Jpn. J. Cancer Res. **90**, 788–793, July 1999

Multidrug Resistance Phenotype in the RMS-GR Human Rhabdomyosarcoma Cell Line Obtained after Polychemotherapy

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Classical cytotoxic treatment of rhabdomyosarcoma (RMS), the most common soft tissue malignacy in children, is often accompanied by significant morbidity and poor response. Chemotherapy may induce multidrug resistance (MDR) associated with the expression of P-glycoprotein, a drug efflux pump which modifies the sensitivity of tumoral cells to drugs. To analyze MDR in RMS we used the RMS-GR cell line, obtained from an embryonal rhabdomyosarcoma treated *in vivo* with polychemotherapy. The RMS-GR cells showed cross-resistance to vincristine, doxorubicin and actinomycin D, the drugs of choice in the conventional treatment of RMS. Polymerase chain reaction (PCR) analysis showed that these RMS cells overexpressed *mdr1*/P-glycoprotein. The pattern of resistance and the level of P-glycoprotein expression were similar to those found in the resistant RMS TE.32.7.DAC cell line obtained *in vitro*. Southern blot analysis showed that *mdr1* overexpression was not due to amplification of the gene. Our results showed that the *in vivo* treatment of embryonal RMS may induce an MDR phenotype mediated by *mdr1*/P-glycoprotein in RMS cells.

Key words: Multidrug resistance — *mdr1* — P-Glycoprotein — Rhabdomyosarcoma

Cross-resistance between different cytostatic agents which are structurally and functionally dissimilar is a common phenomenon called multidrug resistance (MDR). This phenomenon can limit the treatment of human cancers with antineoplastic drugs,[1)] especially in sarcomas, which are characterized by their frequent refractoriness to chemotherapy.[2)] Although the mechanisms responsible for MDR are still not fully understood,[3)] classical MDR has often been associated with the expression of the multidrug resistance gene (*mdr1*).[4)] The product of this gene, a 170-kDa membrane-associated glycoprotein (P-glycoprotein), is a member of the ABC superfamily of membrane proteins, which alter cellular drug transport and distribution.[5)]

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in patients younger than 21 years of age, accounting for 5–8% of all cases of childhood cancer.[6)] Although advances in modern multimodal treatment, especially polychemotherapy, have improved the survival rate, RMS is characterized by poor response to cytotoxic treatment and significant morbidity.[7)] More recently, the use of differentiation therapy mediated by cytotoxic drugs[8)] has been examined in RMS as a new approach to the treatment of these tumors. Cytotoxic treatment of RMS has shown that these tumoral cells can be induced to reenter the differentiation process,[9)] supporting the hypothesis that RMS arises from muscle cells which are arrested along the normal myogenic pathway to maturation.[6)] However, only partial differentiation has been detected in RMS cells after polychemotherapy *in vivo*[10)] and *in vitro*.[11)] Because P-glycoprotein acts as an energy-dependent drug efflux pump which lowers intracellular concentrations of the drug to sublethal levels,[5)] the failure of cytotoxic drugs to induce cytodestruction or complete differentiation has been related to the development of resistance.[12)] However, limited data have been published on drug resistance mechanisms in RMS during cytotoxic treatment.

We recently established a new RMS cell line (RMS-GR) from an embryonal rhabdomyosarcoma treated *in vivo* with polychemotherapy, which showed a moderate degree of myogenic differentiation.[13)] Using this RMS cell line, we investigated the mechanism of resistance mediated by P-glycoprotein against cytotoxic drugs. Our results showed that classical cytotoxic therapy used in the treatment of embryonal RMS may induce an MDR phenotype mediated by *mdr1*/P-glycoprotein in these tumoral cells, and that the pattern of resistance was similar to those developed by *in vitro* exposure of RMS cells to drugs.

MATERIALS AND METHODS

Tissue samples and cell line Tumor tissue samples were obtained before polychemotherapy by intraoperative biopsy from the primary lesion of a tumor which was histopathologically classified as embryonal RMS. The patient was treated with polychemotherapy, had repeated relapses

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and died 8 months later. The RMS-GR cell line, established from the treated embryonal RMS, and the resistant TE.32.7.DAC cell line, established by exposure to increasing concentrations of actinomycin D in the culture medium, were obtained in our laboratory.[13, 14] The drug-sensitive RMS cell line TE.32.7 was obtained from the ATCC (Rockville, MD) and used as a control for cytotoxicity experiments. The RMS cell lines were grown at 37°C in an atmosphere containing 5% CO_2, with Dulbecco's modified Eagle's medium (MEM) (Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS) (Gibco), 20 m*M* L-glutamine, 3.5 mg/μl sodium bicarbonate, 4.5 g/liter glucose, 250 U/ml ampicillin and 20 μg/ml streptomycin. The resistant cell line grew in medium containing 1.2×10^{-6} m*M* actinomycin D.

