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 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 10⁵–10⁶ 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 immunochemical detection of P-glycoprotein revealed the presence of a M₉ 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 vivo (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 cytogenetic 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 minimal 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 × 10⁵, 5 × 10⁵, 1 × 10⁶, 2.5
Omnicon Feature Analysis System II (Bausch and Lomb, Rochester, CO2 plus 95% air atmosphere, colonies (>50 cells) were counted in an of colonies to the number of cells plated. 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 sivity of parental and progressively ADR-resistant sublines of B16-BL6 mouse melanoma was 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 parent 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 x 10^7/ml in PBS were ruptured in a Stansted 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; mitochondrial 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 μM sucrose and frozen at -70°C after samples were taken for protein determination. Protein concentrations were determined using a modification (14) 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 10/-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 10^3 and 1 x 10^4 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 x 10^3, 1.5 x 10^6 and 3 x 10^7 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 10^5 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.

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 softagar 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 10^4 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 10^4 cells for the 5-, 10-, and 20-
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-

<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>

* Data from a least duplicate experiments with 6 mice/group; median day of death.

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.

Table 2 Spontaneous pulmonary metastases formation in C57BL/6 mice

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ity of the mice. Autopsy of mice revealed extensive tumor in the viscera and no metastases in the lung. The median day of death of the sublines >10-fold ADR resistant were higher than the parental sensitive cells, and it is unclear at the present time if this is possibly due to altered proliferation kinetics in vivo, since doubling times in vitro were identical.

The ability of the parental sensitive and ADR-resistant sublines to form experimental pulmonary metastases following i.v. injection of cells is presented in Fig. 4. At a density of 3 x 10⁵ cells, lungs from mice given injections of parental sensitive cells were confluent with tumor metastases. However, in mice given injections of 3 x 10⁶ cells from progressively ADR-resistant sublines there was a decrease in pulmonary metastases with increasing ADR resistance, and with the 40-fold resistant line it was nearly 10-fold lower. Although the number of pulmonary metastases decreased with lower cell inoculum (3 x 10⁵ > 1.5 x 10⁴ > 0.75 x 10³), the ADR-resistant sublines consistently demonstrated remarkably lower numbers of metastatic foci compared to the parental cells. Overall, it appears that the progressive ADR-resistant sublines of B16-BL6 melanoma are characterized by a markedly impaired ability to form experimental lung metastases.

In Table 2 is outlined the data on spontaneous pulmonary metastases with the parental sensitive and ADR-resistant sublines. The parental cells reproducibly produced spontaneous lung metastases in at least 85% of the mice. However, the number of mice with spontaneous lung metastases decreased to as little as 25% with progressive ADR resistance. Similar to the results on experimental metastases (Fig. 4), there was a decrease in spontaneous metastases with increasing ADR resistance, and sublines >5-fold ADR resistant were spontaneously nonmetastatic, based on the median number of pulmonary metastases in replicate experiments.

The availability of tumor models with metastatic and multidrug-resistant phenotypic characteristics could be a valuable aid for the evaluation of novel chemotherapeutic strategies. The 40-fold ADR-resistant subline of B16-BL6 mouse melanoma as previously described is characterized by a multidrug-resistant phenotype (10) based on (a) a 25-fold cross-resistance to vincristine and vinblastine following 12 h of drug treatment, (b) 10-fold cross-resistance to actinomycin-D and 5-fold cross-resistance to N-trifluoracetyladriamycin-14-valerate (AD32), a DNA nonbinding analogue of ADR, following 3-h drug treatment, and (c) lack of cross-resistance to 1β-D-arabinofuranosylcytosine following 3-h drug exposure. Results from this study suggest that although ADR resistance is fairly stable even in the absence of selection pressure, there is a significant alteration of the metastatic phenotype with progressive ADR resistance. At the present time it is not clear if the diminished metastatic characteristics are due to the selection of ADR-resistant cells in vitro by continuous exposure to ADR, or related to membrane alterations characteristic of ADR-resistant cells. A decrease in the formation of experimental pulmonary metastases with a 140-fold ADR-resistant subline of murine fibrosarcoma UV-2237M-ADM⁸ selected in vitro has been observed by Giavazzi et al. (20, 21), but these resistant cells, however, were reported to be spontaneously metastatic.

In spite of the altered metastatic behavior of progressively ADR-resistant B16-BL6 sublines, there was no loss in tumorigenicity (Table 1). The decrease and/or loss in tumorigenicity reported with multidrug-resistant Chinese hamster lung DC-3F cells (7, 8), may be a characteristic of this particular cell type, or the degree of resistance, since our results and those of Giavazzi et al. (21) do not demonstrate the emergence of a nontumorigenic 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 characteristic 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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Characterization in Vitro and in Vivo of Progressively Adriamycin-resistant B16-BL6 Mouse Melanoma Cells

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