Immunohistochemical Detection and Quantitation of P-Glycoprotein in Multiple Drug-Resistant Human Myeloma Cells: Association With Level of Drug Resistance and Drug Accumulation

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Using several multiple drug-resistant human myeloma cell lines as standards, we developed an immunohistochemical staining technique and means of quantitating P-glycoprotein in individual myeloma cells. The level of staining intensity for P-glycoprotein in individual myeloma cells was quantitated by measuring the average optical density of each cell with a microscopic computerized cell analysis system. Using this system, we observed that the level of P-glycoprotein for individual cells within a cell population of known drug sensitivity was very homogeneous (coefficient of variation ≤ 13%). Analysis of cell lines with gradually increasing levels of multidrug resistance (8226/S, 8226/Dox, and 8226/DoxR) demonstrated a close association between the level of resistance to doxorubicin, defined by the mean lethal dose (DL) and the amount of P-glycoprotein on individual cells determined by the optical density (r = 0.82, P < 0.0005). Intracellular doxorubicin (DOX) accumulation in the individual cell lines was inversely related to the level of drug resistance expressed as D0. P-glycoprotein was also detected in the marrow-derived myeloma cells of patients with drug refractory disease using immunohistochemical staining. The amount of P-glycoprotein in the cells of one patient was directly compared to the amount found in the simultaneously stained standard cell lines (8226/DoxR and 8226/DoxR0) by comparing the optical densities for individual cells. Using this immunohistochemical technique to detect and quantitate P-glycoprotein in patient myeloma cells and comparing it to standard multidrug resistant myeloma cell lines may be of value in determining the contribution of P-glycoprotein to clinical drug resistance in patients with multiple myeloma.

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Multiple myeloma is a plasma cell neoplasm with a high initial response rate to chemotherapy. Unfortunately, acquired drug resistance ultimately renders the disease incurable. Determining mechanisms of drug resistance and developing means of identifying drug resistant cells could provide the basis for important advances in the treatment of multiple myeloma. One type of resistance that is frequently described for drug-resistant tumor cell lines is termed multiple-drug resistance (MDR). In this setting, tumor cells develop a resistance to numerous drugs that are very heterogeneous in structure and mechanism of action. Tumor cell lines that have the MDR phenotype frequently overexpress a 170,000 dalton membrane glycoprotein termed P-glycoprotein or P-170. The multidrug-resistant gene (mdr 1) has been isolated and found to be responsible for the expression of P-glycoprotein. Dechares et al have also shown that the high expression of P-glycoprotein is the only alteration necessary to induce multidrug resistance. The mechanism of resistance appears to be due to decreased drug accumulation secondary to enhanced efflux of drugs, which is energy dependent. The precise role of P-glycoprotein in acquired drug resistance is uncertain; however, recent evidence suggests that it may directly bind drugs and act as an efflux pump. An alternative explanation likens the mammalian P-glycoprotein to the bacterial transport protein, hemolysin B, which transports the toxin alpha-hemolysin. In this setting, drugs would bind a carrier protein and the drug-protein complex would be actively transported from the cell. In either case, P-glycoprotein should be in high concentrations in the plasma membrane if it is involved in the transport of drugs out of the cell.

The first monoclonal antibody with a high affinity to P-glycoprotein that proved useful in detecting this transmembrane protein was developed by Ling et al. Several others have recently described the development of monoclonal antibodies that appear useful in the detection of P-glycoprotein. Scheper et al recently characterized the monoclonal antibody JSB-1, and demonstrated its ability to detect P-glycoprotein using immunoprecipitation and immunohistochemistry. In this study, we present a method for the detection and quantitation of P-glycoprotein in individual myeloma cells. A means of standardization and comparison of P-glycoprotein in clinical specimens is provided by using well characterized multidrug-resistant myeloma cell lines with varying degrees of resistance and levels of P-glycoprotein.

