Characterization in Vitro and in Vivo of Progressively Adriamycin-resistant B16-BL6 Mouse Melanoma Cells

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ABSTRACT

Adriamycin (ADR)-resistant sublines of B16-BL6 mouse melanoma selected by exposure to increasing concentrations of ADR were characterized in vitro for growth properties and in vivo for tumorigenicity and pulmonary metastases. The progressively ADR-resistant sublines adapted to grow in the presence of 0.025, 0.05, 0.1, and 0.25 μg/ml ADR in monolayer culture were found to be 5-, 10-, 20-, and 40-fold ADR-resistant, respectively, compared to the parental sensitive cells, using a soft agar colony assay and continuous ADR treatment for 7 days. The doubling time in monolayer culture of the parent sensitive and progressively ADR-resistant sublines of B16-BL6 melanoma cells was approximately 16–18 h. Although the colony-forming efficiency in soft agar of parental sensitive cells was only 0.5–4%, the 5-, 10-, 20-, and 40-fold ADR-resistant sublines had colony-forming efficiencies of 15, 20, 30, and 77%, respectively. Tumorigenicity in C57BL/6 mice of progressively ADR-resistant sublines was similar to parental sensitive cells following s.c. and i.p. implantation of 10^6 tumor cells. Experimental pulmonary metastases were significantly lower in ADR-resistant sublines with progressive resistance. Additionally, unlike the parental sensitive and 5-fold ADR-resistant B16-BL6 cells, the 10-, 20-, and 40-fold ADR-resistant sublines were spontaneously nonmetastatic. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunobiochemical detection of P-glycoprotein revealed the presence of a M, 170,000 plasma membrane glycoprotein in the 40-fold ADR-resistant subline and its counterpart maintained for 1 year in ADR-free medium. Results from this study suggest that progressively ADR-resistant B16-BL6 mouse melanoma cells selected in vitro demonstrate a marked increase in colony formation in soft agar and a decrease in the ability to produce pulmonary metastases, without alterations in tumorigenicity.

INTRODUCTION

The successful treatment of human malignancies is often limited by the emergence of drug-resistant and/or metastatic tumor clones (1, 2). Although surgical techniques contribute to successful management of the primary tumor, the design of effective therapeutic modalities to combat drug resistant and/or metastatic disease continues to be a serious challenge. The selection of drug-resistant and/or metastatic cells is also compounded by the heterogeneous nature of tumor cell populations which contain subpopulations varying in their inherent sensitivity to cytotoxic chemotherapy (3). ADR, an anthracycline antibiotic, is widely used in the treatment of a number of human malignancies, and is probably one of the most effective agents for solid tumors (4, 5). In spite of its potent antitumor activity, the selection of ADR-resistant tumor cells is often encountered with repeated courses of therapy.

A number of studies have attempted to identify mechanisms of resistance to ADR, with the goal of designing therapeutic modalities to circumvent resistance. The ADR-resistant phenotype is also of considerable clinical importance, since ADR-resistant cells exhibit cross-resistance to other potent antitumor drugs which are distinctly different in structure and mechanism of action (6). Cells selected in vitro with the multidrug-resistant phenotype are also reported to be significantly altered in their tumorigenicity and growth characteristics in vitro (7, 8).

To understand the mechanism(s) of tumor cell resistance to ADR in a metastatic solid tumor model which could be utilized for studies in vitro and in vivo, we have utilized the B16-BL6 mouse melanoma tumor system (9). In an earlier report we have described the selection and cytogentic evaluation of progressively ADR-resistant B16-BL6 mouse melanoma cells (10). In this study we describe the growth characteristics in vitro, metastatic behavior (experimental and spontaneous pulmonary metastases), tumorigenicity in vivo, and the expression of the P-glycoprotein (11), a marker for the multidrug-resistant phenotype, in progressively ADR-resistant B16-BL6 tumor sublines.

MATERIALS AND METHODS

Cell Line and Culture Conditions. The in vitro cell line of B16-BL6 mouse melanoma was a gift from Dr. Isaiah J. Fidler, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD. Cells were maintained in vitro at 37°C in a humidified 5% CO2 plus 95% air atmosphere using E-MEM supplemented with nonessential amino acids, sodium pyruvate, vitamins, 2 mm L-glutamine, and 5% FBS. All media and supplements were obtained from M. A. Bioproducts, Walkersville, MD, and FBS was obtained from Hyclone Laboratories, Logan, UT. The cells were maintained in vitro as a monolayer culture using plastic tissue culture flasks and subcultured weekly using 0.25% trypsin-0.02% EDTA. The cell line was routinely examined and found free of Mycoplasma using the technique of Chen (12). The doubling time of the parent B16-BL6 cells in vitro under these conditions was approximately 16–18 h.

