Expression of the Multidrug Resistance Associated Protein and P-Glycoprotein in Doxorubicin-Selected Human Myeloid Leukemia Cells

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Drug-resistant sublines of the human U-937 myeloid leukemia cell line were selected in doxorubicin concentrations of 10, 40, and 200 ng/mL (designated U-A10, U-A40, and U-A200, respectively). Northern blot analysis showed overexpression of the multidrug resistance–associated protein (MRP) gene, but not MDR1, in U-A10 cells as compared with parental U-937 cells. Prolonged passage of U-A10 cells in 10 ng/mL of doxorubicin had little effect on MRP RNA levels, but increased MDR1 expression. The U-A40 and U-A200 cells, derived by selection of U-A10 cells, showed high levels of both MRP and MDR1 expression. None of the drug-resistant cell lines showed MRP or MDR1 gene amplification as judged by Southern blot analysis. U-A10 cells exhibited minimal decreased net accumulation of anthracycline, whereas U-A40 and U-A200 cells showed more significantly decreased drug accumulation as compared with U-937 cells. Subcellular anthracycline accumulation in U-937 cells as determined by fluorescence microscopy showed daunorubicin fluorescence predominantly in the nucleus. However, the drug-resistant cell lines showed minimal nuclear drug accumulation with marked redistribution of drug into a vesicular compartment. Treatment with sodium azide/2-deoxyglucose, 2,4-dinitrophenol, or monensin, but not verapamil, abolished the vesicular accumulation. These studies in doxorubicin-selected U-937 cells indicate that induction of MRP overexpression occurs before that for the MDR1 gene. In addition, the drug-resistant cells possess an energy-dependent redistribution of anthracyclines into a nonnuclear vesicular compartment.

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MATERIALS AND METHODS

Cell culture and subline derivation. The human myeloid leukemia cell line, U-937, was obtained from the American Type Tissue Culture (Rockville, MD) and was grown in suspension culture in RPMI medium containing 10% fetal bovine serum in a 5% CO2 atmosphere. Doxorubicin-selected, drug-resistant sublines were derived by inoculating U-937 cells into medium containing 5.0 ng/mL (10 nmol/L) of doxorubicin. Subsequently, sublines capable of growing in 10, 40, and 200 ng/mL of doxorubicin were selected and designated U-A10, U-A40, and U-A200, respectively. The U-A10 cell line after 10 (U-A10/p10) or 50 (U-A10/p50) passages in 10 ng/mL of doxorubicin was cryopreserved in liquid nitrogen for later study.

Drugs and chemicals. Doxorubicin was purchased from Adria Laboratories (Columbus, OH), daunorubicin from Wyeth-Ayerst Laboratories (Philadelphia, PA), and [3H]-daunorubicin (specific activity, 1.4 Ci/mmol) from DuPont-New England Nuclear (Boston, MA). The chemicals were from Sigma Chemical Co (St Louis, MO).

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and were used at the following final concentrations: 2-deoxyglucose (50 mmol/L), 2,4-dinitrophenol (2 mmol/L), monensin (5 μg/mL), sodium azide (15 mmol/L), and verapamil (5 μg/mL). Silicone oil, adjusted to a specific gravity of 1.045 to 1.048, was from William F. Nye Co (New Bedford, MA).

**Drug sensitivity assay.** Sensitivity of the cell lines to doxorubicin was determined after 96 hours of drug exposure using an MTT assay performed in quadruplicate at least three times. The IC₅₀ represents the drug concentration producing 50% inhibition of growth. The fold resistances were calculated from the ratio of the IC₅₀ of the drug-resistant cell line to the IC₅₀ of parental U-937 cells.

**DNA probes for hybridization.** The human MRP probe, 10.1, was kindly provided by Dr Susan Cole and Dr Roger Decle (Queens University, Kingston, Ontario, Canada); a human MDR1-specific probe, the 1.3-kb fragment obtained after Xba I/EcoRI restriction endonuclease digestion of plasmid pGEM3Zf(−) Xba I-MDR1/l, was kindly provided by Dr Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). DNA probes for β-actin (V. Stanton, Massachusetts Institute of Technology, Cambridge, MA) and for interleukin-2 (IL-2) were used to control for gel loading. All probes were labeled in gel with a random-primer labeling kit (Boehringer-Mannheim, Indianapolis, IN) to a specific activity of 1 to 2 × 10⁶ cpm/μg of DNA.