Materials and Methods

Cell lines. RPMI 8226 myeloma cells obtained from the American Type Culture Collection (Rockville, MD), were selected for resistance to doxorubicin (Dox) (Adria Labs, Columbus, OH) by gradually increasing Dox exposure over a 2-year period. The 8226/DoxR cell line was selected for resistance by gradually exposing the cells to 6 x 10-6 M Dox, a concentration six times the initial concentration (1 x 10-7 M) used in the selection process. The 8226/DoxR0 cell lines were gradually exposed to a maximum concentration of 4 x 10-5 M Dox, representing a 40-fold increase in the initial drug exposure concentration. Cells were maintained as a suspension in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% penicillin (100 U/mL), 1% streptomycin (100 μg/mL), and 1% (v/v) L-glutamine (Grand Island Biological Co., Grand Island, NY).

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Island, NY). Cells were kept at 37°C in a 5% CO₂-95% air atmosphere and subcultured every seven days. Once established, resistance has been stable in the Dox-resistant cell lines for at least 1 year.

In vitro drug sensitivity testing. Techniques for preparing cells for drug exposure by plating cells in soft agar have been reported previously.34 Exponentially growing cells were continuously exposed to varying concentrations of Dox by diluting the drug in media and adding agar. Cells were plated in triplicate in 35 mm petri dishes at 20,000 cells per plate. Plates were then incubated at 37°C in a humidified atmosphere of 5% CO₂ for ten days. Colonies greater than 60 μm were counted by inverted microscopy or by a computerized image analyzer (Omnicon FAS II; Bausch and Lomb, Rochester, NY).

Immunoblot analysis. Plasma membrane preparations from 8226/S, 8226/Doxin, and 8226/Dox40 were purified according to the method of Riordan and Ling.35 Membrane protein determinations were assayed by a modification of Bradford36 using 1.0 M NaOH pretreatment and standardized with bovine serum albumin. Fifty micrograms of membrane protein were loaded in each well. Polyacrylamide gel electrophoresis was performed according to the method of Fairbanks et al.35 with slight modification.37 The procedure of Towbin38 was used to transfer proteins from the gel to nitrocellulose paper, which was then probed with a monocolonal antibody. The murine monoclonal antibody JSB-1 was used as the primary antibody and 125I-rabbit anti-mouse IgG (New England Nuclear Corp, Boston, MA) was used as second antibody for detection by autoradiography (X-Omat AR film, Kodak, Rochester, NY). To quantitate the relative amounts of P-glycoprotein for each cell line, autoradiograms were scanned along the linear portion of the film using a model 620 Video Densitometer (Bio-Rad Laboratories, Richmond, CA).

Immunohistochemical staining. To detect P-glycoprotein on individual cells, the murine monoclonal antibody JSB-1 (subclass IgG1) was used as the primary antibody using the biotin-avidin conjugated immunoperoxidase method of Warnke and Levy with some modifications.39 This antibody detects a highly conserved epitope on P-glycoprotein and has been described in a separate publication.40 Tumor cells were suspended in RPMI media at approximately 200,000 cells/mL and four drops were placed in a cytocentrifuge apparatus (Cytospin 2, Shandon Co., Sewickley, PA) along with one drop of 22% bovine serum albumin (BSA; Gamma Biological, Houston, TX). Cytocentrifuge preparations were dipped in cold acetone (4°C) for less than five seconds, air-dried, and stored at −70°C. JSB-1 antibody from ascitic fluid was used at a dilution of 1/200 in 2% BSA in phosphate-buffered saline (PBS). The second stage employed biotin-conjugated rabbit anti-mouse IgG (H&L) (Accurate Chemical, Westbury, NY) diluted to 1/1200 in 2% normal human serum in PBS. The third stage employed avidin-D conjugated with horseradish peroxidase (Vector Labs, Burlingame, CA). The color reaction was produced using DAB (diaminobenzidine) (Sigma, St. Louis, MO) followed by five minutes in 0.5% copper sulfate in 0.85% NaCl to intensify the color. A negative control slide repeated all these steps excluding the primary antibody and substituted an irrelevant, isotype-matched, monoclonal antibody. A counterstain of 1% methylene blue was used as a final step. To compare the amount of P-glycoprotein on patient specimens with the amount on cell lines, cells from both the patient and drug-resistant cell lines were stained simultaneously to control for variability in staining technique. Cytocentrifuge preparations were then analyzed by light microscopy at 630× power or by the following image analysis system.

