Overexpression of the Multidrug Resistance-associated Protein (MRP) Gene in Vincristine but not Doxorubicin-selected Multidrug-resistant Murine Erythroleukemia Cells

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ABSTRACT

Multidrug-resistant sublines of the murine erythroleukemia cell line PC4 were sequentially selected in increasing vincristine concentrations (5–160 ng/ml). The low- and intermediate-level resistant cell lines, selected in ≤40 ng/ml of vincristine, demonstrated resistance to Vinca alkaloids and to an epipodophyllotoxin but little or none to an anthracycline. The expression of murine mdr genes, as analyzed by Northern blotting, revealed a baseline expression of murine mdr2 in parental cells that was unchanged in the drug-resistant cell lines. Overexpression of mdr3 was observed only in the highest-level resistant cell line, PC-V160, whereas mdr1 mRNA was not detected in any of the cell lines. The polymerase chain reaction, using mdr3-specific primers, excluded the possibility that low levels of P-glycoprotein expression contributed to the resistance phenotype in the low and intermediate-level resistant cell lines. Northern blot analysis using a human complementary DNA probe for the multidrug resistance-associated protein (MRP) demonstrated overexpression of murine mrp in each of the vincristine-selected sublines. Genomic amplification of the mrp gene was coincident with mrx overexpression. The expression of mrp was also examined in two series of previously characterized doxorubicin-selected cell lines derived from parental PC4 and C7D murine erythroleukemia cells. In contrast to the vincristine-selected cell lines, overexpression of mrp was not detected. These studies demonstrate that, in murine erythroleukemia cells selected for vincristine resistance, overexpression of murine mrp occurred prior to that for murine mdr. In contrast to human MRP, selection for vincristine, but not doxorubicin resistance, resulted in the overexpression of mdr3.

INTRODUCTION

Resistance to chemotherapeutic agents is a major cause of failure in the treatment of human malignancies. Selection of resistant cells in vitro by a single anticancer agent, such as an anthracycline, a Vinca alkaloid, or a taxane often leads to cells with cross-resistance to all of these drugs (1–3). In many instances, these multidrug-resistant cell lines overexpress a M, 150,000–180,000 plasma membrane protein, termed P-glycoprotein. Resistant sublines show decreased intracellular drug accumulation associated with energy-dependent efflux of cytotoxic agents, linked to one or more members of the family of P-glycoproteins (4). The murine and human mdr genes which code for P-glycoprotein have been isolated, the cDNAs cloned, and the sequences determined (5, 6).

Multidrug resistance in the clinical setting appears to be multifactorial and may involve mechanisms besides P-glycoprotein overexpression (7). In fact, analysis of drug-resistant cell lines, particularly those selected in anthracyclines or epipodophyllotoxins, has uncovered other resistance mechanisms (8–13). In this regard, the cDNA encoding the MRP was cloned from a doxorubicin-selected, non-P-glycoprotein expressing, human lung carcinoma cell line (14). Transfection of the full-length cDNA into a parental, drug-sensitive cell line has been shown to result in diminished sensitivity to doxorubicin, vincristine, and etoposide (15).

Few non-P-glycoprotein expressing cell lines have been described after selection for Vinca alkaloid resistance (16). In fact, only cells selected in anthracyclines or epipodophyllotoxins, but not Vinca alkaloids, have been reported to overexpress MRP (17–22). Furthermore, the role of MRP in acquired drug resistance in murine cell lines is unknown since a mouse cell line that overexpresses MRP has also yet to be characterized.

We have previously reported a series of multidrug-resistant, murine erythroleukemia cell lines sequentially selected in increasing concentrations of doxorubicin (23). In these cell lines, overexpression of P-glycoprotein occurred only as a late event in highly resistant sublines. We now describe multidrug-resistant cells of the same parental line PC4, selected in increasing concentrations of vincristine. Again, only in the highest-level vincristine-selected cell line was P-glycoprotein overexpression, attributable to murine mdr3, detected. In contrast, overexpression of mrp was evident even in the low-level vincristine-selected cell lines. Overexpression of mrp was not detected in the doxorubicin-selected murine erythroleukemia cells.

