Characterization of a New Drug-resistant Human Myeloma Cell Line That Expresses P-Glycoprotein

William S. Dalton, Brian G. M. Durie, David S. Alberts, James H. Gerlach, and Anne E. Cress

Departments of Medicine [W. S. D., B. G. M. D., D. S. A.] and Radiation Oncology [A. E. C.J., Cancer Center Division, The University of Arizona, Tucson, Arizona 85724, and The Ontario Cancer Institute, Department of Medical Biophysics [J. H. G.] University of Toronto, Toronto, Ontario, M4X 1K9, Canada

ABSTRACT

Multiple myeloma is a disease with a high initial chemotherapeutic response but virtually no cures due to emergence of drug resistance. A doxorubicin-resistant human myeloma cell line (8226/Dox) has been selected from the myeloma cell line RPMI 8226 by continuously exposing cells to gradually increasing doses of doxorubicin. The resistant phenotype has been retained for over 2 months despite growth in drug-free medium. The resistant subline was cross-resistant to mitoxantrone, acracycline, etoposide, and vincristine. The 8226/Dox cell line remained sensitive to melphalan but acquired collateral sensitivity to dexamethasone. Intracellular doxorubicin accumulation, as measured by [3H]doxorubicin and high-performance liquid chromatography, was decreased by 54% at 1 hr for 8226/Dox compared to the sensitive line. Efflux of doxorubicin was significantly greater in the resistant subline as compared to the sensitive parent cell line. Membrane analysis using immunoblotting techniques detected increased expression of the integral membrane protein P-glycoprotein (Mr, 170,000) in the resistant subline. Cytogenetic analysis of 8226/Dox revealed a 7q-anomaly not seen in the sensitive (8226/S) cells.

INTRODUCTION

Multiple myeloma is a tumor in which the majority of patients have an objective response after induction therapy (1, 2). Unfortunately, all patients eventually relapse and become refractory to chemotherapy despite this initial response (3, 4). In addition to developing resistance to previously administered drugs, myeloma cells also become refractory to drugs which differ in chemical structure and mechanism of action. This has also been observed with in vitro drug testing (5, 6). A similar type of multiple-drug resistance has been observed in experimental tumor models using various mammalian cell lines and has been given the term pleiotropic drug resistance (7–9).

The anthracycline antibiotic DOX3 is an effective chemotherapeutic drug in myeloma refractory to alkylating agents and is frequently used as a part of combination chemotherapy (10–12). However, myeloma patients treated with DOX eventually relapse and become resistant to multiple drugs (6). Variant mammalian cell lines, which have been selected in vitro for resistance to the anthracyclines, frequently develop cross-resistance to vincristine alkalooids as well as other unrelated compounds (13–16). The exact mechanism of resistance is unknown; however, variant sublines appear to accumulate less drug in association with cell membrane changes (17–19). Whether this same phenomenon occurs in human tumors and/or is clinically relevant remains to be determined. In this study, we describe the characteristics of a DOX-resistant human myeloma cell line derived from the well-established human myeloma cell line RPMI 8226 (20).

MATERIALS AND METHODS

Cell and Culture Conditions. The RPMI 8226 human myeloma cell line was obtained from the American Type Culture Collection (Rockville, MD). It was originally derived from the peripheral blood of a 61-year-old male with multiple myeloma (20). Cells were grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% (v/v) penicillin (100 units/ml), streptomycin (100 μg/ml), and 1% (v/v) l-glutamine (all from Grand Island Biological Co.). Cells were maintained at 37°C in 10% CO2-90% air atmosphere and subcultured every 6 days.

Drugs. DOX and 4-demethoxydaunorubicin were obtained from Adria Laboratories (Columbus, OH). [14C]DOX (21.0 μCi/mg) was obtained from SRI International (Menlo Park, CA). Purity of [14C]DOX was >95% by HPLC and thin-layer chromatography. Mitoxantrone was obtained from the Medical Research Division, American Cyanamid Co. (Pearl River, NY); melphalan from Burroughs Wellcome Co. (Research Triangle Park, NC); vincristine and acracycline from Lilly Laboratories (Indianapolis, IN); and dexamethasone sodium phosphate from Lypho-Med, Inc. (Chicago, IL).

