Cross-Resistance to Cisplatin in Cells Resistant to Photofrin-mediated Photodynamic Therapy

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Abstract

This study shows that a Photofrin-induced photodynamic therapy-resistant variant (RIF-8A) of a radiation-induced fibrosarcoma-1 cell line (RIF-1) is cross-resistant to cis-diamminedichloroplatinum(II) (cisplatin). This is the first study to show cross-resistance to cisplatin in photodynamic therapy-resistant variants in vitro. Resistance does not appear to be the result of elevated glutathione levels since neither the resistant variant (RIF-8A) nor the parental line (RIF-1) varied in total glutathione levels. However, cisplatin-DNA adduct levels differed significantly between the two cell types. Immediately following a 1-h exposure to cisplatin (50 μM), RIF-1 cells contained 44.6 ± 2.0 (SEM) pg platinum/μg DNA while RIF-8A cells contained 24.8 ± 6.3 pg platinum/μg DNA. In addition, the variant resistant had decreased plasma and mitochondrial membrane potentials. The plasma and mitochondrial membranes of the resistant variant accumulated 3- and 3.6-fold less rhodamine 123, respectively. The difference in rhodamine 123 accumulation could not be attributed to elevated P-glycoprotein expression because both the parental line and the variant contained similar amounts of P-glycoprotein. In conclusion, alterations in the plasma and/or mitochondrial membrane potentials may provide cells with a survival advantage when challenged with either photodynamic therapy or cisplatin in vitro. This appears to be a novel mechanism of resistance.

Introduction

Cisplatin is considered one of the most effective antitumor agents for a large number of solid tumors, but its usefulness is limited by the frequent growth of cisplatin-resistant cell populations (1). Due to the difficulties of investigating drug resistance in vivo, the mechanisms involved in cisplatin resistance have traditionally been studied in vitro in variants selected through a process of incremental drug exposure over an extended period of time (1). These cisplatin-resistant variants are often 10–30 times more resistant to cisplatin than their parental lines. Such selection procedures have been shown to induce multiple changes that contribute to cisplatin resistance including altered drug transport, increased metallothionein or glutathione levels, and altered DNA adduct formation and/or repair, thereby making it difficult to determine the relative importance of each mechanism (2). Alternatively, variants that possess a more modest level of resistance provide a more appropriate model system to study mechanisms of cisplatin resistance, since resistance greater than about 5-fold may not be clinically relevant (3).

In this paper, we present a novel model system to study cisplatin resistance in vitro, utilizing cells resistant to PDT and which demonstrate cisplatin cross-resistance. Previously, we characterized RIF-1 and its PDT-resistant variant (RIF-8A) and demonstrated morphological and functional mitochondrial differences (4). The structural changes in the mitochondria of RIF-8A cells strongly resemble those observed in C13* cells (5), a cisplatin-induced resistant variant of the human ovarian carcinoma cell line (2008), in that the mitochondria in the resistant variants appeared to be in a condensed state with densely stained cristae compared to their respective parental line. The mitochondrial changes in C13* cells contributed to cisplatin resistance through an undetermined mechanism (5, 6). Therefore, RIF-8A cells were further investigated to determine if the mitochondrial structural alterations provided similar functional changes as observed in C13* cells (5) and if the mitochondrial alterations contributed to the cisplatin cross-resistance.

Materials and Methods

Materials. cis-Diamminedichloroplatinum(II), 5-sulfosalicylic acid, NADPH, 5,5'-dithiobis(2-nitrobenzoic acid), glutathione reductase, and reduced glutathione were obtained from Sigma Chemical Co., St. Louis, MO. Rhodamine 123 and H33258 were obtained from Eastman Kodak, Rochester, NY, and Calbiochem, La Jolla, CA, respectively. Alkaline phosphatase conjugate, α-minimal essential medium, molecular weight standards, and Mycotect kits were purchased form Gibco BRL, Burlington, Ontario, Canada. The monoclonal antibody for P-glycoprotein (P-glycMab C219) was purchased from Cantocor, Malvern, PA.

