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Original article

MGr1-Ag is associated with multidrug-resistant phenotype of gastric cancer cells

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Abstract

Background. **MGr1-antigen (Ag) was previously reported as an upregulated protein in multidrug-resistant (MDR) gastric cancer cells. The aim of this study was to characterize the role of MGr1-Ag in the multidrug resistance of gastric cancer cells.**

Methods. **Laser scanning confocal microscopy (LSCM), two-dimensional electrophoresis, and Western blot were used to detect MGr1-Ag in gastric cancer cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to determine the sensitivity of the MDR gastric cancer cells, SGC7901/VCR, to chemotherapeutic drugs. Adriamycin accumulation and retention in SGC7901/VCR cells were analyzed using flow cytometry.**

Results. **LSCM showed that MGr1-Ag localized mainly on the membrane and partly in the cytoplasm of SGC7901/VCR cells. Western blot showed that the expression level of MGr1-Ag in SGC7901/VCR cells was higher than that in its parental cells, SGC7901, and that the apparent molecular weight and isoelectric point of MGr1-Ag were 42 kDa and pH 4.8, respectively. After incubation with MGr1 antibody, SGC7901/VCR cells showed significantly decreased IC_{50} values for adriamycin (from 0.887 ± 0.081 mg/l to 0.607 ± 0.084 mg/l; $P < 0.05$), vincristine (from 0.707 ± 0.055 mg/l to 0.557 ± 0.042 mg/l; $P < 0.05$), and 5-fluorouracil (from 4.367 ± 0.407 mg/l to 2.630 ± 0.644 mg/l; $P < 0.05$), as well as slightly increased IC_{50} values for mitomycin (from 0.183 ± 0.045 mg/l to 0.198 ± 0.048 mg/l; $P > 0.05$). In addition, incubation with MGr1 significantly enhanced adriamycin accumulation and retention in SGC7901/VCR cells.**

Conclusion. **Overexpression of MGr1-Ag is associated with the MDR phenotype of gastric cancer cells.**

Key words MGr1-Ag · Neoplasm · Stomach · Drug resistance · Multiple

Introduction

Malignant tumor cells often develop multidrug resistance by overexpressing such drug transporters as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), lung resistance protein (LRP), and breast carcinoma-resistant protein (BCRP) [1–4]. However, it has been confirmed that not all gastric cancer cells or tissues express these transporters. Gurel et al. [5] and Yeh et al. [6] found, by immunohistochemical staining, that the positive rate for P-gp in gastric cancer tissues was 87% in Turkey and 13.3% in Taiwan district, respectively. According to Takebayashi et al. [7], only 34.1% of gastric cancer tissues from 103 patients expressed MRP. Alexander et al. [8] found, by immunohistochemical staining, that the positive rates for P-gp, MRP, and LRP in 20 gastric cancer tissues were 0%, 55%, and 10%, respectively. In another report, Fan et al. [9] performed reverse transcription-polymerase chain reaction to detect the mRNA of P-gp, MRP, and LRP in 50 gastric cancer tissue specimens and found that the positive rates were 10.0%, 12.0%, and 10.0%, respectively. All the data listed above suggest that there are still some unknown molecules involved in the multidrug resistance of gastric cancer, although P-gp, MRP, and LRP play important roles in the multidrug resistance of gastric cancer.

We prepared a multidrug-resistant (MDR) gastric cancer cell line, SGC7901/VCR, which was derived from the human gastric cancer cell line SGC7901 by stepwise selection in vitro, using vincristine as inducing reagent [10]. Subsequently, we prepared a monoclonal antibody, MGr1, by a hybridoma technique, using SGC7901/VCR cells as the immunogen [11,12]. Immunoelectronmicroscopy and immunofluorescence staining showed that the antigen corresponding to MGr1 (MGr1-Ag) localized mainly on the membrane and partly in the cytoplasm of gastric cancer cells [12,13]. Immunohistochemical staining revealed that SGC7901/

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VCR cells exhibited much stronger staining with MGr1 than its parental cells SGC7901 [12], which suggested a possible relationship between MGr1-Ag and the multidrug resistance of gastric cancer. The aim of this study was to characterize the role of MGr1-Ag in the multidrug resistance of gastric cancer cells.