PCR evaluation of *mdr1* mRNA levels Total RNA was obtained according to Maniatis *et al*.[15] Amounts of *mdr1* mRNA were estimated relative to *β-actin* mRNA using a modification of the RNA polymerase chain reaction (PCR) method.[16] Reverse transcription was done with total cellular RNA as follows: each tube contained a total volume of 100 μl, composed of 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 3 m*M* $MgCl_2$, 0.01% gelatin, 800 μM each of the four deoxyribonucleotide triphosphates, and 1 μM *mdr1* or *β-actin* primers. The primers used were: *mdr1*, 5′-primer nt 3007 to 3026, 3′-primer nt 3141 to 3160; and *β-actin*, 5′-primer nt 1854 to 1873, 3′-primer nt 2151 to 2170.[17] One microgram of total cellular RNA and two units of reverse transcriptase (Stratagene, La Jolla, CA) were added to individual tubes and the reaction was allowed to proceed at 42°C for 45 min. Thermostable DNA polymerase (Pharmacia, Piscataway, NJ) was added to each tube (2.5 units), and *mdr1* or *β-actin* cDNA products, if present, were amplified by PCR. The PCR products were loaded onto 2% agarose gels and visualized by ethidium bromide staining.

Southern blot analysis of *mdr1* gene amplification Genomic DNA was isolated according to Maniatis *et al*.[15] Twenty micrograms of DNA was digested with *Eco*RI (Amersham, Arlington Heights, IL) and electrophoresed on a 0.6% agarose gel. DNA was denatured and transferred to Hybond-N hybridization membranes (Amersham). Hybridization of nylon membranes was done in 50% formamide, 5×SSC, 20 m*M* sodium phosphate (pH 6.5), 200 μg/ml sonicated salmon sperm DNA, 10% dextran sulfate plus ^{32}P-labeled oligoprobes (10^6 dpm/ml) recognizing *mdr1* (cDNA sequence 3027–3049) and *β-actin* sequences (cDNA sequence 1874–1898). Intensity of the autoradiographic bands was determined by densitometry, and the signal of each sample was normalized to adjust for experimental variation in DNA loading by comparisons with the *β-actin* signal of each sample.

Preparation of RMS-GR cells for FACScan Briefly, 10^6 RMS cells were transferred to universal screw cap tubes containing sterile phosphate-buffered saline (PBS), then washed and centrifuged at 225*g* for 5 min. The supernatant was discarded, and the washing and centrifugation steps were repeated twice. To determine P-glycoprotein expression, the cells were fixed with 2% formaldehyde for 10 min at −20°C and immediately washed three times in PBS at 4°C. The cells were permeabilized with Triton X-100 (0.05%) at room temperature for 10 min, then washed three times in PBS and once in distilled water. The cells were incubated for 30 min at 4°C with the monoclonal antibody (mAb) JSB-1 (5 μl),[18] then washed twice with cold PBS and reincubated with fluorescein isothiocyanate (FITC)-conjugated antimouse immunoglobulin (Sigma, St. Louis, MO) (1:50) for 30 min at 37°C. The expression of P-glycoprotein was assessed using the mAb C-219 according to the basic protocol recommended by the manufacturer (Centacor, Inc., Malvern, PA). The tumor tissue sample was cut into pieces and transferred to universal screw cap tubes containing PBS, then washed and centrifuged at 225*g* for 5 min; the supernatant was discarded. The washing and centrifugation steps were repeated twice, after which the whole tissue sample was placed in tubes containing 1 ml of 0.5% trypsin solution in PBS. The tubes were cooled on ice to 4°C and incubated for 4–8 h at this temperature, after which the trypsin was carefully removed. The rest of the procedure to determine P-glycoprotein expression with JSB-1 and C-219 was done as described above. The results were expressed as mean fluorescence.

Cytotoxicity experiment Briefly, RMS cells (2×10^5/ml) were treated with different concentrations of actinomycin D, vincristine or doxorubicin in four replicate samples in the absence or presence of the verapamil at a nontoxic concentration (10 μM) for 1 h.[19] After 72 h, cells were harvested by trypsinization and counted in a model ZBI Coulter counter (Hialeah, FL). Cell viability was determined with trypan blue dye exclusion. The dose that inhibited 50% of growth (ID_{50}) was calculated from the curve for the percentage of cell survival at different concentrations of the drug.