Selection of Progressively Adriamycin-resistant Sublines of B16-BL6 Mouse Melanoma in Vitro. Parent sensitive B16-BL6 cells as monolayer cultures in Eagle's minimum essential medium with Hanks' salts supplemented with nonessential amino acids, sodium pyruvate, vitamins, 2 mm L-glutamine, and 5% FBS were treated with increasing extracellular concentrations of 0.025–0.25 μg/ml ADR (10). B16-BL6 cells adapted to grow in the presence of 0.025, 0.05, 0.1, and 0.25 μg/ml ADR were maintained in ADR-free medium for at least 2 weeks prior to preparation of stock frozen cultures. Resistant sublines following selection were not maintained in the presence of ADR during subsequent experiments. The extracellular concentration of ADR during the selection process was increased to obtain cells at a higher level of resistance, when the doubling time and growth characteristics of the resistant cells treated with ADR in monolayer culture were comparable to the parent sensitive B16-BL6 cells. In general, the progressively ADR-resistant B16-BL6 cells were adapted to grow in the various concentrations of ADR outlined earlier for approximately 12–14 weeks.

Colony Forming Efficiency in Soft-Agar. The colony forming efficiency of parental and progressively ADR-resistant sublines of B16-BL6 mouse melanoma was determined using a soft-agar colony assay. Cells from log-phase cultures of parental and ADR-resistant sublines were trypsinized, resuspended in supplemented E-MEM with 5% FBS, and recovered by centrifugation (80 × g); 1 × 10^3, 5 × 10^3, 1 × 10^4, 2.5
RESULTS AND DISCUSSION

The doubling time in monolayer culture of the sensitive and progressively ADR-resistant sublines of B16-BL6 cells was approximately 16–18 h. A soft-agar colony assay using continuous exposure to various concentrations of ADR for 7 days was utilized to determine cytotoxic effects of ADR in the sensitive and progressively ADR-resistant sublines of B16-BL6 mouse melanoma cells. As shown in Fig. 1, the ADR-resistant sublines adapted to grow in monolayer culture in the presence of 0.025, 0.05, 0.1, and 0.25 μg/ml ADR were found to be approximately 5-, 10-, 20-, and 40-fold ADR resistant, respectively, compared to the parental sensitive cells. In the remainder of studies to be described, for convenience the sublines of B16-BL6 mouse melanoma will be referred to as 5-, 10-, 20-, and 40-fold ADR resistant.

The colony-forming efficiency in soft agar of the parental and ADR-resistant sublines of B16-BL6 mouse melanoma cells is shown in Fig. 2. The parental sensitive cells had a colony-forming efficiency which was between 0.5 and 4% over the range of cells plated. Although the ability of parental sensitive cells to form colonies was not dependent on the number of cells plated, we have observed that a maximal colony-forming efficiency of 20–25% can be achieved by five successive reclonings of the best-growing colonies. In the 5-, 10-, and 20-fold ADR-resistant sublines, a trend towards increased colony formation with higher number of cells plated was apparent, and at a cell density of 1 x 10⁶ cells the maximal colony-forming efficiency was 26–34%. In contrast to these results, the colony-forming efficiency of the 40-fold ADR-resistant subline was 62–82% and independent of the number of cells plated. Colony formation with cell densities >1 x 10⁴ cells for the 5-, 10-, and 20-

PROGRESSIVELY ADR-RESISTANT B16-BL6 MOUSE MELANOMA CELLS

× 10⁴, 5 × 10⁴, 1 × 10⁵, and 2.5 × 10⁵ viable cells/Petri dish (trypan blue dye excluding cells) in 1 ml supplemented E-MEM containing 20% FBS and 0.3% agar were plated over a 1-ml base layer of supplemented Eagle's MEM containing 20% FBS and 0.5% agar in 35- x 10-mm Petri dishes. Following incubation for 7 days in a humidified 5% CO₂ plus 95% air atmosphere, colonies (>50 cells) were counted in an Omnicon Feature Analysis System II (Bausch and Lomb, Rochester, NY). Colony-forming efficiency was expressed as a ratio of the number of colonies to the number of cells plated.

Adriamycin Sensitivity in Soft-Agar Colony Assay. The ADR sensitivities of parental and progressively ADR-resistant sublines of B16-BL6 mouse melanoma were determined using a soft-agar colony assay. Briefly, cells trypsinized from log-phase cultures of parental and ADR-resistant sublines were plated in the presence of 0.001-2.0 μg/ml ADR as described earlier for colony-forming efficiency studies. After incubation in a humidified 95% air plus 5% CO₂ atmosphere, colonies (>50 cells) in control and ADR-treated Petri dishes were counted on Day 7 using an Omnicon Feature Analysis System II (Bausch and Lomb).

