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Mitomycin C cross-resistance induced by Adriamycin in human ovarian cancer cells in vitro

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Summary. We prepared Adriamycin-resistant cancer cells by exposing an ovarian serous cystadenocarcinoma cell line to the drug. The resistant cells also showed cross-resistance to a wide variety of other compounds, including vincristine, vinblastine, actinomycin D, daunorubicin, mitomycin C and carboquone. Against vincristine, the cells showed a >5,000-fold increase in resistance, far surpassing their resistance to the selection drug. The resistant cells displayed a decrease in intracellular Adriamycin content and an increase in the mRNA of the *mdr*-1 gene coding for P-glycoprotein, with no amplification of the DNA. In revertant cells, resistance to Adriamycin was lost, but that to mitomycin C was maintained. Adriamycin resistance was partially overcome by the addition of verapamil or cyclosporin A, but cross-resistance to mitomycin C was not influenced at all. These results strongly suggest that the resistance to mitomycin C observed in our Adriamycin-resistant cells was due to some other mechanism than that causing multidrug resistance.

Introduction

The introduction of potent chemotherapy has dramatically improved the prognosis for ovarian cancer. However, due to disease recurrence the phenomenon of drug resistance has become an important issue in the treatment of ovarian cancer. In our clinical investigations of chemotherapy for ovarian cancer, we have attempted to study the characteristics of restistant cancers by preparing Adriamycin-resistant cells from human ovarian serous cystadenocarcinoma. The multidrug-resistant (MDR) phenotype for Adriamycin-resistant cancer cell lines has been found; it shows cross-resistance to various anticancer agents with different mechanisms of action. The P-glycoprotein in the cell membrane is considered to act as a drug efflux pump in MDR, and its gene (*mdr*-1) has also been identified [7, 12, 17–19].

Our Adriamycin-resistant ovarian serous cystadenocarcinoma cells (TAOV/A0.1) showed relatively low resistance to Adriamycin but exhibited cross-resistance to a wide variety of other drugs, such as vincristine, vinblastine, actinomycin D, and daunorubicin. In particular, these cells displayed a >5,000-fold increase in resistance to vincristine, far exceeding their specific resistance to the selection drug Adriamycin. More interestingly, this cell line also showed cross-resistance to mitomycin C and carboquone. The mechanism of the development of resistance by these cells was investigated in the present study.

Materials and methods

Drugs. Adriamycin, mitomycin C, and 5-fluorouracil were produced by Kyowa Hakko Kogyo Ltd. (Tokyo, Japan). Vincristine and vinblastine were obtained from Shionogi Co. Ltd. (Osaka, Japan), and cisplatin, carboplatin, and bleomycin were received from Nippon Kayaku Ltd. (Tokyo, Japan). Carboquone and nimustine hydrochloride were obtained from Sankyo Ltd. (Tokyo, Japan), and methotrexate was furnished by Lederle Japan Ltd. (Tokyo, Japan). Cytarabine was obtained from Nippon Shinyaku Ltd. (Kyoto, Japan); daunorubicin, from Meiji Seika Ltd. (Tokyo, Japan); and actinomycin D, from Banyu Pharmaceutical Industries Ltd. (Tokyo, Japan). Verapamil hydrochloride was purchased from Eisai Ltd. (Tokyo, Japan) and cyclosporin A was obtained from Sandoz Japan Ltd. (Tokyo, Japan). Adriamycin hydrochloride tagged with carbon 14, sp. act. 56 mCi/mmol) and deoxycytidine 5′-[α-^{32}P]-triphosphate, triethylammonium salt ([^{32}P]-dCTP; sp. act., 3,000 Ci/mmol) were purchased from Amersham Japan Ltd. (Tokyo, Japan). All reagents were products of high purity.

Cell culture. The serous cystadenocarcinoma cell line TAOV, established from a surgical specimen taken from a patient not undergoing chemotherapy, was exposed to Adriamycin by addition to minimal essential medium (MEM) containing 10% fetal bovine serum (FBS, General Scientific Laboratories). The concentration of Adriamycin was initially 0.0125 μg/ml, and four lines of resistant cells were prepared (TAOV/A0.025, TAOV/A0.05, TAOV/A0.1, TAOV/A0.2) by a stepwise selection method. Next, by growing the adriamycin-resistant TAOV/A0.1 cells in culture broth not containing the drug, three lines of revertant cells were obtained (TAOV/A0.1/R6W, TAOV/A0.1/R11W,

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Table 1. Relative drug resistance levels of Adriamycin-resistant ovarian cancer cells

	Relative resistance[a]:	
	TAOV/A0.05	TAOV/A0.1
Methotrexate	2.2	1.8
5-Fluorouracil	0.9	2.5
Cytarabine	0.6	1.6
Carboquone	1.6	14
Nimustine hydrochloride	1.0	2.4
Daunorubicin	0.3	6.3
Vincristine	14	>5000
Vinblastine	1.4	4.1
Cisplatin	0.9	1.8
Carboplatin	0.8	1.4
Bleomycin	0.5	2.1
Mitomycin C	0.8	11
Adriamycin	2.0	9.2
Actinomycin D	1.8	8.3

