Levels of Multidrug Resistance (MDR1) P-Glycoprotein Expression by Human Breast Cancer Correlate with in Vitro Resistance to Taxol and Doxorubicin

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ABSTRACT
To determine whether multidrug resistance (MDR1) P-glycoprotein (Pgp) expression correlated with clinical MDR1-related drug resistance, we established a protocol for quantitative measurement of Pgp expression and in vitro drug resistance in doxorubicin resistant MCF7 breast cancer cell lines and 359 freshly resected specimens of breast carcinoma. Pgp expression was detected with 4E3, UIC2, and JSB-1 monoclonal antibodies using flow cytometry and immunohistochemistry (IHC). Pgp function was determined using PSC833 in a drug resistance-reversal assay and with a three-dimensional agarose-based extreme drug resistance assay. MCF7 calibrator cell lines expressed Pgp, which was functional and in proportion to the degree of drug resistance. Flow cytometry, UIC2 shift assays, IHC scores, and determination of absorbance products by image analysis were all highly correlated (r > 0.9). Overall Pgp expression increased from 11% in untreated patients to 30% in patients who had previously received chemotherapy. Compared with Pgp-negative tumors, a significant increase in doxorubicin and Taxol resistance was seen for breast cancers that expressed Pgp, regardless of prior treatment. A strong correlation between the degree of Pgp expression and in vitro resistance to Taxol and doxorubicin (but not to 5-fluorouracil) was found when either IHC scores or image analysis-based methods were used to quantify Pgp expression (n = 185, P < 0.0001). The degree of Pgp expression strongly correlated with the degree of drug resistance in the clinical specimens studied. These data suggest that (a) Pgp contributes to clinical MDR1-related drug resistance, and (b) both intrinsic and acquired expression of Pgp in breast cancer may contribute in part to therapeutic failure and relapse.

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INTRODUCTION
Although newly diagnosed metastatic breast cancer is initially responsive to chemotherapy, 5-year survival for disseminated breast carcinoma is less than 20% because of relapse with drug-resistant disease (1). Recently, specific mechanisms of drug resistance have been elucidated, which may explain why initially sensitive tumors become drug resistant (2–5). Although various mechanisms of drug resistance have been characterized in breast cancer cell lines, including alterations in glutathione metabolism, increased thymidylate synthase levels, altered topoisomerase, mutations in p53, and increased expression of MRP, the best understood is MDR1-encoded Pgp (2–14). Pgp has been suggested to contribute to resistance to natural product-based chemotherapeutics, including taxanes, anthracyclines, Vinca alkaloids, podophyllotoxins, and camptothecins (4–6). Although it is relatively straightforward to study mechanisms of drug resistance in cell lines, characterization of resistance processes in clinical tumor specimens has been problematic. One approach to testing the clinical role of Pgp in resistance has been to study the capacity of Pgp inhibitors to chemosensitize tumors in refractory patients. Clinical trials evaluating the MDR1 reversal agents verapamil, cyclosporin A, and PSC833 have indicated that these may potentiate drug activity in previously resistant patients (14–17). However, systemic clearance of MDR1 drugs is in part dependent on the Pgp family of transporters, and Pgp antagonists have caused increased levels (measured as area under the curve) of these drugs in patients enrolled in resistance-reversal trials, complicating interpretation of their results (15–17).

Although several reports suggest that Pgp is clinically relevant in hematological malignancies, its contribution to clinical drug resistance in solid tumors is less clear (2). Pgp expression by breast cancer has been previously reported in several small series, and it has been hypothesized that this may contribute to treatment failure (7–13). Studies that have examined series of at least 50 cases of breast carcinoma using RNA detection methods or IHC have indicated that newly diagnosed breast carcinoma shows a 0–29% incidence of Pgp expression, but that this can increase to 71% at relapse (7–10). The incidence of Pgp expression in breast carcinoma becomes increasingly relevant with the heightened use of taxanes and doxorubicin in the treatment of breast cancer (18–22). Toxicity stemming from the use of these chemotherapeutic agents is not
trivial, and they may be among the more avid substrates for Pgp (3–5, 22–23).

Existing immunohistochemical- and RNA-based MDR1 detection methods are often incompatible and give conflicting results, making it difficult to delineate the role of Pgp in clinical breast cancer resistance (12, 23–26). The most clinically relevant parameter of Pgp is its drug efflux function. Although there is one report in the literature comparing breast cancer Pgp expression and in vitro resistance (10), no studies have been published combining highly sensitive quantitative Pgp detection methods with tests for the functional MDR1 drug transporter phenotype and an in vitro drug resistance assay calibrated to clinical response.