Cell Lines and Culture Conditions. RIF-1 cells were obtained originally from Dr. B. Henderson, Roswell Park Memorial Institute, Buffalo, NY. The PDT-resistant variant (RIF-8A) was generated in our laboratory by eight selection cycles with Photofrin and light as described previously (7). CHO cells and CHO-MDR were provided by Dr. V. Ling, Ontario Cancer Institute, Toronto, Ontario, Canada. The CHO-MDR line, containing the P-glycoprotein gene (CHP(C)), was derived from the AUX B1 parent line as described by Ling and Thompson (8). RIF-1, RIF-8A, CHO, and CHO-MDR cells were grown in α-minimal essential medium containing deoxyribonucleosides and ribonucleosides and supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. All cultures were maintained at 37°C and 5% CO2 in a humidified incubator. RIF-1 and RIF-8A were tested with a Mycotect kit and were shown to be Mycoplasma negative.

Survival Assays. Cells in log-phase growth were seeded overnight at a cell density of approximately 100 cells/well in 6-well plates and were treated, in triplicate, for 1 h with various concentrations of cisplatin (prepared fresh for each experiment in PBS, pH 7.2). At this point, the drug-containing medium was removed and replaced with drug-free medium and the cells were incubated at 37°C for 5 days. Surviving colonies were stained with methylene blue and colonies containing 20 or more cells were counted.

P-glycoprotein Determination. Cells in log phase growth were trypsinized, centrifuged at 400 x g for 5 min, and washed once with phosphate-buffered saline. Plasma membranes from these cultures were prepared using the method of Gerlach et al. (9). Thirty μg total protein, as determined by the Pierce Micro BCA spectrophotometric protein assay (10), and
prestained protein molecular weight standards were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9) and transferred to nitrocellulose filters (11). The protein was detected with biotinylated primary antibody specific to P-glycoprotein (1:10,000 dilution of P-glycoMAb in Tris-buffered saline with 0.05% Tween 20) using a modification of the method of Blake et al. (12). The blots were photographed and analyzed using an optical densitometer (Bio-Rad Laboratories, Richmond, CA).

Glutathione Assay. RIF-1 and RIF-8A cells were prepared as described previously (13). The total glutathione content was determined by the method of Akerboom and Sies (14) at a wavelength of 412 nm on a Beckman DU-7 spectrophotometer. Data are expressed as nmol/mg protein.

Quantification of Platinum-DNA Adducts. Cells in log-phase growth were seeded overnight in 150- x 25-mm dishes at a density of 8 x 10⁶ cells/plate. Following a 1-h exposure to cisplatin (50 μM in PBS, pH 7.2), cells were washed with drug-free media and harvested by cell scraping with a rubber policeman. The cells were centrifuged at 150 X g for 5 min, resuspended in PBS, and again centrifuged at 150 X g for 5 min. The supernatant was discarded and the cell pellets were stored at -70°C. DNA was isolated from the cell pellets by resuspending them in digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA (pH 8), 0.5% sodium dodecyl sulfate, 100 μg/ml proteinase K, and 2.5 units/μl RNase T1). Cell digests were incubated at 37°C for 2 h followed by an 18-h digestion at 50°C. Samples were extracted twice with phenol:chloroform (1:1) and centrifuged at 1700 X g for 10 min. The aqueous phase was reextracted with chloroform:isomyl alcohol (25:1) and dialyzed against H₂O for 24 h at 4°C. The samples were lyophilized and reconstituted in PBS (calcium and magnesium free), and DNA content was determined by UV absorbance at 260 nm. Platinum-DNA adducts were determined by flameless atomic absorption spectroscopy with Zeeman background correction, using a Perkin-Elmer 5100 ZL atomic absorption spectrophotometer. For each sample, 250 μg of DNA were digested with nitric acid: perchloric acid (3:1) at ambient temperature for 18 h. After acid digestion, samples were evaporated to dryness and reconstituted in 250 μl of 10% nitric acid, and 25-μl aliquots were injected into the atomic absorption spectroscopy unit. Platinum-DNA adduct levels were expressed as pg platinum/μg DNA as determined from a platinum standard curve prepared in 10% nitric acid. The lower limit of detection was 250 pg of platinum (10 pg platinum/μg DNA).