[a] Relative resistance = resistant-cell IC_{50}/parent-cell IC_{50}. The drug sensitivity was tested by MTT assay in triplicate, and the mean was determined by repeating the experiment at least three times

TAOV/A0.1/R30W) following culture for 6, 11, and 30 weeks, respectively. Another human ovarian serous cystadenocarcinoma cell line, KUOV2, was also continuously exposed to Adriamycin by addition to RPMI 1640 medium with 10% FBS, and Adriamycin-resistant cells (KUOV2/A0.8) were prepared.

Sensitivity testing and modulation of drug resistance. The drug sensitivity of resistant cells was investigated by MTT assay [3, 22]. After 5,000 cells per well had been seeded on a 96-well microplate (Corning Cell Well 25860), the cells were cultivated for 4 days in a culture broth (MEM, 10% FBS) containing the test drug at 37° C in an atmosphere containing 5% CO_2. After the addition of 50 µl tetrazolium salt (MTT, Sigma Chemical Co.) as a 2 mg/ml phosphate-buffered saline (PBS) solution, culture was continued for a further 4 h at 37° C. The supernatant was then aspirated, and the resulting pigment was dissolved in dimethyl sulfoxide. The absorbance at an optical density of 550 nm was measured by means of an Easy Reader (EAR 400 RW, SLT-Labinstruments, Austria), the percentage of survival was calculated, and the dose-response curve was plotted. All cultures were carried out in triplicate, and the mean was determined by repeating the measurement at least three times. Drug resistance was calculated by the formula, relative resistance = resistant cell IC_{50}/parent cell IC_{50}, using the Ic_{50} value obtained from the MTT assay. In addition, after the addition of verapamil or cyclosporin A together with the anticancer agent, a similar MTT assay was done after culture for 4 days at 37° C, and changes in the dose-response curve were determined.

Extraction of cellular DNA and Southern blot analysis. After a cell suspension of 4×10^7 cells had been treated with proteinase K and 10% sodium dodecyl sulfate (SDS), phenol/chloroform extraction was performed, followed by dialysis for 3 days. Then, the cells were treated with RNase A, phenol/chloroform extraction was repeated, and dialysis was again carried out for 3 days. About 1 ml genomic DNA solution was obtained after dialysis. After digestion of 0.5 µg DNA with restriction enzymes (Eco R I or Hind III), 1.2% agarose gel electrophoresis was done. After the gel had been irradiated with UV and denatured with 0.5 *M* NaOH, it was transferred to a nitrocellulose membrane (Schleicher & Schuell) over 16 h [21]. The membrane obtained was hybridized with the [^{32}P] – labelled *mdr*–1 gene using the Multiprime DNA Labelling System (Amersham). The conditions of hybridization were as follows: 6.5 × SSC, 2.7 × Denhardt's solution, 0.1 *M* sodium phosphate (pH 7.0), 0.3 m*M* TRIS-HCl (pH 8.0), denatured salmon-sperm DNA, a temperature of 65° C, and hybridization time of 16 h [21]. Following hybridization, the membrane was washed twice in 2 × SSC and 0.1% SDS at 57° C for 20 min, then twice more in 0.2 × SSC and 0.1% SDS at 57° C for 20 min. It was then exposed to an X-ray film for 2 days at –70° C using an intensifying screen, following which the film was developed. The MDR gene (*mdr*-1) DNA used was obtained from the pMDR-1 gene [28], which was presented by Dr. I. B. Roninson (University of Illinois, College of Medicine) to Dr. K. Kawashima (Nagoya University), and was used after treatment with Eco R I and Hind III.

Purification of messenger RNA and Northern blotting. From samples of 4×10^7 cells, the total RNA content was extracted using the acid-guanidium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi [5]. The cells were treated with 0.5 g/ml guanidium thiocyanate, 0.5% sarcosyl, sodium acetate, and 2-mercaptoethanol. Immediately afterwards, 2 *M* sodium acetate (pH 4.0), phenol, and chloroform-iso-amylalcohol (49 : 1) were added to the sediment under centrifugation. Then, the total RNA content was extracted from the supernatent by isopropanol precipitation. Messenger RNA (mRNA) was refined from the total RNA by using Messenger Activated Paper (Takara Shuzo, Japan). Then, 10 µg mRNA was denatured in glyoxal and dimethyl sulfoxide, subjected to 1% agarose gel electrophoresis, and transferred to a Gene Screen (DuPont) over 16 h [21]. The membrane was then hybridized with [^{32}P]-labelled *mdr*-1 gene DNA. The conditions for hybridization were as follows: 50% formamide, 5 × Denhardt's solution, 5 × SSPE, 1% SDS, denatured herring-sperm DNA, a temperature of 42° C, and hybridization time of 24 h. After hybridization, the membrane was washed twice in 2 × SSC for 5 min at room temperature, twice in 2 × and 1% SDS for 20 min at 65° C, and once in 0.1 × SSC for 10 min at room temperature. It was then exposed to an X-ray film for 2 days at –70° C using an intensifying screen, after which the film was developed.