Selection of DOX-resistant Cells. RPMI 8226 cells were initially exposed to DOX at a concentration of 1.0 x 10⁻⁸ M, which represented the approximate IC₅₀ dose. Drug was added when the medium was changed (approximately three times weekly). The DOX concentration was increased gradually over 10 months to a final concentration of 1 x 10⁻⁷ M. Cells were maintained in drug-free medium for 1 week prior to drug sensitivity assays or drug accumulation studies.

In Vitro Drug Assays. Drug sensitivity testing was performed using a two-layer, soft-agar culture system (21, 22). Drug exposure was either for 1 hr prior to plating or continuously by incorporating the agent into the agar. Cells were plated in triplicate at a concentration of 2.0 x 10⁴ cells/35-mm tissue culture dish (Falcon Plastics, Division of Becton Dickinson and Co., Oxnard, CA). Tumor cell colonies were evaluated using inverted microscopy 10–14 days after plating. Percentage of survival was based upon the ratio of the plating efficiency of treated to control cells. The IC₅₀ for each particular drug was defined as the concentration of the drug which reduces colony formation to 50% of untreated control cells. A relative resistance index was expressed as the ratio of the IC₅₀ of the resistant (8226/Dox) cells to the IC₅₀ of the sensitive (8226S) cells.

Tritiated Thymidine-labeling Index Studies. Labeling index studies were performed on the sensitive and resistant cell lines. Cell suspensions containing 1.0 x 10⁶ cells/ml of suspension were incubated for 1 hr at 37°C with high specific activity (40–60 Ci/mmol) tritiated thymidine (5.0 μCi/ml of cell suspension). Cytocentrifuge smears were then prepared and fixed with methanol. The tritiated thymidine-labeling index was then determined using a high-speed scintillation autoradiography method (23).

Cytotoxicity assays were performed on a population of multiple myeloma (24, 25). The cellular level of these lysosomal enzymes has been directly correlated with disease activity in multiple myeloma (24, 25).
Cytogenetic Studies. Following a 1-h incubation with Colcemid (GIBCO, Lawrence, MS) (10 μg/ml), 8226/S and 8226/Dox cells were harvested and banded as described previously (26). G-Banded karyotypes of the sensitive and resistant strains from a similar passage number were used for comparison.

Immunoblot Analysis for P-Glycoprotein. Plasma membranes from 2 × 10^6 cells were purified according to the method of Riodran and Ling (27). Polyacrylamide gel electrophoresis was performed according to the method of Fairbanks et al. (28) with slight modifications as described previously (29). The method of Towbin et al. (30) was used to transfer protein profiles from sodium dodecyl sulfate-polyacrylamide gels onto nitrocellulose filter paper. This was then probed with a 125I-labeled monoclonal antibody, C219, as described previously (31). Membrane preparations from the multidrug-resistant human lymphoid cell line, CEM/VLB100, and its drug-sensitive parent cell line, CCRF-CEM, were used as controls for P-glycoprotein expression (cell lines were obtained from W. T. Beck, St. Jude's Children's Research Hospital, Memphis, TN). For the 8226/Dox and 8226/S preparations, 40 μg of membrane protein were loaded in the application well, while 20 μg of membrane protein were applied for CEM/VLB100 and CCRF-CEM.

Drug Accumulation and Efflux Studies. To determine cellular accumulation of [3H]DOX in 8226/S and 8226/Dox, cells were studied in the exponential growth phase. Cells were washed free of medium and resuspended at a cell concentration of 2.0 × 10^6 cells/ml in RPMI 1640 medium containing 2% fetal calf serum and 1% l-glutamine. [3H]DOX was added to each tube of cells at a concentration of 0.5 μM, and cells were incubated as a suspension in tubes submerged in a water bath at 37°C. At specific time points following the addition of [3H]DOX, the cells were pelleted and washed twice in cold PBS. After the PBS wash, 1 × 10^6 cells were layered on silicone oil (SF 1250; General Electric Co., Inc., Waterford, NY) in microcentrifuge tubes according to the method of Vistica et al. (32).

To compare the efflux of [3H]DOX from 8226/S and 8226/Dox cells, cells were incubated with 0.5 μM [3H]DOX in a manner similar to that described above. Following this incubation period, the cells were washed once in PBS and then resuspended in drug-free medium and placed in a 37°C water bath. At intervals, aliquots of cells (1 × 10^6) were centrifuged through silicone oil and radioactivity determined.