In the present paper, we have established an in vitro protocol for the parallel quantitative measurement of levels of Pgp expression and in vitro drug resistance in breast carcinoma cell lines and freshly resected breast tumor specimens. This approach included immunohistochemical staining to detect Pgp on paraffin-embedded tissue sections and by flow cytometry, as well as a three-dimensional agarose-based in vitro drug resistance assay system that accurately identifies patients whose tumors express EDR (27, 28). Patients treated with an agent to which their tumor expresses EDR have less than a 3% probability of clinical response (27). In this study, we used three tests to determine breast cancer Pgp function. Our protocol was based on the use of a set of calibrator cell lines that expressed differing levels of Pgp. These lines were found to be resistant in the in vitro EDR assay. We then used PSC833 as a resistance reversal agent in the EDR assay to confirm that calibrator cell line Pgp expression was responsible for the doxorubicin-resistant phenotype. Finally, we evaluated Pgp function with the UIC2 shift conformation assay (29, 30). The UIC2 mAb binds more avidly to a Pgp epitope altered during drug efflux. These calibrator lines and methods were validated and standardized, and they were subsequently used to assess the Pgp expression and drug resistance of freshly resected breast carcinoma specimens. In this study, we found that the levels of breast carcinoma Pgp expression directly correlated to levels of tumor resistance to the Pgp substrates Taxol and doxorubicin. The data also revealed that prior therapy was associated with increased expression of Pgp at relapse.

MATERIALS AND METHODS

Tissue Culture

The human breast carcinoma cell line MCF7-WT was obtained from ATCC. Cell lines with low (MCF7-P4), intermediate (MCF7-P10), and high (MCF7-P19) levels of MDR1 expression were selected by passaging MCF7 cell lines in increasing concentrations of doxorubicin. The Pgp status of the calibrator cell lines was confirmed by Northern blot (31), immunohistochemistry, and flow cytometry. All cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Gemini Bioproducts, Inc., Calabasas, CA), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 2 mm l-glutamine (all purchased from Irvine Scientific, Irvine, CA; complete medium). Cells were harvested with 0.25% trypsin (Life Technologies, Inc.) after washing twice with PBS (Irvine Scientific); washed with complete medium, counted, and checked for viability using trypan blue; and processed for Pgp expression and function assays.

In Vitro Drug Response Assay

We used an agarose-based culture system, the EDR assay, with thymidine incorporation as the end point (27, 28). Viable tumors were placed into medium by the pathologist at the referring institution immediately after surgery and shipped to our laboratory by overnight delivery. Immediately upon receipt, three small fragments representing different areas of the carcinoma were removed from the tumor and fixed in formalin for sectioning and pathologists’ review. The remainder was processed for the EDR assay. Tumors were cut with scissors into pieces of 2 mm or smaller in a petri dish containing 5 ml of complete medium. The resultant slurry was mixed with complete medium containing 0.03% DNase (2650 Kunitz units/ml) and 0.14% collagenase I (both enzymes from Sigma Chemical Co., St. Louis, MO), placed into 50-ml Erlenmeyer flasks with stirring, and incubated for 90 min at 37°C in a humidified 5% CO₂ atmosphere. After enzymatic dispersion into a near single cell suspension, tumor cells were filtered through nylon mesh and washed in complete medium. A portion of the cell suspension was used for cytospin slide preparation and stained with Wright-Giemsa stain for examination in parallel with H&E-stained tissue sections by a medical pathologist to confirm the diagnosis and to determine the tumor cell count and viability. Tumor cells were then suspended in soft agarose (0.13%) and plated at approximately 20,000 cells/well onto an agarose underlayer (0.4%) in 24-well plates. Cells were incubated under standard culture conditions for 4 days in the presence or absence of antineoplastic drugs used in the treatment of breast cancer. The final drug concentrations used in the EDR assay were as follows: 2.45 μM for Taxol (paclitaxel; Calbiochem, La Jolla, CA), 0.17 μM for doxorubicin (Adriamycin; Adria, Columbus, OH), and 23 μM for 5-FU (Pharmacia & Upjohn, Kalamazoo, MI). In MDR1 reversal experiments, 1 μM PSC833 (Sandoz, East Hanover, NJ) was added 2 h prior to drug treatment, and IC₅₀,₅ for the calibrator cell lines incubated with different concentrations of doxorubicin were determined as described previously (32). Treatment with 1 μM PSC833 did not affect the proliferation of control cells. Cells were pulsed with tritiated thymidine (New Life Science Products, Boston, MA) at 5 μCi/ well for the last 48 h of the culture period. After labeling, cell culture plates were heated to 90°C to liquefy the agarose, and the cells were harvested with a microharvester (Brandel, Gaithersburg, MD) onto glass fiber filters. The radioactivity trapped on the filters was counted with an LS-6500 scintillation Counter (Beckman, Fullerton, CA). Untreated cells served as a negative control. In the positive (background) control group, cells were treated with a supratherapeutic dose of cisplatin (33 μM), which caused 100% cell death. Detectable cpm for this group is considered nonspecific background related to debris trapping of tritiated thymidine on the filter. After subtracting background control values, PCI of proliferation was determined by comparing thymidine incorporation by the treatment group with incorporation by the negative control group: PCI = 100% × [1 – (cpm of treatment group ÷ cpm of control group)]. The data from at least three experiments are presented for the calibrator cell lines. Determinations of drug effects on tumor proliferation