RESULTS

Determination of *mdr1* expression by PCR As shown in Fig. 1A, cell line RMS-GR clearly showed a high level of *mdr1* mRNA expression, in contrast with the embryonal RMS tissue sample, in which no expression was found. RMS-GR cells yielded a slightly larger PCR product for *mdr1* than those found in the resistant line TE.32.7.DAC, which was developed *in vitro* and used as a control. To demonstrate the integrity of the RNA preparations, PCR was done using *β-actin* primers. In all cases the expected product was easily identified, indicating that the absence of *mdr1* transcripts in the RMS tissue and normal striated

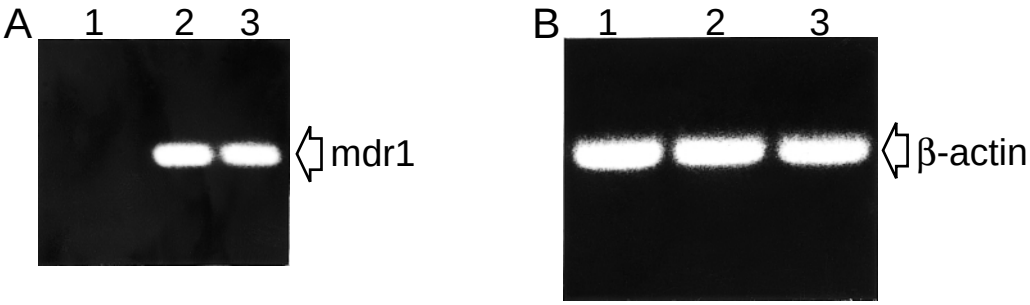

Fig. 1. PCR analysis of *mdr1* expression. A, Agarose gel electrophoresis with ethidium bromide staining of PCR products using primers for the amplification of an *mdr1* fragment (154 bp) of cDNA. B, Agarose gel electrophoresis with ethidium bromide staining of PCR products using primers for the amplification of a β-actin fragment (317 bp) of cDNA. The lanes correspond to: 1, embryonal RMS tissue sample; 2, RMS-GR cell line; 3, TE.32.7.DAC resistant cell line.

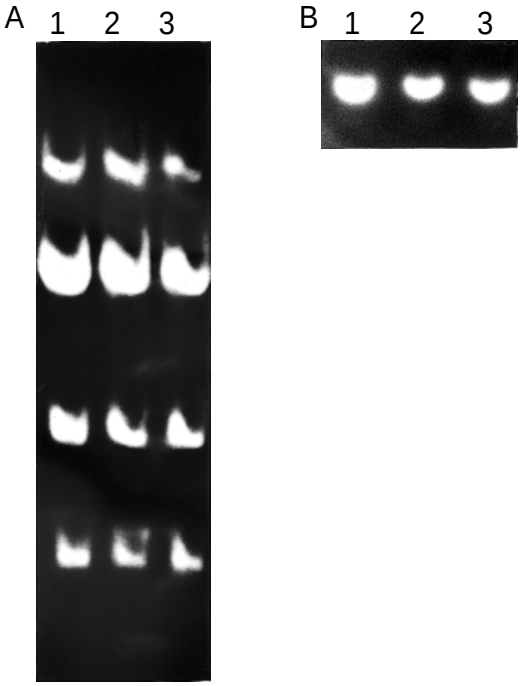

Fig. 2. Southern blot analysis of the *mdr1* gene. A, Hybridization of the blot with the *mdr1* oligoprobe. B, Hybridization of the blot with the *β-actin* oligoprobe. The lanes correspond to: 1, embryonal RMS tissue sample; 2, RMS-GR cell line; 3, TE.32.7.DAC resistant cell line.