**RNA extraction and Northern hybridization.** Total cellular RNA was prepared by lysis of cells with guanidium isothiocyanate and then centrifugation through a cesium chloride cushion. The RNA was extracted with phenol/chloroform and precipitated with ethanol. The RNA was transferred to Gene-Screen Plus hybridization membranes (New England Nuclear, Boston, MA) by blotting with 10× saline sodium citrate (SSC). Membranes were prehybridized for 4 hours then hybridized overnight at 42°C in 50% formamide, 1.0× SSC, 1.0× dextran sulfate, and 1.0 mg/mL denatured salmon sperm DNA. The blots were washed to a final stringency of 0.1× SSC/0.1% SDS at 56°C before autoradiography at −70°C. The blots were hybridized with 0.1 pg/mL of [3H]-daunorubicin in Dulbecco’s phosphate-buffered saline (PBS) supplemented with glucose (1,000 mg/L) at 37°C in the presence or absence of verapamil (5 μg/mL). After 60 minutes, drug accumulation was terminated by sedimenting 200 μM samples through silicone oil as described. Cell-associated radioactivity was determined by scintillation counting.

**Fluorescence microscopy.** Cells (1 × 10⁶/mL) were suspended in PBS-glucose in the presence of daunorubicin (0.05 to 0.5 μg/mL) for 60 minutes at 37°C. In some experiments, cells were treated with PBS (control) or inhibitors for 15 minutes before the addition of daunorubicin (0.5 μg/mL). The cells were then washed twice in PBS (4°C), kept on ice until slides were made, and immediately photographed through an Olympus BH-2 fluorescence microscope (Olympus Optical Co, Tokyo, Japan). Exposures for 4 seconds were made with Kodak TMAX ASA 3200 film or for 40 seconds with Kodak Tri-PanX ASA 400 film.

**RESULTS**

**Doxorubicin sensitivity in U-937 cell lines.** Sensitivity to doxorubicin was determined for U-937, U-A10/p10, U-A10/p50, U-A40, and U-A200 cells utilizing a standard MTT assay. The effect of verapamil treatment (5 μg/mL; final concentration) was determined by adding this agent just before doxorubicin. Verapamil had no detectable effect on doxorubicin sensitivity of parental U-937 cells (Table 1). U-A10/p10 cells showed modest (sevenfold) resistance to doxorubicin that was partially modulated by verapamil (Table 1). After 50 passages in doxorubicin, U-A10 cells showed less sensitivity to doxorubicin (24-fold resistance), which was also partially modulated by verapamil. Selection in 40 ng/mL and 200 ng/mL of doxorubicin resulted in cell lines showing 190-fold and 790-fold resistance to doxorubicin, respectively. In terms of the reduction in fold-resistance, verapamil had the greatest sensitizing effect in the U-A40 and U-A200 cell lines (Table 1).

**Expression of MRP and MDR1 in U-937 and U-A10 cells.** Total RNA from U-937 and U-A10 cells was analyzed for expression of MRP and MDR1. Parental U-937 cells constitutively expressed a 6.5-kb transcript that hybridized to the MRP probe 10.1 (Fig 1). When compared with parental cells, U-A10/p10 cells showed an approximate eightfold increased level of MRP expression (Fig 1). Exposure to doxorubicin for an additional 40 passages resulted in a cell line (U-A10/
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Fig 1. Expression of MRP and MDR1 in U-937 cells. Total RNA (20 μg/lane) from parental cells and U-A10 cells after 10 or 50 passages at 10 ng/mL of doxorubicin. U-A40 cells, and U-A200 cells was resolved by electrophoresis, transferred to a nylon membrane, and then hybridized with 32P-labeled human MRP and MDR1 probes. The blot was reprobed with β-actin to assess RNA loading.

