Relationship between Platinum-DNA Adduct Formation and Removal and Cisplatin Cytotoxicity in Cisplatin-sensitive and -resistant Human Ovarian Cancer Cells

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ABSTRACT

We examined several aspects of platinum-DNA adduct formation and repair in cisplatin-sensitive and -resistant human ovarian cancer cell lines. The formation of cisplatin-interstrand crosslinks (ICLs) was measured in five DNA sequences by renaturing agarose gel electrophoresis. There were considerable differences (up to 4-fold) in ICL levels in these DNA sequences following a 4-h incubation with cisplatin; however, the pattern of ICL formation did not depend on whether the region was transcriptionally active or gene encoding. Incubation of purified DNA with cisplatin yielded an ICL pattern with considerably less variability between the regions examined.

Cisplatin ICL and total DNA platination levels were significantly higher (up to 20- and 40-fold, respectively) in cisplatin-resistant cell lines as compared to the parental, cisplatin-sensitive cell line at equivalent levels of cisplatin cytotoxicity. Under cisplatin exposure conditions which yielded similar initial levels of sequence-specific ICLs, the cisplatin-resistant cells removed up to 2.5 times more ICLs by 12-h posttreatment than the parental cell line. Increased removal of the individual platinum-deoxyribonucleosides of platinum-DNA adducts was also observed in the highly resistant C200 cell line as determined by high performance liquid chromatography separation and quantitation by atomic absorption spectrometry. These results indicate that DNA repair contributes significantly to cisplatin resistance and that increased DNA-damage tolerance may also be a component of the resistance phenotype in this model system.

INTRODUCTION

The major limitation to the successful treatment of ovarian neoplasms with platinum-based chemotherapeutic regimens is the emergence of drug resistance (1). Mechanisms of cisplatin resistance can be classified into two major categories: those which prevent active drug from damaging DNA and those which limit cytotoxicity following DNA damage (2). The first category includes mechanisms such as decreased cellular drug accumulation (3–5) and inactivation via protein (6–9) and non-protein sulfhydryls (10–15), while the second may include enhanced DNA repair (16–20), alteration in the types of platinum-DNA adducts formed (21, 22), and damage tolerance, which could result from an inability to undergo programmed cell death (23–25).

In order to more fully elucidate the multiple mechanisms responsible for cisplatin resistance, we have established a series of cisplatin-resistant cell lines by treating a human ovarian cancer cell line (A2780) with incremental, increasing concentrations of cisplatin. This treatment has resulted in a panel of cell lines with a range of cisplatin resistance (up to 1000-fold; Ref. 15). Using these cell lines, we have now more comprehensively examined the relationship between DNA repair and cytotoxicity in these cell lines. These studies include the determination of ICL repair capacity under conditions of equivalent, initial ICL levels. We have also measured the formation and removal of the various types of platinum-DNA adducts using HPLC separation and quantitation by AAS. These studies provide further insight into the relative contribution of DNA repair to cisplatin cytotoxicity in this model system.

MATERIALS AND METHODS

Materials. Cisplatin was obtained from Bristol-Myers Squibb (Syracuse, NY). Chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Cell culture reagents were obtained from Gibco (Grand Island, NY).

Cell Culture. Cell lines were maintained at 37°C in a humidified incubator containing 5% CO2 in RPMI 1640 supplemented with 10% (v/v) fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin, 0.3 mg/ml glutamine, and 0.3 units/ml insulin (porcine).

Cytotoxicity. Cisplatin cytotoxicity was determined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (28). Cells were plated in 150 μl of medium/well in 96-well plates (Corning Co., Corning, NY). Following overnight incubation, cells were exposed to various concentrations of cisplatin. For 4-h IC50 determination, the cells were incubated for 4 h in cisplatin-containing medium; then the medium was removed and replaced with fresh medium. For the 72-h IC50 determination, cisplatin was added directly in 10-μl volumes. Following a 72-h incubation, 40 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added per well. After 2 h at 37°C, the cells were lysed by adding 100 μl of 20% (w/v) sodium dodecyl sulfate, and 50% (v/v) N,N-dimethylformamide (pH 4.7) and incubated overnight at 37°C. The absorbance at 570 nm was determined for each well using a Bio-Rad model 3550 microplate reader. The reported IC50 is the result of triplicate determinations on at least two separate occasions.