MATERIALS AND METHODS

Cell Culture and Subline Derivation. The murine erythroleukemia cell line PC4 (obtained from D. Housman, Massachusetts Institute of Technology, Cambridge, MA) is a subclone of MEL cell line 745 derived by C. Friend (Mt. Sinai Hospital, NY). It was grown in suspension culture at 37°C using Eagle’s basal medium supplemented with 10% heat-inactivated fetal calf serum in a 5% CO₂ atmosphere. Drug-resistant cell lines were obtained by inoculating PC4 cell lines into culture medium containing 5 ng/ml of vincristine sulfate (Eli Lilly, Indianapolis, IN). After 10 passages at 5 ng/ml, the culture was split with a portion continued at 5 ng/ml and a portion inoculated into 10 ng/ml. After every 10 passages, a new subline was initiated into a 2-fold higher vincristine concentration. The resulting sublines, PC-V5, PC-V10, PC-V20, PC-V40, PC-V80, and PC-V160, were named according to the drug concentration producing 50% inhibition of growth; poly(A)⁺, polyadenylated; SSPE, saline-sodium phosphate-EDTA; SDS, sodium dodecyl sulfate; RT-PCR, reverse transcription-polymerase chain reaction.
sublines were grown in the absence of drug for a minimum of 1 month prior to further studies. Sublines selected in 40 and 160 ng/ml were subcloned by limiting dilution, and five clones from each concentration were generated. When assayed for vincristine sensitivity, all clonal sublines demonstrated less than a 10% variation as compared with the uncloned cell lines selected at the same drug concentration. For all additional studies, the uncloned sublines were used. Doxorubicin-selected sublines of the MEL cell lines, PC4 and CTD, were described previously (23).

Drug Sensitivity Assays. Sensitivity of each cell line to vincristine, vinblastine (Cetus Corp., Emeryville, CA), doxorubicin (Adria Laboratories, Columbus, OH), and etoposide (Bristol-Myers Co., Evansville, IN) was determined in a 48-h growth inhibition assay (23), while that for colchicine was determined in a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (24). The IC50 was the drug concentration producing 50% inhibition of growth. The fold resistances were calculated from the ratio of the IC50 of the resistant cell line to the IC50 of the parental cell line.

DNA Probes for Hybridization. The nonspecific murine mdr probe, pcDR1.3, was provided by D. Housman; the murine mdr1, mdr2, and mdr3 gene-specific probes were provided by D. Housman and P. Gros (McGill University, Montreal, Quebec, Canada); the human mdr probe, 10.1, was provided by S. Cole and R. Deele (Queen’s University, Kingston, Ontario, Canada); the probe for human β-actin was obtained from V. Stanton (Massachusetts Institute of Technology); and that for the murine JH5 gene was provided by K. Barnett (V. A. Medical Center, Boston, MA). All probes were labeled in-gel (Seaplaque agarose; FMC, Rockland, ME) to a specific activity of 1–2 × 106 cpm/μg DNA with a random primer labeling kit (Boehringer Mannheim, Indianapolis, IN).

RNA Extraction and Northern Hybridization. Total cellular RNA was prepared by lysis of cells with guanidine isothiocyanate and then centrifugation through a cesium chloride cushion (25). Poly(A)+-enriched RNA was prepared from total RNA extracted from 1 × 109 logarithmically grown cells as described previously (26). Approximately 1200–1500 μg of total RNA were loaded onto a 100-μl oligodeoxythymidylate cellulose column (Collaborative Research, Bedford, MA). The eluted RNA was quantified by absorbance at A260. After treatment with glyoxal buffer, poly(A)+ RNA (5 μg/lane) or total RNA (20 μg/lane) was subjected to electrophoresis through 1% agarose and then blotted in 10× SSC (0.15 M NaCl, 0.15 M Na citrate) to Gene-Screen Plus (New England Nuclear, Boston, MA). The membranes were prehybridized for 4 h, then hybridized overnight in 50% formamide, 0.1× SSC-0.1% SDS, and then transferred by blotting with 10× SSC. The membrane was prehybridized, hybridized, and then washed as described above before autoradiography at −70°C.

Statistical Analyses. Confidence intervals of 95% were determined for the fold resistance values by calculating the average differences and variance of the log values of the IC50s and then estimating the fold resistance and 95% confidence intervals, assuming log normal distributions (23). All statistics were calculated with software programs 1–2–3 (Lotus Development Corp., Cambridge, MA) or StatView (Brainpower Inc., Calabasas, CA).