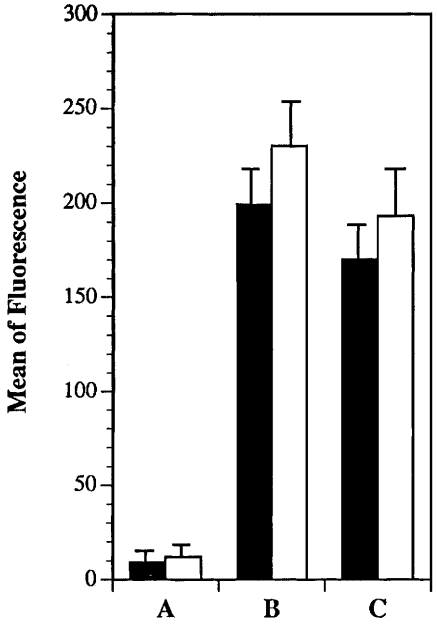

Fig. 3. Determination of P-glycoprotein expression by FACScan using the mAbs JSB-1 (■) and C-219 (□). A, embryonal RMS tissue sample; B, RMS-GR cell line; C, TE.32.7.DAC resistant cell line. JSB-1 and C-219 staining confirmed the MDR phenotype of cell lines RMS-GR and TE.32.7.DAC.

muscle was not due to degradation of the RNA (Fig. 1B).

Determination of *mdr1* gene amplification by Southern blot Blot analyses of DNA from RMS-GR cells were compared with those of the tumor tissue to see whether gene amplification had occurred. Densitometric analyses of the Southern blot bands did not show *mdr1* gene amplification in RMS-GR cells in comparison with DNA obtained from a biopsy of tumor tissue (Fig. 2A). Moreover, when we compared the genomes of the RMS-GR cells and TE.32.7.DAC resistant cells, the number of copies of the *mdr1* gene was similar. The finding of identical amounts of DNA in all lanes was verified by hybridization of the filter with an oligoprobe that recognized *β-actin* (Fig. 2B).

Table I. Comparison of the ID_{50} in RMS-GR, TE.32.7.DAC and TE.32.7 Cells. Effect of Verapamil Treatment (10 μM) on Drug Cytotoxicity

Drugs	ID_{50} (n*M*)					
	RMS-GR		TE.32.7.DAC		TE.32.7	
	Without verapamil	With verapamil	Without verapamil	With verapamil	Without verapamil	With verapamil
Actinomycin-D	175±13	3.3±0.35 (53)	30.14±0.42	0.9±0.09 (33.4)	1.53±0.15	0.8±0.07 (1.9)
Vincristine	5.8±0.51	0.075±0.01 (77)	7.45±0.90	1.7±0.08 (4.3)	0.12±0.003	0.085±0.02 (1.4)
Doxorubicin	17±2.36	1.8±0.17 (9)	162.4±15	2.3±0.026 (70.4)	4.01±0.06	1.62±0.21 (2.4)

The values are means±SEM of four separate experiments. Values in parentheses, decrease (*x*-fold) in the ID_{50} values as compared to the ID_{50} obtained in the absence of verapamil.

Determination of P-glycoprotein expression by FACScan To determine the level of P-glycoprotein expression, RMS-GR cells were stained with the mAb JSB-1 and analyzed by FACScan. JSB-1 staining confirmed that the MDR phenotype of cell line RMS-GR was characterized by increased levels of P-glycoprotein expression, which were similar to those found in the resistant TE. 32.7.DAC cell line used as a control (Fig. 3). Analyses with the C-219 mAb confirmed the high expression of P-glycoprotein in both RMS-GR and TE.32.7.DAC cell lines. In contrast, P-glycoprotein expression was practically undetectable with both JSB-1 and C-219 mAbs in the tumor tissue (Fig. 3).

Level of drug resistance in RMS-GR cells Cytotoxicity experiments with the drugs used to treat the primary tumor (doxorubicin, vincristine and actinomycin-D) were done to determine the ID_{50} of RMS-GR cells. Cultures of TE.32.7 and TE.32.7.DAC cell lines were used as controls. RMS-GR cells showed a pattern of cross-resistance similar to those found in the resistant RMS TE.32.7.DAC cell line obtained *in vitro* (Table I). However, RMS-GR cells showed an ID_{50} for actinomycin-D six fold as high as the resistant TE.32.7.DAC cells, whereas the ID_{50} for doxorubicin was one-ninth of this value. Both cell lines showed a similar ID_{50} for vincristine (Table I).

Effect of the pharmacologic blockade of P-glycoprotein in RMS cells To determine the action of P-glycoprotein as a drug efflux pump, we evaluated the change in drug cytotoxicity in RMS-GR cells after blockade with verapamil. An approximately 53-, 77-, and 9-fold increase in the effect of actinomycin-D, vincristine and doxorubicin, respectively, occurred in RMS-GR cells with verapamil as compared to the ID_{50} values (Table I). Verapamil at the same nontoxic concentration greatly enhanced the cytotoxicity of drugs for resistant TE.32.7.DAC cell line. In contrast, slight modifications in drug cytotoxicity were found in the drug-sensitive TE.32.7 cell line (Table I).

