Relationship of Multidrug Resistance to Rhodamine-123 Selectivity between Carcinoma and Normal Epithelial Cells: Taxol and Vinblastine Modulate Drug Efflux

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Abstract

Preferential retention and cytotoxicity of Rhodamine-123 (Rho-123) was originally reported in a number of carcinoma cell types isolated from a variety of tissues as compared to normal epithelial cells from a limited number of other tissues. In the present study, we have examined Rho-123 selectivity in normal and tumor cell lines isolated from the same tissue source, i.e., human breast. We found that: (a) in matched pairs of normal and carcinoma breast cells, Rho-123 displays no preferential retention in either cell type; (b) there is no preferential toxicity in carcinoma as compared to normal breast cells; in fact, one of the carcinoma cell lines (MDA-MB231) shows moderate resistance to this dye; (c) all of the human breast cell lines do not express P-glycoprotein-mediated multidrug resistance; (d) the normal monkey kidney epithelial cell line CV-1, which was originally used as a model to demonstrate the relative resistance of normal epithelial cells to this drug, is found to express high levels of the mdr-1 gene, is resistant to other multidrug-resistant drugs (taxol and vinblastine), and its resistance to Rho-123 as well as decreased Rho-123 retention can be reversed by verapamil; and (e) taxol and vinblastine are found to block increased Rho-123 efflux in CV-1 cells. Thus, overall the data suggest that preferential retention and cytotoxicity of Rho-123 in carcinoma versus normal epithelial cells is related to the differential expression of the mdr-1 gene.

Introduction

In the early 1980s, Rho-123, a positively-charged xanthene dye, was shown to be unusually retained by mitochondria in cardiac muscle cells as compared to cocultured cardiac fibroblasts (1). At that time, similar elevated retention of Rho-123 was also observed in a large variety of carcinoma cells as compared to a more limited number of normal epithelial cells (1). When both cell types were exposed for prolonged periods to Rho-123, only the carcinoma cells were growth inhibited or killed by this drug (2-4). This result led to the successful treatment of carcinomas in animals with Rho-123 (5). Thus, there was reason to believe that Rho-123 differentiated between certain tumor and normal cell types, which could possibly be exploited for the clinical treatment of cancer. The basis for the differential retention of Rho-123 in carcinoma versus normal epithelial cells and hence its selectivity, however, was not clearly understood at that time.

It was subsequently shown that membrane potentials (ΔΨ) were elevated in carcinoma cells that displayed high retention and sensitivity to Rho-123 as compared to normal epithelial cell lines which showed lowered retention and found to be relatively resistant to this mitochondrial agent (6). The relevance of these findings is that Rho-123 carries a delocalized positive charge and thus cells with elevated ΔΨ could be expected to attract and accumulate more of these types of positively-charged compounds as compared to cells with lowered ΔΨ.

However, working with variants of Friend leukemia cells which express different levels of the P-gp responsible for MDR (7), we found that Rho-123 was recognized by cells displaying the MDR phenotype (8, 9). P-gp-mediated MDR is thought to occur by an active pumping-out mechanism which results in lowered intracellular accumulation and hence increased resistance to a wide variety of antitumor agents as well as other agents (10, 11). Moreover, P-gp has been shown to act as a chloride channel (12), which could explain the ΔΨ differences found between cells which express P-gp-mediated MDR and those that do not.

Thus, the aim of the present study was to determine whether the selectivity of Rho-123 originally found in carcinoma versus normal epithelial cell lines (1-4) could be related to the presence or absence of MDR. Using matched pairs of carcinoma and normal epithelial cells isolated from similar tissue, i.e., human breast, each of the cell lines’ mdr-1 gene expression was investigated and compared to their Rho-123 sensitivity and ability to retain this dye. Similar analyses were made in the model tumor and normal cell lines in which Rho-123 selectivity was first demonstrated, i.e., MCF-7 isolated from human breast carcinoma and CV-1 isolated from normal monkey kidney epithelium. In addition, we investigated whether certain anticancer agents which are recognized by MDR cells, i.e., taxol and Vbl, could also act as modulators of Rho-123 retention as had been found for Vpl.

Materials and Methods

Cells. The human breast carcinoma (MDA-MB-231 and MCF-7) and normal (HBL-100) cell lines were obtained from the American Type Culture Collection. The newly immortalized non-tumorigenic epithelial cell line NPM-14 T4/9, derived from a benign human breast tissue, was established and characterized as described previously (13). The non-tumorigenic epithelial cell line CV-1, derived from normal monkey kidney, was kindly provided by Dr. H. Lazarus (University of Miami, Miami, FL). All cell types were grown in Eagle’s MEM supplemented with 10% calf serum.