Immunohistochemical Detection of P-Glycoprotein. CH*C5, a colchicine-resistant Chinese hamster ovary cell line from drug-sensitive parental line AUXB1 as described previously by Ling and Thompson (13) was used as a reference standard. Cell suspensions from frozen stocks of CH*C5, AUXB1, parental B16-BL6, 40-fold ADR-resistant B16-BL6 subline, and 40-fold ADR-resistant B16-BL6 subline maintained in ADR-free medium for 1 year were rapidly thawed at 37°C and placed on ice. All subsequent steps were carried out at 4°C. Cells were washed three times with PBS. Washed cells at a concentration of 5 × 10⁷/ml in PBS were ruptured in a Stannsted Cell Disruptor, Model A0512, with a No. 716 disrupting valve. Disrupting pressures were chosen to rupture about 80% of the cells. Pressures yielding this level of breakage were found to be 400 psi for parental B16-BL6 and 300 psi for Adriamycin-resistant B16-BL6. AUXB1 and CH*C5 were disrupted at 350 and 300 psi, respectively. Following passage through the pump the following differential centrifugation steps were applied: nuclear spin, 300 x g for 10 min; mitochondrial spin, 4,000 x g for 10 min; microsomal spin, 35,000 x g for 30 min. The plasma membrane-enriched, microsomal pellet was resuspended in 5 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose and frozen at −70°C after samples were taken for protein determination. Protein concentrations were determined using a modification of the procedure of Lowry et al. (15).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using a modification of the procedure of Fairbanks et al. (16) as described previously (17). Protein profiles on sodium dodecyl sulfate-polyacrylamide gels were replica electroblotted (Western blotted) onto nitrocellulose filter paper, essentially as described by Towbin et al. (18). Following transfer, filters were incubated overnight at 37°C with 3% (w/v) bovine serum albumin in Tris-buffered saline to block free protein binding sites. The filter was overlaid with 1:21-labeled murine monoclonal antibody (C219) against P-glycoprotein (19). After overnight incubation at 4°C, the filter was washed five times for 10 min with 100 ml PBS, dried, and placed on X-ray film with intensifying screens at −70°C.

Tumorigenicity and Metastasis Formation. Log phase cultures of parental sensitive and ADR-resistant sublines of B16-BL6 mouse melanoma were trypsinized, resuspended in supplemented E-MEM with 5% FBS, and centrifuged (80 x g). Cells were washed twice at 4°C in 0.9% sterile sodium chloride solution and recovered by centrifugation (80 x g). Cells with >95% viability based on trypan blue dye exclusion were used in all in vivo experiments. For tumorigenicity studies 1 x 10⁶ and 1 x 10⁵ viable cells from parental or ADR-resistant sublines of B16-BL6 melanoma were injected i.p. into 6- to 8-week-old C57BL/6 mice in groups of six matched for age, weight, and sex. Mortality of mice was followed on a daily basis and the median day of death determined. The ability of cells from parental or ADR-resistant sublines of B16-BL6 melanoma to form experimental lung metastases following i.v. injection was determined by injecting (0.1 ml) 0.75 × 10⁴, 1.5 × 10⁴ and 3 × 10⁴ viable cells as a single cell suspension in 0.9% sodium chloride solution into the tail vein of unanesthetized, 6- to 8-week-old C57BL/6 mice in groups of six matched for age, weight, and sex. Mice were killed 21 days later, and the number of pulmonary metastases in both lungs was counted under a stereo dissecting microscope. Each experiment was replicated at least twice and the median number of metastases determined.

The ability of cells from parental or ADR-resistant sublines of B16-BL6 melanoma to form spontaneous lung metastases was determined by giving C57BL/6 mice, in groups of six matched for age, weight, and sex, injections (0.05 ml) s.c. of 1 x 10⁵ viable cells in 0.9% sodium chloride solution in the footpad of the hind leg. The "primary" tumor was amputated at the level of the knee joint when the tumor weight was approximately 325 mg. Mice were subsequently killed 21 days after amputation of the tumor-bearing leg, and the number of pulmonary metastases was counted under a stereoscopic dissecting microscope. Mice were obtained from the Animal Genetics and Production Branch, Frederick Cancer Research Facility, National Cancer Institute, Fort Detrick, MD.

![Fig. 1. Survival in soft agar of sensitive and progressively Adriamycin-resistant B16-BL6 mouse melanoma cells treated continuously for 7 days with Adriamycin. Bars, SE; W/O, without.](image-url)
amplification of DNA sequences homologous to P-glycoprotein in the 40-fold resistant subline and its counterpart maintained in drug-free medium but not in the parent sensitive cells (data not presented).

