Characterization of a Human Squamous Carcinoma Cell Line Resistant to cis-Diaminedichloroplatinum(II)

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ABSTRACT

We have developed a human head and neck squamous cell carcinoma cell line (SCC-25/CP) which is relatively stably resistant to cis-diaminedichloroplatinum(II) (CDDP) after repeated exposure to escalating doses of the drug. The studies reported elucidate the mechanism(s) by which the SCC-25/CP cell line is resistant to CDDP. The SCC-25/CP cell line is approximately 30-fold resistant to CDDP, approximately 10-fold resistant to carboplatin, and about 9-fold resistant to iproplatin.

Using [115mPt]CDDP, we examined the levels of platinum in whole cells and cellular fractions of both the SCC-25 and SCC-25/CP cells after 1 h exposure to 100 μM drug. The SCC-25 cells took up 30 pmol of platinum/10^6 cells; of this, 41% was in the nucleus and 21% in the cytosol. The SCC-25/CP cells took up 7 pmol of platinum/10^6 cells; of this, 41% was in the nucleus and 33% in the cytosol. The SCC-25 cell nuclei contained 331 pmol of platinum/mg protein and the cytosol 21 pmol of platinum/mg protein, whereas the SCC-25/CP cell nuclei contained 47 pmol of platinum/mg protein and the cytosol 8.1 pmol/mg protein. The release of drug from both cell lines followed a very similar course and was most rapid over the first 6 h.

There was no difference in the non-protein sulfhydryl content of the cell lines. The protein sulfhydryl content, as measured by Ellman's procedure, indicated that the SCC-25/CP cell line has approximately a 2-fold increase in protein sulfhydryl content compared to the SCC-25 cell line. The SCC-25/CP cell line is about 2-fold resistant to cadmium chloride at 50% cell kill and about 2.5-fold resistant at 1 log kill compared to the SCC-25 cell line. Glutathione transferase activity in crude cytoplasmic extracts was measured and found to be approximately 2- to 3-fold higher in the CDDP resistant cells. The isoelectric point of the glutathione transerase isozyme was 4.8 in both the sensitive and resistant cell lines, suggesting induction of the predominant isozyme present in the parent cell line. By alkaline elution there was greater cross-link formation by CDDP in the SCC-25 cell line than in the SCC-25/CP cell line at the same drug concentrations. In conclusion, the mechanism of resistance of the SCC-25/CP cell line to CDDP is multifactoral, involving plasma membrane changes, increased cytosolic binding, and decreased DNA cross-linking.

INTRODUCTION

CDDP has demonstrated a broad range of activity against several malignancies in humans (1). CDDP is classified with the antitumor alkylating agents because it forms bidentate adducts with DNA (2-4). It is believed that DNA is the critical intracellular target of CDDP and that DNA cross-linking is the lethal lesion caused by this drug (2, 5, 6). Like many antineoplastic agents, CDDP is a potent mutagen, inducing frame shift and base substitution mutations in both bacterial and human cells (7-10). CDDP-sensitive and -resistant L1210 cell lines have been examined by several laboratories. These studies have described changes in the plasma membrane (11-13) and changes at the level of DNA adduct formation (14) in the resistant cell lines.

Experimental studies of other alkylating agents have indicated that resistance to these drugs may occur by a variety of mechanisms. Goldenberg and Begleiter (15) and Goldenberg et al. (16) found resistance to nitrogen mustard to be associated with a transport defect in the choline carrier impeding uptake of nitrogen mustard into the cells. Resistance to PAM has also been attributed to a transport defect, probably involving the leucine carrier (17, 18). In other studies, PAM resistance was associated with an elevation in intracellular glutathione (19-22). Multifactorial resistance to PAM involving rate of drug efflux, sulfhydryl levels, and DNA interstrand cross-link formation and repair has been reported in several cell lines (23, 24). Hilton and Colvin (25) have reported that cyclophosphamide-resistant human and rodent cell lines have increased levels of an aldehyde dehydrogenase that inactivates the aldophosphamide metabolite of the drug. Increased repair of DNA monoadducts via the action of guanine O6-methyltransferase has been described as the mechanism of resistance in 1,3-bis(2-chloroethyl)-1-nitrosourea-resistant bacterial and human cell lines (26-29).

We have developed a human head and neck squamous cell carcinoma line (SCC-25/CP) which exhibits relatively stable resistance to cis-diaminedichloroplatinum(II) after repeated exposure to escalating doses of the drug (30). The studies reported here were designed to elucidate the mechanism(s) by which the SCC-25/CP cell line are resistant to CDDP.