Analysis of intracellular Adriamycin content. A cell suspension of 2×10^5 cells was seeded onto a 35-mm plate (Falcon 3001). After 48 h, Adriamycin hydrochloride tagged with carbon 14 was added to each plate, to a final concentration of 150 n*M*. At intervals after the addition of radiolabelled drug, cells were removed from the plate using trypsin, then collected and suspended in 2 ml PBS. This cell suspension was aspirated and fixed to a glass microfiber filter (Whatman GF/A). The filter was washed twice in ice-cold PBS and dried. A liquid scintillation counter (Beckman LS7500) was then used to determine the accumulation of Adriamycin per 10^5 cells. To study the effects of verapamil and cyclosporin A on intracellular Adriamycin content, either the medium alone or medium with verapamil or cyclosporin A was applied simultaneously with the radiolabelled Adriamycin. Thus the final cultures either were drug-free or contained verapamil (5 or 10 µ*M*) or cyclosporin A (0.5 or 1 µ*M*). Cells were recovered and counted as described above.

Results

Adriamycin-resistant cells were prepared by exposing human ovarian cancer cells to the drug at a low concentration and using the stepwise selection method in accordance with the conventional procedure. Then, the adriamycin-resistant cell lines TAOV/A0.05 and TAOV/A0.1 underwent sensitivity testing for 14 different anticancer agents (Table 1). The TAOV/A0.05 cells showed Adriamycin resistance twice that of the parent cell line (TAOV) and vincristine resistance 14-fold that of TAOV cells. In contrast, the TAOV/A0.1 cells showed a 9.2-fold resistance to Adriamycin and also displayed cross-resistance to a number of other drugs: carboquone, daunorubicin, vincristine,

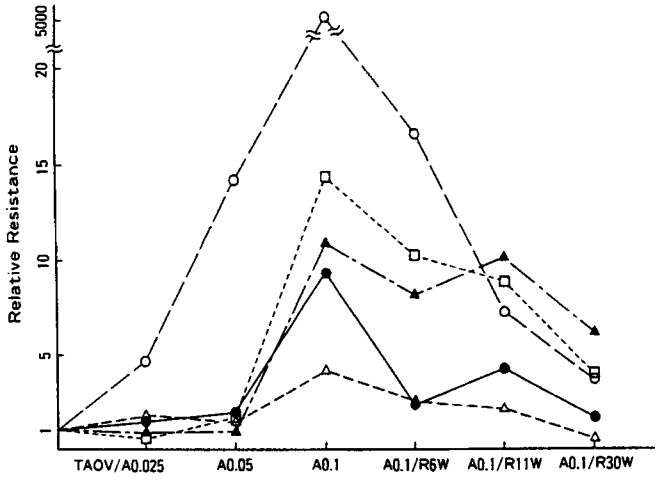

Fig. 1. Acquisition and loss of drug resistance by ovarian cancer cells. Variations of cross-resistance to drugs are shown by plots of the relative resistance to Adriamycin (●), vincristine (○), vinblastine (△), mitomycin C (▲), and carboquone (□) in Adriamycin-resistant (*TAOV/A0.025, TAOV/A0.05, TAOV/A0.1*) and revertant (*TAOV/A0.1/R6W, TAOV/A0.1/R11W, TAOV/A0.1/R30W*) cells

Fig. 3. *Mdr*-1 mRNA expression of Adriamycin-sensitive and -resistant cells in Northern blot analysis. Amounts of 10 μg mRNA from the parent cells (*TAOV*) and Adriamycin-resistant cells (*TAOV/A0.1*) were denatured with glyoxal and dimethylsulfoxide, subjected to 1% agarose gel electrophoresis, transferred to a Gene Screen, and hybridized with *mdr*-1 gene DNA labelled with phosphorus 32