Rhodamine 123 Uptake. RIF-1 and RIF-8A cells in log-phase growth were seeded overnight in 24-well plates at a density of 5 X 10⁶ cells/well and treated with Rh123 (100 μM) for 1 h. The Rh123 accumulation was determined in normal potassium media (whole cell uptake), media supplemented with 137 mM potassium chloride (for selective depolarization of the plasma membrane potential), or normal potassium media supplemented with 5 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (for selective depolarization of mitochondrial membrane potential) (5). Following Rh123 removal, the wells were washed twice with phosphate-buffered saline, and deionized water was added (300 μl/well). The plates were then stored, protected from light, at room temperature for a minimum of 2 h. In order to determine the DNA content in each well, 100 μl of the DNA fluorochrome, H33258 (40 μg/ml in TNE buffer), were added to each well (15). The plates were read on a Cytofluor 2350 fluorescent plate scanner (Millipore, Mississauga, Ontario, Canada) using excitation/emission wavelengths of 485/530 nm for Rh123 and 360/460 nm for the DNA fluorochrome (H33258). Cell number/DNA and Rh123 standard curves were generated to enable the fluorescent readings to be converted to fmol of Rh123/cell.

Statistics. All values report the mean ± SEM unless otherwise specified. Statistical analysis was carried out using an unpaired Student’s t test; P < 0.05 was considered statistically significant.

Results and Discussion

Clonogenic survival studies were performed to compare cisplatin sensitivity in RIF-1 and RIF-8A cells (Fig. 1). The 90% inhibitory concentrations for a one h cisplatin exposure were 9.0 μM for RIF-1 cells and 14.6 μM for RIF-8A cells. The resistant variant was approximately 1.6-fold resistant to cisplatin. Since this is the first time cross-resistance to cisplatin in a PDT-induced resistant variant in vitro has been demonstrated, the focus of this study was to investigate possible mechanism(s) involved in the cross-resistance to cisplatin.

It has been demonstrated previously that elevated levels of glutathione, a known scavenger of cisplatin, can contribute to cisplatin resistance (16). However, there was no apparent difference in total cellular glutathione (oxidized glutathione and reduced glutathione) content inasmuch as RIF-1 contained 5.1 nmol/mg protein while the variant RIF-8A contained 5.4 nmol/mg protein (Table 2). Our observations are consistent with those of Luna and Gomer (17), who found no differences in reduced glutathione and glutathione peroxidase in RIF-1 cells compared to their PDT-resistant variants.

Another common form of drug resistance in tumor cells involves MDR resistance. The main component of MDR is the M₇, 170,000 plasma membrane protein, P-glycoprotein, which is an energy-dependent efflux pump with an affinity for a variety of chemotherapeutic agents (18). We have shown previously that there is no difference in the survival of RIF-1 and RIF-8A cells following Adriamycin administration (7), and the present study demonstrates that there are no significant differences in P-glycoprotein content between RIF-1 cells and the variant RIF-8A. Western blots were used to quantitate P-glycoprotein levels in four different cell types: RIF-1; RIF-8A; CHO; and CHO-MDR cells (Table 1). CHO-MDR cells were used as a positive control and contained approximately 3 times the amount of P-glycoprotein found in CHO cells; however, there was no significant difference in P-glycoprotein content between RIF-1 and RIF-8A. Although P-glycoprotein (M₇, 170,000) is not believed to play a role in cisplatin resistance, Rh123 has been shown to be actively extruded by this protein (19). Since Rh123 was used as an indicator of membrane potential in this study, it was crucial to demonstrate that a P-glycoprotein-dependent mechanism did not influence the Rh123 results.