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**Fig 1.** The effect of Dox by continuous exposure on soft-agar colony formation of sensitive (8226/S; •) and resistant (8226/Doxin; △; 8226/Dox40; □) human myeloma cells. Points, mean of three replicates.

**Fig 2.** Western blot analysis of plasma membrane components of sensitive (8226/S) and resistant cells (8226/Doxin and 8226/Dox40). Equal amounts (50 μg) of protein were placed in each lane and fractionated by sodium dodecylsulfate-gel electrophoresis. Components were transferred to nitrocellulose paper and then probed with a monoclonal antibody JSB-1 specific for P-glycoprotein.
DETECTION AND QUANTITATION OF P-GLYCOPROTEIN

Image analysis. As a means of quantitating the amount of P-glycoprotein in individual myeloma cells, the average optical density of individual cells stained was measured with a Cell Analysis System, Inc., CAS 100 optical microscope image analysis system. 

The system consists of a Reichert-Jung Diastar model 420 microscope (Cambridge Instruments) with the microscopic image collected by a charge-coupled device (CCD) video camera. The CCD is attached to the microscope and cellular images are sent to an image processing board contained within an 8 MHz Intel 80286 central processing unit based microcomputer containing 512 kilobytes of random access memory, a math coprocessor and an internal 30 megabyte fixed-disk. Digitized images are displayed directly on a Barco high-resolution RGB monitor directly under software control. The CAS 100 and its software were purchased from Becton-Dickinson Immunocytometry Systems. Each cell's optical density (which represents the sum of optical density/total number of pixels in each cell's image) was determined using the 40× objective and a 480 nm filter (20 nm bandwidth) and the cell measurement program (CMP) purchased from Becton-Dickinson. The data are expressed as the mean optical density ± SEM for approximately 50 cells per sample (8226/S, 8226/Dox, 8226/Dox4, and patient sample).

Drug accumulation studies. Cellular accumulation of radiolabeled 14C-Dox (sa 23.3 mcg/mmol; SR1 International, Menlo Park, CA) was determined as described previously for this cell line. 

Radiolabeled Dox was incubated with cells in RPMI medium for one hour, washed in cold PBS, and then layered on silicone oil as described by Vistica et al. 

Cells were then solubilized with 1 M NaOH and neutralized with HCl. Radioactivity was determined by adding liquid scintillation fluid and counting with a beta scintillation counter.

RESULTS

Cytotoxicity. The cell survival curves of the three 8226 cell lines following continuous exposure to varying doses of Dox in soft agar are shown in Fig 1. The mean lethal dose (D50) of Dox required to reduce survival to 1/e (37%) of the initial cell population was determined using the negative reciprocal slope of the linear portion of the survival curves. 

D50 was found to be 0.012 μM for 8226/S, 0.12 μM for 8226/Dox4, and 0.73 μM for 8226/Dox4. These values reflect a ten-fold and 61-fold increase in resistance for the 8226/Dox4 and 8226/Dox4 cell lines, respectively.

Cellular analysis of P-glycoprotein. Fig 2 shows a Western blot analysis of the sensitive and two-drug-resistant cell lines using the JSB-1 monoclonal antibody. Densitometric analysis of the P-170 bands demonstrated a progressive increase in amount of P-glycoprotein as the cell lines become more drug resistant. P-glycoprotein was not detectable in the sensitive cell lines. The 8226/Dox4 cell line contained seven times the amount of P-glycoprotein compared to the 8226/Dox4 cell line as determined by the densitometric scanning of the autoradiogram. Similar results were obtained using the monoclonal antibody C-219 originally developed by Dr. Victor Ling, Ontario Cancer Institute, Ontario, Canada and obtained commercially from Centocor (Malvern, PA).