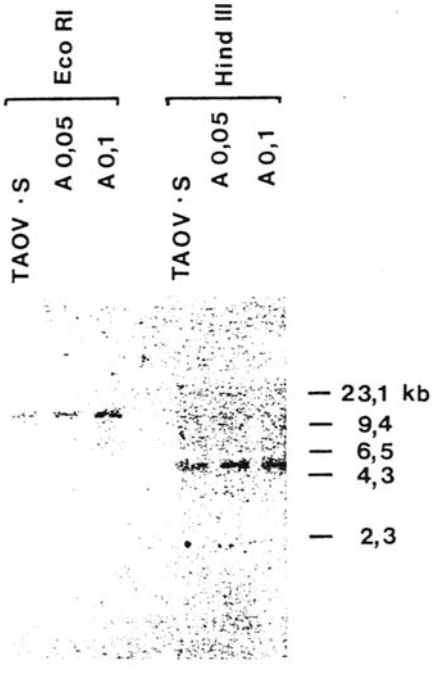

Fig. 2. Southern blot analysis for *mdr*-1 gene DNA expression in genomic DNA derived from parent cells (*TAOV-S*) and Adriamycin-resistant cells (*TAOV/A0.05, TAOV/A0.1*). Amounts of 1 μg each of genomic DNA from TAOV, TAOV/A0.05, and TAOV/A0.1 cells were digested with Eco RI and Hind III, subjected to 1.2% agarose gel electrophoresis, transferred to nitrocellulose membrane, and hydridized with *mdr*-1 gene DNA labelled with phosphorus 32

vinblastine, mitomycin C, and actinomycin D. Its cross-resistance to vincristine was increased >5,000-fold and was far superior to its resistance to the selection drug. Since this cell line showed cross-resistance to vincristine, vinblastine, daunorubicin, and actinomycin D, the role of MDR was suggested, but, more peculiarly, it also exhibited resistance to carboquone and mitomycin C.

Using revertant cells of the TAOV/A0.1 line, the time course of the process of gaining and losing cross-resistance was studied (Fig. 1). It was found that exept for vincristine, cross-resistance to other drugs gradually increased with increasing resistance to Adriamycin. For vincristine, high resistance was observed at a very early stage, and exceptionally high maximal resistance (a 5,000-fold increase) was seen. However, the loss of resistance to vincristine was rapid, with the increase in resistance falling to 3.6-fold after 30 weeks. Resistance to Adriamycin, the selection drug, declined to 1.6 times that of the parent line after 30 weeks. The behavior of cross-resistance to vinblastine was similar to that for Adriamycin and vincristine, but the cells maintained a certain level of resistance to mitomycin C and carboquone. In particular, it was noticeable that resistance to mitomycin C was still 6.1 times that of parent cells after 30 weeks of culture. In the more resistant cells of the TAOV/A0.2 line, the resistance to Adriamycin was increased 26-fold, but that to mitomycin C was about the same as that of the TAOV/A0.1 cells (a 10-fold increase) (Figs. 4, 5).

To investigate the development of resistance to Adriamycin, the intracellular adriamycin concentration in TAOV/A0.1 cells was measured using Adriamycin labelled with carbon 14 (Fig. 6). A notable decrease in Adriamycin content was seen at all times in Adriamycin-resistant cells as compared with the parent cells. The role of P-glycoprotein which acts as a drug efflux pump, in the decrease in intracellular drug concentrations was investigated by molecular biological studies. Southern blotting was carried out using the *mdr*-1 gene as a probe; hybridizing bands were noted in both parent and resistant cells, but remarkable DNA amplification of the *mdr*-1 gene in resistant cells was not observed in comparison with the parent cells (Fig. 2). In the Northern blotting analysis of mRNA from the parent cell line (TAOV) and resistant cells

