A New Functional Role for P-Glycoprotein: Efflux Pump for Benzo(a)pyrene in Human Breast Cancer MCF-7 Cells

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Abstract

We propose that the cellular burden of certain carcinogens may be mitigated by P-glycoprotein (P-gp), the putative drug efflux pump. In a series of multidrug resistant human breast cancer MCF-7 cells with increasing P-gp expression we examined this hypothesis using benzo(a)pyrene, a widely distributed environmental and dietary carcinogen. We found that multidrug resistant cells were cross-resistant to benzo(a)pyrene and the rates of efflux for benzo(a)pyrene were higher in multidrug resistant cells than in wild type cells. Evidence supporting the involvement of P-gp included the inhibition of azidopine binding to P-gp by benzo(a)pyrene and the inhibition of benzo(a)pyrene efflux by Adriamycin and verapamil. Our findings suggest that P-gp may play a role in the cellular defense to carcinogens. The expression of P-gp and the modulation of its function may affect the susceptibility of normal tissues to transformation by carcinogens.

Introduction

The factors determining susceptibility of human cells to environmental and dietary carcinogens remain a fundamental question. Investigators have emphasized activation and detoxification mechanisms and have focused on the liver as a first line of defense against ingested agents. We propose an alternative or complementary mechanism; tissue cells can become resistant by pumping out carcinogens to which they have been exposed. An attractive candidate as the mediator for this mechanism is the putative drug efflux pump mediated by a plasma transmembrane glycoprotein.

The Mr 170,000 P-gp encoded by the MDR genes was originally described by Ling et al. (1, 2). In this model, P-gp in cancer cells functions as an energy dependent efflux pump for chemotherapeutic drugs, thereby conferring cellular resistance to these drugs (3, 4). The possibility that it may work as an efflux pump for carcinogens in normal cells is suggested by several known associations. First, in drug resistant cell lines the cellular defense to carcinogens. The expression of P-gp and the modulation of its function may affect the susceptibility of normal tissues to transformation by carcinogens.

Materials and Methods

Materials. Adriamycin and benzo(a)pyrene were purchased from Sigma Chemical Co., St. Louis, MO. [3H]Azidopine was purchased from Amersham Corp., and C219 monoclonal antibody against P-gp was purchased from Centocor, Inc., Malvern, PA. Enlightning—Rapid Autoradiography Enhancer was from New England Nuclear Co.

Cells. Human breast cancer cell line MCF-7 WT and Adriamycin resistant subclone lines were plated at an initial concentration of 3 to 4 x 10^5 cells/25-cm^2 plastic tissue culture flask and grown for 3 or 4 days in RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal bovine serum and 2 mM-glutamine at 37°C in a humid atmosphere containing 5% CO_2. Drug resistant MCF-7 cells were developed from the WT cells by continuous exposure to increasing concentrations of Adr. The IC_50 for WT, R12, R65, and R300 are 0.004, 0.05, 0.258, and 1.2 μM Adr, respectively. The resistant cells are designated for their 12-, 65-, and 300-fold resistance. All cells were tested once a year and found free of Mycoplasma (NCI-FCRDC). The Adr resistant cell line was maintained in continuous exposure to Adr for three passages, and the cells were subsequently passaged in drug free medium at least 3 times prior to experimental use. For all experiments, Adr resistant cells in the 4th to 15th passage (3 population doublings/passage) without drug were replicate plated. Our drug resistant cells are relatively stable. The IC_50 for Adr did not decrease for up to the 25th passage.

Membrane Preparations. Crude membranes were prepared from confluent cells. Cells were homogenized in 10 mL Tris-HCl, pH 7.4-250 mM sucrose-0.1 mM phenylmethylsulfonyl fluoride and centrifuged at 3000 x g. The supernatant fraction was centrifuged at 100,000 x g for 1 h. The cell pellet was resuspended in 50 mL Tris-HCl, pH 7.4, with protease inhibitors (100 units/ml Trasylol, 1 μg/ml pepstatin A, and 30 μM leupeptin).
P-Glycoprotein Determination by Western Immunoblotting. Crude membranes from drug sensitive and resistant cells were separated and analyzed by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transblotted onto a nitrocellulose membrane. P-gp was identified by monoclonal antibody C219 and detected by alkaline phosphatase conjugated second antibody. After enzyme mediated color development, relative P-gp levels were estimated by densitometry.

