In Situ Hybridization Analysis of Acquisition and Loss of the Human Multidrug-Resistance Gene

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ABSTRACT

The extent of multidrug-resistance of human KB carcinoma cell lines has been shown to be proportional to the level of expression of the MDR1 gene. Using an in situ hybridization analysis with 35S-labeled RNA probes, we have found that there is some heterogeneity in expression of the MDR1 gene from cell to cell, but that the average level of expression is proportional to the resistance of the cell line. In the absence of selective pressure, a colchicine-selected multidrug-resistant population with a highly amplified MDR1 gene loses its resistance in parallel with the loss of the amplified gene. Loss of resistance also parallels a decrease in MDR1 RNA expression in the whole cell population. Loss of MDR1 expression in this population is highly heterogeneous, with small clusters of cells maintaining expression even after the population as a whole has become relatively sensitive. This heterogenous loss of expression of the MDR1 gene is consistent with random segregation of amplified DNA segments in the selected cells. The analysis of MDR1 RNA expression by in situ hybridization which is validated by this study should be useful in the study of normal human tissue and tumor samples expressing the MDR1 gene.

INTRODUCTION

The intrinsic or acquired resistance of tumor cells to multiple chemotherapeutic agents is a major barrier to successful cancer chemotherapy. In tissue culture model systems, resistance has been shown to be proportional to the level of expression of the mdr gene, which encodes the cell membrane P-glycoprotein (1–5). Recent sequence data and direct drug and ATP-binding data indicate that P-glycoprotein is likely to be an energy-dependent drug efflux pump (6–12). Expression of cloned cDNAs for the human and mouse mdr genes confers multidrug resistance on sensitive cells (13–14), indicating that levels of expression of P-glycoprotein are limiting for development of the multidrug resistance phenotype.

Several normal human tissues have been shown to express the MDR1 gene at moderate levels, including the adrenal, kidney, liver, and colon (15). Intrinsically drug-resistant tumors derived from these tissues, as well as some tumors which have acquired drug resistance during the course of chemotherapy, also express the MDR1 gene (15). It is important to develop in situ hybridization techniques for the analysis of expression of the MDR1 gene in individual cells to determine which cell types in specific organs are expressing this gene and to use as a sensitive diagnostic test for the expression of the MDR1 gene in human tumors. To validate such an assay, we have used in situ hybridization to study expression of the MDR1 gene in a series of sequentially selected multidrug-resistant human KB carcinoma cell lines. At the highest levels of resistance, these cell lines contain multiple single and double minute chromosomes and highly amplified MDR1 genes (2, 16). In a series of revertants derived from the most highly resistant line, the copy number of the amplified MDR1 gene decreases as MDR1 RNA expression falls. Our results confirm the validity of the in situ hybridization assay for MDR1 mRNA and also reveal that reversion to a more drug-sensitive phenotype in the absence of selective medium occurs in a highly heterogeneous manner, a possible result of the unequal segregation of extrachromosomal elements to daughter cells.

MATERIALS AND METHODS

Cell Culture and Isolation of Revertants. The human KB carcinoma cell line subclone KB-3-1 and its multidrug-resistant derivative KB-C6 [selected and maintained in medium containing 6 μg of colchicine (Sigma) per milliliter] were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO) and 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (Flow) (2, 17–19). Revertant populations of KB-C6 cells were studied at intervals of from 20 to 150 days while maintaining cells in colchicine-free medium.

Cell Killing. For the colchicine-resistance test, KB-C6 cells and its revertants were plated at 5 x 10^4/35-mm dish, and the drug was added 16 h after cells were seeded. Cell numbers were determined with a Coulter Counter after 3 days of incubation at 37°C. Relative percentage of cell survival in the presence of colchicine was calculated by dividing the LD_50 value (level of dose reducing cell number by 50%) for KB-C6 and its revertants by the LD_50 value for the parental cell line KB-3-1. This assay gives similar results to cell killing assays based on cloning efficiency (17).