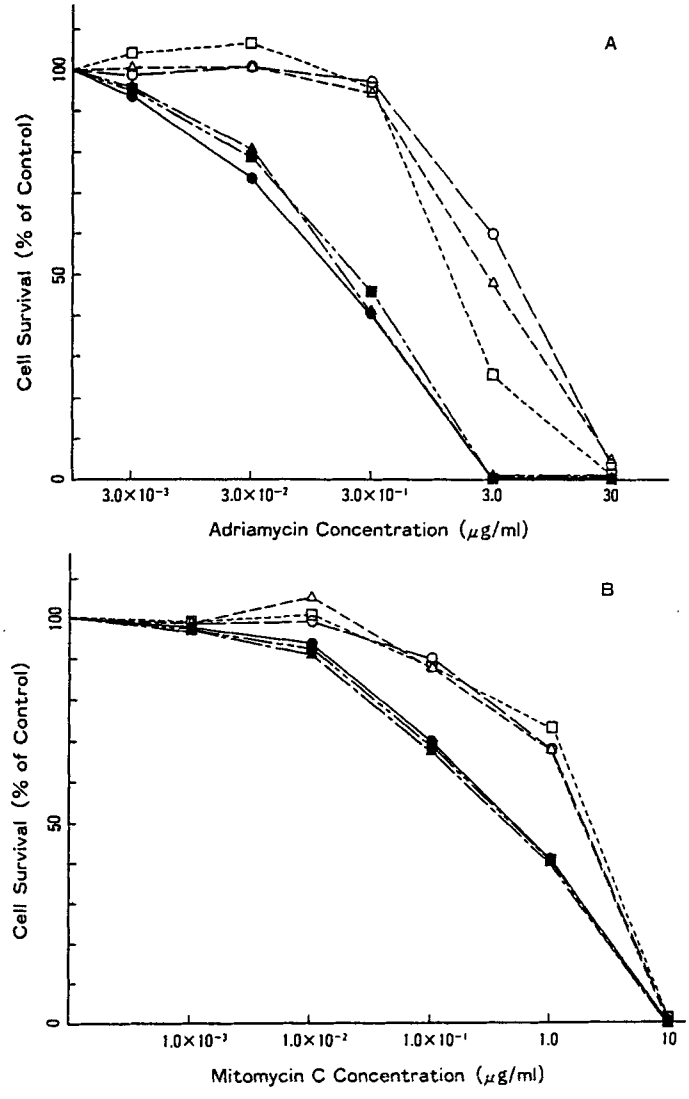

Fig. 4. Effects of verapamil on the cytocidal actions of Adriamycin or mitomycin C. A cell suspension of 5,000 parent cells (*TAOV*) or Adriamycin-resistant cells (*TAOV/AO.2*) was seeded and then verapamil was applied simultaneously with A Adriamycin or B mitomycin C. After 4 days of culture, an MTT assay was conducted. A TAOV cells: Adriamycin alone (●), Adriamycin with 0.5 μ*M* verapamil (▲), and Adriamycin with 5 μM verapamil (■). TAOV/A0.2 cells: Adriamycin alone (○), Adriamycin with 0.5 μ*M* verapamil (△) and Adriamycin with 5 μ*M* verapamil (□). B TAOV cells: mitomycin C alone (●), mitomycin C with 0.5 μ*M* verapamil (▲), and mitomycin C with 5 μ*M* verapamil (■). TAOV/A0.2 cells: mitomycin C alone (○), mitomycin C with 0.5 μ*M* verapamil (△), and mitomycin C with 5μ*M* verapamil (□)

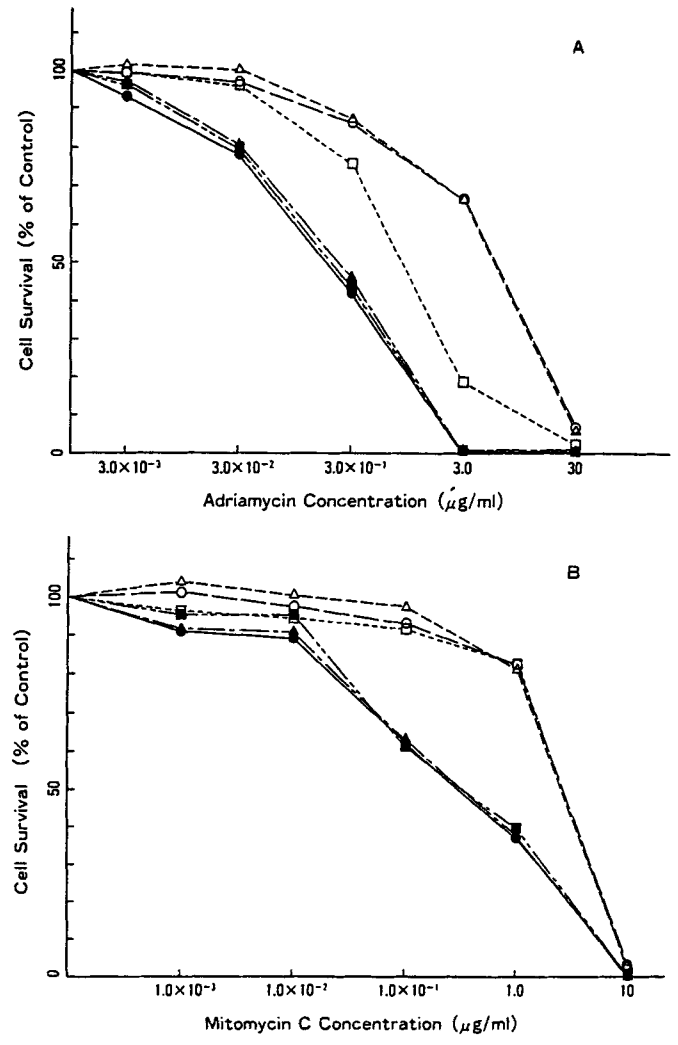

Fig. 5. Effects of cyclosporin A on the cytocidal actions of Adriamycin or mitomycin C. A cell suspension of 5,000 parent cells (*TAOV*) and Adriamycin-resistant cells (*TAOV/AO.2*) was seeded and then cyclosporin A was applied simultaneously with A Adriamycin or B mitomycin C. A TAOV cells: Adriamycin alone (●), Adriamycin with 0.1 μ*M* cyclosporin A (▲), and Adriamycin with 0.5 μ*M* cyclosporin A (■). TAOV/A0.2 cells: Adriamycin alone (○), Adriamycin with 0.1 μ*M* cyclosporin A (△), and Adriamycin with 0.5 μ*M* cyclosporin A (□). B TAOV cells: mitomycin C alone (●), mit mitomycin C with 0.1 μ*M* cyclosporin A (▲), and mitomycin C with 0.5 μ*M* cyclosporin A (■). TAOV/A0.2 cells: mitomycin C alone (○), mitomycin C with 0.1 μ*M* cyclosporin A (△), and mitomycin C with 0.5 μ*M* cyclosporin A (□)

(TAOV/A0.1), a dense band was noted at the 4.5-kb location in the resistant lane, which suggested the role of an increase in mRNA of the *mdr*-1 gene in the development of resistance (Fig. 3).