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were performed in duplicate or triplicate. Tumor cell lines tested in the EDR assay were handled in a fashion comparable to solid tumors. Cell lines were harvested with trypsin and washed twice in PBS prior to their addition to the culture plates.

**Pgp Detection Methods**

Pgp expression by a series of MCF7-derived doxorubicin-resistant cell lines was determined by quantitative (immuno-) containing the cells under analysis and presented as number of molecules/cell. Functional (the UIC2 shift assay) flow cytometry, as well as by standard IHC (IHC scores and image analysis) on sections cut from fixed, paraffin-embedded cell lines and tumors. For each method, standard curves were established for low, intermediate, and high levels of Pgp expression based on correlating the Pgp expression levels of the calibrator cell lines determined by flow cytometry with those determined by tissue section analysis. Tumor specimens were analyzed by IHC in parallel with the calibrator cell lines, and patient's results were fit to the standard curves. Our preliminary experiments showed that the harvesting procedures did not affect the expression and function of Pgp on the surface of MCF7 calibrator cell lines and tumor cells.

**Quantitative Determination of Pgp Expression by Flow Cytometry.** The number of Pgp molecules/cell was determined by flow cytometry using the 4E3 mAb specific for external epitopes of Pgp (Signet Laboratories, Dedham, MA; Ref. 33). 1 × 10^6 cells were washed once in PBS containing 1% FCS, resuspended in 1 ml of 2% paraformaldehyde in PBS for 1 h at 4°C, and then centrifuged and resuspended in PBS containing 1% FCS (PBS/FCS). Cells were then washed once in PBS/FCS and incubated for 60 min at 37°C in IgG2/IgG1 isotypic controls (Becton Dickinson, San Jose, CA) or 4E3 mAb. Cells were washed twice in ice-cold PBS/FCS and incubated for 30 min at 4°C with goat F(ab')2 antismouse IgG-FITC. The cells were then washed twice in PBS/FCS, and data were acquired in the list mode on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 mW argon laser. Fluorescence emission (488 nm excitation) was collected after passing through band pass filters (530 nm for FITC). Data (10,000 events) were collected and analyzed on a FACScan interfaced model 340 Hewlett Packard computer using LYSYS II or Cell Quest software (Becton Dickinson). Pgp grading was based on median cell fluorescence, percentage of cells staining, and number of binding sites/cell (34, 35). Quantum Simply Cellular Calibration microbeads (Flow Cytometry Standards Corp., Research Triangle Park, NC) were used according to manufacturer's instructions to quantitate the fluorescence intensity associated with the number of binding sites. Pgp quantitation was performed using internal standards run in the tubes containing the cells under analysis and presented as number of molecules/cell.