Fig 3 illustrates our immunohistochemical assay of P-glycoprotein in a myeloma patient sample using the monoclonal antibody JSB-1. As illustrated, our assay employs negative controls and positive standards along with the patient specimen. Fig 3A is a negative control for 8226/Dox4. Fig 3B and 3C show the immunohistochemical staining results for the drug-resistant cell lines 8226/Dox4 and Dox4, respectively. As the cells become more resistant, the intensity of staining (brown coloration) of individual cells also increases. The staining is particularly intense in the plasma membrane of individual cells with occasional cells

Fig 3. Immunohistochemical assay of P-glycoprotein in a patient with myeloma. Using a monoclonal antibody (JSB-1) and a peroxidase method, we detect P-glycoprotein (evidenced as brown coloration) in patient myeloma cells (D-G). This assay is always compared to established cell lines (A-C) expressing known amounts of P-glycoprotein. Methylene blue counter stain is employed to highlight cells. A. Cell line negative control: the monoclonal antibody JSB-1 is substituted with an irrelevant control antibody (see text). The absence of brown coloration indicates background staining is absent; B. 8226/Dox4 cell line showing moderate P-glycoprotein expression; C. 8226/Dox4 cell line showing strong P-glycoprotein expression; D. patient marrow aspirate containing atypical plasma cells (Wright's stain); E. patient myeloma cells negative control substituting primary monoclonal antibody with an irrelevant antibody; F. patient myeloma cells show detection of P-glycoprotein using JSB-1; G. patient myeloma cells showing P-glycoprotein with a strong Golgi distribution.
showing increased staining of the plasma cell "hof" (Golgi apparatus) (see Fig 3C and G).

The results of cell staining for a patient with drug refractory, end-stage multiple myeloma are shown in Figs 3D, E, F, and G. Fig 3D shows the typical cell morphology of a patient with myeloma. The negative control for this patient's cells are seen in Fig 3E. Figs 3F and G are typical P-glycoprotein positive cells seen in this patient specimen; while plasma membrane staining predominates, we occasionally noted the intense staining in the Golgi apparatus of some plasma cells (Fig 3G). At the time of biopsy, this 43-year-old female patient with IgA kappa multiple myeloma had developed progressive disease after treatment with multiple drugs including vincristine and Dox.

To date, we have tested the myeloma cells of 13 patients with drug refractory disease and seven of these patients had P-glycoprotein positive cells. All 13 patients tested had prior chemotherapy containing vincristine and Dox and were considered to be drug refractory. Six of these patients were treated with combination chemotherapy consisting of continuous infusion vincristine and Dox with oral decadron (VAD) in combination with verapamil and are described in greater detail in a separate report.21

**Cellular quantitation of P-glycoprotein.** Using the computerized cell analysis system (CAS), the amount of staining intensity for the individual cells was quantitated by measuring the average optical density of each cell. Approximately 50 cells were analyzed for each cell line or patient specimen. Fig 4 shows the mean optical density and SEM for the sensitive and drug-resistant cell lines. In general, the intensity of staining for individual cells within each cell line was uniform (coefficient of variation of 12.6% and 13.5% for 8226/Doxø and 8226/Doxøø, respectively). Linear regression analysis of all data points revealed a strong correlation ($r = 0.82$, $P < 0.0005$) between the level of P-glycoprotein (optical density) and mean Dox for each individual cell line.

The amount of P-glycoprotein seen in the patient specimen (Fig 3) in comparison to the standard drug-resistant cell lines is shown in Fig 5. This particular patient's cells appeared to have slightly more P-glycoprotein than the Doxø cell line that is 10-fold resistant compared to 8226/S. Visual observation by light microscopy (Fig 3) appears to confirm this comparison. In this particular patient with drug refractory disease, virtually all the cells were P-glycoprotein positive. Total cellular RNA analysis for P-glycoprotein expression confirmed the immunohistochemical results (data not shown).

**Doxorubicin accumulation.** The amount of Dox accumulated in one hour for the individual cell lines at two different drug concentrations is shown in Table 1. Generally, as the cells become more resistant, the amount of Dox is less compared to the sensitive cell lines. At the 1 μM concentration, the Doxø and Doxøø cell lines accumulate 48% and 71% less Dox than the sensitive cell line. This relationship is depicted graphically in Fig 6. As the degree of resistance increases (increase in mean lethal dose, $D_L$), the amount of P-glycoprotein (optical density) increases, and the intracellular accumulation of doxorubicin decreases.