Sequence-specific Interstrand Cross-Link Formation and Repair. Cisplatin-interstrand cross-link formation and repair studies were performed by incubating duplicate flasks of cells (1–3 × 108) for 4 h with cisplatin, followed by incubation in drug-free medium for 4, 8, or 12 h. Very little, if any, new DNA is synthesized during the repair times chosen under the cisplatin exposure conditions used. DNA was isolated by the phenol/CHC13 method, restriction-digested, and subjected to RAGE as described previously (29). Briefly, 1–10 μg of DNA were incubated at 60–65°C for 5 min in 0.2% NaOH and immediately placed on ice. Samples were loaded onto 0.5% agarose gels prepared in 90 mM Tris-borate buffer containing 2 mM EDTA and electro-
phosphorylated for 16 h at 35–40°C. Following Southern blotting, membranes were hybridized with the ABE 2 probe (provided by Dr. J. E. Sylvester, University of Pennsylvania) which recognizes a 17-kilobase HindIII fragment of the 28S ribosomal RNA gene (30), the pHD84 probe which recognizes a 23-kilobase HindIII fragment of the DHFR gene (31), the interferon-β1 probe, which recognizes a 14-kilobase HindIII fragment of the β-interferon gene (32), the 144D6 probe (D17S34, obtained from the American Type Culture Collection, Rockville, MD) which recognizes a 23-kilobase HindIII fragment of a nontranscribed variable number tandem repeat region on chromosome 17p, or the KKA35 probe (D17S75, obtained from the American Type Culture Collection), which recognizes a 38-kilobase HindIII fragment of a nontranscribed variable number tandem repeat region on chromosome 17q. Histograms were generated for each lane using an AMBIUS radioanalytic imaging system (AMBIUS Systems, San Diego, CA), and the fraction of cross-linked strands was determined by weight analysis of the peaks. The average number of interstrand cross-links/fragment was calculated using the Poisson distribution equation, 
\[ -\ln(1 - F_c) \]  where \( F_c \) is the fraction of DNA strands containing cross-links (29). DNA platination was measured relative to a standard curve for elemental platinum using a Perkin-Elmer model 3100 atomic absorption spectrometer equipped with a graphite furnace.

Separation of Platinum-DNA Adducts by HPLC. The separation of platinum-DNA adducts from A2780 and C200 cells was performed essentially as described by Eastman (33, 34). Cells were treated with 25 μM (A2780) or 1200 μM cisplatin for 4 h and either lysed or incubated in fresh medium for 12 h. Purified DNA (300–500 μg) was digested to deoxyribonucleosides sequentially with 5 units of RQ1 DNase (Promega, Madison, WI), 5 units nuclease P1, and 25 units Escherichia coli alkaline phosphatase for 16–20 h at 37°C. The individual platinum-DNA adducts were resolved by reverse phase HPLC (Hewlett Packard model 1090) on a Whatman Partisil 5 ODS-3 column (4.6 × 25 cm) using a 0–35% methanol gradient in 1 mM ammonium acetate (pH 5.5) for 35 min followed by a 30–100% methanol gradient for 5 min in 1 mM ammonium acetate (pH 5.5) at a flow rate of 1 ml/min. Equivalent amounts of DNA were injected for each cell line based on 260 nm absorbance measurements. Fractions (0.5 ml) were collected, vacuum-dried, and resuspended in 60 μl of 5% HCl. Following incubation at 90–95°C for 20 min, platinum was confirmed by AAS. The positions of the individual adducts were confirmed by determining the retention times of authentic standards which were prepared in vitro as described (34), and the relative amounts of the individual adducts were determined by weight analysis of the Pt-DNA adduct profile.