To see whether Adriamycin resistance was affected by the calcium antagonist verapamil or the immunosuppressant cyclosporin A, the cell line showing a 26-fold increase in resistance to Adriamycin (TAOV/A0.2) was investigated. Adriamycin resistance was partially overcome in the presence of 5 μ*M* verapamil (Fig. 4A), but that to mitomycin C was not influenced at all (Fig. 4B). Cyclosporin A was more potent than verapamil in its effect on Adriamycin resistance (Fig. 5A), but it also had no effect on mitomycin C resistance (Fig. 5B). The same effect of cyclosporin A on Adriamycin was also observed in KUOV2/A0.8 cells, showing a 22-fold resistance to Adriamycin and a 2.4-fold cross-resistance to mitomycin C (Fig. 6A) but no effect on mitomycin C (Fig. 6B. Both verapamil (Fig. 7A) and cyclosporin A (Fig. 7B) elevated intracellular Adriamycin concentrations in Adriamycin-resistant cells, but this effect was only slight and the level in the parent cells did not change (data not shown).

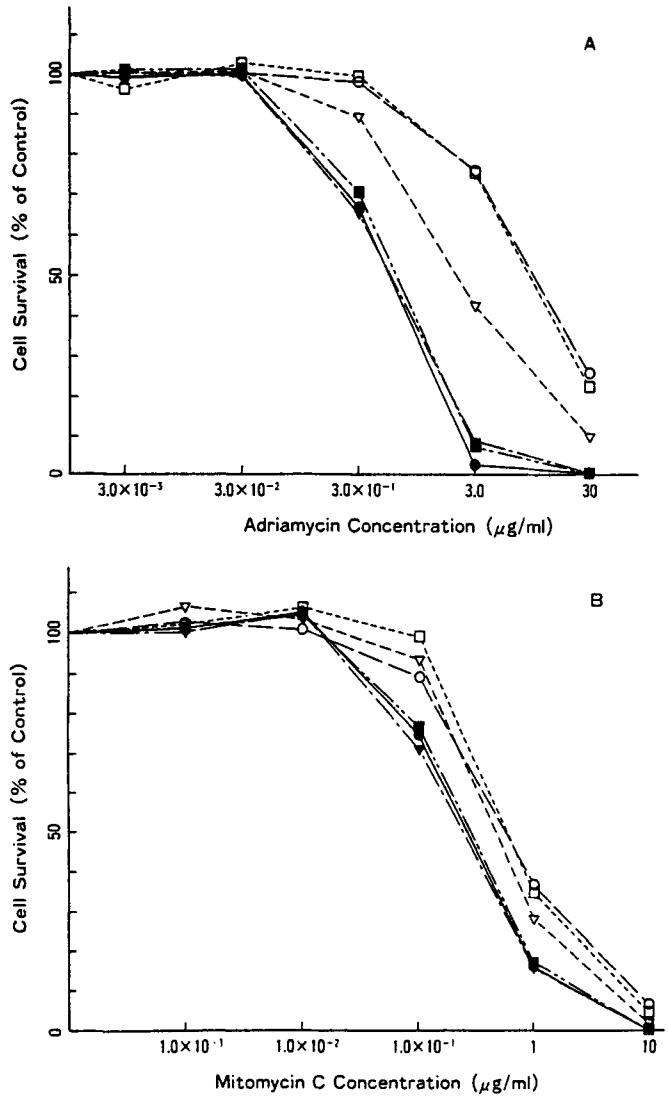

Fig. 6. Effects of cyclosporin A on the cytocidal actions of Adriamycin or mitomycin C. A cell suspension of 5,000 parent cells (*KUOV2*) and Adriamycin-resistant cells (*KUOV2/A0.8*) was seeded and then cyclosporin A was applied simultaniously with **A** Adriamycin or **B** mitomycin C. A KUOV2 cells: Adriamycin alone (●), Adriamycin with 0.5 μ*M* cyclosporin A (■), and Adriamycin with 1.0 μ*M* cyclosporin A (▼). KUOV2/AO.8 cells: Adriamycin alone (○), Adriamycin with 0.5 μ*M* cyclosporin A (□), and Adriamycin with 1.0 μ*M* cyclosporin A (▽). **B** KUOV2 cells: mitomycin C alone (●), mitomycin C with 0.5 μ*M* cyclosporin A (■), and mitomycin C with 1.0 μM cyclosporin A (▼). KUOV2/A0.8 cells: mitomycin C alone (○), mitomycin C with 0.5 μ*M* cyclosporin A (□), and mitomycin C with 1.0 μ*M* cyclosporin A (▽)

