ISOLATION AND CHARACTERIZATION OF AN ADRIAMYCIN-RESISTANT BREAST TUMOR CELL LINE

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SUMMARY

An adriamycin-resistant human breast tumor cell line MDA-A1* was generated by step-wise selection in increasing concentrations of drug from the parent cell line MDA-MB-231. MDA-A1* cells grow as loosely attached cell aggregates with a doubling time of 28-32 h; the MDA-MB-231 parent cell line grows as a standard monolayer culture with a 20-h doubling time. The MDA-A1* cell line is highly resistant to adriamycin compared to the parent cell line, and is cross-resistant to velban and colchicine suggestive of a multidrug resistance (MDR) phenotype. MDA-A1* cells exhibit reduced net adriamycin content as compared to the parent cell line. The MDR-associated P-glycoprotein gene is amplified approximately 10- to 30-fold in MDA-A1* cells. P-glycoprotein sequences are overexpressed in the resistant cells and are stable for up to 13 wk after drug removal. Moreover, MDA-A1* cells show the presence of very high levels of P-glycoprotein. MDA-A1* is thus an in vitro model system to study the mechanism of MDR in human breast cancer.

Key words: adriamycin; drug-resistance; breast tumor cells; p170; in vitro; growth kinetics.

INTRODUCTION

Adriamycin (ADM) is a cytotoxic anthracycline that binds to DNA and inhibits nucleic acid synthesis (29). ADM has been used successfully to produce regression in a variety of disseminated neoplastic diseases, including breast cancer (2,9,32). Patients treated with ADM often become cross-resistant to other antineoplastic agents as well, such as velban and colchicine (9). In vitro resistance to the above drugs is associated with the overexpression of the 170 kilodalton (kDa) surface membrane glycoprotein (P-glycoprotein), and amplification of the corresponding gene sequences (24,25).

Numerous reports describe selection of ADM-resistant sublines from a variety of human and other mammalian cell lines, which model pleiotropic cross-resistance to chemotherapeutic agents (7,12,23,34). A number of different mechanisms have been proposed to explain drug resistance, including reduced drug uptake or enhanced drug efflux due to alterations in membrane transport (8,10,35). A model has been proposed in which P-glycoprotein functions as an energy-dependent export pump that reduces intracellular levels of antineoplastic drugs (4,13,16).

A recent report from our laboratory indicated that ADM-resistant MDA-MB-231 breast tumor cells did not exhibit amplified P-glycoprotein gene sequences, but did overexpress the gene (12). To study the phenomena of ADM-resistance and to produce a human breast tumor cell subline with the classic MDR phenotype, we methodically isolated and followed an ADM-resistant subline of MDA-MB-231 (14). This paper describes the isolation and characterization of the ADM-resistant subline referred to as MDA-A1* according to classification criteria as previously reported (27).

MATERIALS AND METHODS

Cell Culture

The human breast tumor cell line MDA-MB-231 was obtained from the American Type Culture Collection (Rockville, MD). This cell line was originally derived from the pleural effusion of a breast adenocarcinoma (3). MDA-MB-231 cells were cultured as a continuous monolayer cell line at 37°C in 6% CO2:94% air in minimum Eagle's medium (MEM) supplemented with 10% heat-inactivated HyClone fetal bovine serum (Sterile Systems, Logan, UT), 6 ng/ml insulin, 2 mM L-glutamine, and 25 μg/ml Gentamycin sulfate (Schering Corporation, Kenilworth, NJ). Cultures were fed twice weekly and subcultured at confluence at a ratio of 1:10 using 1 mM EDTA-phosphate buffered saline (PBS). Cell lines were tested at various passages for Mycoplasma as previously reported (36).

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Drugs. Adriamycin (dox orbitin hydrochloride, Adria Laboratories Inc., Columbus, OH), velban (Eli Lilly, Indianapolis, IN), and colchicine (Sigma, St. Louis, MO) were rehydrated in sterile water at 1 mg/ml, aliquoted, and stored frozen at −70°C. Before use, drugs were resterilized by filtration through a 0.2 μm filter (Schleicher and Schuell, Keane, NH), maintained at 4°C, and protected from light.