RESULTS

The 4-h IC50 of cisplatin in the A2780/C-series were determined in order to compare platinum-DNA adduct and interstrand cross-link formation and removal rates to cisplatin cytotoxicity. The IC50 for 4- and 72-h cisplatin exposures in the A2780, CP70, C30, and C200 cell lines were determined using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Table 1). The 4-h IC50 were 2- to 4-fold higher in all of the cell lines examined relative to the 72-h values, and the magnitude of resistance remained similar, regardless of the duration of drug exposure, except in the C200 cell line, which exhibited a 2-fold increase in resistance for the 4-h versus the 72-h exposure time. It should be noted that the range of resistance (~500-fold), determined with 72-h drug exposures, was lower than that reported previously (15). This is due to a change in the cisplatin sensitivity of the A2780 cell line used in these studies, which exhibited an IC50 of 0.46 μM, in contrast to the previously observed value of 0.19 μM (15). The IC50 values for cisplatin in the CP70, C30, and C200 cell lines has remained relatively similar for at least 2 years.

The conditions necessary to achieve similar levels of cisplatin-interstrand cross-links and total DNA platination were established by treating A2780 and the resistant variants with a range of cisplatin concentrations (80–800 μM) for 4 h. DNA was isolated and subjected to RAGE and AAS as described in "Materials and Methods."

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The conditions necessary to achieve similar levels of cisplatin-interstrand cross-links and total DNA platination were established by treating A2780 and the resistant variants with a range of cisplatin concentrations (80–800 μM) for 4 h and measuring the sequence-specific formation of interstrand cross-links using RAGE and total DNA platination by AAS. Based on these data, we were able to determine the relationship between total DNA platination, ICL formation, and cisplatin cytotoxicity in these cell lines. Fig. 1 shows the formation of interstrand cross-links in five DNA fragments of C30 cells relative to DNA platination. The 38-kilobase noncoding region (KKA35) of chromosome 17q contained approximately 2-fold more cross-links than the DHFR gene, 28S ribosomal RNA gene, or the 23-kilobase noncoding region (144D6) located on chromosome 17p. The β-interferon gene, which is nontranscribed in these cells (data not shown), formed the fewest number of interstrand cross-links, approximately 4-fold lower than the 38-kilobase noncoding region. This pattern of interstrand cross-link formation in C30 cells is representative of the pattern observed in all of the cell lines examined (data not shown). Interstrand cross-link formation was also measured in purified DNA which was incubated with cisplatin in vitro (Fig. 2). In this case, the level of interstrand cross-links was similar in all of the regions examined.

The data obtained from the formation of total platinum-DNA adducts and cisplatin-interstrand cross-links were used to determine the amount of total platinum-DNA adducts and interstrand cross-links produced by the concentration of cisplatin required to kill 50% of the cells (IC50) in the A2780/C-series. Fig. 3 indicates that 2.5 pg Pt/μg DNA is calculated to be present in the parental, cisplatin-sensitive A2780 cells at their 4-h IC50 cisplatin concentration, whereas the cisplatin-resistant CP70, C30, and C200 cells contained 3.0, 22, and 101 pg Pt/μg DNA, respectively, at the corresponding 4-h IC50. This indicates that approximately 40-fold more total DNA platination is required to kill the most cisplatin-resistant C200 cells as compared to the cisplatin-sensitive A2780 cells.