Isolation of Nucleic Acids and Filter Hybridization. The human genomic DNAs were prepared from KB-3-1, KB-C6 and its revertants by proteinase K-SDS digestion, followed by extraction with buffer-saturated phenol-chloroform-isooamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). RNA was digested with RNase (200 μg/ml) (Sigma) at 37°C for 45 min. DNA was precipitated with sodium acetate and ethanol and dissolved in TE buffer. Total cellular RNA was isolated by the methods of Chirgwin et al. (20) and Maniatis et al. (21). Cells were washed twice with phosphate-buffered saline and lysed with guanidine thiocyanate buffer, followed by homogenization in the same buffer. Each homogenate was layered over a CsCl cushion and centrifuged for 20 h at 32,000 rpm in a Beckman SW-40 rotor. RNA pellets were purified with phenol-chloroform and then with chloroform-butanol, precipitated twice, and dissolved in TE buffer.

HindIII was purchased from Bethesda Research Laboratories, Inc., and digestions were carried out under conditions recommended by the supplier. Digested DNA samples were analyzed by electrophoresis on 0.8% agarose in 1 x TBE buffer and transferred from the gel to nitrocellulose paper by the method of Southern (22). RNA samples were analyzed by using a slot blot apparatus (Schleicher & Schuell, Inc.). Filters were baked in a vacuum oven for 2 h at 80°C. The probe used was labeled to specific activities of 2 x 10^6 dpm/μg by nick translation (19). Baked filters were incubated for 4 to 6 h at 42°C in 50% formamide/5X SSC/10X Denhardt's solution, 0.1% SDS/100 μg of sonicated salmon sperm DNA per milliliter and then hybridized overnight in the same solution containing 35S-labeled probe. Filters were washed three times for 10 min each time at room temperature in 0.1% SDS.

Received 9/21/87; revised 12/31/87, 4/26/88; accepted 4/28/88.

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The abbreviations used are: SDS, sodium dodecyl sulfate; SSC, standard saline citrate (0.15 M sodium chloride: 0.015 M sodium citrate, pH 7.4); TE, tris-EDTA (0.01 M Tris-Cl, pH 7.4, 0.1 mM EDTA); TBE, tris-borate-EDTA (6.05 M Tris-Cl, 0.05 M Boric acid, 1 mM EDTA).

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MDR RNA DETECTION BY IN SITU HYBRIDIZATION

RESULTS

Acquisition of Multidrug Resistance Studied by in Situ Hybridization. Expression of the MDR1 gene in a series of colchicine-selected multidrug-resistant cell populations and in a revertant cloned from this line after several months of growth in nonselective medium has been previously described (2). Using a nick-translated 32P-labeled cDNA probe representing the midportion of the MDR1 gene (clone 5A; 23) it was possible to detect expression of the MDR1 gene in blots of total RNA from all resistant cell lines, including cell line KB-8, which is only twofold resistant to the selecting drug (Table 1) (2). Use of a 35S-labeled RNA probe made from the same cDNA gave similar sensitively by in situ hybridization (Fig. 1 and Table 1). Several observations can be made from the representative in situ hybridizations shown in Fig. 1 and quantitated in Table 1: (a) the number of grains observed per cell on the average increases with increasing drug-resistance of the cell lines. This conclusion is confirmed by counting total 35S-labeled RNA associated with the slides (Table 1, last column); (b) there is some heterogeneity of expression of the MDR1 gene, even in cell lines such as KB-8, KB-8-5, and KB-8-5-11 which are derived from cloned cells and have been kept in selective medium for several months; and (c) there is no obvious variation in MDR1 expression seen between mitotic and interphase cells. This study validates the in situ hybridization technique as a way to semiquantitatively determine expression of the MDR1 gene in cultured cells.