Mitochondrial alterations have been shown to be associated with cell transformation, but little is known about their role in drug resistance (20). Our previous characterization of RIF-1 and RIF-8A cells revealed structural changes in the mitochondria of the resistant variant

Table 1 P-glycoprotein content in RIF-1, RIF-8A, CHO, and CHO-MDR cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>CHO</th>
<th>CHO-MDR</th>
<th>RIF-1</th>
<th>RIF-8A</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-glycoprotein*</td>
<td>0.75 ± 0.02</td>
<td>2.29 ± 0.22</td>
<td>0.60 ± 0.12</td>
<td>0.70 ± 0.07</td>
</tr>
</tbody>
</table>

* Values are expressed as arbitrary absorbance units/mg protein (n = 3).

b p < 0.05 for CHO versus CHO-MDR.
(4). Interestingly, the mitochondrial structure of RIF-8A was strikingly similar to that of a human ovarian cisplatin-induced resistant variant, C13* (4, 5). Furthermore, we have shown C13* cells to be cross-resistant to Photofrin-mediated PDT (4). Thus, the observation that RIF-8A cells are cross-resistant to cisplatin suggests possible common mechanisms of resistance related to the mitochondrial alterations. However, the ultrastructural changes of the mitochondria in RIF-8A cells were not accompanied by an increase in mitochondrial membrane potential, in contrast to that observed in C13* cells (Fig. 2).

Rh123, which accumulates in response to membrane potential, was used to compare both the plasma and mitochondrial membrane potentials in RIF-1 and RIF-8A cells (21, 22). The mitochondrial and plasma membranes of RIF-8A cells accumulated 3.6- and 3-fold less Rh123, respectively, relative to RIF-1 cells (Table 2). As a result of a cell size and mitochondrial area difference between RIF-1 and RIF-8A cells, Rh123 accumulation is expressed as amol of Rh123/mm² of mitochondrial area and amol of Rh123/mm² of cytoplasmic area, since a doubling of mitochondrial area could increase Rh123 accumulation independent of a membrane potential difference (5). Mitochondrial and cytoplasmic areas of RIF-1 and RIF-8A were published previously by Sharkey et al. (4).

Even though an increase in mitochondrial membrane potential is believed to indicate an enhanced rate of ATP production via oxidative phosphorylation (22), diminished mitochondrial membrane potential in RIF-8A cells did not compromise total cellular ATP content (4). This observation could be interpreted to suggest that RIF-8A cells have an increased dependence on ATP production via glycolysis; however, both electron microscopy and 2-deoxyglucose sensitivity (4) suggest that this is not the case. Ultrastructural changes associated with RIF-8A mitochondria appear similar to those described by Hackenbrock et al. (23) as being in a condensed state indicative of elevated oxidative phosphorylation activity, in contrast to the “orthodox-like” mitochondria in RIF-1 cells. Also, RIF-1 cells are more sensitive than RIF-8A cells to 2-deoxyglucose toxicity, an inhibitor of glycolytic ATP production, suggesting that RIF-8A cells have a reduced relative dependence on glycolysis (4).

Another possible explanation for the observed decrease in mitochondrial membrane potential in RIF-8A without a corresponding decrease in total cellular ATP is that the mitochondria in RIF-8A cells express a greater portion of their electrochemical gradient as a pH gradient rather than as a membrane potential. The driving force behind ATP synthesis is the mitochondrial electrochemical gradient, which consists of both a membrane potential and a pH gradient (22). Thus, the mitochondria in RIF-1 and RIF-8A cells may have similar electrochemical gradients but vary in the relative contribution of the two aforementioned components. A final possible explanation is that the mitochondria of RIF-8A are more tightly coupled than those of RIF-1 and thus have increased their ATP production efficiency. Not all protons extruded by the electron transport chain return via the $F_0F_1$-ATPase, thus producing ATP; some protons leak across the inner mitochondrial membrane reducing the efficiency of ATP synthesis (24). The hypothesis of an increased efficiency is supported by previous work which demonstrated that RIF-8A cells utilize less oxygen than RIF-1 cells to maintain similar intracellular ATP pools (4).

