MATERIALS AND METHODS

Cell lines and culture conditions

The MKN45 human gastric cancer cell line originated from a poorly differentiated adenocarcinoma of the stomach.[7] The resistant MKN45R0.05 and MKN45R0.8 cells were established by continuous exposure of MKN45 cells to stepwise increasing concentrations of ADM (up to 0.05 and 0.8 μg/ml, respectively). All the cell lines were grown as monolayers in RPMI-1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FBS (Gibco, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37℃.

Drugs and chemicals

ADM was obtained from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), bromodeoxyuridine (BrdU), ribonuclease A (RNase), Tween 20 and propidium iodine (PI) were obtained from Sigma (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU monoclonal antibody was purchased from Becton Dickinson (San Jose, CA, USA). The monoclonal antibody to P-glycoprotein, MRK16, and normal mouse IgG2a were obtained from Kyowa Medex Co. Ltd. (Tokyo, Japan) and Chemicon International Inc. (Temecula, CA, USA), respectively. FITC-conjugated rabbit anti-mouse IgG was purchased from Dakopatts (Copenhagen, Denmark). VPM was obtained from Eisai Co. Ltd. (Tokyo, Japan).

Drug sensitivity assay

Drug sensitivity was determined by using the MTT assay as described by Mosmann[8] and Carmichael et al.[9] Briefly, the cells were seeded into 96-well microplates (Corning Glass Works, Corning, NY, USA) at a density of 3×10^4 cells per well and incubated with or without varying concentrations of ADM. After 48 h incubation, 50 μl of an MTT stock solution (2 mg/ml) was added to each well and incubated for 4 h at 37℃. DMSO (200 μl per well) was added to each well and the plates were agitated on a plate shaker to solubilize the formazan crystals generated in viable cells. The absorbance in each well was measured at 540 nm on a Model 450 scanning microplate spectrophotometer (Bio-Rad Japan, Tokyo, Japan). The IC_{50} value was defined as the drug concentration to inhibit the growth of tumor cells by 50%, and was determined from a plot of percentage surviving cells (compared with control cells) versus drug concentrations. To examine the effect of VPM on ADM cytotoxicity, the cells were treated with ADM in the absence or presence of 0.5 or 1 μg/ml of VPM. The ability of VPM to modify ADM resistance was expressed as the dose modifying factor [(DMF) = IC_{50} in absence of VPM/IC_{50} in presence of VPM].

Cell cycle analysis by flow cytometry

Cell cycle distribution was determined by BrdU/DNA flow cytometry as described by Dolbeare et al.[10] In brief, the cells exposed to ADM for 48 h were pulse-labeled with 10 μM BrdU for 30 min and fixed with 70% ethanol. After the removal of ethanol, the cells were treated with 0.1% RNase for 20 min at 37℃ and then incubated with FITC-conjugated anti-BrdU monoclonal antibody for 20 min at room temperature. The cells were then reacted with 20 μg/ml PI for 15 min at 4℃ in the dark and analyzed on a FACScan analyzer (Becton Dickinson). LYSIS II software (Becton Dickinson) was used to estimate the percentage of cells in each phase of the cell cycle.

Cellular uptake and efflux of ADM

Exponentially growing cells were treated with 4 μg/ml ADM for 30 and 60 min. The cells were then collected and washed with cold phosphate buffered saline (PBS) at the end of the incubation period. In the efflux study, cells were similarly treated with 4 μg/ml ADM for 60 min, washed with drug-free medium and incubated at 37℃ for 2 to 9 h. Then the cells were collected and washed with cold PBS. 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.[11] 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 of P-glycoprotein

FCM analysis of quantification for P-glycoprotein was performed according to the methods developed by Hamada and Tsuruo[12] and Heike et al.[13] Exponentially growing cells were fixed in 70% methanol at −20℃ for 24 h. They were washed twice with cold PBS, and incubated with 100 μg/ml of MRK 16 or 100 μg/ml of normal mouse IgG2a as a negative

control for 30 min at 4℃. The cells were then washed twice with cold PBS containing 10% FBS (PBS/FBS), and incubated with FITC-conjugated rabbit anti-mouse IgG for 30 min at 4℃. They were washed with cold PBS/FBS and PBS, then treated with 10 mg/ml RNase for 20 min at 37℃, and incubated with 20 μg/ml PI for 15 min at 4℃ in the dark. Indirect immunofluorescence analysis was carried out on a FACScan analyzer.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test. Differences were considered significant at $P<0.05$.

