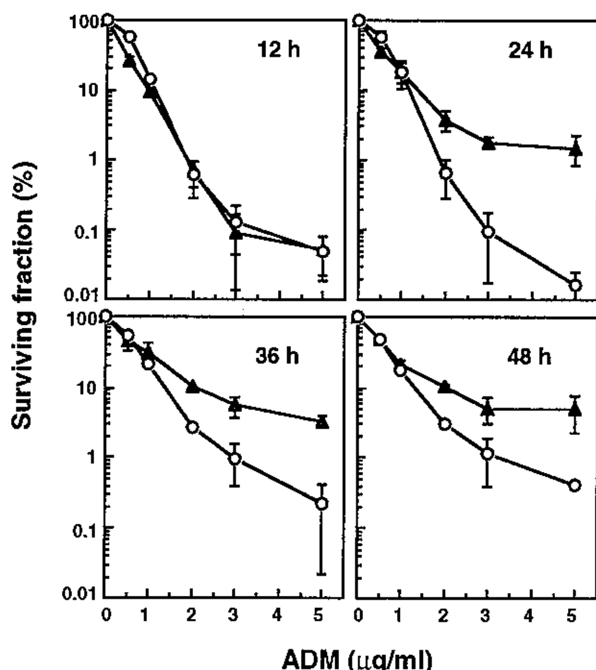

Fig. 3. Effect of recovery time on the development of ADM-resistance in HT-29T9 cells. HT-29T9 cells were pretreated for 1 h with ADM at 0.3 μg/ml and incubated for 12, 24, 36, or 48 h in drug-free complete medium. Control (open symbols) and ADM-pretreated cells (closed symbols) were exposed for 1 h to ADM at between 0 and 5 μg/ml. The survival curves were determined by assay of colony-forming ability. Points represent mean values and bars indicate the SD of quadruplicate determinations in two independent experiments performed in duplicate.

after the ADM pretreatment reached near confluency under our experimental conditions. As the sensitivity of HT-29 cells to anthracyclines has been reported to drop with confluence,[34, 35] we chose the 24 h recovery period for further studies.

Effect of ADM-pretreatment on the induction of resistance to various antitumor agents We examined the induction of drug resistance to various antitumor agents after ADM pretreatment. HT-29T9 cells, with or without ADM-pretreatment, were exposed to 11 other drugs, and the individual survival curves are complied in Fig. 4. ADM-pretreated HT-29 cells exhibited several degrees of resistance to daunomycin, VP-16, and VM-26 (Fig. 4A). These drugs, and ADM, act on the nuclear enzyme DNA topoisomerase II. Cytotoxicity was enhanced by ADM-pretreatment for 5-fluorouracil, cisplatin, bleomycin, and methotrexate (Fig. 4B). Among them, 5-fluorouracil was most efficiently potentiated by ADM pretreatment. HT-29T9 cells did not show significant resistance to ACNU, mitomycin C, camptothecin, or vincristine after the ADM-pretreatment (Fig. 4C). In these experiments, HT-29T9 cells were exposed for 24 h to methotrexate, bleomycin, vincristine, and camptothecin because a 1-h exposure to these drugs did not induce measurable cytotoxicity in HT-29T9 cells. Similar induction of resistance to ADM could be observed when the HT-29T9 cells were treated for 24 h and 1 h (compare Fig. 4A and Fig. 2).

Induction of ADM-resistance by the pretreatment of HT-29T9 cells with other agents The above results indicate that ADM itself can induce resistance to DNA topoisomerase II inhibitors. We next examined the effects of pretreatment with other topoisomerase II inhibitors on ADM resistance induction. HT-29T9 cells were pretreated for 1 h with daunomycin, VP-16, or VM-26 at sublethal doses, and then incubated for 24 h without these agents. The colony-forming efficiency of the pretreated cells is summarized in Table I. Pretreatment of HT-29T9 cells with daunomycin, an analog of ADM, induced an ADM-resistant subpopulation (Fig. 5A). However, pretreatment of cells with VP-16 or VM-26 did not induce significant resistance to ADM (Fig. 5B and C). While ADM and daunomycin, both free radical-producing compounds, induced ADM-resistance as mentioned above, hydrogen peroxide (50 and 100 *μM*) did not induce ADM-resistance (data not shown).