Table 1 Sensitivity of the A2780/C-series to cisplatin at 4-h and 72-h drug exposure times

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin IC50 (72-h)</th>
<th>Relative resistance</th>
<th>Cisplatin IC50 (4-h)</th>
<th>Relative resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>0.46 μM</td>
<td>1</td>
<td>1.10 μM</td>
<td>1</td>
</tr>
<tr>
<td>CP70</td>
<td>8.0</td>
<td>17</td>
<td>12.5</td>
<td>11</td>
</tr>
<tr>
<td>C30</td>
<td>66.0</td>
<td>143</td>
<td>145.0</td>
<td>132</td>
</tr>
<tr>
<td>C200</td>
<td>225.0</td>
<td>489</td>
<td>950.0</td>
<td>864</td>
</tr>
</tbody>
</table>

*The IC50 is the concentration of cisplatin that inhibited growth by 50%. The values are the average of triplicate determinations measured on at least two separate occasions.*
PLATINUM-DNA ADDUCT FORMATION AND REPAIR

Conditions of equivalent ICL levels in sensitive and resistant human ovarian cancer cells. Three DNA sequences were examined; two were gene encoding (ribosomal RNA and DHFR) and one was a non-gene sequence (KKA35). The cisplatin exposure conditions chosen yielded approximately 0.3 ICL/kilobases of the KKA35 non-gene encoding sequence and approximately 0.2 ICL/kilobase for the ribosomal and DHFR gene sequences (Fig. 5; Table 2). We found that, under these conditions, the cisplatin-resistant cells (CP70, C30, and C200) removed significantly more lesions than the cisplatin-sensitive A2780 cells. The rate of removal was related to the degree of cisplatin resistance such that the parental A2780 cells showed essentially no removal during the initial 4 h after cisplatin exposure in the three sequences examined but by 12 h had removed 30–36% of the cross-links in all three sequences. In contrast, the resistant cells removed 17–63% of the interstrand lesions during the 4-h postincubation time, which was related to the degree of resistance. This removal continued such that by 12 h after cisplatin exposure, the most resistant C200 cells had removed 82–86% of the interstrand lesions, while the two cell lines with intermediate resistance had removed 54–66% of these lesions. Similar removal rates were observed in all of the DNA sequences examined.

The formation and repair of individual platinum-DNA adducts were examined under conditions of similar levels of total DNA platination. Following the enzymatic digestion of purified DNA to deoxyribonucleosides and platinum-deoxyribonucleoside adducts, HPLC was used to separate the individual lesions in A2780 and C200 cells, and AAS was used to measure platinum (Fig. 6; Table 3). Five major platinum-DNA adducts could be separated using this technique including: (a) dG-Pt monoadducts; (b) dA-Pt monoadducts; (c) d(GpG)Pt intrastrand cross-links; (d) d(ApG)Pt intrastrand cross-links; and (e) (dG)₂Pt adducts, which are the digestion products of (dG)₂Pt interstrand cross-links or d(GpNpG)Pt intrastrand cross-links (Fig. 6A). The peak that eluted in the void volume is likely to result from platinum-containing protein, which was not completely removed from the DNA samples. Following a 4-h incubation of A2780 cells with 25 μM cisplatin, the largest peaks formed were the dG-Pt and dA-Pt monoadducts, which accounted for 39 and 18% of the total adduct profile, respectively. Significant levels of d(GpG)Pt (15%), d(ApG)Pt (13%), and (dG)₂Pt (11%) cross-links were also observed (Fig. 6A;...
Table 2: Removal of cisplatin interstrand cross-links from (a) the ribosomal RNA gene, (b) the DHFR gene, and (c) a noncoding gene region (KKA35) of cisplatin-sensitive and resistant human ovarian cancer cell lines.

<table>
<thead>
<tr>
<th>Repair Time (h)</th>
<th>A2780</th>
<th>CP70</th>
<th>C30</th>
<th>C200</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.204 (1)</td>
<td>0.126 (1)</td>
<td>0.165 (1)</td>
<td>0.186 (1)</td>
</tr>
<tr>
<td>4</td>
<td>0.210 (2)</td>
<td>0.111 (2)</td>
<td>0.118 (2)</td>
<td>0.082 (2)</td>
</tr>
<tr>
<td>8</td>
<td>0.162 (2)</td>
<td>0.100 (2)</td>
<td>0.083 (2)</td>
<td>0.058 (2)</td>
</tr>
<tr>
<td>12</td>
<td>0.143 (3)</td>
<td>0.083 (3)</td>
<td>0.075 (3)</td>
<td>0.034 (3)</td>
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</table>