Rho-123 Retention Assay. Living cells, grown on 12-mm round glass coverslips, were exposed to 26 μM of Rho-123 in the presence or absence of 20 μM of Vpl for 10 min. After extensive rinsing in dye-free medium (±Vpl), coverslips were mounted onto silicone rubber chambers in dye-free medium as described previously (14). At indicated times, Rho-123 fluorescence was monitored under an Olympus epifluorescence microscope equipped with a photomicrographic system. In other experiments, taxol and Vbl were used under the same experimental conditions as Vpl.

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4 The abbreviations used are: Rho-123, Rhodamine 123; P-gp, P-glycoprotein; Vbl, Vinblastine; Vpl, Verapamil; MDR, multidrug resistance.
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Cytotoxicity Assay. Cells were seeded in 24-well Falcon plates at a concentration of 2 x 10^4 cells/well. After 24 h, the culture medium was removed, and various concentrations of Rho-123, taxol, or Vbl were added in 2 ml. After 3 days, cells were washed once with PBS, trypsinized, and counted in a hemocytometer in duplicate. The ID_{50} was defined as the concentration which gave 50% reduction of cell growth as compared to untreated control cells. To assay for reversal of resistance, cells were exposed to increasing doses of Rho-123 in the presence or absence of nontoxic doses of the following drugs: Vpl (20 μM), taxol (0.12 μM), and Vbl (0.006 μM).

Assay of mdr-1 Gene Expression by Reverse Transcription-PCR. Total RNA was purified by acid guanidinium thiocyanate/phenol chloroform extraction (15). One μg of RNA was used for cDNA synthesis as follows. RNA was heated, chilled on ice, and then incubated with 200 units of M-MLV reverse transcriptase, 500 μM dodeoxynucleotide triphosphate, 10 μM of random primer and RT buffer (GIBCO-BRL) for 1 h at 37°C. For PCR, the primer was the mdr-1-specific sequence GGAGTGTCCGTGGATCACAAG (residue 1909-1903) and TGTTCAGGATCATCAATTCTTGT (residue 2218-2241) (Ref. 16), kindly provided by Dr. M. T. Kuo (UT M. D. Anderson Cancer Center, Houston, TX). These primers were selected at the regions which are 36.4 and 37.5% similar to the corresponding region of mdr3 cDNA. Thus, it should not recognize the mdr-3 gene. The resulting PCR product from these primers was 232 base pairs. Glyceraldehyde-3-phosphate dehydrogenase was used as internal standard, and the amplimers used for glyceraldehyde-3-phosphate dehydrogenase were CCACCACCGTGTGTGCTAGCC (antisense) and GTCTTGACCACCCATGGAGAAGGC (sense). These primers yield a 676-base pair PCR product. The PCR was carried out using the cDNA in PCR buffer according to the Perkin-Elmer Cetus protocol. Amplification was performed in sequential cycle at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min for 30 cycles using a Perkin-Elmer thermocycle. The PCR product was electro-phoresed in 1 X Trisacetate EDTA buffer on 2% Nusieve agarose/1% agarose gel. The gel was then stained with ethidium bromide and photographed.

Drugs. Rho-123 was obtained from Eastman Kodak (Rochester, NY) and diluted in sterile water. Vpl and Vbl were purchased from Sigma Chemical Co. (St. Louis, MO), and both were made to stock by diluting in sterile water. Taxol was a gift from Napro, Inc. (Boulder, CO) and was diluted in DMSO.

Results

Retention of Rho-123 and Modulation by Vpl in Normal and Tumor Cells. In contrast to previously published results (1-4), no differences in Rho-123 retention could be observed between normal epithelial and carcinoma cell lines isolated from human breast (Fig. 1). In both cell types, mitochondrial staining of Rho-123 was initially bright and remained intense 2 h after drug removal (Fig. 1). Furthermore, in the normal and carcinoma breast cell lines studied, there was

Fig. 1. Analysis of Rho-123 retention in carcinoma and normal human breast cells by fluorescence microscopy. Matched phase-contrast and fluorescence micrographs of MDA-MB-231 carcinoma (A and B) and NPM-14 T4/9 normal cells (C and D) incubated with Rho-123 alone for 10 min and observed 2 h after rinsing in Rho-123-free medium. Normal NPM-14 T4/9 cells were also cotreated with 20 μM of Vpl, rinsed, and incubated for 2 h in Rho-123-free medium in the presence of Vpl (E and F). Bar, 10 μm.