The expression of P-glycoprotein (MDR1) We investigated whether the ADM-pretreatment of HT-29T9 cells could induce the expression of the MDR1 gene, as determined by PCR (Fig. 6A). HT-29T9 cells were pretreated for 1 h with 0.3 μg/ml of ADM and incubated for 24 h without ADM. MDR1 mRNA was not detected in either control (lane 1) or ADM-pretreated HT-29T9 cells (lane 2), whereas it was obviously detected in human ovary carcinoma A2780 (lane 3) and its ADM-resistant 2780^{AD} cell line (lane 4). Drug accumulation experimentation also supported these results, as [^{14}C]ADM accumulation in ADM-pretreated HT-29T9 cells was not reduced, as compared with control cells (intracellular accumulations of [^{14}C]ADM during the incubation period for 2 h were 12.48 ± 0.57 and 12.87 ± 1.09 ng/10^5 cells in control and ADM-pretreated cells, respectively). Furthermore, ADM-induced resistance was not reversed by 10 μg/ml of verapamil, an inhibitor of the P-glycoprotein efflux pump (Fig. 6B). These observations, together with the finding of non-cross-resistance to vincristine (Fig. 4A), indicate that the ADM-induced resistance is not associated with P-glycoprotein (MDR1 mRNA) expression.

Immunoblot analysis of DNA topoisomerases We measured the cellular contents of topoisomerases I and II in ADM-pretreated and untreated HT-29T9 cells by Western blotting (Fig. 7). The amount of topoisomerase I in ADM-pretreated cells was not significantly different

