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Flow Cytometric Analysis of Early Steps in Development of Adriamycin Resistance in a Human Gastric Cancer Cell Line

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We have established a low-level adriamycin (ADM)-resistant human gastric cancer cell line (MKN45R) from the parental cell line (MKN45) by exposure to stepwise increases of ADM concentration (final concentration, 0.026 μg/ml). The purpose of this study was to identify the early steps in the development of ADM resistance in MKN45R by flow cytometric (FCM) analysis. Comparison of the concentration required for 50% growth inhibition, determined by a tetrazolium-based colorimetric assay, showed that MKN45R was about 2.6-fold more resistant to ADM than MKN45. However, the inhibition index values were 89.5% for MKN45 and 86.4% for MKN45R, respectively, showing that ADM was judged to be "effective" against both cell lines. On the other hand, cell kinetic analysis by FCM revealed that the increase of the ratio of G_2M accumulation induced by ADM treatment was significantly lower ($P<0.01$) in MKN45R. Moreover, the efflux of ADM estimated by FCM analysis was significantly increased ($P<0.05$) in MKN45R even though there was no significant increase of P-glycoprotein expression. These results suggest that although ADM was still effective based on a standard drug sensitivity test, the cancer cells were already acquiring resistance to ADM as judged from FCM analysis. Moreover, the mechanism of this ADM resistance is considered to be independent of P-glycoprotein expression. Thus, FCM analysis is useful for detecting the early steps in the development of drug resistance of cancer cells.

Key words: Flow cytometry — MTT assay — Adriamycin — Drug resistance — P-glycoprotein

The emergence of anticancer drug resistance, especially multidrug resistance, remains a major obstacle to effective chemotherapy of cancer. Therefore, it is important to detect the development of resistance to anticancer drugs as early as possible, and to switch to more useful drugs or employ some modulators for overcoming drug resistance during treatment of cancer.

One mechanism of multidrug resistance which has been well characterized is associated with the increased expression of the *mdr*1 gene,[1] which encodes a 170-kilodalton transmembrane protein termed the P-glycoprotein.[2,3] P-glycoprotein is believed to act as an energy-dependent drug efflux pump, leading to reduced drug accumulation.[4] This mechanism has been extensively studied *in vitro* in both animal and human cell lines which have acquired high levels of resistance to anticancer drugs. Only a few studies regarding early steps in the development of multidrug resistance have been reported.[5,6] Moreover, several investigators have recently reported that some multidrug-resistant cells appear in the absence of P-glycoprotein expression[7,8] or that non-P-glycoprotein-mediated drug resistance appears in human lung cancer cells,[9] indicating the presence of other mechanisms for expression of this phenotype. Therefore, the establishment and use of low-level drug-resistant cells seems to be a convenient approach to characterize early steps in multidrug resistance or to develop a system for early detection of drug resistance during clinical treatment of cancer.

In the present study, we have established a low-level adriamycin (ADM)-resistant human gastric cancer cell line and examined the properties of this drug-resistant variant by flow cytometry (FCM). Our results demonstrate that FCM analysis is more useful for early detection of ADM resistance than standard drug sensitivity testing, and indicate that ADM resistance in the early phase is independent of P-glycoprotein expression.

MATERIALS AND METHODS

Cell lines The human gastric cancer cell line MKN45[10] and its ADM-resistant derivative MKN45R were used in these studies. MKN45R was established by exposure of MKN45 cells to stepwise increasing ADM concentrations up to 0.026 μg/ml. Cells were grown as monolayers in RPMI 1640 medium (Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (FBS, GIBCO, NY) and 250 μg/ml kanamycin monosulfate (Meiji Seika, Tokyo) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The degree of ADM resistance was stable in ADM-free medium for at least 2 months in the MKN45R cell line (data not shown).