Materials and methods

Laser scanning confocal microscopy (LSCM)

Human gastric cancer cells, SGC7901 and SGC7901/VCR, were cultured in RPMI 1640 (Gibco, Uxbridge, UK) supplemented with 10% fetal calf serum (Gibco). For LSCM, SGC7901/VCR cells grown on coverslips were fixed with ice-cold acetone for 10 min. After rehydration with phosphate-buffered saline (PBS) for 10 min, the cells were exposed to 0.3% Triton X-100 in PBS for 15 min. After a brief wash, they were soaked in blocking solution (PBS containing 3% normal goat serum) for 30 min. Then the cells were incubated in turn with MGr1 overnight at 4 °C, biotinylated goat anti-mouse IgG (Boster, Wuhan, China) at room temperature for 1 h, and streptavidin-conjugated Texas red (Boster) at room temperature for 1 h. Finally, the samples were analyzed with a laser scanning confocal microscope (MRC 1000; Bio-Rad, Richmond, CA, USA).

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed according to Lee et al. [14] with modification. Briefly, 150 μg total protein extracted from SGC7901/VCR cells was employed for isoelectrofocusing and the final volume was brought to 250 μl, using buffer (pH 9.5) containing 8 mol/l urea (Sangon, Shanghai, China), 1% Nonidet P-40 (NP-40), 0.28% dithiothreitol, and 0.2% bromophenol blue. After being left to stand at room temperature for 1–1.5 h, the protein sample was loaded into the sample groove. A solid gel strip of pH gradient pH 3–10 (Pharmacia, Uppsala, Sweden) was fixed in a Pgphor isoelectrofocusing system (Pharmacia), and isoelectrofocusing was performed under homeothermia at 20 °C according to the program: 0–300 V for 1 h, 300–500 V for 1 h, 500–1000 V for 1 h, 1000–2000 V for 1 h, 2000–4000 V for 1 h, and 4000–8000 V for 4 h. After the isoelectrofocusing, the gel was equilibrated with equilibration buffer I (pH 6.8) containing 6 mol/l urea, 50 mmol/l Tris base, 30% glycerol, and 1% dithiothreitol for 15 min, and with equilibration buffer II (pH 6.8), containing 6 mol/l urea, 50 mmol/l Tris base, 30% glycerol, and 2.5% iodoacetamide (Sangon) for another 15 min. Then, the gel was attached to 12.5% polyacrylamide sodium dodecylsulfate (SDS) gel alongside a low-range protein marker (SABC, Shanghai, China), and subjected to electrophoresis in the second dimension at 15 °–20 °C under a constant current of 20 mA. When bromophenol blue reached the bottom of the gel, the gel was retrieved and proteins were detected by silver staining or, alternatively, the gel was used for Western blot.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot

Equal amounts of total proteins extracted from SGC7901 and SGC7901/VCR cells were separated by SDS-PAGE according to the standard protocol. SDS-PAGE gels and two-dimensional electrophoresis gels were equilibrated with transferring buffer (pH 8.3–8.4), containing 25 mmol/l Tris base, 192 mmol/l glycine, and 20% methanol for 10–20 min. Separated proteins on the polyacrylamide gel were electro-transferred to a nitrocellulose filter (Gene Company, Shanghai, China) at a constant current of 300 mA for 1.5 h. The filter was stained with ponceau S (Sangon) to show protein bands, and bands of protein standards were marked with a marker pen corresponding to their positions. Then the filter was washed with deionized water to remove ponceau S and exposed to 5% defatted milk powder in TBST buffer (10 mmol/l Tris base, 150 mmol/l sodium chloride, 0.05% Tween 20, pH 8.0) at room temperature for 1 h to block nonspecific protein binding sites. The monoclonal antibody MGr1 was used as the primary antibody in TBST buffer containing 5% defatted milk powder overnight at 4 °C. After four washes with TBST buffer, the filter was incubated with the second antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Dako, Glostrup, Denmark) diluted in TBST buffer, containing 5% defatted milk powder, at room temperature for 1.5 h. After four washes with TBST buffer, the filter was submitted to Diaminobenzidine (DAB) (Sigma, St. Louis, MO, USA) solution for 5–30 min to visualize positive signals.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