**ADM-resistant cell selection.** ADM-resistant cells were initially selected from MDA-MB-231 cells cultured in a 25-cm² Corning culture flask with 10 ng/ml ADM for 4 wk. Cells selected by this procedure were slow growing, loosely attached clusters of cells. After the cells became conditioned to growth in drug-containing medium, as described above, ADM was added in step-wise doubling concentration. When cell growth was inhibited or abnormal cell forms were observed, ADM-containing medium was removed. The cells were then fed with drug-free medium until they reached confluence, split, and seeded using the last concentration of ADM that inhibited cell growth. This procedure of periodic drug-free growth combined with increasing concentrations of ADM was continued for 8 mo., after which time the drug-resistant cells were cultured continuously in 1 μg/ml ADM for 5 passages, and were assigned the MDA-A1R nomenclature and a passage number.

Optimum culture conditions for the MDA-A1R subline, as determined by growth, morphology, and P-glycoprotein overexpression, are as follows: cells are routinely split at a ratio of 1:2, seeded with ADM at 1 μg/ml in complete MEM, and then fed drug-free medium every 48 to 72 h until confluent. A 48 h ADM feeding was initially maintained, but this schedule interfered with cellular replication creating bizarre cell morphology usually ascribed to terminal differentiation (36). Therefore, ADM was administered only at the initial seeding of each passage. MDA-A1R has been cultured for over 34 passages in our laboratory under these conditions. Drug-resistant cells were passaged in drug and maintained in drug-free medium for 8 d before drug sensitivity assay or drug content studies unless noted.

**Growth kinetics and drug-resistance studies.** Growth kinetics and drug resistance were determined for both MDA-MB-231 and MDA-A1R cell lines by plating in 16-mm tissue culture wells (Costar, Cambridge, MA) at 5 × 10⁴ cells/well in 2 ml media at Day 0.

Growth kinetics were determined by calculating doubling time. Triplicate wells were harvested every 24 h for 5 d. Cells were fed fresh medium at each time point. Mean cell count and viability was determined by direct cell count in a hemaecytometer using trypan blue dye exclusion. Cell doubling time was determined using linear regression analysis of log-transformed cell counts. Growth curves were obtained by plotting mean cell counts per well against time using a semilogarithmic scale as previously reported (36).

Dose-response curves for resistance to ADM, velban, and colchicine were determined by adding increasing concentrations (0.001 to 100 μg/ml) of drug in fresh medium to cultures in triplicate 24-h after seeding in drug-free medium. The cells were cultured with drug for 48 h, harvested, and counted as above for mean cell count and viability. Drug resistance to ADM and cross-resistance to velban and colchicine were measured by determining the drug concentration inhibiting cell growth by 50% (IC₅₀) for each drug. IC₅₀ was obtained by plotting the logarithm of the drug concentration against viable cell count as percent of control. The resistance factor for MDA-A1R was determined as:

\[ \text{IC}_{50}(\text{MDA-A1R}) / \text{IC}_{50}(\text{MDA-MB-231}) \]

These assays were repeated several times at different passages for each cell line to confirm drug resistance stability.

**Adriamycin Content**

Adriamycin content for MDA-MB-231 and MDA-A1R cells was determined as previously described (26,34). Briefly, tubes containing 1 × 10⁶ cells/2 ml complete medium were incubated with either a range of ADM concentrations (0.5 to 10 μg/ml) for 1 h or 4 μg/ml ADM for 1 to 60 min at 37°C with agitation. The ADM-treated cell pellets were washed twice in complete medium, solubilized in 200 μl 0.1% sodium lauryl sulphate, treated

![Fig. 1. Morphology of MDA-MB-231 p320 (A) and MDA-A1R p12 (B) in culture. MDA-A1R cells were cultured in the presence of 1 μg/ml ADM. Phase contrast ×100.](image-url)