**Quantitative Pgp Determination Using IHC and Image Analysis.** MCF7 cells were fixed in 10% neutral buffered formalin for preparation of an agarose pellet and subsequent cell block preparation (36). Image analysis using the CAS system (Becton Dickinson) was performed to determine the staining intensity of Pgp-positive cell populations using the quantitative cellular and nuclear antigen protocols (37). Image analysis was carried out by technologists blinded to the pathologist's scoring results. The JSB-1 mAb directed against internal epitopes of Pgp (38) was purchased from BioGenex (San Ramon, CA). BioGenex Negative Control was run in parallel for all sections. Paraffin-embedded tissue sections were deparaffinized in Histoclear, incubated in 3% hydrogen peroxide in distilled water for 10 min, and rinsed in tap water and distilled water. Each slide was subsequently incubated for 15 min at room temperature in 100 μl of blocking buffer (Protein Block, BioGenex) in a humid chamber. Excess blocking buffer was shaken off, and the slides were incubated for 30 min with primary mAbs at room temperature in a humid chamber and then rinsed in PBS for 5 min. Tissue sections or cyto spin preps were then incubated in LINK (StrAvigen Super Sensitive Immunodetection System, BioGenex) for 20 min at room temperature in a humid chamber. After rinsing for 5 min in PBS, each section was covered with Label (BioGenex) for 20 min at room temperature, rinsed in PBS, and exposed to Substrate Solution (BioGenex) for 5 min at room temperature. The slides were then rinsed in PBS for 5 min, counterstained with hematoxylin for 1 min, rinsed for 10 min in tap water, dehydrated in ascending ethanol series, cleared in xylene, coverslipped in Permunt, and viewed under ×40 magnification. Only malignant tumor cells were counted for each tissue specimen, and the number of stained cells and intensity was determined. Pgp levels in individual tumor specimens were determined using a modified nuclear masking program and two-color image analysis (CAS 200, Becton Dickinson). A minimum of 10 fields were analyzed. To determine total cellular area, paraffin-embedded sections of each tumor were stained with methyl green (peak absorbance of 600–650 nm), which shows minimal overlap with the major diaminobenzidine absorbance peak of 400–550 nm (CAS Users Manual, version 2.0, 1990). Pgp expression levels were determined as the product of the percentage of cells containing Pgp (percentage of positive area) and the degree of diaminobenzidine absorbance at 500 nm (percentage of positive stain), referred to as the absorbance product. In preliminary experiments, Pgp image analysis determinations were validated against IHC scores determined independently. Results by these two methods showed a correlation of 0.9.

**UIC2 Shift Assay.** The UIC2 shift assay for functional Pgp was performed as described elsewhere (29, 30). The UIC2 mAb (IgG2a) reacts with the extracellular moiety of Pgp and inhibits Pgp-mediated efflux of all tested MDR chemotherapeutic drugs (29, 30). It has been shown that the reactivity of UIC2 mAb with Pgp was enhanced in the presence of MDR1 compounds due to conformational changes in functioning Pgp. This phenomenon was used to develop a highly specific and sensitive method for the detection of Pgp in its functional, biochemically active conformation (29). The standard cell lines were trypsinized, washed twice in PBS at room temperature, and aliquoted in 2 ml plastic tubes, 10^6 cells/tube in 1 ml of prewarmed Ca, Mg-free PBS. The cells were then warmed to 37°C for 10 min, and 5 ml of vinblastine stock solution (4 mg/ml in DMSO) or DMSO (negative drug control) were added with subsequent mixing of the tubes and incubated for another 10 min at 37°C. The final concentration of vinblastine was 25 nm. Fifty-ml aliquots of PE-labeled UIC2 at 30 mg/ml were added, and the tubes were mixed and incubated for another 20 min at 37°C with occasional stirring. After incubation, the cells were washed twice with ice-cold PBS, transferred in PBS with 1
mg/ml of propidium iodide (Sigma) and kept on ice until analysis. A PE-labeled irrelevant mAb of the IgG2a isotype was used at the above concentration as a negative antibody control.

**Statistical Analysis**

Statistical tests were performed using InStat GraphPAD software, version 2.0 (San Diego, CA) and Cell Quest Software (Becton Dickinson). Pearson regression analysis was used to calculate correlation coefficients for the number of Pgp molecules/cell versus flow cytometry and IHC data. Overall incidence of Pgp expression in breast cancer was evaluated using the Fisher’s exact test, and the Mann-Whitney test was used to determine P values for the data on the impact of prior chemotherapy of Pgp expression and function in breast cancer. Spearman correlation coefficient values were calculated to correlate Pgp expression with in vitro drug response data. Unpaired t test for mean ± SD IC50 values was used to evaluate the difference in Pgp expression on calibrator cell lines treated with doxorubicin versus doxorubicin in the presence of PSC833. Two-tailed P values were determined in all statistical methods used in this study; Ps < 0.05 were considered significant.