The sensitivity of gastric cancer cells to anticancer drugs was evaluated using the MTT assay as described previously [15]. Briefly, SGC7901/VCR cells in log phase were harvested and plated into a 96-well plate (1×10^4 cells/well). After overnight culture at 37 °C, adriamycin, vincristine, 5-fluorouracil, and mitomycin were added to the SGC7901/VCR cells. Purified monoclonal antibody MGr1 was also added, to a final concentration of 10 mg/l. Anti-human gastric cancer monoclonal antibody MGb2 was used as the control antibody (MGb2 antibody was prepared in our institute by Fan et al. [16],

using a hybridoma technique, and the human gastric cancer cells KATO III as immunogen. The antigen corresponding to MGb2 was characterized as a glycoprotein localized on the membrane of gastric cancer cells [16]. Further studies revealed that the MGb2 antibody could specifically target anticancer agents to cancer cells [17–19]). Each treated group was done in triplicate. After the cells had been cultured for 72 h, 50 μl of 2 g/l MTT (Sigma) was dropped into each well, and the cells were cultured for another 4 h. Then the supernatants were discarded, and 150 μl of dimethylsulfoxide (DMSO) (Sigma) was dropped into each well to dissolve crystals. The absorbance at 490 nm (A_{490}) of each well was read on a spectrophotometer (MRP-2100; Syntron, Carlsbad, CA, USA). Cell survival rates were calculated according to the formula: survival rate = (mean A_{490} of treated wells/mean A_{490} of untreated wells) $\times 100\%$. Finally, dose-effect curves of the anticancer drugs were drawn on semilogarithm coordinate paper and IC_{50} values were determined.

Flow cytometry

The fluorescence intensity of intracellular adriamycin was determined using flow cytometry, as described previously [15]. In brief, SGC7901/VCR cells in log phase were plated into a six-well plate (1×10^6 cells/well) and cultured overnight at 37 °C. Adriamycin and MGr1 antibody were added to SGC7901/VCR cells to a final concentration of 5 mg/l and 10 mg/l, respectively. Cells continued to be cultured for 1 h. Then the cells were trypsinized and harvested, or, alternatively, cultured in drug-free RPMI 1640 for another 30 min, followed by trypsinization and harvesting. Finally, the cells were washed twice with pre-cooled PBS, and the mean fluorescence intensity of intracellular adriamycin was determined by flow cytometry (Coulter, Miami, FL, USA) with an excitation wavelength of 488 nm and receiving wavelength of 575 nm.

Statistical analysis

Values for results were expressed as means ± standard deviation. The differences between two mean values were evaluated using one-way analysis of variance (ANOVA) test with statistical analysis software (SPSS, version 10.0; Chicago, IL, USA). Differences were considered significant if $P < 0.05$.

Results

LSCM

The subcellular location of MGr1-Ag in SGC7901/VCR cells was detected by indirect immunofluorescence staining. As Fig. 1 shows, the major part of MGr1-Ag was localized in the membrane of SGC7901/VCR cells, with a small part of MGr1-Ag localized in the cytoplasm of the SGC7901/VCR cells.