Loss of the Amplified MDR1 Gene in the Absence of Selective Pressure. We have previously reported that multidrug-resistant KB cells selected in either colchicine, Adriamycin, or vinblastine lose their drug resistance after several months of cultivation in nonselective medium (2). This instability of the drug resistance phenotype has been used to isolate revertants of these multidrug-resistant cell lines (2). In the current study, we determined the time course of loss of resistance of a colchicine-selected line, KB-C6 (2), which has been kept in selective medium (6 μg/ml colchicine) for at least 12 months. As shown in Fig. 2, after 50 days of cultivation in the absence of selective medium, there appears to be a wide range in resistance level of cells (KB-C6-R50), with approximately 10% of the cell population remaining resistant to greater than 1 μg/ml colchicine (i.e., about 500-fold resistant), whereas more than 50% of the cells are sensitive to less than 100 ng/ml colchicine (less than 50-fold resistant). After 170 days of cultivation in the absence of selective medium (KB-C6-R170), the killing curve appears to be much more representative of a uniform population with an LD10 value of 40 ng/ml (10-fold resistant). As we have previously reported (2), even after prolonged incubation in the absence of selective medium and successive subcloning, we have been unable to derive a line from KB-C6 cells which is as sensitive to colchicine as the parental KB-3-1 cell line.

Analysis of Southern blots of DNA derived from KB-C6 cells with an MDR1 cDNA probe during growth in nonselective medium demonstrates that loss of resistance corresponds to reduced copy number of the MDR1 gene (Fig. 3). As quantitated in Table 2, there is very little loss of MDR1 genes during the first 20 days of cultivation in the absence of colchicine (KB-C6-R20), but by 40 days many MDR1 gene copies have been lost, and by 150 days the MDR1 gene is only slightly amplified in the whole population. Loss of MDR1 RNA expression, shown in Fig. 4 and quantitated in Table 2, decreases abruptly after 40 days of cultivation in the absence of selective medium.

The in situ hybridization analysis of KB-C6 cells maintained in nonselective medium for various time periods is shown in Fig. 5. The loss of expression of the MDR1 gene at the cellular level appears to be quite heterogeneous, with islands of cells remaining which continued to express MDR1 at easily detectable levels 30–50 days after plating the cells in nonselective medium (Fig. 5, B and C). This heterogeneity of expression is consistent with the heterogeneity of colchicine-resistance seen in Fig. 2 in the killing curves for KB-C6-R50. The islands of MDR1 RNA positive cells are about the size expected for clones derived from single cells, but a clonal origin for these plasmids has not been proven.

DISCUSSION

Sensitivity and Clinical Usefulness of the in Situ Hybridization Assay. This study, by showing a correspondence between certain...
Table 1  Multidrug-resistant KB cells: drug-resistance, MDRI gene amplification and MDRI expression by RNA blot and in situ hybridization

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Relative resistance to*</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Col</td>
<td>Adr</td>
<td>Vbl</td>
<td>MDRI amplification*</td>
<td>MDRI expression*</td>
</tr>
<tr>
<td>KB-3-1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>KB-8</td>
<td>2.1</td>
<td>1.1</td>
<td>1.2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>KB-8-5</td>
<td>3.8</td>
<td>3.2</td>
<td>6.3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>KB-8-5-11</td>
<td>40</td>
<td>23</td>
<td>51</td>
<td>7</td>
<td>80</td>
</tr>
<tr>
<td>KB-C1.5</td>
<td>320</td>
<td>140</td>
<td>370</td>
<td>15</td>
<td>340</td>
</tr>
<tr>
<td>KB-C6</td>
<td>2100</td>
<td>320</td>
<td>80</td>
<td>820</td>
<td>45 (9–76)</td>
</tr>
</tbody>
</table>

* Relative resistance is taken from Shen et al. (2).

† The amplification of the MDRI gene is taken from Roninson et al. (34).