**RESULTS**

**Breast Carcinoma Calibrator Cell Lines: Correlation between Pgp Expression and Function.** We used a series of MCF7 breast carcinoma-derived cell lines as a model of clinical breast cancer for standardization and comparison of Pgp detection methods. Three calibrator cell lines, MCF7-P4 (low level of MDR1 resistance), MCF7-P10 (low to medium level), and MCF7-P19 (high level), were derived through selection for doxorubicin resistance in clinical and in vitro proliferation of the calibrator cell lines. The levels of Pgp expression in the parental MCF7-WT and doxorubicin-resistant cell lines were determined by quantitative microbead calibration in flow cytometry and are expressed as the number of molecules/cell. As shown in Table 1 and Fig. 1, the number of Pgp molecules/cell correlated (r = 0.78) with doxorubicin resistance of the calibrator cell lines: 0 molecules/cell for MCF7-WT (IC50 = 0.15 μM), 7,491 molecules/cell for MCF7-P4 (IC50 = 1.8 μM), 14,692 molecules/cell for MCF7-P10 (IC50 = 6.0 μM), and 60,037 molecules/cell for MCF7-P19 (IC50 = 7.4 μM). The calibrator cell line also showed a positive correlation between the number of Pgp molecules/cell and their resistance to Taxol (data not shown). Conversion from a Pgp-negative phenotype (MCF7-WT) to a low level expressor (MCF7-P4; 7,491 molecules/cell) was associated with a 12-fold increase in doxorubicin resistance. A 2-fold further increase in Pgp expression by the MCF7-P10 (14,692 molecules/cell) was associated with a 3-fold increase in doxorubicin resistance when compared to the P4 line. A subsequent 4-fold increase in Pgp expression by the MCF7-P19 cell line to 60,037 molecules/cell was associated with only a 1.2-fold increase in doxorubicin resistance when compared to the MCF7-P10 cell line. Compared with the MCF7-WT line, the resistance to doxorubicin of calibrator lines was 12-fold for P4, 40-fold for P10, and 49-fold for P19. Diminishing doxorubicin sensitivity with increasing Pgp expression is shown in Fig. 1. PSC833, a small molecule that competitively inhibits Pgp function, efficiently reversed doxorubicin (Fig. 1) and Taxol (data not shown) resistance of the calibrator cell lines. No statistically significant difference between IC50s calculated for MCF7-WT cells treated with doxorubicin alone or doxorubicin in the presence of PSC833 (P = 0.87) were noted. However, PSC833 treatment resulted in a statistically significant decrease in IC50 values for doxorubicin treated MCF7-P4 (P = 0.033), MCF7-P10 (P = 0.0025), and MCF7-P19 (P = 0.0001) cell lines, returning their IC50 values to a level approximating the MCF7-WT cell line.

To directly demonstrate that Pgp expressed on the membrane of doxorubicin-resistant MCF7 cell lines was functional, we took advantage of the ability of the UIC2 mAb to bind with increased affinity to the biochemically active conformation of functioning Pgp. The calibrator cell lines were stained at 37°C with PE-labeled UIC2 in the presence or absence of 25 μm vinblastine, a Pgp substrate that has been shown to be a potent UIC2 shift inducer (29, 30). Flow cytometric analysis (Table 1)
showed that the addition of vinblastine increased the binding of MCF7-P4, MCF7-P10, and MCF7-P19 to UIC2-PE, increasing their fluorescent intensity compared to binding in the absence of drug. No staining and no reactivity shift were found in the Pgp-negative MCF7-WT cell line. The levels of Pgp expression determined by UIC2-PE direct immunostaining strongly correlated ($r = 0.98$) with the number of Pgp molecules/cell, with or without vinblastine. UIC2 shift was more pronounced for the MCF7-P4 and MCF7-P10 cells, which expressed relatively low levels of Pgp expression, reflecting a higher molar drug:Pgp ratio in these cells, as compared to MCF7-P19, which expressed high Pgp levels.

To test the effects of fixation, embedding in paraffin, and immunostaining, we subjected the calibrator cell lines to the entire immunohistological procedure. As shown in Table 1, IHC scores generated by a pathologist using routine light microscopy ($r = 0.98$) and absorbance products determined by image analysis ($r = 0.97$) positively correlated with the numbers of Pgp molecules/cell obtained by quantitative flow cytometry.

**Incidence of Pgp in Clinical Breast Cancer.** To determine whether the incidence of breast cancer Pgp expression was associated with acquired drug resistance, we correlated Pgp levels with patient treatment status. For the tumor specimens tested, prior treatment status and specific patient history regarding the drugs used were determined by reports obtained from physicians referring specimens to our laboratory. Table 2 summarizes the incidence of Pgp expression and treatment status in 359 breast cancers. Of the tumors evaluated, 68% (244 of 359) were from patients who had not received prior therapy (“untreated”), and 32% (115 of 359) came from those who had received prior therapy (“treated”). Pgp expression was found in 17% (63 of 359) of the cases overall. Untreated patients showed an 11% (28 of 244) incidence of Pgp expression, whereas 30% (35 of 115) of previously treated patients were Pgp positive. The difference in Pgp expression between untreated and treated cases was highly significant ($P = 0.0002$).