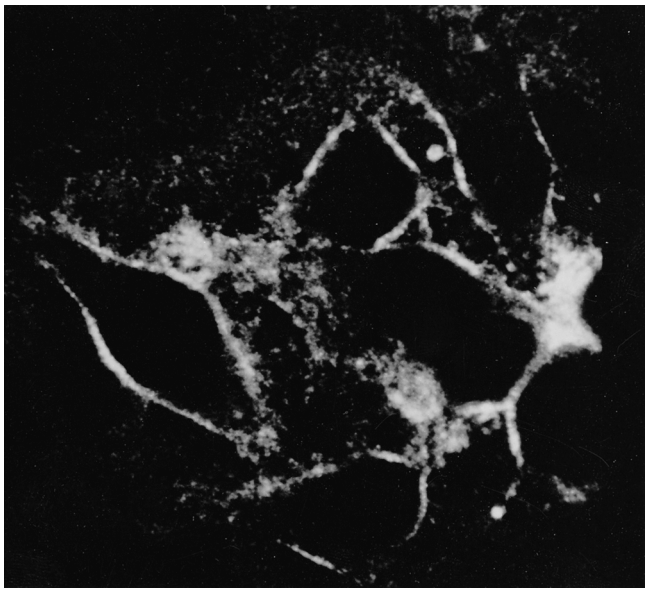

Fig. 1. Subcellular location of MGr1-antigen (Ag) in SGC7901/VCR cells analyzed by laser scanning confocal microscopy. MGr1-Ag was labeled by monoclonal antibody MGr1, and then detected by biotinylated goat anti-mouse IgG and streptavidin-conjugated Texas red

Two-dimensional electrophoresis and Western blot

To determine the molecular weight and isoelectric point of MGr1-Ag, total proteins of SGC7901/VCR cells were separated by two-dimensional electrophoresis and then subjected to Western blot. As Fig. 2A shows, there were about 600 proteins displayed by two-dimensional electrophoresis. After electroblotting to the nitrocellulose filter and detection by the monoclonal antibody MGr1, there was only one positive signal dot (Fig. 2B). When estimated by the relative migration rate, the molecular weight and isoelectric point corresponding to this signal point were about 42 kDa and pH 4.8 respectively.

SDS-PAGE and Western blot

To determine the expression level of MGr1-Ag in gastric cancer cells, equal amounts of total proteins extracted from SGC7901 cells and SGC7901/VCR cells were subjected to SDS-PAGE and Western blot. As Fig. 3 shows, compared with its parental cell line, SGC7901, SGC7901/VCR cells exhibited increased expression of MGr1-Ag.