MATERIALS AND METHODS

Drugs. CDDP, diammine [1,l-cyclobutenedicarboxylato(2)]-O,O'-platinum(II) (carboplatin), and cis-dichloro-trans-dihydroxybis(isopropylamine)platinum(IV) (iproplatin) were gifts from Johnson Matthey, Inc. (West Chester, PA). [155mPt]cis-diaminedichloroplatinum(II) in isotonic saline was made available by Drs. J. D. Hoescchele and F. F. Knapp, Jr. at Oak Ridge National Laboratories (Oak Ridge, TN) (31, 32). CdCl2 was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Cell Lines. SCC-25 and SCC-25/CP human squamous carcinoma of the head and neck cells grow as monolayers in Dulbecco-Vogt modified Eagle's minimum medium supplemented with antibiotics and 5% FBS (30). These cell lines have a plating efficiency of 10-30% and a doubling time of about 48-50 h in vitro (33). For cloning, SCC-25 and SCC-25/CP cells were suspended by trypsinization, diluted in complete growth medium, and plated into 60- x 15-mm tissue culture dishes containing 5 ml of complete growth medium. Colonies grow to a countable size (>50 cells) in 2 weeks.

The SCC-25/CP cell line has been maintained for 9 months in the absence of exposure to CDDP and the resistance of this cell line to CDDP has remained stable for that period.

Survival Studies. SCC-25 and SCC-25/CP cells in exponential growth were exposed for 1 h to concentrations of CDDP, carboplatin, or iproplatin, or CdCl2, ranging from 1-1000 μM in media without sera. The cells were then washed three times with PBS and plated for colony formation as described above. Each survival curve was determined in three independent experiments.

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Cellular Fractionation. [195mPt]CDDP was received with a specific activity of 145–165 mCi/mmol. The half-life of [195mPt] is 4.02 days, and therefore experiments were carried out immediately upon receipt of the drug. Two million SCC-25 and SCC-25/CP cells/plate were incubated with 100 μM [195mPt]CDDP in media without serum for 60 min at 37°C in humidified air with 8% CO2. Cells were washed six times with PBS, fresh medium containing serum was added, and the plates were replaced in a 37°C/8% CO2 incubator for 0, 3, 6, or 24 h. At the end of each time period, cells were suspended using 0.5% EDTA/0.125% trypsin, centrifuged at 500 × g, resuspended in water, and lysed by sonication on ice.

The fractionation procedure of Sharma and Edwards (34) was performed at 4°C and the final pellets were resuspended in water. Lysed cells were centrifuged at 3500 × g for 10 min and the mitochondrial pellet was obtained. The supernatant from the first 1000 × g spin was centrifuged at 3500 × g for 10 min to obtain the mitochondrial pellet. The second supernatant was centrifuged at 16,000 × g for 20 min to obtain the lysosomal pellet. The third supernatant was centrifuged at 100,000 × g for 60 min to obtain the microsomal pellet. This final supernatant contained the cytosol. The purity of the subcellular fractions from each cell line was checked by electron microscopy. [195mPt] content was determined using a Beckman Gamma 4000 counter. The amounts of [195mPt]CDDP were determined by comparison to standards. Each point is the mean of duplicate determinations in two independent experiments. The range of duplicate determinations was ±8%.

Protein Determinations. Protein was measured using a modified biuret assay (Sigma Diagnostics, Sigma Chemical Co., St. Louis, MO) following the cell fractionation procedure described above.

Sulfhydryl Measurements. SCC-25 and SCC-25/CP cells in exponential growth (4–10) were lyzed in 0.02 M EDTA (6 ml). For non-protein sulfhydryl determinations, the cell lysate (0.5 ml) was treated with 10% trichloroacetic acid (5 ml), then centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was neutralized then passed through a 0.2-μm filter before derivatization. Glutathione was assayed at four dilutions of the supernatants.

For the fluorescence assay, 0.1 ml of the sample was added to 3 ml of 0.1 M potassium phosphate buffer containing 5 mM EDTA (pH 8), then 0.15 ml of an OPT (Aldrich Chemical Co., Milwaukee, WI) solution (1 mg/ml OPT in methanol) was added. The derivatization was allowed to continue for 15 min at room temperature in the dark. Fluorescence was measured with an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The calibration curve was linear from 0.05–50 nmol of GSH/ml (35, 36). The data shown are the mean of five determinations.

Glutathione was also determined by the method of Hissay et al. (37). Glutathione was derivatized by monochromobimane and quantitated by peak height on high-performance liquid chromatography. Non-protein and total sulfhydryl contents were determined using a modification of the Ellman method (38–42). SCC-25 and SCC-25/CP cells (105) were lysed in 0.02 M EDTA (6 ml). For non-protein sulfhydryl determination, the cell lysate (5 ml) was treated with 10% trichloroacetic acid (5 ml), then centrifuged at 500 × g for 5 min at 4°C. The supernatant (4 ml) was adjusted to pH 8.9 with 0.2 M Tris buffer. Ellman's reagent [0.01 M 5,5'-dithiobis(2-nitrobenzoic acid)] in methanol (Aldrich) (12.5 μl/ml sample) was added and color was allowed to develop for 30 min at room temperature. After bringing the sample volume to 3 ml with methanol, the color was allowed to develop for 30 min at room temperature. All of the samples were filtered (0.45 μm) and absorbance was read at 412 nm. Protein sulfhydryl content was determined from the difference between the total sulfhydryl content and non-protein sulfhydryl content. The measurement was repeated three times.