**Impact of Prior Therapy and Pgp Status on in Vitro Drug Response.** Of the 359 cases examined for Pgp expression, 185 breast tumors were then evaluated in the EDR assay to determine whether MDR1 status or their treatment history influenced their *in vitro* drug response to antineoplastic drugs (Table 3). For all evaluable specimens, the untreated and treated tumors were subdivided based on their Pgp staining by IHC and compared for their *in vitro* response to the Pgp substrates doxorubicin and Taxol (MDR1 drugs) and to 5-FU, a non-MDR1 drug. The values represent the mean PCI ± SD in tumor specimens evaluated by the EDR assay. In the untreated group, Pgp-positive tumors (PCI = 58 ± 21) were significantly more resistant to doxorubicin than Pgp-negative tumors (82 ± 15; $P < 0.001$). Similarly, in the treated group, doxorubicin resistance was higher in tumors expressing Pgp (56 ± 26) than in Pgp-negative tumors (82 ± 15; $P < 0.001$). With respect to Taxol response, for the untreated group, Pgp-positive tumors (PCI = 15 ± 18) were 3.5-fold more resistant to Taxol than Pgp-negative tumors (PCI = 53 ± 19; $P < 0.0001$). In the treated group, Pgp-positive tumors (58 ± 17) were 2.2-fold more resistant to Taxol than Pgp-negative tumors (26 ± 22; $P < 0.0001$). No difference in response to 5-FU was seen between untreated and treated cases or between Pgp-negative and Pgp-positive tumors. These data indicate that Pgp-positive breast carcinomas demonstrated increased resistance to Taxol and doxorubicin but not to 5-FU. No significant difference in Taxol or doxorubicin response was noted between Pgp-positive tumors from untreated or previously treated cases.

**Impact of Pgp Expression on in Vitro Drug Resistance.** To quantitate Pgp expression by clinical specimens, we generated IHC scores and absorbance product measurements for Pgp-stained tissue sections from the 185 untreated and treated breast carcinoma cases described in Table 3. Plots of the PCI of their IHC score and absorbance product arc shown in Fig. 2. A statistically significant correlation ($P < 0.0001$) between Pgp expression determined by either
Pgp Expression and Function in Breast Cancer

Table 3  Impact of prior chemotherapy on Pgp expression and function in breast cancer

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<tr>
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<th>Untreated</th>
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<tr>
<td></td>
<td>Pgp-positive</td>
<td>Pgp-negative</td>
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<tr>
<td>Doxorubicin</td>
<td>58 ± 2</td>
<td>82 ± 15</td>
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<td>Taxol</td>
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<td>5-FU</td>
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R ≥ -0.52, n = 172) or absorbance product (r = -0.47, n = 170) and in vitro resistance to doxorubicin was observed. Correlation coefficients were higher for tumors treated with Taxol in vitro: r = -0.70, n = 129 for IHC score, and r = -0.80, n = 128 for absorbance product per cell (P < 0.0001 for both detection methods). There was no statistically significant correlation between Pgp expression measured by IHC score (r = -0.078; n = 163; P = 0.32) or image analysis (r = -0.10; n = 161; P = 0.21), and response to 5-FU in the EDR assay.

Using calibrator cell line Pgp expression level results from quantitative flow cytometry and image analysis (Table 1), a standard curve was generated for fitting tumor sample Pgp expression levels as molecules/cell. Fig. 3 illustrates tumor Pgp levels (expressed as molecules/cell), determined by fitting tumor Pgp levels to the standard calibrator cell line curve, correlated with in vitro response to doxorubicin, Taxol, and 5-FU. As shown in Fig. 3, there was a strong correlation (P < 0.0001) between Pgp levels and in vitro resistance to doxorubicin (r = -0.47) and Taxol (r = -0.79) but not to 5-FU (r = -0.12; P = 0.12; data not shown). Essentially the same relationship was observed when the fitting curves of tumor samples were generated based on molecules/cell, IHC score, or absorbance product. Taken together, these data demonstrated that the degree of breast cancer resistance to Taxol and doxorubicin in vitro highly correlated with levels of Pgp expression determined by IHC.

DISCUSSION

Various mechanisms have been postulated to contribute to breast cancer resistance to chemotherapy, including overexpression of the membrane associated pumps MRP and Pgp, increased levels of thymidylate synthase, altered expression of DNA topoisomerase II, and enhanced detoxification of alkylating agents by glutathione-linked enzyme systems (2–7). MDR1-associated MDR is the best characterized and understood molecular mechanism used by tumor cells to evade the cytotoxic effects of doxorubicin and Taxol, two agents widely used in the treatment of breast cancer (2–4). Despite encouraging results from cell culture experiments, animal work, and clinical trials, the contribution of Pgp to the treatment failure of breast cancer patients has yet to be proven (2–11).