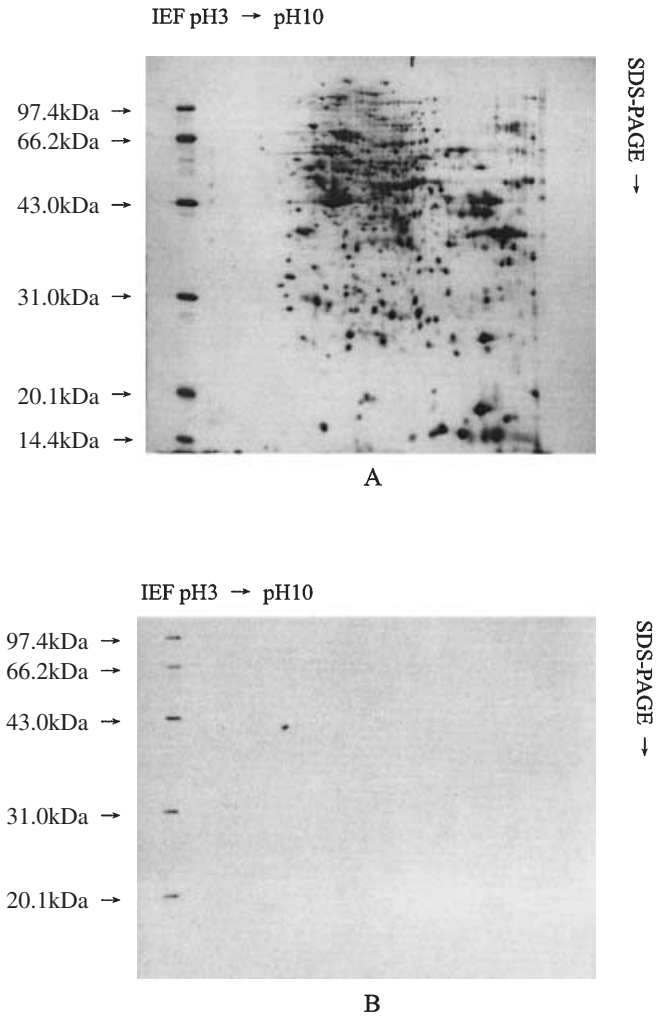

Fig. 2A,B. Molecular weight and isoelectric point of MGr1-Ag determined by two-dimensional electrophoresis and Western blot. Total proteins of SGC7901/VCR cells were separated by two-dimensional electrophoresis (**A**) and then subjected to Western blot (**B**). Monoclonal antibody MGr1 was used as the primary antibody. Horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody. Positive signals were visualized with Diaminobenzidine (DAB). *Arrows* on the *left* in **A** and **B** indicate positions of protein standards and their apparent molecular weights. *IEF*, Isoelectric focusing; *SDS-PAGE*, sodium dodecylsulfate-polyacrylamide gel electrophoresis

Drug sensitivity assay in vitro

The MTT assay was performed to evaluate the effects of the MGr1 antibody on the sensitivity of SGC7901/VCR cells to chemotherapeutic drugs. As Table 1 shows, the IC_{50} values of SGC7901/VCR cells to adriamycin, vincristine, 5-fluorouracil, and mitomycin were 0.887 ± 0.081 mg/l, 0.707 ± 0.055 mg/l, 4.367 ± 0.407 mg/l, and 0.183 ± 0.045 mg/l, respectively. After treatment with the monoclonal antibody MGr1, the IC_{50} values of SGC7901/VCR cells to adriamycin, vincristine, and 5-fluorouracil decreased to 0.607 ± 0.084 mg/l ($P < 0.05$), 0.557 ± 0.042 mg/l ($P < 0.05$), and 2.630 ± 0.644 mg/l ($P < 0.05$), respectively. Although the IC_{50} value of mitomycin increased slightly, to 0.198 ± 0.048 mg/l, there was no significant difference between the MGr1-treated and non-treated groups. The control antibody MGb2 had no significant influence on the IC_{50} values of SGC7901/VCR cells to adriamycin, vincristine, 5-fluorouracil, and mitomycin.

Fluorescence intensity assay of intracellular adriamycin

The effects of the MGr1 antibody on adriamycin accumulation and retention in SGC7901/VCR cells were determined using flow cytometry. After incubation with adriamycin for 1 h, the fluorescence intensity of adriamycin accumulated in SGC7901/VCR cells was

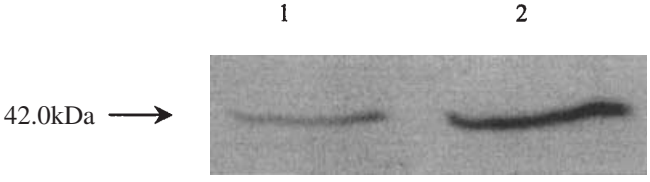

Fig. 3. Expression of MGr1-Ag in gastric cancer cells determined by Western blot. Equal amounts of total proteins extracted from SGC7901 cells (*lane 1*) and SGC7901/VCR cells (*lane 2*) were subjected to SDS-PAGE and Western blot. Monoclonal antibody MGr1 was used as the primary antibody. Horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody. Positive signals were visualized with DAB

Table 1. IC_{50} values of SGC7901/VCR cells for anticancer drugs

Drug	IC_{50} (mg/l)		
	SGC7901/VCR	SGC7901/VCR + MGr1	SGC7901/VCR + MGb2
Adriamycin	0.887 ± 0.081	0.607 ± 0.084*	0.879 ± 0.067
Vincristine	0.707 ± 0.055	0.557 ± 0.042*	0.717 ± 0.069
5-Fluorouracil	4.367 ± 0.407	2.630 ± 0.644*	4.352 ± 0.398
Mitomycin	0.183 ± 0.045	0.198 ± 0.048	0.189 ± 0.046