RESULTS

Resistance Phenotypes of Vincristine-selected PC4 Sublines. The PC-V5 and PC-V10 cell lines showed stable vincristine resistance (5.5- and 11-fold), vinblastine resistance (4.8-fold for both cell lines), etoposide resistance (2.8- and 3.8-fold), and colchicine resistance (2.5- and 3.1-fold) but were without significant doxorubicin resistance (Table 1). Passage of PC-V10 cells into 20 ng/ml of vincristine yielded a cell line, PC-V20, that modestly increased its vincristine resistance but led to little or no change in vinblastine or colchicine susceptibility and a slight, although statistically significant, doxorubicin resistance. Etoposide resistance increased nearly 5-fold. With selection of sublines in progressively higher concentrations of vincristine, resistance to vincristine and vinblastine increased in a stepwise manner, whereas etoposide and colchicine resistance appeared to plateau. Modestly decreased doxorubicin sensitivity was followed by more marked resistance in the highest vincristine-selected cell line, PC-V160.

Expression of the Murine mdr Gene Family. The murine genome contains three mdr genes known to be expressed in a tissue-specific manner (25). In order to assess mdr gene expression, poly(A)+-enriched RNA from each of the cell lines was probed sequentially by Northern blot analysis with the mdr gene-specific probes. Neither the PC4 cell line nor any of the vincristine-selected sublines expressed a detectable level of mdr1, even with prolonged film exposure (data not shown). The parental PC4 cell line expressed a 4.5-kilobase message that hybridized with the mdr2 gene-specific probe. The expression of mdr2 appeared unchanged in any of the vincristine-selected sublines (Fig. 1). Hybridization with the mdr3 gene-specific probe detected a 4.5-kilobase and a 5.0-kilobase message that was present only in PC-V160 cells (Fig. 1).

Analysis of mdr3 Expression by PCR. To examine for the presence of mdr3 transcripts not detected by Northern blotting, poly(A)+-enriched RNA was reverse transcribed to cDNA in the PC4-WT, PC-V40, PC-V80, and PC-V160 cell lines before amplification using
Table 1 Drug Resistance in vincristine-selected sublines

<table>
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<th>Fold resistancea</th>
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<th>VBL</th>
<th>DOX</th>
<th>VP-16</th>
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<td>1</td>
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<td>PC-V5</td>
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<td>(0.8-1.4)</td>
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<tr>
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<td>4.8</td>
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<td>(10-12)</td>
<td>(4.6-5.1)</td>
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</table>

a The results are reported as fold resistant over the parental cell line. The numbers in parentheses represent the 95% confidence intervals for the fold resistance.

b VCR, vincristine; VBL, vinblastine; DOX, doxorubicin; VP-16, etoposide; COL, colchicine.

mdr3-specific primers. PC4-WT and PC-V40 cells demonstrated no detectable PCR product after electrophoresis and ethidium bromide staining (Fig. 2A). The PC-V80 subline showed a barely detectable 708-base pair PCR product that was easily seen in the PC-V160 line. The reverse transcription and amplification was repeated with mdr3 primers as well as positive control RNA and positive control primers. Similar results were obtained with no detectable mdr3 mRNA in PC4-WT or PC-V40 cells, but a positive control band of appropriate size was generated in near-equal intensity in all the cell lines (data not shown). To verify that the 708-base pair product generated from the PC-V80 and PC-V160 cell lines was solely due to mdr3, we digested a portion of the product with PmlI and another portion with NspI. PmlI digested the product to completion and yielded two fragments of approximately 120 and 590 base pairs in length, while NspI digested the product to completion and produced approximately 280- and 430-base pair products. To increase sensitivity, the PCR products were transferred to a membrane and probed with the radiolabeled mdr3-specific PCR product generated from the PC-V160 cell line (Fig. 2B). The reaction from the PC-V160 subline showed some size heterogeneity not appreciable on the ethidium bromide-stained gel. A band of markedly decreased intensity was present in PC-V80 cells. However, PC-V40 and PC4-WT cells had no bands detected by autoradiography even with prolonged film exposure.