Glutathione Transferase Measurements. SCC-25 and SCC-25/CP cells were maintained in cell culture and harvested by standard techniques. The cells were washed three times with PBS and suspended in 50 mM sodium phosphate buffer, pH 6.5. The cell suspensions were kept at 4°C, disrupted by sonication (Sonifier 200; Bronson, Inc.), and centrifuged at 16,000 × g for 30 min. GST activity in the supernatant was measured by the method of Habig et al. (42) using 1 mM 1-chloro-2,4-dinitrobenzene as the electrophilic substrate. GST activity is expressed as nanomoles of GSH-1-chloro-2,4-dinitrobenzene conjugate formed per min per mg protein. The results presented are from cells harvested on three separate occasions.

Cell extracts were analyzed by isoelectric focusing on 0.5 mm 5% polyacrylamide gels through a pH 3-10 ampholine gradient (Serva). One hundred to 500 μg of protein was applied to each gel. Gels were impregnated with 0.25 M sucrose/1.8 mM CaCl2/1% Triton X-100 solution. An equal volume of 0.34 M sucrose/0.18 mM CaCl2 solution was added to the bottom of the tube, pushing up the lighter solution. After centrifugation at 600 × g for 10 min, the nuclear pellet was obtained. The supernatant from the first 1000 × g spin was centrifuged at 3500 × g for 10 min to obtain the mitochondrial pellet. The second supernatant was centrifuged at 16,000 × g for 20 min to obtain the lysosomal pellet. The third supernatant was centrifuged at 100,000 × g for 60 min to obtain the microsomal pellet. This final supernatant contained the cytosol. The purity of the subcellular fractions from each cell line was checked by electron microscopy. [195mPt] content was determined using a Beckman Gamma 4000 counter. The amounts of [195mPt]CDDP were determined by comparison to standards. Each point is the mean of duplicate determinations in two independent experiments. The range of duplicate determinations was ±3% (SE) and the range of experimental values was ±5%.

RESULTS

The SCC-25/CP cell line was developed by repeated treatment with escalating concentrations of CDDP and is generally not cross-resistant to alkylating agents of the nitrogen mustard and nitrosourea classes as determined by median inhibitory concentration ratios (30). However, it was not known whether the SCC-25/CP line would be resistant to other platinum complexes. The survival of SCC-25 and SCC-25/CP cells exposed to various concentrations of CDDP and two second-generation platinum complexes, carboplatin and iroplatin, is shown in Fig. 1. A 30-fold higher concentration of CDDP is required to kill 90% of SCC-25/CP cells compared to SCC-25 cells. Carboplatin and iroplatin are less potent cytotoxic agents
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Fig. 1. Survival of SCC-25 (A) and SCC-25/CP (C) cells treated with various doses of CDDP (A), carboplatin (B), or iproplatin (C). Points, means of three independent determinations ± SE (bars).

Fig. 2. Subcellular distribution of [195mPt]CDDP in SCC-25 and SCC-25/CP cells. Cells were exposed to 100 μM [195mPt]CDDP for 1 h. The zero time is immediately after drug removal. △, cytosol; ●, nuclei; ○, mitochondria; ■, lysosomes; □, microsomes.

Reduced uptake of CDDP through the plasma membrane of SCC-25/CP cells as indicated by the levels of platinum in these cells after 1 h exposure to the drug provides these cells with one mechanism of resistance. However, the amount of platinum which is found in the nuclei of SCC-25/CP cells is lower than expected on the basis of the plasma membrane barrier alone. Therefore, we examined the cytosol of both cell lines for possible differences which could account for the increased differential in nuclear platinum content. The non-protein sulphydryl content of both cell lines was assayed using OPT derivatization and fluorescence measurement for total non-protein sulphydryls and monomobromobimane derivatization, high-performance liquid chromatography, and fluorescence detection for GSH (Table 1) (35–37). There was no difference in the non-protein sulphydryl content between the cell lines, and the ratio of GSH levels between the cell lines was 1.11 ± 0.13. However, the protein sulphydryl content, as measured by Ellman’s procedure, indicated that the SCC-25/CP cell line has approximately a 2-fold increase in protein sulphydryl content compared to the SCC-25 cell line (38–41). Metallothioneins, which contain about 30% cysteine, can comprise a large percentage of cellular protein sulphydryl content. Resistance to the cytotoxic effects of cadmium chloride has been used as an indicator for an increased metallothionein content (46–49). The survival of SCC-25 and SCC-25/CP cells exposed to various concentrations of cadmium chloride for 1 h is shown in Fig. 3. The SCC-25/CP cell line is about 2-fold resistant to cadmium chloride at 50% cell kill and about 2.5-fold resistant at 1 log kill compared to the SCC-25 cell line. This finding correlates very well with the increase in protein sulphydryl content of the SCC-25/CP cells.