* $P < 0.05$ vs SGC7901/VCR group

Table 2. Fluorescence intensity of intracellular adriamycin in SGC7901/VCR cells

	Mean fluorescence intensity		
	SGC7901/VCR	SGC7901/VCR + MGr1	SGC7901/VCR + MGb2
Accumulation	2.19 ± 0.71	2.46 ± 0.79*	2.17 ± 0.69
Retention	0.95 ± 0.44	1.42 ± 0.58*	0.93 ± 0.45

* $P < 0.01$ vs SGC7901/VCR group

2.19 ± 0.71. When culture continued in fresh RPMI 1640 for another 30 min, the fluorescence intensity of adriamycin retention in SGC7901/VCR cells was still 0.95 ± 0.44. However, when the cells were simultaneously incubated with MGr1 and adriamycin, the fluorescence intensity of adriamycin accumulation and retention in SGC7901/VCR cells increased to 2.46 ± 0.79 ($P < 0.01$) and 1.42 ± 0.58 ($P < 0.01$), respectively (Table 2). The control antibody MGb2 had no significant effect on adriamycin accumulation and retention in SGC7901/VCR cells.

Discussion

The main issue of chemotherapy is the development of multidrug resistance in tumor cells. It has been reported that the increased expression of thymidine kinase, equilibrative nucleoside transporter 1, and methylenetetrahydrofolate dehydrogenase, as well as the decreased expression of orotate phosphoribosyltransferase, thymidine phosphorylase, uridine phosphorylase, cytidine deaminase, and integrin alpha 3 were correlated with a 5-fluorouracil-resistant phenotype of gastric cancer cells [20,21]. In another report, overexpression of annexin I and thioredoxin was found to be associated with daunorubicin-resistance, and overexpression of thioredoxin was found to be associated with mitoxantrone-resistant phenotypes of gastric cancer cells [22]. These data strongly suggested that there might be diverse regulatory systems in gastric cancer cells under drug attack.

To better understand the regulatory network underlying multidrug resistance in gastric cancer cells, we prepared a vincristine-resistant gastric cancer cell line, SGC7901/VCR, which developed resistance not only to vincristine but also to adriamycin, 5-fluorouracil, and mitomycin [10]. The present study demonstrated that MGr1-Ag was overexpressed in the drug-resistant gastric cancer cells SGC7901/VCR, and that blockage of MGr1-Ag could partly reverse the MDR phenotype of the SGC7901/VCR cells. These data indicated an important role for MGr1-Ag in the multidrug resistance of SGC7901/VCR cells. We have detected MGr1-Ag by an immunohistochemical method in specimens from 68 gastric cancer patients and 56 colon cancer patients. The results showed positive rates of 35.29% and 33.93% for MGr1-Ag in gastric cancer and colon cancer, respectively (unpublished data). We speculate that MGr1-Ag might confer drug resistance not only to gastric cancer cells but also to colon cancer cells.

Previous studies have found that SGC7901/VCR cells express slightly increased levels of P-gp and MRP and exhibit significantly decreased adriamycin accumulation and retention in comparison with SGC7901 cells [10,23]. The present study found that MGr1 enhanced adriamycin accumulation and retention in SGC7901/VCR cells. This could lead to the postulation that MGr1-Ag may be a drug transporter. But the apparent molecular weight of MGr1-Ag is just 42 kDa. In general, the apparent molecular weight of transporter proteins is much higher. Even the half-transporter BCRP exhibits an apparent molecular weight of 95 kDa [4]. Therefore, we speculate that MGr1-Ag is a modulator that could interfere with the functions of P-gp or MRP.

In conclusion, we have characterized MGr1-Ag as a 42 kDa protein, and confirmed the association of MGr1-Ag with the MDR phenotype of gastric cancer cells. Gene cloning, structural analysis, and functional identification of MGr1-Ag are ongoing.

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