Anticancer drugs and chemicals ADM and mitomycin C (MMC) were obtained from Kyowa Hakko Kogyo

Co., Ltd., Tokyo. *cis*-Diamminedichloroplatinum(II) (CDDP) was purchased from Nippon Kayaku Co., Ltd., Tokyo. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), bromodeoxyuridine (BrdU), ribonuclease A (RNase), Tween 20 and propidium iodide (PI) were obtained from Sigma Chemical Co., Ltd., St. Louis, MO.

Drug sensitivity test The drug sensitivity of each cell line to various anticancer drugs was determined by the MTT assay as described by Mosmann[11] and Carmichael *et al.*[12] Briefly, exponentially growing cells were plated in 96-well flat-bottomed microplates (Corning, NY) at a density of 4×10^4 cells per well and incubated with or without varying concentrations of anticancer drugs. After 48 h incubation, 50 μl of an MTT stock solution (2 mg/ml) was added to each well of the plate, which was then incubated for 4 h at 37°C. The formazan crystals generated in viable cells were solubilized in DMSO (200 μl per well) and mixing was done on a microplate mixer. The OD_{540} was measured on an enzyme-linked immunosorbent assay reader (Japan Bio-Rad Laboratories, Inc., Tokyo). The drug concentration resulting in 50% inhibition of MTT dye formation, compared to untreated controls (IC50) was estimated from the plot of percentage of control OD_{540} versus the logarithm of drug concentration. The efficacy of each drug was defined as the inhibition index (I.I.)[13] at a concentration 10 times greater than the peak plasma concentration (PPC, 0.4 for ADM, 1.0 for MMC and 2.0 μg/ml for CDDP, respectively)[14] determined by the following formula:

$$\text{I.I.} = (1-T/C) \times 100\ (\%)$$

where T is OD_{540} of treated cells and C is OD_{540} of control cells. When the I.I. was greater than or equal to 50%, the anticancer drug was judged to be effective and when it was less than 50%, the drug was judged to be ineffective.

Cell kinetics by FCM Cell kinetics were analyzed by bivariate BrdU/DNA flow cytometry according to the method developed by Dolbeare *et al.*[15] The cells exposed to ADM for 24 to 48 h were pulse-labeled with 10 μM BrdU for 30 min. They were collected and fixed in cold 70% ethanol. After removal of ethanol, the cells were treated with 0.1% RNase for 20 min at 37°C, and DNA was denatured with 4 N HCl for 40 min at room temperature. The HCl was neutralized with 0.1 M sodium tetraborate (Sigma), washed with cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS(−)] containing 0.5% Tween 20, incubated with FITC-conjugated monoclonal anti-BrdU antibody (Becton Dickinson, Mountain View, CA) and reacted with 20 μg/ml PI for 15 min at 4°C in the dark. Fluorescence was analyzed with the use of a FACScan analyzer (Becton Dickinson) with excitation at 490 nm. DNA content (red fluorescence) was measured at 580 nm, and amount of incorporated BrdU (green fluorescence) was measured at 530 nm. The bivariate BrdU/DNA distributions were displayed as contour plots. The values of cell cycle distributions were analyzed using LYSIS II software (Becton Dickinson).

ADM influx and efflux Exponentially growing cells were treated with various concentrations of ADM, ranging from 0.04 to 4.0 μg/ml, for 30 to 180 min. Then, the cells were collected and washed with cold PBS(−) at the end of the incubation period. In efflux studies, cells were treated with 4.0 μg/ml ADM for 60 min, washed with drug-free medium and incubated at 37°C for 3 to 24 h. Then the cells were collected and processed as above. To quantify the intracellular ADM content, FCM analysis was carried out using a FACScan analyzer with excitation at 488 nm (argon ion laser) under conditions similar to those reported by Krishan and Ganapathi[16] and Sasaki *et al.*[17] The data obtained from FCM analysis of 1×10^4 cells were displayed in the form of a histogram plotting cell number versus fluorescence intensity. The intracellular ADM content was estimated as the ADM fluorescence intensity per cell (that is the mean channel number of fluorescence intensity).