The tumorigenicity of parental sensitive and ADR-resistant sublines of B16-BL6 mouse melanoma cells is outlined in Table 1. It is apparent that the sensitive cells and ADR-resistant sublines were indeed tumorigenic and resulted in 100% mortal-

![Fig. 2. Colony-forming efficiency in soft agar of sensitive and progressively Adriamycin-resistant B16-BL6 mouse melanoma cells. 1 x 10^3, 5 x 10^3, 2 x 10^4, 5 x 10^4, 2 x 10^5, and 2.5 x 10^5 cells were plated and colonies (>50 cells) counted as described in "Materials and Methods." Data for 40-fold Adriamycin-resistant cells cultured without (W/O) Adriamycin for 1 year is from a representative experiment. Bars, SE.](image)

![Fig. 3. Immunochemical detection of P-glycoprotein (p) in Adriamycin-resistant B16-BL6 melanoma. Lane a, CH₃C5; lane b, AUXBI; lane c, parental sensitive B16-BL6; lane d, 40-fold Adriamycin-resistant B16-BL6; lane e, 40-fold Adriamycin-resistant B16-BL6 maintained in ADR-free medium for 1 year.](image)

![Fig. 4. Experimental pulmonary metastases formation by sensitive and progressively Adriamycin-resistant B16-BL6 mouse melanoma. Data on median number of metastases are from at least duplicate experiments. Analysis of the data was carried out using the Wilcoxon rank sum test with Bonferroni correction for multiple pairwise testing. P < 0.005 was the boundary for statistical significance. a, significantly different from sensitive, P < 0.005; b, significantly different from 5-fold Adriamycin-resistant subline, P < 0.005; c, significantly different from 10-fold Adriamycin-resistant subline, P < 0.005; d, significantly different from 20-fold Adriamycin-resistant subline, P < 0.005.](image)

Table 1 Tumorigenicity of parent sensitive and progressively Adriamycin-resistant B16-BL6 cells injected i.p. in C57BL/6 mice

<table>
<thead>
<tr>
<th>Cell inoculum/mouse</th>
<th>1 x 10^3</th>
<th>1 x 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent sensitive</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>5-Fold ADR-resistant</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>10-Fold ADR-resistant</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>20-Fold ADR-resistant</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>40-Fold ADR-resistant</td>
<td>25</td>
<td>19</td>
</tr>
</tbody>
</table>

* Data from a representative experiment with at least 6 mice/group; median day of death.

Table 2 Spontaneous pulmonary metastases formation in C57BL/6 mice

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Median no. of pulmonary metastases (range)</th>
<th>No. of animals with metastases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent sensitive</td>
<td>53 (0–500)</td>
<td>28/33 (85)*</td>
</tr>
<tr>
<td>5-Fold ADR-resistant</td>
<td>2 (0–13)</td>
<td>11/16 (69)</td>
</tr>
<tr>
<td>10-Fold ADR-resistant</td>
<td>0 (0–8)</td>
<td>4/14 (29)</td>
</tr>
<tr>
<td>20-Fold ADR-resistant</td>
<td>0 (0–2)</td>
<td>5/10 (50)</td>
</tr>
<tr>
<td>40-Fold ADR-resistant</td>
<td>0 (0–5)</td>
<td>2/8 (25)</td>
</tr>
</tbody>
</table>

* Pooled data from at least duplicate experiments with 6 mice/group.
Giavazzi et al. (21) do not demonstrate the emergence of a non-tumorigenic phenotype. The reason for altered colony formation in soft agar but not growth characteristics in monolayer culture of the progressively ADR-resistant sublines is presently unknown. Based on the recently described association between epidermal growth factor receptor expression with ADR treatment (22) and multidrug resistance (23), we are currently characterizing the expression of epidermal growth factor receptors in these progressively ADR-resistant sublines of B16-BL6 melanoma cells. The 40-fold ADR-resistant cells maintained in ADR-free medium for a year were similar to the 40-fold ADR-resistant subline maintained in ADR-free medium for <4 weeks with respect to characteristics of colony formation in soft agar (Fig. 2), experimental, and spontaneous lung metastases and tumorigenicity (results not shown). However, some decrease in ADR sensitivity of the cells maintained in the absence of selection pressure (Fig. 1) seemed to be associated with reduced expression of P-glycoprotein (Fig. 3).

In summary, results from the present study demonstrate that progressively Adriamycin-resistant B16-BL6 melanoma cells demonstrate alterations in growth properties in vitro based on colony formation in soft agar, and changes in metastatic characteristics but not tumorigenicity in vivo. P-glycoprotein, a marker for the multidrug-resistant phenotype, was expressed in the resistant subline even in the absence of selection pressure for prolonged periods, demonstrating the stability of resistant phenotype. The metastatic and tumorigenic features of the progressively Adriamycin-resistant B16-BL6 cells should be useful for studies in vitro and in vivo characterizing mechanism(s) of ADR resistance as well as evaluation of calmodulin inhibitors or calcium channel blockers as potential agents for modulating the multidrug-resistant phenotype.

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