In addition to metallothionein binding, conjugation of CDDP to GSH by glutathione-S-transferase could account for the reduced nuclear content of CDDP. GST activity in crude cytoplasmic extracts of both cell lines was measured and GST activity was found to be 2- to 3-fold higher in the SCC-25/CP cell line rather than induction of a new or previously undetectable isozyme.

It is believed that the major mechanism of the cytotoxicity of CDDP is the formation of cross-links in DNA (2, 5, 6). The formation of DNA cross-links by CDDP in the SCC-25 and SCC-25/CP cells was assessed using alkaline elution (45) at three concentrations (50, 10, and 2 μM) after 1 h exposure to the drug (Fig. 4). The formation of DNA cross-links was followed for 48 h. Overall, there was greater cross-link formation in the SCC-25 than in the SCC-25/CP cell line. Although the formation of cross-links in the SCC-25/CP cell line appears to plateau after 24 h, there was no evidence of removal of the cross-links formed up to 48 h. When the levels of cross-links formed at the various drug concentrations are compared, the cross-linking factor in SCC-25/CP cells is approximately 2-fold lower than that of the SCC-25 cell line at the same drug concentration.

DISCUSSION

CDDP has gained wide clinical use as an antineoplastic alkylating agent (1, 50). The goal of this study is to understand the mechanism(s) by which the SCC-25/CP human squamous carcinoma cell line is resistant to CDDP. These mechanisms may also account for the cross-resistance to carboplatin and iproplatin in this cell line. Cross-resistance with CDDP analogues has previously been reported in a human ovarian cancer...
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Table 1  Protein and non-protein sulfhydryl content and glutathione transferase specific activity in SCC-25 and SCC-25/CP cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Non-protein sulfhydryl*</th>
<th>Protein sulfhydryl*</th>
<th>Glutathione transferase activity†</th>
<th>Isoelectric point‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/10⁶) cells</td>
<td>(nmol/mg protein)</td>
<td>(nmol/min)</td>
<td></td>
</tr>
<tr>
<td>SCC-25</td>
<td>25 ± 2 (55 ± 5)</td>
<td>23 ± 3 (48 ± 7)</td>
<td>231 ± 25 (503 ± 55)</td>
<td>4.8</td>
</tr>
<tr>
<td>SCC-25/CP</td>
<td>21 ± 3 (54 ± 7)</td>
<td>39 ± 2 (100 ± 5)</td>
<td>447 ± 73 (1145 ± 186)</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Measured by fluorescence emission at 420 nm of an OPT derivative.
† Measured by the difference between total sulfhydryl content and non-protein sulfhydryl content using Ellman's method. Absorbance was measured at 412 nm.
‡ For the principal glutathione transferase isozyme present in these cells.
§ Mean ± S.E.
∥ Numbers in parentheses, nmol per 10⁶ cells per mg protein.
¶ Numbers in parentheses, nmol per mg protein.

Fig. 3. Survival of SCC-25 (●) and SCC-25/CP (O) cells treated with various doses of cadmium chloride. Points, means of three independent determinations ± SE (bars).

Fig. 4. DNA cross-linking in SCC-25 and SCC-25/CP cells at various times after drug exposure. The concentrations of CDDP used were: ●, 50; O, 10; and □, 2 µM. The cross-linking factor was calculated according to the method of Kohn et al. (43). Data were derived from three independent experiments ± SE (bars).

In conclusion, the mechanism of resistance of the SCC-25/CP cell line to CDDP is multifactorial: (a) there are reduced intracellular levels of platinum in the SCC-25/CP compared to SCC-25 cells, implying altered plasma membrane properties in the resistant line; (b) there is a 2-fold increase in protein sulfhydryl content, a 2-fold increase in GST, and a 2-fold reduction in sensitivity to CdCl₂, implying altered cytosolic binding and metabolism of CDDP in the resistant line; and (c) there is a reduced level of DNA cross-linking by CDDP in the SCC-25/CP line compared to the parent line. In addition to these changes which we have measured, there may be other alterations in the SCC-25/CP cell line which result in the 30-fold resistance observed.

* E. Frei III, B. A. Teicher, and C. A. Cucchi, unpublished observations.
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