Expression of MRP and MDR1 in cell lines selected in increasing doxorubicin concentrations. The U-937 sublines, selected in doxorubicin concentrations of 40 and 200 ng/mL, were examined for MRP and MDR1 expression by Northern blotting. Both U-A40 and U-A200 cells showed about a 12-fold increased level of MRP expression as compared with parental U-937 cells. Expression of MDR1 was observed in U-A40 and U-A200 cells at levels markedly higher (15-fold) than those observed in U-A10/p50 cells (Fig 1). Taken together, these results suggest that MRP overexpression is an early event in the process of acquired multiple-drug resistance and occurs in cells selected in clinically relevant concentrations of drug after relatively brief exposure times. Cell lines with high-level MDR1 expression are selected in relatively high drug concentration. Modest MDR1 expression can also be seen after prolonged passage at lower drug concentrations.

Analysis of MRP and MDR1 gene amplification. Overexpression of MRP in other doxorubicin-selected cell lines has been associated with MRP gene amplification.25 To assess the presence of MRP gene amplification, genomic DNA was isolated and digested with EcoRI before analysis by Southern blot methods. The MRP probe 10.1 hybridized to a single band of greater than 23 kb in DNA from U-937 cells (Fig 2). The finding that there was no difference in the intensity of the signal in U-A10/p10 (9.9-fold compared with parental U-937 cells) or U-A10/p50 cells (onefold compared with U-937 cells) indicated that the MRP gene was not amplified in these cell lines. Likewise, there was no detectable MDR1 gene amplification in these same cell lines (1.0- and 1.1-fold respectively) (Fig 2). Analysis of the U-A40 and U-A200 cell lines also showed no MRP gene amplification (data not shown).

Analysis of P-glycoprotein expression. P-glycoprotein expression was examined by flow cytometry after staining with MoAb 4E3. The U-A10/p50 cell line showed increased P-glycoprotein expression as compared with parental cells (Fig 3). Progressively more P-glycoprotein was noted in U-A40 and U-A200 cells.

Analysis of anthracycline accumulation. Net accumulation of anthracycline was measured using radiolabeled daunorubicin after 60 minutes of drug exposure. The U-A10/p10
Fig 3. P-glycoprotein expression in U-937 cell lines. Expression of P-glycoprotein was analyzed in the parental cell line (thin line) and the drug-resistant sublines (thick line) by flow cytometry after staining with MoAb 4E3.

Fluorescence Intensity

Cell Number

U937/U-A10

U937/U-A40

U937/U-A200

and the U-A10/p50 cell lines showed minimal decreases in daunorubicin accumulation compared with parental U-937 cells (85% ± 5.4% and 82% ± 7.2%, respectively) that was increased by exposure to verapamil (Fig 4). The U-A40 cell line showed 57% ± 5% and the U-A200 cell line 51% ± 6% of the daunorubicin accumulation observed in parental cells. Net drug accumulation in these sublines was also increased by treatment with verapamil although the steady-state levels were still less than that observed in parental U-937 cells (Fig 4).

Subcellular anthracycline distribution. Daunorubicin distribution was assayed by fluorescence microscopy; used because of its superior fluorescence profile compared with that of doxorubicin. After 60 minutes of daunorubicin loading (final concentration 0.05 to 0.1 μg/mL), parental U-937 cells showed daunorubicin-associated fluorescence in a focal, nonnuclear compartment (Fig 5A). This nonnuclear accumulation was susceptible to photo-bleaching and was most effectively photographed using high-speed film. At higher daunorubicin concentrations (0.2 to 0.5 μg/mL), increased nuclear staining was observed as compared with the subcellular, focal accumulation (Fig 5B and C). Although the amount of vesicular accumulation observed in parental U-937 cells appeared less, the general pattern of daunorubicin accumulation was similar to that previously noted in parental K562 cells.

In contrast with parental cells, U-A10/p10 cells showed marked vesicular daunorubicin accumulation into a nonnuclear compartment that was readily observed at the lowest daunorubicin concentrations of 0.05 to 0.1 μg/mL (Fig 5D). This pattern was noted in essentially every U-A10 cell examined and was resistant to photo-bleaching. Compared with parental U-937 cells, the vesicular compartment appeared to be greatly expanded (Fig 5, A through C compared with D through F). At higher daunorubicin concentrations, substantially less nuclear staining was visible in U-A10 cells compared with parental cells (Fig 5, A through F). This general pattern of expanded vesicular daunorubicin accumulation with redistribution of drug away from the nucleus was also noted in U-A10/p50, U-A40, and U-A200 cells (data not shown).