FCM analysis for P-glycoprotein Flow cytometric analysis of quanification for P-glycoprotein was performed according to the methods developed by Epstein *et al.*[18] and Krishan *et al.*[19] Exponentially growing cells were fixed in 70% methanol at −20°C for 24 h. They were washed twice with cold PBS, and incubated with 10 μg/ml of the monoclonal antibody to P-glycoprotein, C219 (Centocor, Inc., Malvern, PA) or 10 μg/ml of normal mouse IgG2a (Chemicon International, Inc., Temecula, CA) for 1 h in a volume of 100 μl at 4°C. The cells were then washed twice with cold PBS containing 1% bovine serum albumin (BSA, GIBCO) (PBS/BSA), and incubated with 10 μg/ml of FITC-conjugated rabbit anti-mouse IgG (Dakopatts, Denmark) for 1 h in a volume of 100 μl at 4°C. They were washed with cold PBS/BSA and PBS, then treated with 10 mg/ml RNase for 20 min at 37°C, and incubated with 20 μg/ml PI for 15 min at 4°C in the dark. Indirect immunofluorescence analysis was carried out on a FACScan analyzer.

Statistical analysis All values are given as the mean ± SD unless otherwise indicated. Statistical significance of differences was determined by one-way ANOVA, with $P<0.05$ as the criterion.

RESULTS

Characteristics and drug sensitivity of MKN45 and ADM-resistant MKN45R cell lines An ADM-resistant cell line, MKN45R, was derived from MKN45 parental cells. The doubling time was 25 for MKN45 and 26 h for MKN45R, with no significant difference between the two

cell lines. Moreover, the morphology observed by phase contrast microscopy did not change in MKN45R (data not shown). We tested the drug sensitivity of each cell line by MTT assay. The semilogarithmic dose-response curves for MKN45 and MKN45R following 48 h of exposure to various anticancer drugs are shown in Fig. 1. The I.I. and IC50 values for each anticancer drug estimated from these curves are summarized in Table I. The I.I. values of ADM, MMC and CDDP for the parental and resistant cell lines were more than 50%, so all these drugs were judged to be effective. However, the IC50 values for ADM on MKN45 and MKN45R were 0.42±0.10 and 1.09±0.08 μg/ml, respectively, demonstrating that MKN45R was more resistant to ADM than MKN45,

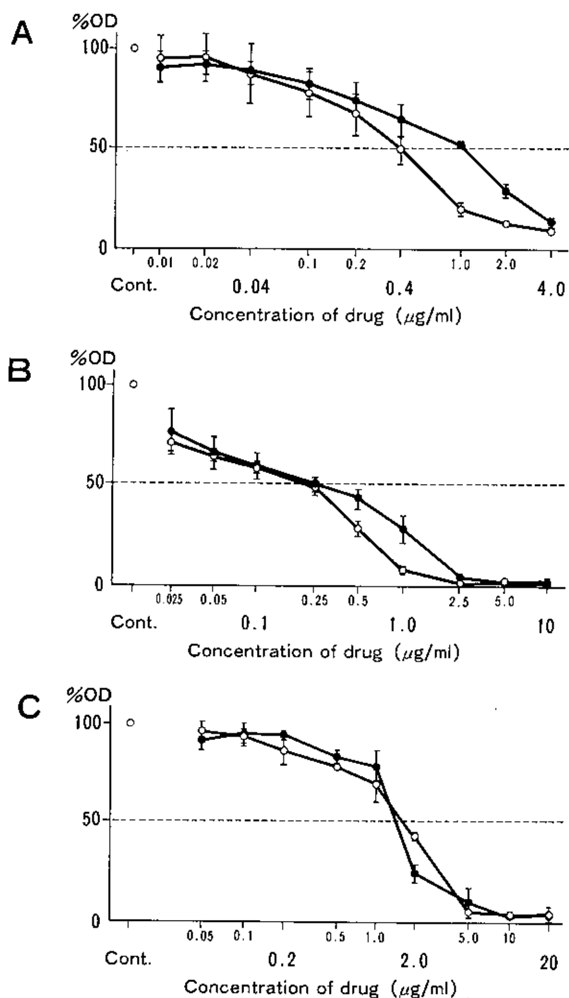

Fig. 1. Dose-response curves of ADM, MMC and CDDP against MKN45 and MKN45R cells. A, ADM; B, MMC; C, CDDP; ○, MKN45; and ●, MKN45R. Values represent the mean±SD of four separate assays.