(a) Ribosomal RNA gene

<table>
<thead>
<tr>
<th>Repair Time (h)</th>
<th>A2780</th>
<th>CP70</th>
<th>C30</th>
<th>C200</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.180 (1)</td>
<td>0.176 (1)</td>
<td>0.146 (1)</td>
<td>0.173 (1)</td>
</tr>
<tr>
<td>4</td>
<td>0.160 (1)</td>
<td>0.129 (1)</td>
<td>0.110 (1)</td>
<td>0.064 (1)</td>
</tr>
<tr>
<td>8</td>
<td>0.144 (2)</td>
<td>0.113 (2)</td>
<td>0.057 (2)</td>
<td>0.038 (2)</td>
</tr>
<tr>
<td>12</td>
<td>0.116 (3)</td>
<td>0.097 (3)</td>
<td>0.061 (3)</td>
<td>0.024 (3)</td>
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</tbody>
</table>

(b) DHFR gene

<table>
<thead>
<tr>
<th>Repair Time (h)</th>
<th>A2780</th>
<th>CP70</th>
<th>C30</th>
<th>C200</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.297 (1)</td>
<td>0.197 (1)</td>
<td>0.250 (1)</td>
<td>0.313 (1)</td>
</tr>
<tr>
<td>4</td>
<td>0.290 (1)</td>
<td>0.184 (1)</td>
<td>0.211 (1)</td>
<td>0.124 (1)</td>
</tr>
<tr>
<td>8</td>
<td>0.233 (2)</td>
<td>0.134 (2)</td>
<td>0.118 (2)</td>
<td>0.087 (2)</td>
</tr>
<tr>
<td>12</td>
<td>0.204 (3)</td>
<td>0.132 (3)</td>
<td>0.103 (3)</td>
<td>0.055 (3)</td>
</tr>
</tbody>
</table>

(c) Noncoding region (KKA35)

The values reported are the average ICL/10 kilobase pairs determined from DNA isolated from duplicate flasks of cells; kbp, kilobase pairs.

In our previous study (22), we examined the rate of removal of total platinum-DNA adducts from cisplatin-susceptible human ovarian cancer cell lines. We demonstrated an increase in the overall removal of platinum-DNA adducts in the resistant cells of the A2780/C-series as well as an increase in the overall removal of cisplatin ICLs from the ribosomal RNA gene and a noncoding DNA sequence. In the present study, we used cisplatin exposure conditions, which yielded similar levels of sequence-specific ICL levels. The cisplatin-resistant CP70, C30, and C200 cell lines showed an increase in ICL removal as compared to cisplatin-sensitive A2780 cells.

DISCUSSION

To more fully understand the contribution of the various cisplatin resistance mechanisms to cisplatin sensitivity, we studied the relationship between platinum-DNA adduct formation and removal and cisplatin cytotoxicity in the A2780/C-series. The 4-h IC50s for cisplatin in this panel of cell lines were determined in order to facilitate comparison with the 4-h DNA platination and interstrand cross-link levels. The formation of cisplatin interstrand cross-links was linear with respect to cisplatin concentration. A considerable variation existed in the level of cisplatin interstrand cross-links formed in a specific genomic sequence, up to 4-fold in the fragments analyzed. ICL formation does not appear to be dependent on the transcriptional status of the gene since the DHFR and ribosomal RNA genes formed similar levels of ICLs to the 144D6 noncoding region. The variation in ICL levels observed in the DNA isolated from cisplatin-treated cells was reduced considerably when purified DNA was incubated with cisplatin in vitro. This suggests that overall chromatin structure and not nucleotide sequence is responsible for the observed differences. Heterogeneous gene-specific ICL formation in cells treated with cisplatin (21, 22), nitrogen mustard (21, 35), and psoralens (36) has been reported previously.
compared to the parental A2780 cell line. The removal of sequence-specific ICLs was similar within the same cell line, regardless of the DNA fragment analyzed. If the kinetics of ICL repair in the KKA35 noncoding fragment represent that of bulk DNA, then it is likely that there is an increase in overall genome ICL repair rather than sequence-specific ICL repair in the resistant cell lines. This is in contrast to a report which found differences in overall genome ICL repair by alkaline elution and sequence-specific ICL repair (27). It is possible that the preferential repair of interstrand cross-links in transcriptionally active regions of DNA may be observed at lower lesion densities (21).