Several papers have described Pgp expression in breast cancer (7–13). The majority of these studies have found that Pgp is expressed by 0–75% of cases, depending on the methods (IHC versus RNA detection) and the thresholds used. Because of technical and design limitations, it is difficult to compare the results of most studies that evaluated the incidence of Pgp expression in breast cancer. With the exception of the report by Broxterman et al. (26) describing Pgp-mediated drug transport in mouse xenografts of human MDR1-expressing cell lines, no published studies have evaluated human breast carcinoma Pgp expression and function in parallel using a standardized reference method. We used a combination of IHC, flow cytometry and in vitro drug response assays to establish a reference laboratory procedure for parallel quantitation of Pgp expression levels and drug resistance in Pgp-negative and Pgp-positive MCF7 breast cancer cell lines and freshly resected specimens of breast carcinoma. The ultimate goal of the study was to determine whether there was a relationship between Pgp expression, Pgp function, and drug resistance in clinical samples of breast carcinoma.

For quantitative detection of Pgp expression, we chose antibody-based techniques (immunohistochemistry and flow cytometry) over RNA analysis. It has been demonstrated that nonmalignant stromal cells in breast tumors, as well as peripheral blood lymphocytes, express detectable levels of Pgp, which can contribute to the total RNA in a cellular homogenate, confounding the results (24–26). RNA-based detection methods are also limited by stringent handling requirements because of the extreme lability of RNA, particularly in clinical specimens. In contrast, cell surface Pgp is relatively stable, and amenable to detection by both flow cytometry and IHC (24, 26). Another advantage of flow cytometric and immunohistochemical techniques is that they can discriminate between Pgp expression by malignant cells versus expression by adjacent stroma and infiltrating blood cells (24).

MCF7 breast carcinoma calibrator cell lines were selected for differing levels of Pgp expression in doxorubicin containing media. Their Pgp expression levels were determined by flow cytometry using 4E3 and UIC2 mAbs directed against external epitopes of Pgp. These antibodies proved to be specific and sensitive reagents for the detection of Pgp (24, 37). As shown in Table 1, the number of Pgp molecules/cell for the four calibrator cell lines determined by flow cytometry using 4E3 strongly correlated (r > 0.9) with UIC2 staining by flow cytometry and with IHC staining using JSB-1 measured by IHC scores or absorbance product per cell line by image analysis. Although close correlations between different Pgp antibody-based detection methods were previously reported by Leith et al. (39) for hematological malignancies, our cell line correlations allowed us to generate standard curves for fitting tumor sample Pgp expression levels. As depicted in Fig. 1, the Pgp expression levels of the calibrator cell lines correlated well with their in vitro drug response. However, we also noted that the contribution of Pgp expression above 15,000 molecules/cell to drug resistance was significantly diminished, suggesting that above a certain point, further increases in Pgp expression made only a
Fig. 2 Correlation between doxorubicin (DOXO) and Taxol resistance (expressed as PCI in the EDR assay) with Pgp expression measured by standard IHC score or image analysis (absorbance product) in breast tumors. Spearman correlation coefficient (r) values were determined in the group of 185 freshly resected breast carcinoma specimens, and two-tailed Ps were calculated for the slope of each curve. In vitro resistance to doxorubicin and Taxol (but not to 5-FU) correlated with the degree of Pgp expression. Ps < 0.05 were considered significant.
modest contribution to drug resistance in our assay system. This finding emphasizes the importance of using low Pgp-expressing calibrator cell lines, such as MCF7-P4 (7,491 molecules/cell) and MCF7-P10 (14,692 molecules/cell), when analyzing MDR1 levels in clinical breast cancer.

It has been reported that some tumor specimens may express Pgp that is not functional (39). To exclude the possibility that our calibrator cell lines might express nonfunctional Pgp, their Pgp function was assessed by three different methods. First, the EDR assay showed that increased Pgp expression was related to increased resistance to doxorubicin (Table 1 and Fig. 1). Second, in resistance-reversal studies, PCS833, a competitive inhibitor of Pgp, almost completely reversed doxorubicin resistance in all three Pgp-positive calibrator cell lines (Fig. 1). Third, in the UIC2 shift assay, flow cytometric analysis of the calibrator cell lines in the presence of 25 μM vinblastine showed increased UIC2 binding to a biochemically active conformation of Pgp only for the Pgp-positive lines (Table 1). The extent of Pgp functionality in these assays strongly correlated with the degree of Pgp expression, expressed as molecules/cell, in all calibrator cell lines. Taken together, these data demonstrated a close relationship between Pgp expression and function in our experimental system and provided us with a useful reference system to apply to the analysis of freshly resected specimens of breast carcinoma.