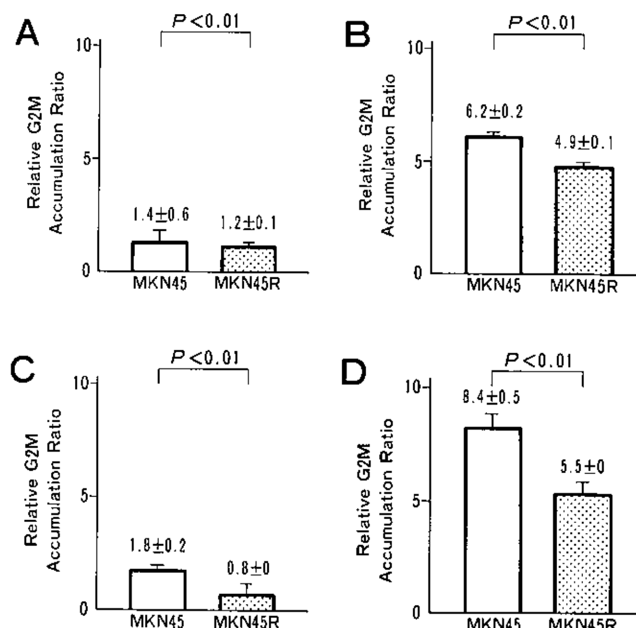

Fig. 2. Comparison of the relative G_2M accumulation ratio (percentage of G_2M phase in ADM-treated cells/pecentage of G_2M phase in untreated cells) of MKN45 to that of MKN45R cells. A) 24 h after ADM (0.4 μg/ml) treatment, B) 24 h after ADM (4.0 μg/ml) treatment, C) 48 h after ADM (0.4 μg/ml) treatment, and D) 48 h after ADM (4.0 μg/ml) treatment. Columns and bars show the mean±SD of triplicate experiments.

Table I. Inhibition Index and IC50 Values Determined by the MTT Assay

Anticancer drug	Inhibition index (%)		IC50 (μg/ml)	
	MKN45	MKN45R	MKN45	MKN45R
ADM	89.5±1.3	86.4±3.3	0.42±0.10	1.09±0.08[a]
MMC	98.0±2.6	97.4±2.2	0.24±0.04	0.28±0.06
CDDP	95.9±0.9	95.9±4.4	1.71±0.15	1.52±0.06

a) $P<0.01$ compared to value for MKN45 by one-way ANOVA. Each value is the mean±SD of four separate assays.

with a relative resistance (IC50 of resistant cell line/IC50 of parental cell line) of 2.6. The MKN45R cell line did not show cross-resistance to MMC or CDDP.

Analysis of cell kinetics by FCM The effect of ADM on cell cycle traverse of MKN45 and MKN45R cells is summarized in Table II. Treatment with 0.4 and 4.0 μg/ml of ADM caused a significant accumulation of cells in the G_2M phase at 24 or 48 h compared to the untreated control in both cell lines ($P<0.01$). Under identical culture conditions, the relative G_2M accumulation ratio (percentage of the G_2M phase in ADM-treated cells/percentage of the G_2M phase in untreated cells) was significantly ($P<0.01$) less in MKN45R than in the parental cell line (Fig. 2).