Southern Blot Analysis of mdr Gene Family. Genomic DNA was extracted from the PC4-WT cell line and each of the vincristine-selected sublines. Southern blot analysis using the nonspecific murine mdr probe pCDR1.3 and the mdr2 and mdr3 gene-specific probes showed no mdr gene amplification in any of the cell lines. Shown in Fig. 3 are the results from PC4-WT, PC-V80, and PC-V160 cell lines. Taken together, these results show that the murine mdr gene is overexpressed in PC4 cell lines selected for high-level resistance.

Expression of mdr in Vincristine-selected Sublines. The parental line and the vincristine-selected sublines were examined for the expression of mdr. Transcripts of mdr were not detected in PC4 cells by Northern blotting of total RNA (Fig. 4). By contrast, an approximate 6.0-kilobase message hybridized with the human MRP probe, 10.1, in each of the vincristine-selected sublines (Fig. 4). The level of expression was modest in PC-V5 (data not shown) and PC-V10 cells but distinctly higher in PC-V20 cells. In PC-V40, PC-V80, and PC-V160 cells, the levels of mdr expression were comparable to PC-V20 cells (Fig. 4).

Analysis of mdr Gene Amplification. Overexpression of MRP in human cell lines has been associated with amplification of the MRP gene (14). Amplification of the murine mdr gene was assessed by Southern blot methods after isolation of genomic DNA and restriction endonuclease digestion with EcoRI. A single band of approximately 8.5 kilobases was seen in DNA from parental PC4 cells, which was increased in PC-V5 and PC-V10 cells (Fig. 5). A further increase was noted in PC-V20 cells, which likewise remained at high levels in PC-V40, PC-V80, and PC-V160 cell lines (Fig. 5). Taken together, these results indicate that the murine mdr gene is overexpressed in PC4 cell lines selected for low, intermediate, and high-level vincristine resistance. Associated with mdr overexpression is amplification of the mdr gene.

Expression of mdr in Doxorubicin-selected Sublines. Since mdr expression has been predominately associated with doxorubicin-selected cell lines, we examined the expression of mdr in two doxorubicin-selected sublines, including one series derived from PC4 cells that we have described previously (23). Northern hybridization of total RNA revealed no detectable mdr message in the parental or the doxorubicin-selected PC4 or C7D sublines (not shown). To increase sensitivity, the PCR products were transferred to a membrane and probed with the radiolabeled murine mdr gene-specific probes. Reprobing the same blot with a β-actin probe confirmed near-equal loading of all the lanes.

Fig. 1. Expression of mdr genes in parental and vincristine-selected cell lines. Poly(A)+-enriched RNA (5.0 µg/line) was resolved by electrophoresis through 1% agarose, transferred to a nylon membrane, then sequentially hybridized to 32P-labeled murine mdr gene-specific probes. Reproducing the same blot with a β-actin probe confirmed near-equal loading of all the lanes.
sion appeared to be moderately decreased compared to parental cells. In C7D cells, a 6.0-kilobase message was also visible after hybridization with the human MRP probe (Fig. 7). The level of expression was similar in C7D-5 and C7D-10 cells. These studies demonstrate that the PC4 and C7D cell lines, when selected for doxorubicin resistance, do not overexpress *mrp*.

**DISCUSSION**

The series of vincristine-selected murine erythroleukemia cells described in this report provide new insights into the process of acquired drug resistance. Efforts to determine how tumor cells become insensitive to chemotherapeutic agents have resulted in the identification of several novel drug resistance mechanisms (14, 29, 30). The relationship between the expression of these individual mechanisms and how they may interact to yield a complex multidrug-resistant phenotype are areas of active investigation. Both the P-glycoprotein and the MRP are members of the ATP-binding cassette superfamily of transport proteins; each has been demonstrated to cause multidrug resistance in vitro (15, 31). Both contain 12 transmembrane spanning domains but share only limited sequence similarity near the regions of the ATP-binding cassette (14, 32). The relationship, if any, between the expression of these two genes in multidrug-resistant tumors is currently unknown.