DOX Metabolism Studies. The amount of intracellular DOX present as parent compound was compared between 8226/S and 8226/Dox by using reversed-phase HPLC. Cells (2 × 10^6) were incubated with DOX (0.5 μM) in RPMI complete medium at 37°C for 1 h. At the end of the incubation period, the cells were rinsed twice with cold medium and once with cold PBS. The pelleted cells were resuspended in 1 ml of double-distilled water and sonicated for 10 s. The lysed cells were prepared for HPLC analysis according to the method of Peng et al. (33). Daunorubicin was added to each sample as an internal standard prior to analysis by HPLC.

The HPLC apparatus consisted of a Waters Associates Model 6000A solvent delivery system, and a Waters Associates C18-Bondapak reversed-phase column. Compounds were eluted isocratically at ambient temperature with acetonitrile:0.2 M ammonium acetate, pH 4.0 (25:75) as solvent at a flow rate of 2.0 ml/min. Fluorescent measurements of DOX and daunorubicin were made with a Perkin-Elmer LS-1 fluorescence detector (excitation, 480 nm; emission, 550 nm).

RESULTS

Selection of DOX-resistant Clones. DOX-resistant 8226 myeloma cells were selected by continuously exposing cells to gradually increasing concentrations of DOX. Despite the development of resistance, the 8226/Dox subline retained several biological characteristics of the sensitive parent line (8226/S), as illustrated in Table 1. All cells of both lines had a plasma cell morphology and expressed the plasma cell-associated antigen, PCA-1, that is characteristic of mature plasma cells (34). The plating efficiency in soft agar of both cell types was similar; however, the labeling index of the resistant subline was consistently less than that of the parent cell line. Two standard cytochemical studies for myeloma cells revealed that the acid phosphatase scores were similar but that the β-glucuronidase score was consistently higher for the 8226/Dox subline.

The survival curves for the 8226/Dox and 8226/S in the presence of varying concentrations of DOX are shown in Figs. 1 and 2. Comparing the respective IC50 concentrations, a 10-fold resistance to DOX was observed in the 8226/Dox subline when cells were exposed to DOX for 1 h, whereas the level of resistance was 17-fold when cells were continuously exposed to drug in the two-layer, soft agar culture system. In addition, this degree of resistance was observed to be stable for over 2 months (approximately 10 passages) following the removal of DOX from the growth media (data not shown).

Cross-Resistance Studies. As shown in Table 2, 8226/Dox demonstrated cross-resistance to a number of other drugs that are both similar and dissimilar in structure and mechanism of action. Cross-resistance was observed for 4-demethoxydaunorubicin, mitoxantrone, acracynine, vincristine, and etoposide. Unlike DOX, when cells were continuously exposed to the

Table 1 Comparison of biological characteristics of 8226/S and 8226/Dox

<table>
<thead>
<tr>
<th>Biological characteristics</th>
<th>8226/S</th>
<th>8226/Dox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>100% plasma cells, PCA-1 positive</td>
<td>100% plasma cells, PCA-1 positive</td>
</tr>
<tr>
<td>Plating efficiency (%)</td>
<td>5.18 ± 0.85</td>
<td>4.82 ± 0.81</td>
</tr>
<tr>
<td>Labeling index (%)</td>
<td>58 ± 4.9</td>
<td>38.3 ± 5.1</td>
</tr>
<tr>
<td>Doubling time (h)</td>
<td>26.7 (12-43)</td>
<td>28.9 (19-39)</td>
</tr>
<tr>
<td>β-Glucuronidase score</td>
<td>142 ± 9.9</td>
<td>204 ± 13.5</td>
</tr>
<tr>
<td>Acid phosphatase score</td>
<td>298 ± 1.4</td>
<td>300 ± 1.0</td>
</tr>
</tbody>
</table>

*PCA, plasma-cell-associated antigen.  
Mean ±SD of at least 3 separate experiments.  
95% confidence limits.
**Vinca** alkaloid, vincristine, as opposed to 1-h exposure, the level of resistance decreased markedly from a level of 197-fold to a level of 10-fold. The resistant and sensitive 8226 cells were equally sensitive to the alkylating agent melphalan; however, collateral sensitivity was observed for the corticosteroid dexamethasone, with the 8226/Dox subline being approximately 10-fold more sensitive.