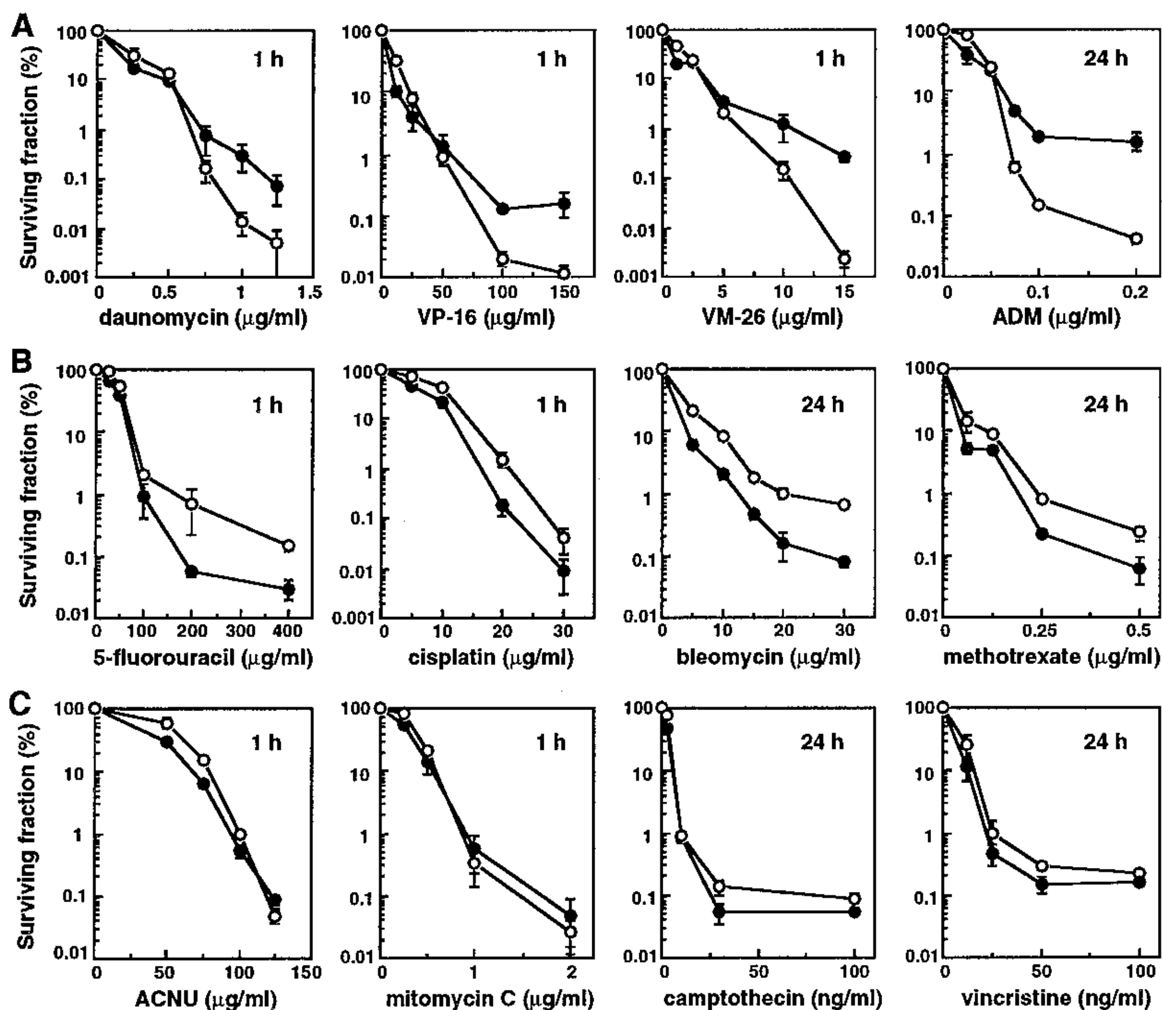

Fig. 4. Effect of ADM-pretreatment on sensitivity of HT-29T9 cells to various antitumor agents. HT-29T9 cells were pretreated for 1 h with ADM at 0.3 μg/ml. After a 24-h recovery period, control (open symbols) and ADM-pretreated cells (closed symbols) were exposed for the indicated time to various antitumor drugs. A, associated resistance; B, sensitization; C, no effect. Points represent mean values and bars indicate the SD of quadruplicate determinations in two independent experiments performed in duplicate.

from that in untreated cells (Fig. 7B). On the other hand, topoisomerase II content was significantly increased in ADM-pretreated cells, as determined by Western blots with an anti-topoisomerase II antibody 2H5 (Fig. 7A). A similar result was obtained by using another anti-topoisomerase II antibody, KF4 (data not shown).

DISCUSSION

To develop effective therapeutic approaches, induction mechanisms of drug resistance and means for their prevention must be clarified. In this study we examined whether resistance could be acquired in response to drug exposure, as with thermotolerance to heat shock.[19] We established a model system in which ADM induced resistance to topoisomerase II inhibitors. In this system, the first step was pretreatment with ADM at sublethal doses (0.1–0.3 μg/ml). Under these conditions, more than 50% of HT-29 cells could survive, as determined by colony formation. The second step was incubation without ADM for a short recovery period (24 h), which almost corresponded to one cell generation of HT-29 cells (18–20 h). These two steps were designed to induce ADM-resistance. The third step was to estimate resistance induction in terms of colony formation after treating the cells with ADM for 1 h. Under these conditions,

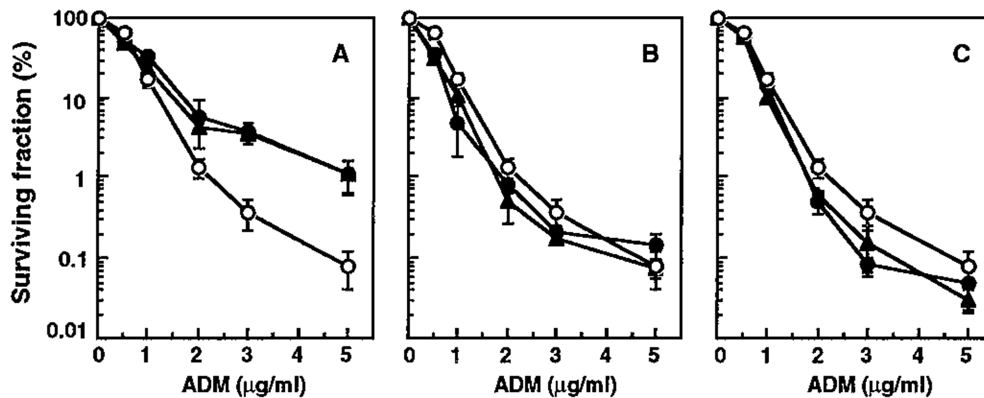

Fig. 5. Effect of the pretreatments of HT-29T9 cells with topoisomerase II inhibitors on the induction of ADM resistance. HT-29T9 cells were pretreated for 1 h with A, daunomycin at 0.05 (▲) or 0.1 μg/ml (●); B, VP-16 at 5 (▲) or 10 μg/ml (●); or C, VM-26 at 0.5 (▲) or 1 μg/ml (●). After a 24-h recovery period, control (open symbols) and pretreated cells (closed symbols) were exposed for 1 h to ADM at between 0 and 5 μg/ml. Points represent mean values and bars indicate the SD of quadruplicate determinations in two independent experiments performed in duplicate.