Of the 359 different cases of breast carcinoma that we studied, Pgp expression was found in 11% of untreated and 30% of previously treated tumors (Table 2). Breast carcinoma has not been thought to belong to the group of tumors derived from tissues that intrinsically express Pgp, such as colon and renal cell carcinoma (2–4). Our data showing that 11% of untreated cases express Pgp suggest that transformation of terminal duct cells to breast carcinoma may in some instances involve activation of a stress-response cassette that includes Pgp (40–42).

The 3-fold increase in the incidence of Pgp expression by previously treated tumors may be due to induction of Pgp by chemotherapy and/or selection of Pgp overexpressing malignant clones. Such induction with subsequent selection has been previously reported for MDR1 cell lines treated with a variety of chemotherapeutic drugs in vitro (6). We previously found that serial sampling of paired breast carcinoma cases for Pgp expression using IHC before and after treatment demonstrated that previously negative cases can become positive and that positive cases can increase their intensity of expression. Thus, both induction and selection may be involved. Alternatively, previously negative cases may have been positive for Pgp at a level beneath the limits of our detection methods. Additional studies of breast tumors, sampled and assayed by highly sensitive detection methods before and after treatment with MDR1 drugs, are needed to better understand the contribution of intrinsic versus acquired Pgp-mediated drug resistance in breast cancer.

Irrespective of how Pgp expression is controlled, we found that Pgp-positive tumors were significantly more resistant in vitro to both doxorubicin and Taxol (P < 0.0001, n = 185, Table 3). It is interesting to note that the degree of drug resistance was almost identical for both untreated and treated Pgp-positive tumor samples. The degree of in vitro resistance to 5-FU, which is not recognized by Pgp, was unaffected by the Pgp expression status of the tumors, regardless of their treatment history. These data strongly imply that Pgp expression by breast carcinoma is a clinically relevant mechanism of drug resistance to MDR1 drugs.

The relationship between Pgp expression and drug resistance of breast carcinoma was further confirmed when the same patient cohort was analyzed by linear regression analysis comparing levels of Pgp expression, determined by IHC scores, absorbance product, or fitting to a standard curve generated from the calibrator lines, and drug response in the EDR assay. Figs. 2 and 3 demonstrate that a significant correlation existed between Pgp expression levels and in vitro tumor response to doxorubicin and Taxol but not to 5-FU. A good correlation was also observed between Pgp levels detected by routine IHC and image analysis. Calibrator cell line Pgp expression levels used to generate a standard curve for fitting solid tumors encompassed the levels detected on clinical specimens. The calibrator cell lines therefore expressed clinically relevant levels of Pgp, supporting their value as standards. On the basis of previously published correlations between EDR assay results and clinical

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4 Unpublished results.
response, the degree of resistance conferred by Pgp expression above an IHC score of 200 would be associated with less than a 3% probability of clinical response to Taxol (27, 28). It must be noted that in vitro tumor response to Taxol correlated better with levels of Pgp expression than that of doxorubicin. The higher correlation between tumor Pgp levels and Taxol versus doxorubicin resistance may be related to the multifactorial mechanisms responsible for doxorubicin resistance (MRP, altered topoisomerase II, and GSH-linked systems; Refs. 2 and 3). On the other hand, Taxol has been proven to be one of the most avidly transported Pgp substrates, suggesting that very low levels of MDR1 expression may confer clinical resistance to this drug (13). As with doxorubicin, however, we found a wide range of tumor responses to Taxol for specimens that did not express detectable Pgp. This finding may be explained by an inadequate sensitivity of our assay system to detect functional Pgp and/or by alternative mechanisms of Taxol resistance, such as altered tubulin binding, or others that have not yet been characterized (3). The UIC2 shift assay, in which the Pgp expression signal of the two calibrator cell lines with relatively low Pgp levels was enhanced 4-fold in the presence of vinblastine (Table 1), may provide a highly sensitive and specific tool for improved detection of functional Pgp.

In conclusion, the data presented in this study strongly suggest that Pgp confers resistance to MDR1 cytotoxic drugs in breast cancer and that the degree of Pgp expression may significantly contribute to the level of clinical resistance to Taxol and doxorubicin. Prior therapy with MDR1 chemotherapeutic agents may induce Pgp expression and select for Pgp-overexpressing clones at relapse.

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