Intracellular accumulation and efflux of ADM The kinetics of ADM accumulation are shown in Fig. 3. Accumulation of ADM increased in a dose-dependent manner in both cell lines. When cells were incubated with 0.04 or 0.4 μg/ml ADM, intracellular ADM content

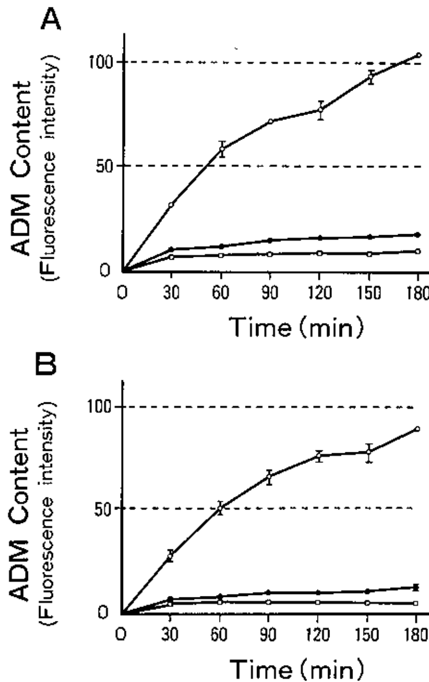

Fig. 3. Intracellular influx of ADM in MKN45 and MKN45R cells. The intracellular ADM content was determined by FCM as described in "Materials and Methods." A, MKN45; B, MKN45R; □, ADM 0.04; ●, 0.4; and ○, 4.0 μg/ml. Values represent the mean±SD of triplicate experiments.

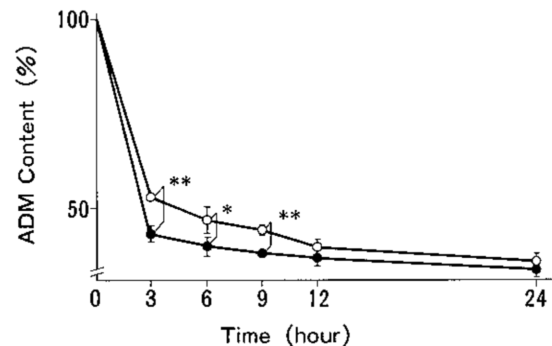

Fig. 4. Efflux of intracellular ADM from MKN45 and MKN45R cells. The intracellular ADM content was obtained from FCM as described in "Materials and Methods" and its value as a percentage of the initial intracellular ADM content at various time points was determined. ○, MKN45; and ●, MKN45R. Values represent the mean±SD of triplicate experiments. *, $P<0.05$; **, $P<0.01$. Significant difference between MKN45 and MKN45R cells.

Table II. Effect of ADM on the Cell Cycle Traverse of MKN45 and MKN45R Cells

Time after ADM exposure (h)	ADM (μg/ml)	Cells in each cell cycle phase (%) MKN45				Cells in each cell cycle phase (%) MKN45R			
		G_0G_1	G_2M	S_0[a]	S	G_0G_1	G_2M	S_0[a]	S
0	Control[b]	48.8±1.1	9.5±0.3	0.6±0.1	41.1±0.8	52.6±0.9	9.4±0.3	0.6±0.1	37.4±0.9
24	Control[b]	55.0±0.5	7.2±0.3	1.0±0.01	36.8±0.8	50.9±0.8	10.2±0.2	0.6±0.1	38.3±0.8
	0.04	42.8±1.5	6.4±0.3	1.1±0.2	49.7±1.8	56.2±0.7	8.6±0.3	0.9±0.2	34.3±1.1
	0.4	61.9±0.9	12.6±0.4[c]	1.9±0.4	23.6±0.6	58.2±0.5	12.7±0.6[c]	0.9±0.2	28.2±0.4
	4.0	42.9±0.6	44.4±1.0[c]	0.9±0.1	11.8±1.3	28.9±1.0	50.1±0.8[c]	2.1±0.5	18.9±0.6
48	Control[b]	51.9±0.2	5.1±0.4	1.5±0.3	41.5±0.7	45.8±1.1	10.2±0.1	0.7±0.1	43.3±1.0
	0.04	58.6±1.9	5.9±0.3	2.3±0.6	33.2±2.5	70.0±0.9	4.4±0.4	0.4±0.1	25.2±0.6
	0.4	61.4±0.4	9.4±0.5[c]	1.4±0.1	27.8±0.7	67.1±0.6	8.4±0.1	1.1±0.2	23.4±0.5
	4.0	41.0±1.4	44.0±1.0[c]	0.9±0.1	14.1±0.3	22.9±0.3	56.2±0.3[c]	2.8±0.3	18.1±0.3