Table I. Colony-forming Ability of HT-29T9 Cells Pretreated with Topoisomerase II Inhibitors

Drug	Concentration (μg/ml)	Colony-forming ability[a] (% of control)
ADM	0.3	57.5±4.0
Daunomycin	0.05	89.4±7.3
	0.1	56.0±4.7
VP-16	5	84.8±4.1
	10	24.7±3.7
VM-26	0.5	82.3±5.4
	1	28.7±1.9

HT-29T9 cells were pretreated for 1 h with drugs at the indicated concentrations. After a 24-h recovery period, the cells were trypsinized and plated in a 100-mm dish at 500 cells per dish. After 7 days of incubation in drug-free complete medium, the formed colonies were fixed, stained, and counted.

a) Colony-forming ability of drug-treated cells was corrected for the plating efficiency of untreated cells. The values are means±SD of two independent experiments performed in triplicate.

ADM pretreatment (0.3 μg/ml) of HT-29 cells induced ADM resistance in a small fraction of the treated cells (Fig. 1). Similar resistance induction occurred in 5 of 7 subclones, e.g., HT-29T9, isolated from the parent HT-29 cells (Fig. 2). This observation suggests that most of the clones in the parental HT-29 population could be transformed to the ADM-resistant phenotype. Pretreating HT-29T9 cells with ADM could induce resistance not only to ADM but also to daunomycin, VP-16, and VM-26 (Fig. 4). These drugs have the same cellular target, topoisomerase II; therefore, the ADM-induced resistance in our system can be regarded as resistance to topoisomerase II inhibitors.

The question then arises of whether the induction of the resistance perhaps results from cytotoxic selection of preexisting resistant cells to topoisomerase II inhibitors. The possibility of cytotoxic selection, however, can be ruled out because pretreating HT-29T9 cells with VP-16 or VM-26 did not induce ADM-resistance (Fig. 5), and further, if ADM-resistant cells preexisted among HT-29T9 cells, they could not expand enough to form such a resistant subpopulation within a 24-h recovery period, as used in this experiment. Therefore, mechanisms other than cytotoxic selection should be responsible for the emergence of the ADM-resistant subpopulations in this experiment.

The ADM pretreatment of the cells induced resistance only to topoisomerase II inhibitors, among the drugs examined (Fig. 4). We could not observe the induction of MDR1 (encoding P-glycoprotein) mRNA expression (Fig. 6) that would confer resistance to topoisomerase II inhibitors and *Vinca* alkaloids.[2-4] Topoisomerase II inhibitors, such as ADM and VP-16, stabilize the cleavable complex between the DNA and topoisomerase II, increase DNA breakage and inhibit the DNA-rejoining reaction.[5-7] We observed that the content of topoisomerase II was slightly increased in ADM-pretreated HT-29T9 cells (Fig. 7). However, increased level of topoisomerase II gene expression has been reported to have a positive correlation with increased sensitivity to ADM, VP-16, and VM-26 in human lung cancer cell lines.[36] In our previous study, we reported that increased levels of topoisomerase II protein, mRNA, and activity coincided with increased sensitivity to ADM in a variant