‡ Expression of MDRI RNA is from Shen et al. (2). ND, not detected in this assay. By more sensitive tests, the amount of MDRI RNA in KB-3-1 cells appears to be 1/40th that of KB-8-5 cells.

§ Average grains per cell was determined as described in “Materials and Methods.” The number in parenthesis is the range of grain counts observed in 50 randomly selected cells.

After hybridization and washing as described in “Materials and Methods,” cells were solubilized in Aquasol (New England Nuclear), and [35S]RNA radioactivity was determined in a liquid scintillation counter.

Fig. 1. KB-3-1 and its colchicine-selected multidrug-resistant derivatives. Autoradiographs of Giemsa-stained cells at 1600× magnification hybridized in situ with 35S-labeled MDRI riboprobe 5A. A, parental KB-3-1 cells; and the colchicine-resistant sublines: B, KB-8; C, KB-8-5; D, KB-8-5-11; E, KB-C1.5; F, KB-C6.

Counts, extent of drug-resistance, and MDRI RNA expression, demonstrates the feasibility of using in situ hybridization analysis to analyze single cells for expression of the human multidrug resistance gene. We have recently reported that MDRI RNA is expressed at higher levels in normal adrenal, kidney, colon, small intestine and liver than in many other tissues (15). P-glycoprotein, the product of the MDRI gene, can be localized to the luminal surface of epithelial cells of the intestine, the brush border of kidney proximal tubules, biliary hepatocytes, and diffusely to the cells of the adrenal (29). It will be of interest to confirm that these cell types in these tissues are expressing the MDRI RNA. Although the assay is not entirely linear at high MDRI levels (see Tables 1 and 2), the sensitivity of the in situ hybridization analysis (Table 1) should make it possible to detect cells with levels of MDRI RNA equal to or greater than KB-8-5 cells, which are 4-fold resistant to colchicine, 6-fold resistant to vincristine, and 6-fold resistant to Adriamycin.
**Fig. 2.** Killing curves showing resistance of KB-C6 and some of its revertants and the original parental cell line KB-3-1 to colchicine. Cell survival was calculated using the monolayer growth assay as described in "Materials and Methods." ○, KB-3-1; □, KB-C6-R170; △, KB-C6-R50; ●, KB-C6.

**Fig. 3.** Analysis by Southern hybridization of the revertants isolated from KB-C6 cells. DNA was digested with HindIII and probed with nick-translated mdr1 clone 5A. 2 µg of DNA per lane was analyzed on a 0.8% agarose gel.

**Table 2 Loss of MDR1 gene expression and amplification during reversion of the KB-C6 cell line**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MDR1 gene copy number</th>
<th>MDR1 RNA expression</th>
<th>Whole population excluding foci grain/cell (range)</th>
<th>Foci grain/cell (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB-C6</td>
<td>37</td>
<td>820</td>
<td>109 (46–420)</td>
<td>ND</td>
</tr>
<tr>
<td>KB-C6-R20</td>
<td>26</td>
<td>190</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KB-C6-R30</td>
<td>ND</td>
<td>120</td>
<td>17 (3–44)</td>
<td>100 (40–173)</td>
</tr>
<tr>
<td>KB-C6-R40</td>
<td>12</td>
<td>12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KB-C6-R50</td>
<td>ND</td>
<td>6</td>
<td>5 (0–12)</td>
<td>33 (12–49)</td>
</tr>
<tr>
<td>KB-C6-R60</td>
<td>4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KB-C6-R100</td>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KB-C6-R150</td>
<td>1</td>
<td>ND</td>
<td>4 (0–8)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*The number of copies of the MDR1 gene was estimated by densitometry of the Southern blots shown in Fig. 3 and expressed relative to a copy number of 1 for KB-3-1 cells. ND, not determined.

*The expression of MDR1 RNA was estimated by densitometry of the slot blots shown in Fig. 4. KB-C6 RNA levels were set at 820, our previously published estimate based on comparison to KB-8 cells taken as 1 (2).