Effect of inhibitors on subcellular anthracycline distribution. To characterize further the subcellular daunorubicin accumulation observed in drug-resistant cell lines, the effect of a various inhibitors known to deplete cellular adenosine triphosphate (ATP) levels and/or disrupt membrane pH gradients was analyzed in U-A10/p10 cells by fluorescence microscopy. All inhibitors were added 15 minutes before a 60-minute daunorubicin exposure (0.5 μg/mL). The combination of 2-deoxyglucose/sodium azide abolished the subcellular, focal accumulation (Fig 5, B and C). Although the amount of vesicular accumulation observed in parental U-937 cells appeared less, the general pattern of daunorubicin accumulation was similar to that previously noted in parental K562 cells.

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Fig 5. Subcellular distribution of daunorubicin in U-937 cells. Parental U-937 cells (A through C) and U-A10/P10 cells (D through F) were exposed to daunorubicin at 0.1 μg/ml (A and D), 0.2 μg/ml (B and E), and 0.5 μg/ml (C and F). After 60 minutes, the cells were washed, visualized by fluorescence microscopy, and then photographed. Shown are photomicrographs of typical single cells.

lular vesicular accumulation (Fig 6A). This finding suggested the vesicular accumulation was an energy-dependent process. It was further verified by the use of 2,4-dinitrophenol, a proton ionophore and uncoupler of oxidative phosphorylation, which also eliminated daunorubicin vesicular accumulation (Fig 6B). Monensin, a carboxylic ionophore that disrupts vesicular proton gradients associated with lysosomal compartments, but does not uncouple oxidative phosphorylation, similarly eliminated vesicular daunorubicin accumulation (Fig 6C). Verapamil, a P-glycoprotein modulating agent, had no effect on the pattern of vesicular accumulation in U-A10 cells (Fig 6D). Examined together, these results suggest that the vesicular daunorubicin accumulation observed in the drug-resistant cell lines is an energy-requiring process that is dependent on membrane proton gradients and independent of P-glycoprotein.

DISCUSSION

The full-length cDNA encoding MRP was cloned from a doxorubicin-selected human lung carcinoma cell line, H69AR, that does not overexpress P-glycoprotein. H69AR cells show significant cross-resistance to other anthracyclines, vinca alkaloids, and epipodophyllotoxins that is not reversed by treating with classic P-glycoprotein modulating agents, such as verapamil. Although MRP and P-glycoprotein are both members of the ATP-binding cassette superfamily (ABC) of proteins, they share only limited sequence identity in regions involving the nucleotide binding domains. The function of MRP in mediating drug-resistance is unclear because MRP overexpressing cells do not consistently show changes in net drug accumulation.

That MRP may be important in acquired drug resistance in myeloid leukemia has been suggested by independent studies of two doxorubicin-selected sublines of the human leukemia cell line, HL-60. Both drug-resistant isolates express MRP in the absence of P-glycoprotein. Each of these variants shows decreased steady-state accumulation of anthracyclines and possesses changes in subcellular anthracycline transport with a redistribution of drug away from the nucleus into a cytoplasmic compartment.

The doxorubicin-selected U-937 variants we have described are the first report of drug-selected cell lines that simultaneously overexpress MRP and MDRI. The studies also provide potential insights into the temporal relationship between MRP and MDRI overexpression. Selection of U-937 cells in a clinically relevant concentration of doxorubicin
(10 ng/mL)\textsuperscript{47} for as few as 10 passages, resulted in a cell line that overexpressed MRP, but not MDR1 (Fig 1). Overexpression of MRP was not associated with gene amplification (Fig 2). Only when U-A10 cells were exposed to 10 ng/mL of doxorubicin for a prolonged time period (Fig 1) or when selected for relatively high-level doxorubicin resistance, did MDR1 and P-glycoprotein overexpression become apparent (Table I; Figs 1 and 3).