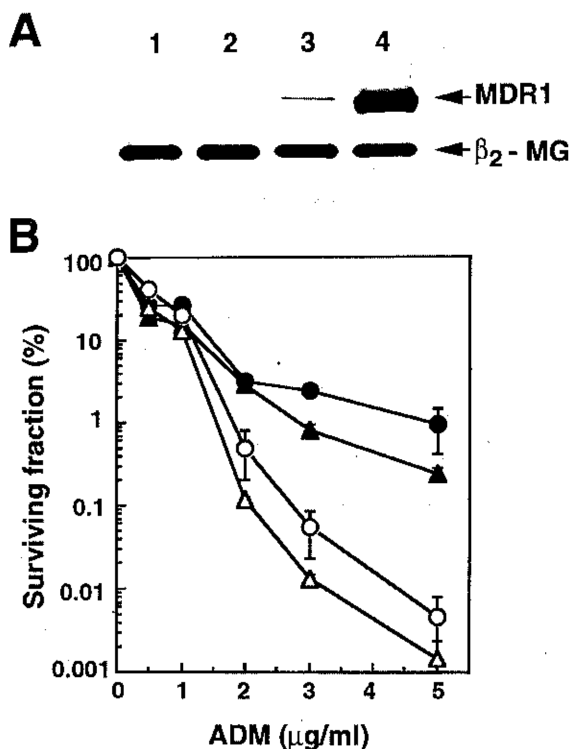

Fig. 6. A: cDNA-PCR analysis of MDR1 mRNA expression in ADM-pretreated HT-29T9 cells. In each lane, the upper band (157 base pairs) corresponds to MDR1, and the lower band (120 base pairs) corresponds to β_2-microglobulin (β_2-MG)-specific PCR products. HT-29T9 cells were pretreated for 1 h with ADM and cultured in drug-free medium for 24 h. A2780 and A2780^{AD} cells were not treated with ADM. Lane 1, HT-29T9, control; lane 2, pretreated with 0.3 μg/ml; lane 3, A2780; lane 4, 2780^{AD}. B: Effect of verapamil on ADM-induced resistance. Control (open symbols) and ADM-pretreated HT-29T9 cells (closed symbols) were exposed for 1 h to ADM at between 0 and 5 μg/ml in the absence (○, ●) or presence (△, ▲) of 10 μg/ml of verapamil. Verapamil was added 1 h prior to the addition of ADM. Points represent mean values and bars indicate the SD of quadruplicate determinations in two independent experiments performed in duplicate.

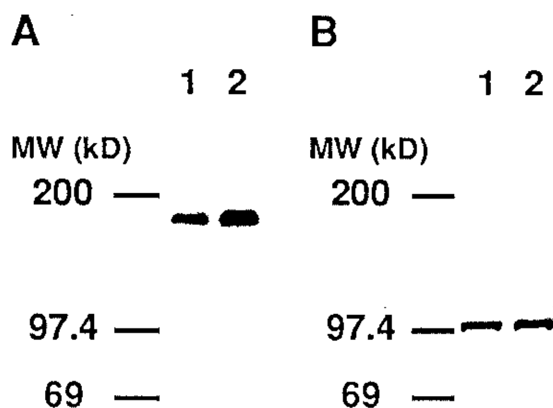

Fig. 7. Immunoblot analysis of topoisomerase II (A) and topoisomerase I (B). Whole cell lysates from control (lane 1) and ADM-pretreated (0.3 μg/ml) HT-29T9 cells (lane 2) were prepared. Fifty μg of each cell lysate was subjected to immunoblot analysis.

of HT-29 cells.[37] These previous studies indicate that increased amounts of topoisomerase II in ADM-pretreated HT-29T9 cells cannot be the mechanism of resistance to topoisomerase II inhibitors. The ADM pretreatment (0.3 μg/ml) of HT-29T9 cells reduced the cell proliferation; the cell density at 24 h after the pretreatment was 70% of the untreated cells' density. However, a similar reduction of cell proliferation occurred in HT-29T9 cells pretreated with VP-16 and VM-26, and in HT-29T17 cells pretreated with ADM. In these cases, ADM resistance was not induced (Figs. 2 and 5). These observations indicate that a reduction of cell proliferation is not the direct cause of the ADM-resistance. At present, the mechanism or mechanisms of the ADM-induced resistance in HT-29T9 cells remain uncertain. Further studies are needed to clarify the underlying mechanisms of the resistance to topoisomerase II inhibitors in ADM-pretreated HT-29 cells.

Another question is whether the induction of resistance to topoisomerase II inhibitors is due to genetic or epigenetic changes in the cells during pretreatment and recovery. ADM may induce a mutation leading to resistance. If a mutation is responsible for the resistance induction, such a mechanism would induce nonspecific resistance to a variety of antitumor agents by a random mutation process. In our system, ADM-induced resistance occurred specifically to topoisomerase II inhibitors, ruling out mutation as a mechanism. However, if ADM could induce a mutation at a topoisomerase II-related specific site on the genome, although this seems unlikely, such a mutation could induce resistance specific to topoisomerase II inhibitors. Alternatively, ADM-induced resistance in HT-29T9 cells could be explained by epigenetic alterations through cellular stress responses by ADM. As shown in Fig. 1, resistance in ADM-pretreated cells occurred only when the cells were pretreated at a cytocidal ADM concentration (0.3 μg/ml), but not at a non-cytocidal ADM concentration (0.1 μg/ml). The ADM resistance could not be induced by VP-16 or VM-26 pretreatment at any dose (Fig. 5). This suggests that the exposure of the cells to cytocidal ADM can induce a specific cellular response that leads to resistance to topoisomerase II inhibitors.

We have shown that pretreating HT-29 cells at cytocidal doses of ADM induced resistance to topoisomerase II inhibitors. This type of resistance-induction could be an obstacle to cancer chemotherapy that aims at total cell kill in tumors. Although the significance of the present findings to the development of clinical drug resistance remains to be seen, this system should be useful in the study of the induction mechanisms of resistance to topoisomerase II inhibitors, including ADM, in clinical situations.

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