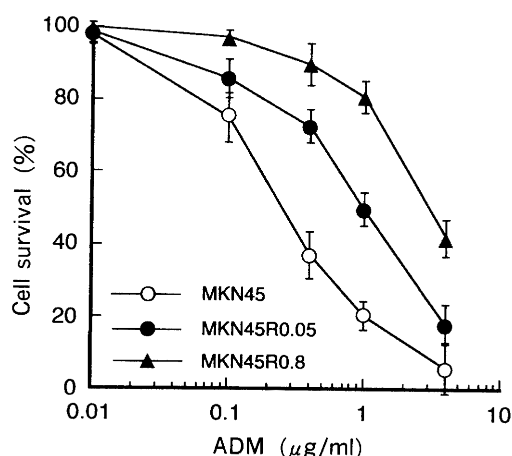

Fig. 1. Survival of MKN45, MKN45R0.05 and MKN45R0.8 cells after a 48-h incubation with ADM determined by the MTT assay. Each point is the mean of three independent experiments. Bars=SD.

RESULTS

Characteristics and drug sensitivity of MKN45, ADM-resistant MKN45R0.05 and MKN45R0.8 cell lines

As shown in Fig. 1, IC_{50} values of ADM for MKN45, MKN45R0.05 and MKN45R0.8 cells were 0.22, 1.06, and 3.33 μg/ml, respectively. Therefore, MKN45R0.05 and MKN45R0.8 cells were 4.8-and 15-fold more resistant to ADM than the parental MKN45 cells. The level of ADM resistance was stable in ADM-free medium for at least 12 months in both resistant cell lines. There were no significant differences in doubling time and morphology observed by phase contrast microscopy among three cell lines (data not shown).

Cell kinetic analysis by flow cytometry

FCM analysis demonstrated that treatment with 0.2 to 4.0 μg/ml ADM resulted in an increase in the percentage of cells in G_2M phase at 48 h compared to the untreated control in each cell line. However, the percentage of cells in G_2M phase was significantly low in MKN45R0.05 and MKN45R0.8 cells when compared with the parental MKN45 cells. Moreover, MKN45R0.8 cells showed a significantly lower accumulation of cells in G_2M phase than MKN45R0.05 cells at 0.2 to 0.4 μg/ml ADM treatment (Table 1).

Intracellular uptake and efflux of ADM

ADM uptake in three cell lines as measured by FCM analysis is shown in Fig. 2. Both resistant cell lines demonstrated reduced uptake, but no difference between MKN45R0.05 and MKN45R0.8 cells was

Table 1. Effect of ADM treatment on the accumulation of cells in G_2M phase of MKN45, MKN45R0.05 and MKN45R0.8 cells

ADM (μg/ml)	Percentage of cells in G_2M phase [a]		
	MKN45	MKN45R0.05	MKN45R0.8
0(control)	3.9±0.7[b]	5.0±0.4	9.6±1.0
0.2	45.7±3.5	26.5±3.2[c]	17.8±1.1[c,e]
0.4	36.9±2.8	33.0±0.5[d]	28.4±0.2[c,e]
4.0	31.0±1.8	32.5±1.2	22.2±0.5[c,e]

[a] Percentage of cells in G_2M phase was determined by FCM analysis after treatment with various concentrations of ADM for 48 h. [b] Mean±SD of three independent experiments. [c] $P<0.01$ compared to MKN45 cells. [d] $P<0.05$ compared to MKN45 cells. [e] $P<0.01$ compared to MKN45R 0.05 cells.