Our previous finding (22) of decreased DNA sequence-specific interstrand cross-links in cisplatin-resistant compared to sensitive cells under conditions of similar total DNA platination suggested that resistant cells might have the ability to alter the formation of platinum lesions at the nucleotide level. Therefore, we examined this facet of platinum-DNA damage and repair. The separation and quantitation of the individual platinum-DNA adducts has been achieved using several techniques (33, 38). Variations in the relative amounts of each individual adduct have been observed, depending on the cisplatin exposure conditions used, the preparation of the DNA, and the method of quantitation. We have found that, using 400–500 μg of DNA, the individual platinum-DNA adducts could be adequately measured by AAS following HPLC separation using the cisplatin exposure conditions used for our ICL formation and repair studies.

In the A2780 cell line, a high proportion of dO-Pt and dA-Pt monoadducts was observed following a 4-h cisplatin exposure. The decrease in dG-Pt monoadducts at 12 h and the resulting increase in d(GpG)Pt intrastrand cross-links and (dG)2Pt intra- and interstrand cross-links suggests that a significant conversion of monoadducts to cross-links has occurred. There were apparent small differences in the relative amounts of the individual platinum-deoxyribonucleoside adducts between C200 and A2780, including lower initial levels of dO-Pt monoadducts (28 versus 42%) and higher levels of d(GpG)Pt intrastrand cross-links (26 versus 15%). Plooy et al. (38) also observed the formation of significant levels of dO-Pt monoadducts and d(GpG)Pt intrastrand cross-links in Chinese hamster ovary cells following incubation with 83 μM cisplatin for 1 h. The C200 cell line demonstrated an enhanced ability to remove all of the platinum lesions by 12 h. Eastman and Schulte (20) have demonstrated previously a significant enhancement of d(GpG)Pt adduct repair in cis...
platin-resistant murine leukemia cell lines relative to the cisplatin-sensitive parental cell line.

Clearly, a higher DNA lesion density is required to kill the cisplatin-resistant cells relative to the cisplatin-sensitive A2780 cells. One reason for this is that resistant cells have a marked ability to repair platinum-DNA damage. The increased repair capacity of the cisplatin-resistant cell lines may not be sufficient, however, to account for the increased level of DNA damage required to kill these cells. For example, if the A2780 and C200 cells are exposed to their respective 4-h IC50 concentrations of cisplatin, the A2780 cells would be expected to remove 31% of the resulting ICLs from their KAA35 noncoding sequence, and the C200 cells would remove 82% of the ICLs from the same region after 12 h, based on our data (Table 2). This would result in approximately 5-fold more ICLs remaining in the C200 cells relative to the A2780 cells. A similar pattern is also observed when total platinum-DNA lesions are considered. These findings suggest that DNA-damage tolerance pathways are also present in the resistant cells. One mechanism by which a cell can exhibit damage tolerance is through increased replicative or transcriptional bypass of a DNA lesion. This has been reported to occur in murine leukemia cells (37) in which a 3- to 4-fold increased ability to synthesize DNA past platinum adducts was observed. Additionally, cisplatin-damaged DNA may no longer serve as a signal for resistant cells to undergo programmed cell death. Therefore, death must be induced by intracellular drug concentrations which are high enough to inhibit other vital cellular processes such as transcription, translation, energy production and/or cell division. The inhibition of apoptosis has been reported to result in reduced sensitivity to many chemotherapeutic agents (39, 40), and this is an area under active investigation by us in this model system.

REFERENCES

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