Drugs. Rho-123 was obtained from Eastman Kodak (Rochester, NY) and diluted in sterile water. Vpl and Vbl were purchased from Sigma Chemical Co. (St. Louis, MO), and both were made to stock by diluting in sterile water. Taxol was a gift from Napro, Inc. (Boulder, CO) and was diluted in DMSO.
Fig. 2. Analysis of Rho-123 retention in normal monkey kidney CV-1 cells by fluorescence microscopy. Matched phase-contrast (A) and fluorescence micrographs of cells incubated with Rho-123 alone and observed immediately (B) and 1 h (C) after rinsing in Rho-123-free medium. Cells were also cotreated with 20 µM of Vpl, rinsed, and incubated for 1 h in Rho-123-free medium in the presence of Vpl (D). Bar, 10 µm.

Fig. 3. Reverse transcriptase-PCR analysis of mdr-1 gene expression. Lanes 1–6, glyceraldehyde-3-phosphate dehydrogenase-specific PCR product from the following cell types was used as an internal cDNA control: Lane 1, NPM-14 T4/9 cells; Lane 2, HBL-100 cells; Lane 3, MCF-7 cells; Lane 4, MDA-MB-231 cells; Lane 5, CV-1 cells; and Lane 6, CEM/VLB cells. Lanes 7–12, MDR-specific PCR products from cells in the same order as above. Note: NPM-14 T4/9, HBL-100, MCF-7, and MDA-MB-231 cells show no MDR product, while CV-1 cells do. Lane 13, PGEM1 marker.
Table 1  Sensitivity of human breast epithelial cell lines to Rho-123, Vpl, Taxol, and Vbl

<table>
<thead>
<tr>
<th>Cells</th>
<th>Doses (μM) yielding 50% cell growth inhibition after 3-day treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Rho-123</td>
</tr>
<tr>
<td>Human breast cells</td>
<td></td>
</tr>
<tr>
<td>NPM-14 T4/9</td>
<td>1.5</td>
</tr>
<tr>
<td>HBL-100</td>
<td>1.8</td>
</tr>
<tr>
<td>MCF-7</td>
<td>0.8</td>
</tr>
<tr>
<td>MDA-MB231</td>
<td>11.0</td>
</tr>
<tr>
<td>Monkey kidney cells</td>
<td></td>
</tr>
<tr>
<td>CV-1</td>
<td>370.0</td>
</tr>
</tbody>
</table>

Growth-inhibitory data represent an average of at least two different experiments with each experimental point done in duplicate. SDs for each point were less than 15%.

no apparent increase in Rho-123 staining after cotreatment of the cells with the classical MDR modulator Vpl (17) at a concentration of 20 μM; cells remained as bright as cells not treated with Vpl (Fig. 1). In comparison, in normal monkey kidney epithelial CV-1 cells, which showed reduced Rho-123 mitochondrial accumulation as described previously (3), Vpl markedly increased the intensity of Rho-123 staining and prevented its rapid loss (Fig. 2).

**mdr-1 Gene Expression in Normal and Tumor Epithelial Cells.**

The extended retention of Rho-123 in the human normal and tumor breast cells tested suggested that these cells did not express the mdr-1 gene which was confirmed by PCR. As illustrated in Fig. 3, none of the human breast cell lines tested (tumor or normal) expressed mdr-1. Contrastingly, normal monkey kidney epithelial cells, CV-1, which did not retain Rho-123, expressed high levels of mdr-1.

**Cytotoxicity of Rho-123, Taxol, Vbl, and Vpl in Normal and Tumor Cells.** Following continuous exposure to Rho-123, taxol, or Vbl, respectively, the ID₅₀ in each of the normal epithelial and carcinoma cell lines isolated from human breast was found to be in a moderately narrow range (Table 1). In comparison, the epithelial cell line isolated from normal monkey kidney, CV-1, was significantly more resistant to the cytotoxic effects of these three drugs, which are known to be recognized by the P-gp-mediated MDR mechanism. For
T4/9 cell line (which is closest to CV-1 in its non-tumorigenic phenotype) is 1.5 μM, while for CV-1, the ID50 was 250 times higher (370 μM). Similarly, CV-1 cells were found to be 150- and 100-fold more resistant to taxol and Vbl, respectively, as compared to human breast cells. In contrast, all cell types, including CV-1 cells, were equally sensitive to the cytotoxic effects of Vpl (Table 1).