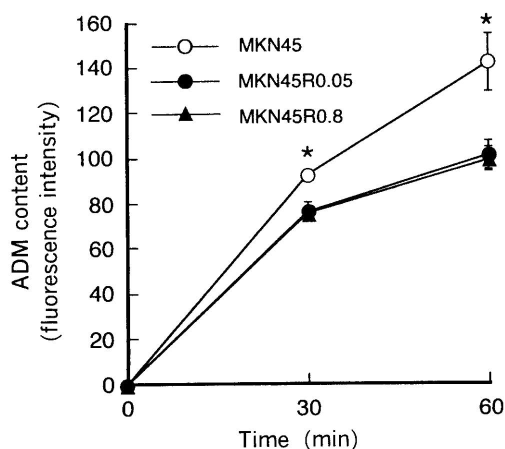

Fig. 2. Intracellular uptake of ADM by MKN45, MKN45R0.05 and MKN45R0.8 cells. The intracellular ADM content was determined by FCM analysis as described in "MATERIALS AND METHODS". Values represent the mean of triplicate experiments. Bars=SD. *P<0.05 compared to values for MKN45R0.05 and MKN45R0.8 cells.

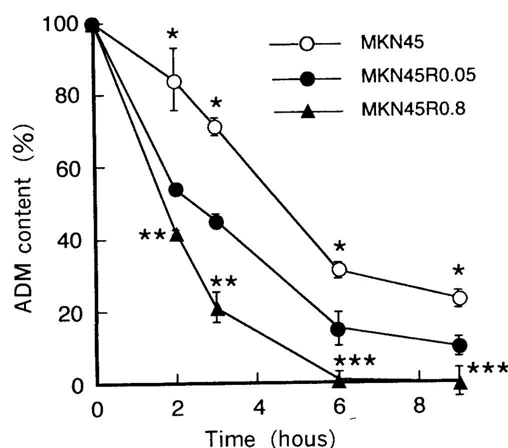

Fig. 3. Efflux of intracellular ADM content from MKN45, MKN45R0.05 and MKN45R0.8 cells. The intracellular ADM content was obtained from FCM analysis as described in "MATERIALS AND METHODS" and its value as a percentage of the initial intracellular ADM content at various time points was determined. Values represent the mean of triplicate experiments. Bar=SD. *P<0.01 compared to values for MKN45R0.05 and MKN45R0.8 cells. **P<0.01 and ***P<0.05 compared to values for MKN45R0.05 cells.

seen for the cellular uptake of ADM. Fig. 3 shows efflux of ADM after exposure to 4 μg/ml ADM for 60 min. Drug efflux was significantly enhanced in both MKN45R0.05 and MKN45R0.8 cells compared to the parental MKN45 cells. Moreover, at each time point, the intracellular ADM content of MKN45R0.8 cells was significantly lower than that of MKN45R0.05 cells and ADM was completely excreted from the MKN45R0.8 cells at 9 h after ADM treatment, although MKN45 and MKN45R0.05 cells had yet preserved intracellular ADM content.

FCM analysis for P-glycoprotein

P-glycoprotein levels were examined by FCM analysis after staining with the anti-human P-glycoprotein antibody, MRK16. All the three cell lines stained similarly with MRK16 and normal mouse IgG2a, indicating that there was no P-glycoprotein expression in these cell lines (Fig. 4).

Effect of VPM on ADM cytotoxicity

Initially, the effect of VPM to inhibit cell growth in the parental MKN45 and ADM-resistant cell lines was studied. Cell growth of three cell lines was not inhibited by treatment with 0.5 and 1 μg/ml VPM (data not shown). These concentrations were then used in combination with ADM. The results obtained with VPM on ADM cytotoxicity to three cell lines are summarized in Table 2. In the parental MKN45 cell line, VPM had no effect on ADM cytotoxicity: the DMF was almost constant at 1. On the other hand, in ADM-resistant cell lines, VPM treatment (at 0.5 and 1 μg/ml) significantly increased the sensitivity of MKN45R0.8 cells to ADM, although in MKN45R0.05 cells, an increased sensitization to ADM was observed only at 1 μg/ml VPM treatment. Moreover, the DMF of MKN45R0.8 cells at 1 μg/ml VPM treatment was significantly higher than that of MKN45R0.05 cells. This stronger modifying effect of VPM on MKN45R0.8 cells was also observed by FCM cell kinetic analysis: VPM treatment resulted in a significant increase in the percentage of cells in G_2M phase in MKN45R0.8 cells but not in the parental MKN45 and MKN45R0.05 cells (Table 2).