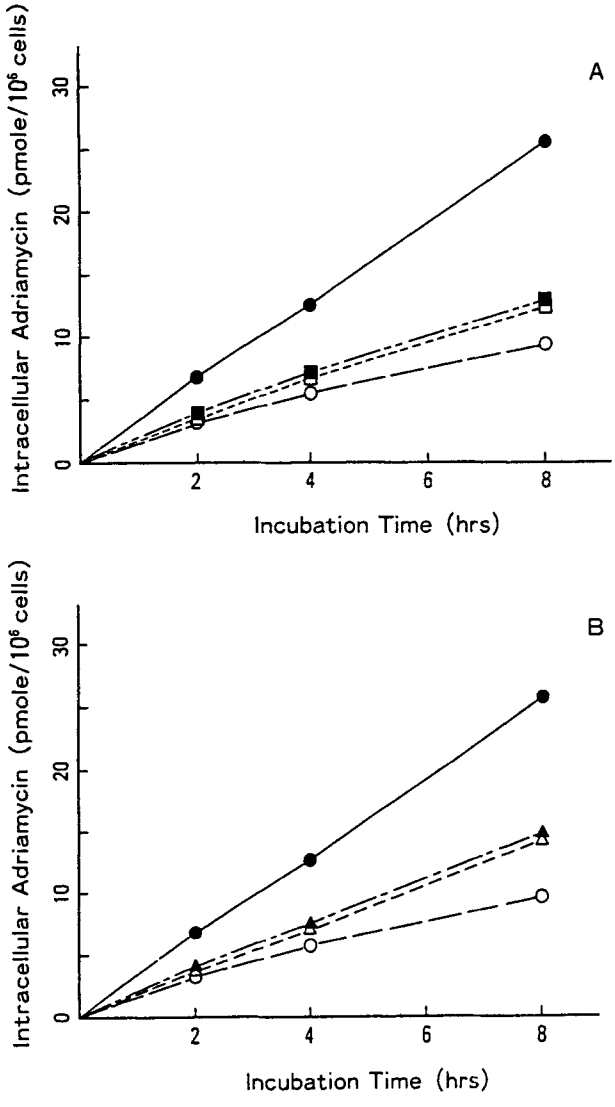

Fig. 7. Effects of verapamil or cyclosporin A on the intracellular Adriamycin content of Adriamycin-sensitive and -resistant cells. To 2▲10^5 cells of the Adriamycin-resistant line TAOV/A0.2, Adriamycin (150 n*M*) labelled with carbon 14 was applied alone (○) or with **A** verapamil [5 μ*M*(□) or 10 μ*M*(■)] or **B** cyclosporin A [0.5 μ*M*(△) or 1 μ*M*(▲)]. Cells were recovered after culture, radioactivity was measured, and the intracellular Adriamycin content per 10^6 cells was calculated. (●) denotes the intracellular Adriamycin content following administration of Adriamycin (150 n*M*) tagged with carbon 14 to the parental TAOV cells

Discussion

Adriamycin, together with cisplatin, is an important agent in the chemotherapy of ovarian cancer, and resistance to it is becoming a very serious clinical problem. Acording to reports published thus far, cancers showing MDR in vitro were mainly those with amplification of the *mdr-1* gene DNA of P-glycoprotein [14, 25, 27]. However, it has recently been shown that resistance may be induced by an increase in the mRNA alone without amplification of the DNA [30].

Furthermore, P-glycoprotein has been sporadically observed in normal tissues (e.g. adrenals, kidneys), and an increase in the mRNA has also been reported [2, 9, 31, 33, 34]. Tumors arising in such tissues have been shown to be resistant to anticancer agents [10]. In clinical chemotherapy we very rarely observe advanced drug resistance that is accompanied by amplification of the DNA of the *mdr*-1 gene as observed in vitro, and an increase in resistance of only several-fold due to an increase in the mRNA level is considered to pose a serious problem.

We prepared Adriamycin-resistant cells (TAOV/A0.1) that showed a 9.2-fold increase in Adriamycin resistance as compared with the parent cell line (TAOV). This level of resistance was relatively low compared with that previously reported in other cell lines [19, 27, 28]. In these cells, P-glycoprotein DNA amplification was not recognized and only an increase in mRNA was noted. However, broad cross-resistance was observed to drugs generally considered to be part of the MDR complex, such as vincristine, vinblastine, daunorubicin, and actinomycin D, and even to agents not belonging to MDR: mitomycin C and carboquone. In revertant cells of the TAOV/A0.1 line, a rapid loss of resistance to the drugs vincristine, vinblastine, daunorubicin, and actinomycin D (which show cross-resistance as part of the MDR complex) was noted along with loss of that to Adriamycin. This implied that the MDR of these cells depended on the increase in mRNA rather than on DNA amplification, and expression of the *mdr*-1 gene was rapidly decreased without exposure to the selection drug [31]. Also resistance to mitomycin C was well maintained in revertant cells, which showed a different behavior toward the MDR complex.