**Intracellular Accumulation and Efflux Studies.** In order to characterize the nature of the resistance, the time course of intracellular [14C]DOX accumulation was measured in the sensitive and resistant sublines of 8226. Figure 3 demonstrates that the net [14C]DOX accumulation was approximately 2-fold less than that observed for the sensitive parent line 8226/S. At 60 min, the 8226/Dox cells had accumulated 54% (range 42-65%) less [14C]DOX than the 8226/S cells at the same time point.

Efflux of [14C]DOX from cells was measured to determine if the ability to retain drug had a significant influence on drug accumulation. Figure 4 compares [14C]DOX retention at various time points for sensitive and resistant cells. The amount of intracellular DOX in sensitive and resistant cells is expressed as the percentage remaining after incubating the cells with 0.5 μM [14C]DOX for 1 h, washing the cells in PBS, and resuspending in drug-free medium at 37°C. After 60 min, the 8226/S cells retain 92% of the [14C]DOX, whereas the 8226/Dox cells retain only 44% of the original [14C]DOX.

**Table 2 Cross-resistance patterns of the 8226/S and 8226/Dox myeloma cell lines to various antitumor agents**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Exposure</th>
<th>IC50 (×10^4 μM) 8226/S</th>
<th>IC50 (×10^4 μM) 8226/Dox</th>
<th>Degree of cross-resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>1 h</td>
<td>0.105</td>
<td>0.110</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>Continuous</td>
<td>0.004</td>
<td>0.108</td>
<td>17.0</td>
</tr>
<tr>
<td>4-Demethoxy- daunorubicin</td>
<td>1 h</td>
<td>0.014</td>
<td>0.110</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>Continuous</td>
<td>0.001</td>
<td>0.108</td>
<td>10.5</td>
</tr>
<tr>
<td>Mitoxantrone</td>
<td>1 h</td>
<td>0.019</td>
<td>0.068</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>0.113</td>
<td>0.77</td>
<td>68.5</td>
</tr>
<tr>
<td>Vincristine</td>
<td>1 h</td>
<td>0.075</td>
<td>1.47</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>Continuous</td>
<td>0.029</td>
<td>0.28</td>
<td>9.6</td>
</tr>
<tr>
<td>Etoposide</td>
<td>1 h</td>
<td>0.51</td>
<td>2.55</td>
<td>5.0</td>
</tr>
<tr>
<td>Melphalan</td>
<td>1 h</td>
<td>1.7</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Continuous</td>
<td>2.55</td>
<td>0.20</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* Determined by dividing the IC50 for the 8226/Dox subline by the IC50 for the parent 8226/S cell line.

DISCUSSION

In this study we have examined the biological characteristics and mechanisms of resistance for a stable variant of the human myeloma cell line RPMI 8226, which is resistant to the antityrocidline DOX. Routine light microscopy and cytochemical stains demonstrated that both the parental cell line and the drug-resistant cell line retained their mature plasma cell appearance with serial passaging; however, the resistant subline contained elevated levels of the enzyme β-glucuronidase. Although increased levels of this enzyme have been associated with active multiple myeloma (25), the significance of this increase in the resistant subline is unknown at this time.

The multidrug-resistant pattern observed for the myeloma variant line is similar in many respects to that seen for a number of other animal and human resistant sublines (17, 19). That is, although the resistant myeloma cell line was selected for its resistance to DOX, it exhibits a cross-resistance to a wide range of compounds. Variable cross-resistance was seen for both structurally related drugs, such as 4'-demethoxydaunorubicin, mitoxantrone, and acrornyline, as well as structurally unrelated
compounds such as vincristine and etoposide. The sensitive and resistant 8226 cells were equally sensitive to the alkylating agent melphalan; non-cross-resistance to alkylating agents has been observed in other cell lines selected for resistance to DOX (18). In addition, enhanced sensitivity to hormones has been observed for multidrug-resistant cell lines (35), as was the case with the 8226/Dox subline being 10-fold more sensitive to the corticosteroid dexamethasone.