Taxol and Vbl Modulate Rho-123 Retention. CV-1 cells which lose their Rho-123 mitochondrial staining shortly after rinsing in Rho-123-free medium remained brightly stained for as long as 2 h when cotreated with either taxol (12 μM) or Vbl (6 μM) (Fig. 4). As described above in Table 1, Vbl and taxol were much more toxic to CV-1 cells than Vpl. Thus, the doses of Vbl or taxol that are necessary to modulate Rho-123 retention in CV-1 cells were highly toxic when these drugs were applied continuously (72 h) in combination with Rho-123. At doses of 0.12 μM of taxol and of 0.006 μM of Vbl which were just below toxicity, there was no significant increase of Rho-123 retention over the amounts of Rho-123 retained in Vpl-treated CV-1 cells. In contrast to taxol, however, activity as modulators of resistance to Rho-123 appears limited. The doses of taxol or Vbl necessary to reverse Rho-123 resistance is toxic to CV-1 cells when either of these drugs are applied alone. Thus, this in vitro data would suggest that the clinical activity of either taxol or Vbl as modulators of resistance would be expected to be less than that of Vpl.

Discussion

Previously, it was shown that Rho-123 accumulates preferentially and selectively kills carcinoma versus normal epithelial cell lines isolated from a variety of different tissues (1–4). Our results here with matched pairs of carcinoma and normal cells isolated from similar tissue (human breast) indicate no preferential retention and minor differences in growth inhibitory activity of Rho-123 between these cell types.

MCF-7 and CV-1 were the cell lines most extensively studied and used as a model to demonstrate Rho-123 selectivity between tumor and normal cells (2–4, 6, 14, 18). MCF-7 is a carcinoma cell line originally isolated from human breast, a tissue which has been shown not to intrinsically express the mdr-1 gene (19). In contrast, the normal epithelial cell line, CV-1, was isolated from monkey kidney which has proven to be a tissue that expresses high levels of the mdr-1 gene (20, 21). Our results here, which show that CV-1 cells express the mdr-1 gene while MCF-7 cells do not, suggests that the selectivity of Rho-123 in these cells is related to the presence or absence of the mdr-1 phenotype.

Using other drugs known to be recognized by the MDR efflux pump, i.e., taxol (22) and Vbl (10), no significant selective cytotoxicity was found between the breast normal and tumor cell types studied. In contrast, the CV-1 cells, which express MDR, demonstrated resistance to both these drugs. The findings that the human breast cell lines (normal and tumor) studied here did not express MDR further suggests that the differences in Rho-123 retention and sensitivity between normal and tumor cells are related to the absence or presence of MDR.

Previous studies in which Friend leukemia cell variants showed lower Rho-123 retention and increased resistance to this mitochondrial dye as a function of increasing levels of P-gp MDR suggested that the MDR efflux pump could recognize this compound (8, 9). It was also shown that Vpl could block the rapid efflux of Rho-123, thereby increasing intracellular Rho-123 retention and reversing resistance to this agent (23). Thus, the data presented here for the CV-1 cell line indicates that its lowered Rho-123 retention, increased resistance, and reversal by Vpl is related to the expression of MDR. In retrospect, most of the normal epithelial cell lines used to show Rho-123 selectivity between carcinoma and normal epithelial cells were isolated from tissue which intrinsically expresses MDR, i.e., kidney (3).

Since the ΔΨ of cells can act as a force to attract positively-charged compounds such as Rho-123, elevated ΔΨ found in MCF-7 as compared to CV-1 appeared to at least partially explain the selectivity of this dye. In addition to the reports which show that cells which express MDR also have reduced ΔΨ as compared to their non-MDR counterparts (24), P-gp MDR has also been shown to act as a chloride ion channel (12). This latter finding could explain altered ΔΨ found in cells with P-gp in their membranes. The relative importance of lowered ΔΨ for the differential accumulation of positively-charged compounds as compared to recognition and transport of these compounds by the P-gp efflux pump remains unclear. Further studies are required to better understand the relative contribution of each of these mechanisms to altered drug accumulation in P-gp-mediated MDR cells.

Additionally, we have found that both taxol and Vbl can block the efflux of Rho-123 from CV-1 cells as well as from Friend leukemia cells which have acquired MDR (data not shown). When applied for short periods (2 h), taxol and/or Vbl increase the amount of Rho-123 retention in CV-1 cells. In contrast to Vpl, however, their activity as modulators of resistance to Rho-123 appears limited. The doses of taxol or Vbl necessary to reverse Rho-123 resistance is toxic to CV-1 cells when either of these drugs are applied alone. Thus, this in vitro data would suggest that the clinical activity of either taxol or Vbl as modulators of resistance would be expected to be less than that of Vpl.

References

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