Resistance to the *Vinca* alkaloids has almost exclusively been associated with P-glycoprotein-mediated multidrug resistance. Various vincristine or vinblastine-selected mammalian cell lines have been derived that demonstrate energy-dependent, decreased accumulation of these agents linked to the overexpression of the P-glycoprotein or...
are capable of simultaneous overexpression of *mrp* and *mdr*, yet during the process of selection for progressive vincristine resistance, only *mrp* was overexpressed at every level of vincristine resistance. Since the process of acquired high-level multidrug resistance may involve more than one acquired resistance mechanism, these data show that *mrp* overexpression may occur as a relatively early event, prior to that of *mdr* expression.

In the low-level resistant sublines, the levels of *mrp* expression and amplification increased progressively with increasing levels of resistance. However, in the intermediate-level resistant cell lines, the levels of *mrp* expression and amplification appeared to reach a plateau; there was little difference in the levels of *mrp* expression between the PC-V20, PC-V40, and PC-V80 cell lines (Fig. 4). The reason for this difference cannot be determined with certainty since the precise mechanism by which *mrp* produces drug resistance is not yet understood. Perhaps when *mrp* is expressed at high levels, small differences in expression that are not detectable by Northern blotting may be sufficient to cause more dramatic differences in the levels of resistance. Another possibility is that, in the intermediate-level resistant cell lines, *mrp* activity may be modulated by posttranslational modifications, such as phosphorylation, that would not be reflected in the Northern analysis. Finally, the increased levels of resistance in the intermediate-level cell lines without apparent change in *mrp* expression may indicate that the cells have acquired other non-*mrp*/non-*mdr* resistance mechanisms.

Examination of the resistance phenotypes in the *mrp*-expressing cell lines revealed that cross-resistance to the anthracycline doxorubicin was not detected in the lowest-level resistant sublines, PC-V5 and PC-V10 (Table 1). Likewise, despite high-level *mrp* expression in the PC-V20 and the PC-V40 cell lines, only minimal resistance to doxorubicin (1.8- and 6.0-fold, respectively) was seen. The lack of significant cross-resistance to doxorubicin was somewhat unexpected since the transfection studies of human *MRP* demonstrated that its overexpression resulted in diminished doxorubicin cytotoxicity (15). Furthermore, doxorubicin has been the most frequently reported se-

Fig. 5. Amplification of the *mrp* gene in vincristine-selected cell lines. Genomic DNA was isolated from each of the cell lines, digested to completion with EcoRI, resolved by electrophoresis, transferred to a nylon membrane, and then probed with 32P-labeled human *MRP* probe, 10.1. Reprobing the blot with a murine *JH*4 probe showed near-equal loading of the lanes.
MRP IN VINCristINE-SELECTED MEL CELLS

Fig. 7. Expression of the mdr gene in doxorubicin-selected C7D cells. Poly(A)\(^+\)-enriched RNA was isolated from parental C7D cells and sublines selected in doxorubicin concentrations of 5 and 10 ng/ml. The RNA was resolved by electrophoresis (5 μg/lane), transferred to a nylon membrane, and then hybridized with \(^{32}P\)-labeled human MRP probe, 10.1. Reprobing with a β-actin probe confirmed equal loading of the lanes.

selecting agent for cells that overexpress MRP (17, 19—22). Until murine mdr is isolated and characterized, the reason for minimal doxorubicin resistance remains speculative. One explanation could be that the native murine mdr gene may preferentially encode for Vinca alkaloid, as opposed to anthracycline, resistance. This possibility was also suggested by the lack of overexpression of mdr when the same parental PC4 cell line and another independently derived murine erythroleukemia cell line were selected for doxorubicin resistance (Figs. 6 and 7). Another possibility is that the mdr gene overexpressed in the vincristine-selected PC4 cell lines has acquired a mutation, resulting in a change in amino acid composition with subsequent preferential Vinca alkaloid resistance. A change in substrate affinity has been linked to an amino acid change for P-glycoprotein in drug-selected cell lines (37, 38).

This series of vincristine-selected sublines, in addition to providing insight into the temporal relationship between the expression of mdr and mdr genes, addresses other issues relevant to understanding acquired drug resistance. The importance of mdr expression that is detected only by using RT-PCR is unclear, especially in the evaluation and non-P-glycoprotein mediated forms of multidrug resistance will help in defining the biochemical and genetic basis of clinically acquired drug resistance and may provide a rational basis for the use of chemosensitizing agents early in the process of acquired multidrug resistance.

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