Concerning cross-resistance to mitomycin C and the alkylating agent melphalan, only a few reports have been published about cell lines with advanced Adriamycin resistance [15, 16, 26]. The induction of cross-resistance to mitomycin C and carboquone, an alkylating agent, at a low (9.2-fold) level of resistance to Adriamycin has not been reported, and this is extremely interesting. Carboquone, one of the most active alkylating agents, resembles mitomycin C structurally and resistance to both agents may depend on the same mechanism. Cross-resistance to mitomycin C was also induced in another 22-fold Adriamycin-resistant ovarian-cancer cell line, KUOV2/A0.8, as shown in Fig. 6. Furthermore, we observed that the 1.4-fold Adriamycin-resistant ovarian-cancer cell line (SKOV/A0.1), established by the same continuous-exposure method, exhibited 4.5-fold cross-resistance to mitomycin C (data not shown). Accordingly, it is not thought to be rare that cross-resistance to mitomycin C develops in human ovarian cancer cells with such a low level of resistance to Adriamycin.

The functioning of P-glycoprotein is impaired by various agents, and the representative one is verapamil [11, 20, 24, 26, 29, 35–37]. Although the details of this mechanism of action are not known, it has recently been found that the immunosuppressant cyclosporin A has a similar effect [23, 32, 38]. Therefore, to determine whether or not the resistance to mitomycin C induced by Adriamycin was based on a mechanism different from MDR, the modification of resistance by verapamil or cyclosporin A was studied in TAOV/A0.1 and KUOV2/A0.8 cells. In both cell lines, these agents reversed Adriamycin resistance; this reversal was stronger with cyclosporin A than with verapamil. We also observed that intracellular Adriamycin accumulation was increased by these agents in TAOV/A0.1 cells. However, these effects were only partial; furthermore, mitomycin C resistance was not affected by these drugs. These results strongly suggest the presence of a mechanism that is not influenced by verapamil or cyclosporin A in the development of resistance to Adriamycin.

Regarding mechanisms of resistance other than MDR, various factors may be considered, such as the promotion of DNA repair [8, 40] or an increased expression of glutathione-S-transferase [1] and cytochrome P450-related enzymes [8]; however, a report has also been published negating the roles of the above-mentioned factors [39], and each drug-resistant cell may have its own respective mechanism. In our Adriamycin-resistant cells the detoxification mechanism, such as glutathione and metallothionein or increased DNA repair activity, may involve mitomycin C resistance. Buthionine sulfoximine, a γ-glutamylcysteine synthetase inhibitor, suppresses glutathione synthesis and exhausts intracellular glutathione [13]. Although we examined the influence of buthionine sulfoximine on resistance to Adriamycin, mitomycin C, and carboquone, no effects were observed (data not shown). It was thought that a glutathione detoxification system might not play an important role in mitomycin C resistance, but a detailed study on the measurement of intracellular glutathione content or glutathione-S-transferase activity should be done. Perhaps a mechanism such as the promotion of DNA repair activity may cause resistance to mitomycin C as well as Adriamycin.

Another important feature of TAOV/A0.1 cells was the development of cross-resistance to vincristine that was markedly higher than that to the selection drug Adriamycin. In MDR, is rare that the induction of cross-resistance to other drugs be higher than resistance to the selection drug. Although an exception has previously been reported [15], such a striking difference (an increase of 9.2-fold in resistance to Adriamycin vs that of >5,000-fold to vincristine) has not previously been described. Moreover, it is also characteristic that there was marked difference between the acquisition of resistance to vinca alkaloids, vincristine, and vinblastine.

Using the KB carcinoma cell line with expression of MDR, Choi et al. [4] discovered that resistance to colchicine increased when the 185th amino acid of P-glycoprotein was valine. Resistance to vinblastine increased when it was glycine, and the pattern of resistance varied with point mutations of the *mdr*-1 gene [4]. Another report has shown that MDR cells showed differences in the degree of resistance to Adriamycin analogues, which depended on the binding affinity to compounds of P-glycoprotein [6]. In TAOV/A0.1 cells it is also possible that P-glycoprotein, recognizing a minimal structural divergence of drug, was produced by a change in the *mdr*-1 gene and exhibited an extremely high affinity for vincristine. Therefore, it would be very interesting to analyze the DNA sequence of this "atypical" P-glycoprotein.

The present study proved that drug-resistant cells, even those showing a relatively low degree of resistance, exhibit various resistance mechanisms, which makes the clinical practice of cancer chemotherapy more difficult.

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