DISCUSSION

In this study, we have established two ADM-resistant human gastric cancer cell lines, MKN45R0.05 and MKN45R0.8, with different levels of drug resistance.

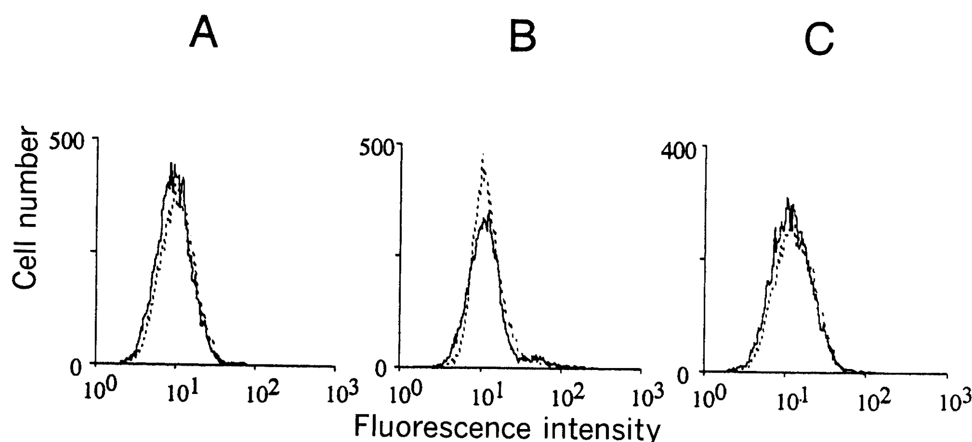

Fig. 4. FCM analysis for P-glycoprotein expression in MKN45(**A**), MKN45R0.05(**B**) and MKN45R0.8(**C**) cells. Each solid and dotted line represents cells reacted with MRK16 and normal IgG2a, respectively.

Table 2. Effect of VPM on ADM sensitivity of MKN45, MKN45R0.05 and MKN45R0.8 cells

Cell line	Treatment[a]	MTT assay		Cell kinetics by FCM	
		IC_{50} of ADM (μg/ml)	DMF[b]	Percentage of cells in G_2M phase	G_2M modification[c]
MKN45	ADM alone	0.22±0.77[d]	1	45.7±3.5[d]	1
	+0.5 μg/ml VPM	0.22±0.01	1	nt[e]	nt
	+1 μg/ml VPM	0.21±0.01	1.1	45.4±0.3	1.0
MKN45R0.05	ADM alone	1.06±0.20	1	26.5±2.8	1
	+0.5 μg/ml VPM	1.05±0.13	1.2	nt	nt
	+1 μg/ml VPM	0.73±0.21	1.6[f]	26.7±1.3	1.0
MKN45R0.8	ADM alone	3.33±0.52	1	17.8±1.1	1
	+0.5 μg/ml VPM	2.92±0.30	1.3[f]	nt	nt
	+1 μg/ml VPM	1.73±0.23	1.9[f,g]	22.1±0.9[f]	1.3[g]

[a] Cells were incubated with ADM in the presence or absence of VPM for 48 h. [b] DMF, dose modifying factor (IC50 in absence of VPM / IC50 in presence of VPM). [c] G_2M modification (percentage of cells in G_2M phase in presence of VPM / percentage of cells in G_2M phase in absence of VPM). [d] Mean±SD of the three independent experiments. [e] nt, not tested. [f] $P<0.05$ compared to value for each cell line treated with ADM alone. g) $P<0.05$ compared to value for MKN45R0.05 cells.