Several investigators have suggested that mitochondria may play a role in determining cisplatin sensitivity. Cisplatin has been shown to bind to mitochondrial DNA with greater affinity than nuclear DNA (25). In addition, combination chemotherapy using cisplatin and agents that alter mitochondrial function (i.e., lipophilic cations) have been shown to enhance cisplatin cytotoxicity in vitro (26) and in vivo (27). Furthermore, Shinomiya et al. (28) demonstrated that Rh123 fluorescence intensity in cells increased following exposure to cisplatin. Finally, a human ovarian cancer cell line (2008) and its resistant variant (C13*) have been shown to respond to cisplatin by elevating their ATP production (29).

Cisplatin-DNA interactions also differed between RIF-1 and RIF-8A cells. Cisplatin-DNA adduct levels were determined by atomic absorption spectrometry immediately following a 1-h exposure to cisplatin (50 μM) (Table 2). RIF-8A cells contained 24.8 ± 6.3 pg platinum/μg DNA which was significantly less than the 44.6 ± 2.0 pg platinum/μg DNA found in RIF-1 cells. Since the cytotoxic actions of cisplatin are believed to arise through its interaction with DNA, a decreased amount of DNA damage could contribute to cisplatin resistance (2). Thus, the 2-fold difference in adduct levels could account for the cisplatin resistance observed (1.6-fold). A number of other studies examining either intra- or interstrand cross-links in cisplatin-resistant variants have also shown adduct levels to be decreased in their resistant variants (30-32), including C13* cells (33). If platinum-DNA adduct levels reflect a net balance between adduct formation and repair, the lower number of adducts in the cisplatin-resistant variants may suggest an up-regulated platinum-DNA adduct repair capacity.

In this regard, the structurally and functionally altered mitochondria in RIF-8A (and C13*) may influence the efficiency of DNA repair. For example, the enzymes involved in DNA synthesis and/or repair may increase the energy requirements of the cell and thus trigger a demand for ATP production. This increased energy demand could result, in turn, in an elevation in mitochondrial activity, similar to the increase in mitochondrial activity observed during normal cell proliferation (20, 28). Therefore, enhanced capacity to produce ATP in response to cisplatin damage may provide the energy required by the enzymes involved in the repair of cisplatin adducts, even though basal

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**Table 2. Comparison of parental and PDT-resistant variant**

<table>
<thead>
<tr>
<th></th>
<th>RIF-1</th>
<th>RIF-8A</th>
</tr>
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<tbody>
<tr>
<td>Glutathione (nmol/mg protein)</td>
<td>5.1</td>
<td>5.4</td>
</tr>
<tr>
<td>Pt-DNA adducts (pg Pt/μg DNA)</td>
<td>44.6 ± 2.0</td>
<td>24.8 ± 6.3</td>
</tr>
<tr>
<td>Mitochondrial Rh123 accumulation</td>
<td>8188 ± 1120</td>
<td>2275 ± 640</td>
</tr>
<tr>
<td>(amol/mm² mitochondrial area)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma membrane Rh123 accumulation</td>
<td>156 ± 20</td>
<td>52 ± 20</td>
</tr>
<tr>
<td>(amol/mm² cytoplasmic area)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a n = 2,  
*b n = 3,  
*c Adduct levels immediately after a 1-h exposure to cisplatin (50 μM).  
*d P < 0.05 for RIF-1 versus RIF-8A.  
*e n = 4.
ATP levels, in the absence of cisplatin, were equivalent in RIF-1 and RIF-8A (4).

In summary, we have demonstrated that a PDT-resistant variant is cross-resistant to cisplatin. One of the mechanisms that apparently contributes to the resistance is a decrease in platinum-DNA adduct levels in the resistant variant. The Rh123 accumulation experiments suggest that both the plasma and mitochondrial membrane potentials are reduced in the cisplatin-resistant variant. This study suggests a novel mode of resistance.

References

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