a) Non BrdU-labeled cells in the S phase.
b) Cells incubated with ADM-free culture medium.
c) $P<0.01$ compared to the respective control by one-way ANOVA. Each value is the mean±SD of triplicate experiments.

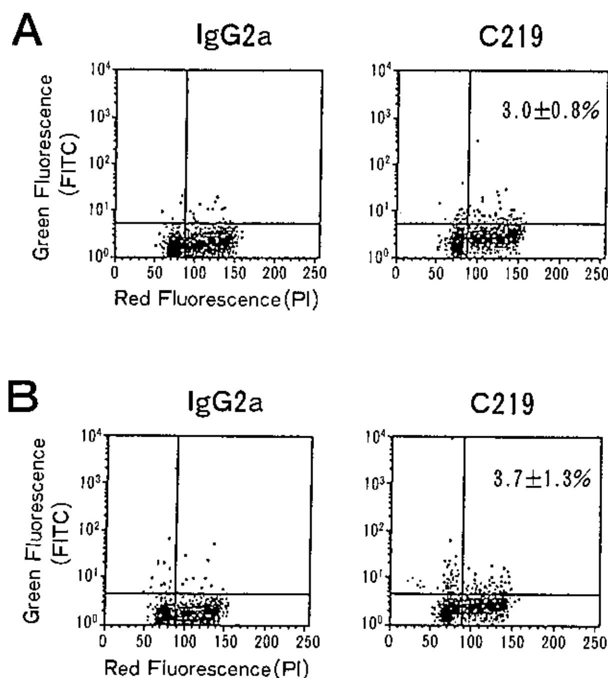

Fig. 5. Scattergrams of cells stained with FITC-C219, an indirect method for measuring P-glycoprotein and with PI, for measuring DNA content. The horizontal line indicates the electronic gate set to exclude 99% of positive cells from the isotype-matched control IgG2a. Cells which had a higher fluorescence than that of the gated population were counted as C219-reactive cells. A, MKN45; and B, MKN45R. Values represent the mean±SD of triplicate experiments. No significant difference was observed between MKN45 and MKN45R cells.

reached a plateau in 60 to 90 min. There was little difference in ADM accumulation between MKN45 and MKN45R. In efflux studies, approximately 50% of the intracellular ADM was excreted repidly from the cells in the first 3 h in both cell lines. The rate of decrease of intracellular ADM content (100 − percentage intracellular ADM content at various time points) in 3 to 9 h was significantly higher in MKN45R compared to the parental cell line ($P<0.01$, $P<0.05$), demónstrating that ADM efflux of MKN45R was faster than that of MKN45 (Fig. 4).

FCM analysis for P-glycoprotein The expression of P-glycoprotein was evaluated by FCM analysis of indirect immunofluorescence-stained cells. Both MKN45 and MKN45R cells display low levels of P-glycoprotein. The values of the ratio of P-glycoprotein-positive cells were 3.0±0.8% for MKN45 and 3.7±1.3% for MKN45R cells, respectively, showing that there was no significant difference in P-glycoprotein expression between the parental and resistant cell lines (Fig. 5).