The resistance factors (IC_{50} resistant subline/IC_{50} parent line) were 4.8 for MKN45R0.05 and 15 for MKN45R0.8 cells, respectively. FCM analysis demonstrated that treatment of cells with ADM resulted in an increase in the percentage of cells in G_2M phase compared with the corresponding percentage for the control in each cell line. In the ADM-resistant cell lines, the percentage of cells in G_2M phase was significantly low when compared with the parental MKN45 cells. Moreover, it was lower as the resistance factor increased. ADM as well as other anticancer drugs, such as *cis*-diamminedichloroplatinum, causes an accumulation of cells in the G_2M phase, and the cells treated with ADM either eventually divide or die after staying in G_2M.[14,15] Therefore, it is conceivable that the change of cell cycle-mediated event (the decrease in the percentage of G_2M phase cells) occurs in the ADM-resistant cell lines. Moreover, our previous findings have shown that the magnitude of accumulation of G_2M phase cells may be a useful indicator of drug sensitivity.[6,16] Accordingly, it correlates also with the level of drug resistance. ADM uptake and efflux could also be examined by FCM analysis. Although both ADM-

resistant cell lines showed similar reduced uptake, the high-level ADM-resistant MKN45R0.8 cells showed a higher efflux rate of ADM compared to the low-level ADM-resistant MKN45R0.05 cells. Thus, FCM analysis is useful for determining the level of drug resistance and drug accumulation in cancer cells. If we can detect drug resistance in clinical samples during chemotherapy and determine the degree of drug resistance, cell kinetics and cellular pharmacokinetics of cancer cells by FCM analysis, they may provide valuable informations for designing the combined use of reversal agents, such as verapamil that is discussed in the last section of this paper, or its analogues.

FCM analysis also revealed that all the ADM-resistant gastric cancer cell lines established by us, including a previously reported line,[6] did not overexpress P-glycoprotein. P-glycoprotein mediated drug resistance is thought to be preceded by another mechanism for MDR.[3] Therefore, our cell lines may be at relatively early steps in the acquisition of MDR and have non-P-glycoprotein mediated mechanism of drug resistance. To date, at least two MDR genes, the P-glycoprotein (*mdr*1)and MDR-associated protein (MRP) genes, have been identified in humans. MRPs as well as P-glycoprotein belong to the ATP-dependent family of transporters, but they only share limited structural homology to P-glycoprotein, and the resulting phenotypes differ significantly from those observed with P-glycoprotein.[5,17-19] Further studies are necessary to elucidate whether overexpression of MRP can account for non-P-glycoprotein mediated drug resistance in our ADM-resistant cell lines.

Considerable effort has been made to discover the specific means to reverse or circumvent MDR. The calcium channel blocker, VPM, and the calmodulin antagonist, trifluoperazine, are the first compounds found to reverse P-glycoprotein mediated drug resistance.[20,21] In addition to these compounds, other chemosensitizers, including cyclosporin A and SDZ PSC 833, steroid hormones, quinidine and quinine, have been able to sensitize P-glycoprotein-positive and drug resistant cell lines to cytotoxic agents.[22-25] These compounds also affect non-P-glycoprotein mediated drug resistance, but their reversal effects are less or vary among different cell lines.[18] In the present study, we showed that the reversal effect of VPM was stronger in high-level ADM-resistant MKN45R0.8 cells than low-level ADM-resistant MKN45R0.05 cells. Moreover, this effect was confirmed by FCM cell kinetic analysis: the accumulation of G_2M phase cells, which is low in ADM-resistant cells when compared with the parental cells, was significantly increased by VPM treatment in MKN45R0.8 cells but not in MKN45R0.05 cells. Thus, the reversal effect of VPM was observed even in our non-P-glycoprotein mediated ADM-resistant cell lines and was greater in high-level ADM-resistant cell lines. Although our ADM-resistant cell lines do not express P-glycoprotein because their degree of drug resistance is still at an early stage, these results indicate that VPM may be more useful in reversal of higher-level drug resistance, in which P-glycoprotein is probably overexpressed. In other words, VPM is much less effective at reversing resistance in low-level drug resistant cell lines, many of which do not express P-glycoprotein. Thus, the reversal of anticancer drug resistance appears to depend directly on the level or the mechanism of resistance.

In summary, we have established two ADM-resistant gastric cancer cell lines with different levels of drug resistance, and explored common features related to ADM resistance and resistance-modifying activity of VPM in both cell lines. FCM analysis may be useful for determining the level of drug resistance, cellular pharmacokinetics or P-glycoprotein expression in cancer cells. VPM has a stronger reversal effect on ADM resistance especially in high-level drug-resistant gastric cancer cells. The results suggest that treatment with reversal agents should be considered for the level or the mechanism of drug resistance in cancer cells.

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