P-Glycoprotein Determination by [3H]Azidopine Photoaffinity Labeling. Cell membranes (20 μg of protein) prepared from human breast cancer MCF-7 drug sensitive and resistant cells were incubated in a final volume of 35 μl with 1 μl of [3H]azidopine (51 Ci/mmol; Amer sham) at 50 nM for 1 h at 28°C and then irradiated for 15 min at 4°C with UV lamp (UVP, Inc. Model R-52G) as described (15). After irradiation, samples were analyzed on a 4–15% gradient sodium dodecyl sulfate-polyacrylamide gel and labeling of the P-gp band was determined by autoradiography with Enlightning solution and measured by densitometry.

Benzo(a)pyrene Growth Inhibition Study. Benzo(a)pyrene concentration for 50% cell inhibition was determined by growth inhibition assay. Cells were plated in duplicate at 500,000 cells/5 ml RPMI complete medium with 10% fetal calf serum and 2 mM glutamine. Benzo(a)pyrene at different concentrations was added after 24 h of plating and duplicates were set for each concentration. Benzo(a)pyrene was dissolved in dimethyl sulfoxide at various concentrations such that 10 μl added to 5 ml medium yielded the desired final concentration of BP. A volume of 10 μl dimethyl sulfoxide was added to control cells. Cells were counted daily for 5 continuous days.

Benzo(a)pyrene Efflux Study. Benzo(a)pyrene efflux study was monitored by laser cytometry of intact cells. Cells were plated at 2 × 10⁴/ chamber in 4-cm² coverglasses of 2 tissue culture chambers (Nunc) in 1 ml RPMI without phenol red (Biofluids) with 10% fetal bovine serum and 2 mM glutamine. Cells were used after 48–72 h of plating and the medium was changed 24 h before the study. The experiments were performed on the microscope stage of the ACAS (Adherent Cell Analysis and Sorting, Meridian, Okemos, MI) at room temperature using laser excitation at 320 nm. Benzo(a)pyrene in dimethyl sulfoxide, final concentrations of 5 μM and 0.1%, respectively, was added to complete medium as above and the fluorescence monitored during uptake. After about 5 min or when fluorescence reached a predetermined level, the medium was removed, the monolayer was washed twice with 1 ml fresh medium without benzo(a)pyrene, and efflux was monitored in 1 ml fresh medium.

RNA Hybridization by Northern Analysis. Total RNA from drug sensitive and resistant cells was isolated by the guanidinium thiocyanate-CsCl method (16) and separated by electrophoresis (17). RNA was transferred to Nytran membrane (Schleicher and Schuell, Keene, NH) by electroblotting. RNA blot was hybridized with a 32p-labeled complementary DNA (MDR1 specific; a generous gift from M. M. Gottesman) probe and the amount of RNA was standardized with α-actin probe. The RNA values were estimated by densitometry.