*Grain counts were obtained as described in "Materials and Methods." Counts for foci with high grain counts and the general population, excluding these foci, are shown.

**Fig. 4.** Reduced expression of human MDR1 RNA in KB-C6 revertants analyzed by slot blot hybridization. The amount of RNA (µg) loaded is indicated at the top of the lanes. Filters were probed with nick-translated MDR1 clone 5A. MDR1 RNA in KB-8 and KB-8-5 are shown for comparison purposes.

MDR RNA detection by *in situ* hybridization

resistant to vinblastine, and 3-fold resistant to Adriamycin (2). Since adrenal and kidney samples, and some colon, small intestine, and liver samples have levels of MDR1 RNA which are in this range and P-glycoprotein expression seems to be concentrated in only a limited number of cells in these tissues, it should be possible to use *in situ* hybridization to localize MDR1 RNA in the positive cells. Many tumors derived from these tissues, and some drug-resistant tumors derived from drug-sensitive cancers, also have levels of MDR1 RNA high enough to detect by *in situ* hybridization. Hence, using this technique, it should be possible to analyze small samples of tumor cells for amounts and heterogeneity of MDR1 expression. This information could be used to design more rational chemotherapy protocols.

**Heterogeneity of Expression.** Despite the application of constant selection pressures, most populations of multidrug-resistant KB cells selected in colchicine showed heterogeneous expression of MDR1 RNA at the single cell level (Fig. 1). This may not be simply a technical artifact, since we have recently been able to confirm this heterogeneity by an immunofluorescence analysis using an antibody to a surface determinant on P-glycoprotein (30–32). It seems unlikely that this heterogeneity reflects cell cycle variations in MDR1 RNA expression, since the high expressing clones have a tendency to cluster, suggesting a clonal origin from a single cell. It seems more likely that this heterogeneity reflects variable expression of the MDR1 segments possibly due to altered transcriptional control in different cell clones or altered numbers of amplified MDR1 segments (see below). In the highly resistant populations, such as KB-C1.5 and KB-C6, which were not cloned after successive steps of selection, heterogeneity of MDR1 expression could reflect the presence of other mechanisms of drug resistance.

**Loss of Resistance is Heterogeneous.** These studies show that, beginning with an uncloned highly drug-resistant population of cells with amplified MDR1 genes, loss of multidrug resistance...
in the absence of selective medium is slow for the first 20 days, and then increases rapidly as the MDR1 gene copy number and MDR1 RNA expression decrease in the population as a whole. The rapid acceleration of loss of MDR1 RNA levels after 20 days appears to correspond to a decrease in cell doubling time, so that between 20 and 40 days there are many more cell doublings than during the first 20 days in the absence of selective medium. We have previously noted that highly multidrug-resistant KB cell lines grow more slowly than their drug-sensitive counterparts, and it is possible that the early slow loss of the amplified MDR1 gene accelerates as the cells begin to grow faster and undergo more doublings. The colchicine-selected KB cell lines are known to contain large numbers of double minute chromosomes (16), which are presumed to represent amplified copies of the MDR1 gene. Random segregation of these double minute chromosomes would produce the heterogeneous patterns seen both during acquisition and loss of multidrug resistance by KB cells. Loss of expression seems, in general, to parallel loss of the amplified segments (Table 2). In contrast, revertants of multidrug-resistant human leukemic K562 cells have been found to retain their amplified segments (33).

Conclusions. This study suggests that it will be feasible to study expression and acquisition of multidrug resistance by some normal human tissues and tumors. Several such tissues and tumors express levels of MDR1 mRNA near the level of KB-8-5 cells which have approximately 40 times as much MDR1 mRNA as drug-sensitive KB-3-1 cells (15). This level of MDR1 RNA should be detectable using this in situ hybridization assay (15). Care, however, will need to be exercised in interpreting results close to the low limit of sensitivity of this assay because of the background of grains on MDR1 RNA negative cells.

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