DISCUSSION

Drug resistance remains a formidable obstacle to the successful treatment of different tumors. There have consequently been many attempts to produce cell lines resistant to antineoplastic agents as *in vitro* models to study MDR.[20] The *in vitro* exposure to progressively increasing amounts of a drug to obtain resistant cell lines induces cross-resistance to chemotherapeutic agents that differ in their chemical structure. However, the *in vitro* selection of these resistant cell lines may not be representative of the clinical situation.[20]

To analyze the effect of classical *in vivo* treatment in RMS, we used the RMS-GR cell line obtained in our laboratory from an embryonal RMS treated with conventional chemotherapy.[13] The RMS-GR cells showed a pattern of cross-resistance to chemically unrelated compounds, such as the antibiotics actinomycin-D and doxorubicin and the vinca alkaloid vincristine, which were used to treat the primary tumor. This pattern of resistance was similar to those found in the resistant RMS TE.32.7.DAC cell line obtained *in vitro*,[14] and was also described in resistant lines derived from other tumor tissues.[20, 21] However, RMS-GR cells showed a higher degree of resistance to actinomycin-D in comparison with cell lines induced *in vitro*, and the resistance to this drug was always greater than resistance to vincristine or doxorubicin. The different levels of drug resistance may be related with the different *in vivo*[22] and *in vitro*[20] drug exposures, or with other mechanisms such as multidrug resistance-associated protein (MRP), which has been detected together with *mdr1* in a few resistant cell lines.[23] In fact, the MDR phenotype alone does not completely account for the resistance of human sarcomas.[24] However, our results support the hypothesis that after drug treatment of RMS *in vivo*, many cancer cells take advantage of their ability to develop a resistant phenotype[25] characterizated by *mdr1* overexpres-

sion and a pattern of resistance similar to those observed *in vitro*.

Analyses of the RMS-GR cell line indicated that *mdr1* mRNA overexpression was slightly greater than that found in the resistant TE.32.7.DAC cell line obtained *in vitro*. The overexpression of P-glycoprotein has been widely described in solid tumors[26] including sarcomas[27, 28] as the mechanism which explains the decrease in the amount of drug that accumulates in the tumor cell cytoplasm. Our findings showed that *mdr1* was involved in the resistance mechanism of RMS-GR cells, and strongly suggest that this mechanism may be responsible for the failure of treatment of primary embryonal RMS with classical cycles of cytotoxics. The demonstrated interrelationship between *mdr1* and genes implicated in cellular differentiation[29, 30] suggests that the presence of this resistance mechanism in RMS cells may be related to the failure of complete reentry in the myogenic differentiation process induced by cytotoxic drugs (differentiation therapy). In fact, previous inmunohistochemical analysis of RMS-GR cells showed only partial differentiation,[13] whereas we have recently shown that low concentrations of actinomycin-D, which do not induce *mdr1* overexpression, led to a terminal process of myogenic differentiation in RMS cells.[31] The mechanism that leads to overexpression of P-glycoprotein may involve amplification, increased stability of the protein, or increased transcription without amplification.[32] Studies in resistant tumor cells induced *in vitro* showed that *mdr1* is often amplified.[30, 33] However, analyses of the mechanism of drug resistance in RMS-GR cells demonstrated that the high expression of *mdr1*/P-glycoprotein was not due to gene amplification.

We showed that *in vivo* treatment of embryonal RMS may induce an MDR phenotype mediated by *mdr1*/P-glycoprotein in RMS cells. This phenotype decreases the cytotoxicity of the different drugs used in classical RMS therapy. This pattern of cross-resistance, which was similar to patterns found in resistant cell lines that developed *in vitro*, suggests that resistance mediated by *mdr1* may be the mechanism responsible for the failure of embryonal RMS treatment with cytotoxics. This resistance mechanism may also be related to the partial differentition found in RMS treated with cytotoxic drugs, although this hypothesis needs futher investigation.

ACKNOWLEDGMENTS

We thank Dr. F. Garrido of the Immunology Service, Virgen de las Nieves Hospital in Granada for providing the oligoprobes and primers to *β-actin* and *mdr1* genes. We thank Karen Shashok for improving the English style of the manuscript. This study was supported by the Fondo de Investigaciones Sanitarias de la Seguridad Social (FIS) through Project no. 95/0974.

(Received March 10, 1999/Revised May 1, 1999/Accepted May 12, 1999)

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