Results and Discussion

We proposed that P-gp may be involved in the resistance to carcinogens. To address this hypothesis, we used a series of cells derived from human breast cancer cells, MCF-7, which manifest multidrug resistance with increasing P-gp expression.
These cells were advantageous because detoxification enzymes, e.g., glutathione S-transferase and glutathione peroxidase, which could confound the interpretation of mechanisms mediating resistance, were unchanged in these cells (18). This series of MDR cells derived for resistance to Adriamycin were designated R12, R65, and R300 for their respective fold increase in concentration of Adriamycin necessary for 50% inhibition of cell growth (IC$_{50}$ 0.05, 0.26, 1.2 $\mu$M, respectively) as compared to WT MCF-7 (IC$_{50}$ 0.004 $\mu$M). In WT, neither MDR specific RNA nor P-gp protein was detectable. By contrast, the level of MDR specific RNA increased with increasing resistance; the level of total RNA was measured by Northern analysis, the levels in WT, R12, and R300 were 0.1, 0.76, 2.12, and $3.1 \times 10^3$ arbitrary units by densitometry, respectively; and the levels of P-gp measured by both Western immunoblotting (Fig. 1a) and $[^3]$H]azidopine labeling (Fig. 1b) also increased with increasing resistance. $[^3]$H]Azidopine, the photoactive compound, specifically binds to P-gp and has been used to radiolabel P-gp in tissue culture studies by others (15, 19, 20). We then characterized the efflux of Adriamycin by interactive laser cytometry. Essentially no drug efflux was observed in WT whereas R12, R65, and R300 showed rapid efflux. Importantly, verapamil, a specific inhibitor of P-gp mediated efflux (21, 22) completely inhibited Adriamycin efflux in R65 (data not shown). Thus, this series of MDR cells had markedly increased IC$_{50}$ for Adriamycin, expressed the MDR gene, showed high levels of P-gp, and exhibited verapamil sensitive drug efflux.

It has been shown that exposure to the hepatic carcinogen 2-acetylaminofluorene selects for foci of transformed cells that express high levels of P-gp (6, 7). However, it has not been shown that these cells are resistant to the carcinogen. Having characterized our MDR cells, we investigated their ability to resist BP, widely distributed environmental and dietary carcinogen for skin, respiratory tract, bladder, stomach, and colon. The IC$_{50}$ for BP for these cells was significantly higher in cells expressing P-gp as compared to WT (Fig. 2a). The IC$_{50}$ for BP in WT, R12, R65, and R300 was 0.2, 0.75, 1.24, and 1.75 $\mu$M, respectively. The cross-resistance to BP in our MDR cells is in agreement with observations by Cowan et al. in cells expressing late stages of Adriamycin resistance (5, 8). However, this resistance may occur by mechanisms other than the P-gp mediated efflux pump. To ascertain the role of P-gp, we first studied the interaction of BP with P-gp in crude membrane preparations of R65. We found that BP competed with azidopine by inhibiting $[^3]$H]azidopine labeling of P-gp in a concentration dependent fashion (Fig. 2b). At a concentration of 25 nM, BP inhibited P-gp labeling by 60%; at 100 nM of BP, labeling of P-gp was essentially undetectable (Fig. 2b).

Although these studies suggested the interaction of BP with P-gp, the critical question remained whether BP efflux could be mediated by P-gp. Using interactive adherent cell laser cytometry we monitored BP fluorescence in individual viable cells in monolayer and the efflux curves are shown in Fig. 3. We found that cells expressing P-gp exhibited rapid efflux of BP in R65 (Fig. 3, bottom) compared to the WT cells (Fig. 3, top). Expressed as fractional disappearance per unit time ($\Delta F/F_0/min$). BP efflux rates in WT, R12, R65, and R300 were 0.016 ± 0.002 (SEM), 0.045 ± 0.004, 0.054 ± 0.003, and 0.053 ± 0.002, respectively (Fig. 4). The difference between the rates in resistant cells compared to WT was highly significant ($P < 0.0005$) but these rates were similar among the resistant cells suggesting that mechanisms other than P-gp may be rate limiting for BP efflux. Nevertheless, these results clearly demonstrated that P-gp expression was a necessary mechanism for mediating the efflux of BP. That P-gp mediated the observed efflux was further supported by the inhibitory effects of either Adriamycin or...
widely distributed in fruits and vegetables, may enhance the function of cellular P-gp (23). The possibility that the P-gp of MDR verapamil sensitive efflux of BP and by the BP inhibition of genesis. For example, flavonoids, nonnutrient xenobiotics efflux of activated and unactivated species are important con-

pharmacological factors exert a modulating effect on carcino-

togen resistance and decreased cancer risk is currently under intensive investigation in our laboratory.

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