**Table 1. Doxorubicin Accumulation (p mol/1 × 10⁶ cells) at One Hour for Drug-Sensitive (8226/S) and Drug-Resistant (8226/Doxæ and 8226/Doxøø) Cell Lines**

<table>
<thead>
<tr>
<th>Dox Dose (μM)</th>
<th>8226/S</th>
<th>8226/Doxæ</th>
<th>8226/Doxøø</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>32.9 ± 4.0</td>
<td>15.8 ± 2.0</td>
<td>13.2 ± 2.6</td>
</tr>
<tr>
<td>1.0</td>
<td>60.7 ± 5.4</td>
<td>31.6 ± 4.5</td>
<td>17.8 ± 5.0</td>
</tr>
</tbody>
</table>

Each value represents the mean of six replicates with standard deviation.
**DISCUSSION**

This study describes the use of a murine monoclonal antibody JSB-1 to detect P-glycoprotein on individual myeloma cells. This particular antibody (an IgG1 isotype) was derived using a Chinese hamster ovary cell line selected for colchicine resistance. Details of development and full characterization of this antibody are described elsewhere. Using this antibody and a standard immunohistochemical technique, we were able to detect P-glycoprotein on individual tumor cells from drug-resistant cell lines, as well as patient specimens. In addition, we have presented a method to quantitate the amount of P-glycoprotein on individual cells by measuring the average optical density of each cell stained immunohistochemically and comparing the values obtained to cell lines expressing known amounts of P-glycoprotein (positive standards) and those without P-glycoprotein (negative control). By using a technique that measures P-glycoprotein in individual cells, one can also perform a differential on tumor specimens, to determine not only the relative staining intensity of individual cells, but the percent of tumor cells that are positive. Ma et al reported an immunohistochemical technique for detecting P-glycoprotein in individual cells of two patients with acute nonlymphoblastic leukemia. Using the monoclonal antibody C-219, they demonstrated that as the disease progressed in these two patients, the percentage of cells that were P-glycoprotein positive increased. In this context, a method that would both determine the percent of positive cells and quantitate the amount of P-glycoprotein on individual cells may be of value in determining the significance of P-glycoprotein in clinical drug resistance.

The immunohistochemical technique used with the JSB-1 monoclonal antibody required preliminary fixation of cells with acetone suggesting that the epitope is internal. We have recently performed similar studies using the monoclonal antibody C-219 developed by Ling et al and observed similar results. Whether these two antibodies recognize the same epitope remains to be determined. Even so, the highest concentration of the antibody reacted to the plasma membrane and occasionally the hof of the plasma cells, suggesting that a high concentration of P-glycoprotein exists in the plasma membrane and perhaps the Golgi apparatus. Willingham et al recently described the location of P-glycoprotein in human multidrug-resistant carcinoma cells using the monoclonal antibody MRK16 and immunofluorescence with electron microscopy. They found that P-glycoprotein was localized to the plasma membrane and the luminal side of the Golgi stack membranes. These findings are consistent with the hypothesis that P-glycoprotein functions as a drug transport protein.

We have previously reported the characterization of a multidrug-resistant human myeloma cell line that overexpresses P-glycoprotein (8226/Dox). The drug-resistant profile for this resistant myeloma cell line was characteristic of multidrug-resistant cell lines, exhibiting resistance to many natural products including Dox, vincristine, and etoposide, but increased sensitivity to steroids, including dexamethasone. This study demonstrates that the degree of resistance to one of these agents, Dox, correlates well with the amount of P-glycoprotein expressed on individual cells. Recently, we observed that when the extracellular concentration of Dox is adjusted to yield equivalent intracellular concentrations for sensitive and resistant cells, that the degree of DNA damage and cytotoxicity obtained are identical. While these studies do not exclude other mechanisms of resistance, such as enhanced DNA repair, they demonstrate that decreased drug accumulation associated with the presence of P-glycoprotein plays a major role in drug resistance in this particular myeloma cell line.

The ability to detect and quantitate P-glycoprotein in individual tumor cells from tumor specimens is an important step in determining the role of P-glycoprotein in clinical drug resistance. Detection of P-glycoprotein in tumor cells may permit prediction of the responsiveness of individual tumor cells to selected chemotherapeutic agents, thereby aiding in the design of treatment protocols.

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