It has been demonstrated in a number of cell lines selected for drug resistance that intracellular drug accumulation is less than that attained by the parent cell line (17–19). In the studies described here, differences in drug accumulation between the sensitive and resistant myeloma cell lines were observed as early as 1 min following drug incubation. The efflux of DOX was considerably greater in the 8226/Dox line as compared to the 8226/S line; however, this difference did not become obvious until after 30 min. Thus, the difference in DOX accumulation observed for the sensitive and resistant lines can be explained, at least in part, by enhanced efflux of drug in the resistant subline. Differences in initial drug uptake may also play a role but cannot be determined by measuring net intracellular DOX accumulation as performed in these studies. Other factors such as intracellular redistribution of drug may also explain differences in cytotoxic potential; studies are ongoing to investigate this possibility.

Because alterations in transport of DOX appears to account, at least in part, for the resistance of the 8226/Dox line, it is logical to examine the plasma membrane for changes in structure that might be associated with membrane permeability. The most frequent and dramatic membrane alteration in multidrug-resistant cell lines is the expression of a high molecular weight glycoprotein, termed P-glycoprotein (36). This glycoprotein was initially detected in Chinese hamster ovary cells resistant to colchicine but has more recently been described in a human CEM-leukemic lymphoblast line resistant to vinblastine (37) and in fresh human ovarian carcinoma cells obtained from patients who are refractory to chemotherapy (38). DNA transfection studies using colchicine-resistant Chinese hamster ovary cell lines have established a strong correlation between multidrug resistance and the expression of P-glycoprotein (29). Using an iodinated monoclonal antibody to probe for P-glycoprotein, we have observed an overexpression of this glycoprotein in the myeloma cells resistant to DOX. The way in which this protein might function in mediating resistance remains to be determined; however, it is likely that it alters membrane permeability and drug transport. Studies are currently ongoing to determine if this glycoprotein is overexpressed in myeloma cells derived from patients who are unresponsive to therapy. Use of 8226/Dox as a predictive model may allow for the development of protocols to circumvent drug resistance known to occur clinically in multiple myeloma (5, 6).
Karyotypic analyses and analysis at the DNA level have indicated that the overexpression of P-glycoprotein is frequently associated with gene amplification (39). No karyotypic evidence of gene amplification has been observed in the 8226/Dox line compared to the 8226/S line. A recurrent observation in the 8226/Dox subline has been the presence of a 7q anomaly that has not been observed in the 8226/S parent cell line. This anomaly has been observed in myeloma cells from patients who have been clinically refractory to chemotherapy. Recently, studies using a DNA probe encoding of a conserved portion of P-glycoprotein cloned from hamster cells have localized human P-glycoprotein gene sequences to the distal long arm of chromosome 7 (40). It is possible, therefore, that the alteration of chromosome 7 noted in the resistant subline may lead to the increased expression of P-glycoprotein and subsequent drug resistance.

Multiple myeloma is a tumor with a high initial chemotherapeutic response rate but virtually no cures due to the development of multiple drug resistance. Twenty years ago, Salmon et al. demonstrated that intermittent high-dose prednisone was effective therapy in patients who had become refractory to standard chemotherapy (41). More recently, Barlogie et al. have reported on a chemotherapeutic regimen that utilizes high-dose dexamethasone combined with a continuous infusion of vincristine and doxorubicin (12). This regimen was reasonably active in patients resistant to more standard regimens containing alkylating agents and doxorubicin. It is of interest that the doxorubicin-resistant myeloma cell line described here is, in fact, more sensitive to dexamethasone than is the sensitive cell line and that, when vincristine is continuously exposed to cells, the degree of relative resistance is markedly decreased. The drug sensitivity profile observed for the resistant myeloma line may make it a valuable tool for discovering drugs that have collateral sensitivity in resistant disease. We believe that these clinical correlations indicate the potential of this cell line as a valuable model for the study of drug resistance in multiple myeloma.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Victor Ling for helpful discussion and for conducting part of this work in his laboratory. We also thank Valerie Baum, Yvette Frutiger, Elizabeth Vela, Janine Einspahr, Deanna Everenden-Porelle, and Tom McCloskey for their excellent technical assistance and Charlene Sass for preparation of the manuscript.

REFERENCES

Characterization of a New Drug-resistant Human Myeloma Cell Line That Expresses P-Glycoprotein


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/10/5125

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.