Radiolabeled drug transport studies showed that U-A10 cells had minimally decreased daunorubicin accumulation compared with the parental cell line (Fig 4). The ability of verapamil to reverse this accumulation deficit suggested that P-glycoprotein was in part responsible; however, U-A10/p50 cells, which have substantially more P-glycoprotein than U-A10/p10 cells, exhibited a similar accumulation profile (Fig 3). Thus, P-glycoprotein expression may only partially explain the transport changes observed in U-A10 cells.

A striking phenotypic change in the drug-resistant cells was a redistribution of anthracyclines into an apparently expanded vesicular compartment. In parental U-937 cells at relatively low daunorubicin concentrations (0.05 to 0.1 µg/mL), daunorubicin fluorescence appeared in a focal pattern suggestive of cytoplasmic organelle accumulation (Fig 5A). As the external daunorubicin concentration was increased (0.2 to 0.5 µg/mL), nuclear staining predominated (Fig 5B and C). However, in U-A10 cells at all drug concentrations, daunorubicin fluorescence appeared distributed into expanded, cytoplasmic vesicles (Fig 5D through F). Inhibitor studies implied this vesicular accumulation was independent of P-glycoprotein and occurred in an energy-requiring manner dependent on vesicular proton gradients (Fig 6).

The anthracyclines traverse the plasma membrane by passive diffusion.\textsuperscript{48,49} It was shown in the human myeloid leukemia cell line, K562, that drug is accumulated in an energy-dependent manner into a subcellular vesicular compartment.\textsuperscript{52} Parental U-937 cells also appear to accumulate anthracyclines in this manner (Fig 5A). This compartment may be part of the trans-Golgi or lysosomal network that is
known to contain an acidic pH. Upon entering such a compartment, the anthracyclines would become protonated and trapped at the low pH. Although the anthracyclines may preferentially accumulate in this vesicular compartment in parental, drug-sensitive cells, the capacity for vesicular drug uptake appears limited. As the external drug concentration is increased, more drug appears to accumulate in the nucleus than the vesicular compartment (Fig 5, A through C).

In drug-resistant sublines, the vesicular compartment appears to have greatly expanded and with it, the potential for total drug accumulation. In U-A10 cells, as the external daunorubicin concentration is increased, nuclear staining is minimal, even at the highest drug concentration tested. Drug appears trapped in this expanded intracellular compartment and may be prevented from reaching its cytotoxic target, topoisomerase II, in the nucleus. Although the ultimate fate of the entrapped anthracyclines is unclear, studies of exocytic vesicular pathways suggest that the drug may eventually be externalized. This process could then contribute to an overall net decreased drug accumulation.

The role of MRP in vesicular anthracycline accumulation has yet to be proven. Indeed, a protein of M, 110,000 has also been identified as possibly involved in this process. The manner in which anthracyclines traverse the intracellular vesicular membrane is also unknown. However, members of the ABC family, to which MRP belongs, transport substrates across a membrane in an ATP-requiring manner. Whether MRP is actively transporting the drug into the vesicles, or is indirectly involved by facilitating the expanded vesicular compartment, or functioning in another manner awaits further investigation. However, to date there has been a consistent correlation between reported cell lines that overexpress MRP and the demonstration of anthracycline subcellular redistribution.

Our studies suggest that in drug-selected cell lines, high-level P-glycoprotein expression occurs relatively late and may be preceded by the expression of other acquired resistance mechanisms. In the case of human myeloid leukemia, MRP may play a significant role. Similar findings where P-glycoprotein expression occurs only in cell lines selected for relatively high-level resistance have been shown in murine leukemia cell lines or have been shown to occur in the setting of acquisition of other non-P-glycoprotein-mediated resistance mechanisms. Furthermore, in drug-resistant HL-60 cells, either P-glycoprotein expression has not been observed, or has been shown to be part of a complex phenotype involved with other cellular changes. Taken together, these in vitro studies suggest that acquired multidrug resistance is multifactorial and that clinical trials that selectively modulate P-glycoprotein are likely to achieve limited success. The present findings in human myeloid leukemia suggest that the role of MRP in acquired resistance may need to be considered as well.

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