Characterization of Low- and High-level Adriamycin-Resistant Human Gastric Cancer Cell Lines: Flow Cytometric Studies and Reversal Effect of Verapamil

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Received March 12 1999; accepted October 28 1999

Summary. We have established two adriamycin (ADM)-resistant sublines with different levels of drug resistance, MKN45R0.05 and MKN45R0.8, from the parental MKN45 human gastric cancer cell line by exposure to stepwise increases in ADM. MKN45R0.05 and MKN45R0.8 cells were 4.8- and 15-fold more resistant to ADM than MKN45 cells, respectively. Flow cytometric (FCM) analysis revealed that MKN45R0.8 cells had significantly lower accumulation of cells in G2M phase and higher efflux rate of intracellular ADM content than MKN45 and MKN45R0.05 cells. There was no significant increase of P-glycoprotein expression in both resistant sublines by FCM analysis. Verapamil (VPM) showed stronger modifying effects on ADM sensitivity and cell kinetics in MKN45R0.8 than MKN45R0.05 cells. These results indicate that FCM analysis is useful for determining the level of drug resistance, drug accumulation and P-glycoprotein expression in cancer cells, and that the differential resistance-modifying activity of VPM depends on the level of drug resistance.

Key words—adriamycin, drug resistance, verapamil, gastric cancer cell line, flow cytometry.

INTRODUCTION

Acquired resistance to anticancer drugs is a major obstacle in the treatment of gastrointestinal malignancies. Resistance to cytotoxic drugs occurs not only to those used in therapy but often to structurally and functionally unrelated classes of anticancer drugs. This type of resistance is called multidrug resistance (MDR). MDR is frequently associated with the overexpression of the mdr1 gene, which encodes a 170-kilodalton transmembrane protein termed the P-glycoprotein.3 P-glycoprotein functions as an energy-dependent efflux pump which decreases net intracellular drug accumulation. There are, however, a number of reports describing cell lines that show an MDR phenotype, but without evidence for P-glycoprotein expression,2^5^ suggesting the existence of other mechanisms for this phenotype. We have previously reported the establishment and characterization of a low-level adriamycin (ADM)-resistant human gastric cancer cell line.6 Our results have demonstrated that the mechanism of ADM resistance in this cell line is independent of P-glycoprotein expression, and that flow-cytometric (FCM) analysis is useful for detecting early steps in the acquisition of drug resistance of cancer cells.

In the present studies, we have established two ADM-resistant human gastric cancer cell lines with different levels of drug resistance. We now report on the differences in cell kinetics, cellular pharmacokinetics, P-glycoprotein expression and resistance modifying activity of verapamil (VPM) among the resistant and parent cell lines.
MATERIALS AND METHODS

Cell lines and culture conditions

The MKN45 human gastric cancer cell line originating from a poorly differentiated adenocarcinoma of the stomach. 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% CO₂ and 95% air at 37°C.

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 and Carmichael et al. Briefly, the cells were seeded into 96-well microplates (Corning Glass Works, Corning, NY, USA) at a density of 3×10⁴ 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°C. 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 IC₅₀ 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) = IC₅₀ in absence of VPM/IC₅₀ 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. 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°C 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°C 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°C 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. The data obtained from FCM analysis of 1×10⁴ 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 and Heike et al. Exponentially growing cells were fixed in 70% methanol at −20°C 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°C. 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°C. They were washed with cold PBS/FBS 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
Statistical analysis was performed using the Student’s t-test. Differences were considered significant at P<0.05.

RESULTS
Characteristics and drug sensitivity of MKN45, ADM-resistant MKN45R0.05 and MKN45R0.8 cell lines
As shown in Fig. 1, IC50 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 G2M phase at 48 h compared to the untreated control in each cell line. However, the percentage of cells in G2M 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 G2M 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

<p>| Table 1. Effect of ADM treatment on the accumulation of cells in G2M phase of MKN45, MKN45R0.05 and MKN45R0.8 cells |</p>
<table>
<thead>
<tr>
<th>ADM (μg/ml)</th>
<th>MKN45</th>
<th>MKN45R0.05</th>
<th>MKN45R0.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>3.9±0.7</td>
<td>5.0±0.4</td>
<td>9.6±1.0</td>
</tr>
<tr>
<td>0.2</td>
<td>45.7±3.5</td>
<td>26.5±3.2</td>
<td>17.8±1.1</td>
</tr>
<tr>
<td>0.4</td>
<td>36.9±2.8</td>
<td>33.0±0.5</td>
<td>28.4±0.2</td>
</tr>
<tr>
<td>4.0</td>
<td>31.0±1.8</td>
<td>32.5±1.2</td>
<td>22.2±0.5</td>
</tr>
</tbody>
</table>

a) Percentage of cells in G2M 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 MKN45R0.05 cells.
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. It was seen that these cells exhibited different levels of intracellular ADM uptake. The intracellular ADM content was determined using 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. 2. Intracellular uptake of ADM by MKN45, MKN45R0.05 and MKN45R0.8 cells.**

P-glycoprotein levels were examined by FCM analysis after staining with the anti-human P-glycoprotein antibody, MRK16. All 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).

**Fig. 3. Efflux of intracellular ADM content from MKN45, MKN45R0.05 and MKN45R0.8 cells.**

**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 G0/M 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.
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. 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. 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, did not overexpress P-glycoprotein. P-glycoprotein mediated drug resistance is thought to be preceded by another mechanism for MDR. 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 (mdr1) 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.

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.

Acknowledgments. We are grateful to Professor Katsuyoshi Hatakeyama for his advice and critical reading of the manuscript. We are also grateful to Mrs Minako Kanada for her technical assistance with flow cytometry.

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