DISCUSSION

In this paper, we describe the usefulness of FCM analysis for early detection of drug resistance using a low-level ADM-resistant human gastric cancer cell line. *In vitro* chemosensitivity testing of anticancer drugs for human tumors has evolved gradually since the report of Hamburger and Salmon[20] on the use of a human tumor clonogenic assay. However, since the assay is highly labor-intensive and some technical problems remain, it is still necessary to develop an assay system which is simple, rapid, inexpensive and applicable to most patients. The MTT assay is a simple colorimetric test of cell proliferation and survival which was developed by Mosmann.[11] A derivative of this method has been applied on a large scale for drug sensitivity testing of human cell lines[21-23] and it is now being adopted for routine use in clinical practice worldwide. However, our primary analyses by MTT assay revealed that there was no significant difference between the parental and resistant cell lines in terms of the I.I., employed as the clinically achievable value of drug sensitivity. Only with respect to the IC50 value for ADM did MKN45R differ from the parental cell line, having a small degree of relative resistance of 2.6.

On the other hand, FCM is a valuable tool for characterizing individual cells, analyzing cell kinetics and studying cell surface markers in a heterogenous tumor population. Several investigators have used FCM to evaluate sensitivity to anticancer drugs from the viewpoint of changes in cell kinetics.[24,25] Barlogie *et al.* reported that ADM caused an irreversible accumulation of cells in the G_2M phase, and resulted in cell destruction,[26] indicating that degree of accumulation in G_2M phase can be regarded as an indicator of the cytotoxicity of ADM to tumor cells. In our present study too, ADM caused a significant increase in G_2M phase in both cell lines at concentrations of 0.4 and 4.0 μg/ml. However, in comparing the cytotoxicity of ADM to various cell lines, it is not always appropriate simply to compare accumulation in G_2M phase, because each cell line has its individual cell cycle distribution. It is necessary to examine how ADM augments accumulation in G_2M phase in treated cells as compared to untreated cells. Therefore we calculated the relative G_2M accumulation ratio in order to compare the cytotoxicity of ADM to MKN45 and MKN45R cells. Comparison of the relative G_2M accumulation ratio showed a significant difference between the parental MKN45 and ADM-resistant MKN45R cell lines. The increase of G_2M accumulation of MKN45R was significantly less than that of the parental cell line. This means that although ADM is considered to be effective against

both cell lines in the standard drug sensitivity test, i.e., MTT assay, the early steps in the development of resistance have already occurred. Thus, cell kinetic analysis by FCM may be useful for detection of early drug resistance.

FCM has also been utilized for studying fluorescent anthracycline levels in cultured cells and in tumor specimens.[27, 28] It can be used to demonstrate changes in drug influx and efflux. Previous publications showed that the efflux of ADM in ADM-resistant cell lines was faster than that in sensitive cells.[29, 30] Moreover, a lower influx of drugs, such as ADM, daunorubicin, vincristine and VP-16, in P-glycoprotein-related drug-resistant cells has been reported.[31-34] Our results demonstrate that although the ADM influx was not reduced in MKN45R, the ADM efflux of MKN45R was significantly faster than that of the parental cell line.

Resistance of cancer cells to several drugs, including anthracyclines such as ADM, and Vinca alkaloids, is frequently due to expression of P-glycoprotein, encoded by the *mdr*1 gene. Since the P-glycoprotein functions as an energy-dependent drug efflux pump, the increase in drug efflux is correlated with P-glycoprotein expression.[35] However, our resistant cell line contained little, if any, P-glycoprotein on the cell surface as evaluated by FCM analysis. It is not entirely surprising that our resistant cell line does not display a significant increase in P-glycoprotein expression, because some drug-resistant cells, especially low-level resistant cells, without P-glycoprotein expression, have been reported[7, 8] and P-glycoprotein expression is correlated with the degree of drug resistance.[1, 5, 36] It is suggested that the increase of ADM efflux in MKN45R is due to some other mechanisms such as a putative ATP-dependent drug efflux[37] or pH gradient across the plasma membrane.[38] Further studies are needed to elucidate the mechanism responsible for drug transport in early resistant cells.

In summary, we could detect early steps in the development of drug resistance by FCM analyses of cell kinetics, drug accumulation and P-glycoprotein expression, using a low-level ADM-resistant cell line which was evaluated as sensitive to ADM in the standard drug sensitivity test. Thus, FCM is a useful tool for early detection of the development of resistance to anticancer drugs.

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