Independent Pathways of p53 Induction by Cisplatin and X-Rays in a Cisplatin-resistant Ovarian Tumor Cell Line

Zahid H. Siddik, Betsy Mims, Guillermina Lozano, and Gerald Thai

ABSTRACT

The p53 tumor suppressor gene is critical in regulating cell proliferation following DNA damage, and disruption of p53 protein function by mutation has been implicated as a factor responsible for resistance of tumor cells to chemotherapeutic agents. Our studies were initiated by asking whether the translational product of the p53 gene is associated with cisplatin resistance in the 2780CP human ovarian tumor model. We have demonstrated by single-strand conformation polymorphism analysis and sequencing that p53 in parental cisplatin-sensitive A2780 cells was wild type. In 2780CP cells, however, a mutation was found in exon 5 at codon 172 (Val to Phe). Interestingly, exposure to X-rays resulted in p53 induction in both A2780 and 2780CP tumor models. The p53 increases by the ionizing radiation were accompanied by concomitant increases in levels of the p53-regulated p21WAF1/Cip1 protein and led to arrest of cells in G1 phase of the cell cycle. A yeast functional assay confirmed that p53 in A2780 was wild type, but, more importantly, it provided evidence that the p53 mutation in 2780CP cells was temperature sensitive and heterozygous. These experiments demonstrate that sensitive and resistant cells have normal p53 functions, despite the presence of p53 mutation in the 2780CP model. In parallel investigations using the Western technique, exposure of A2780 cells to clinically relevant concentrations of cisplatin (1–2 μM) resulted in time- and dose-dependent increases in p53, together with coordinate increases in p21WAF1/Cip1. In contrast, cisplatin did not induce these proteins in 2780CP cells to any significant degree. The results indicate that a defect exists in the signal transduction pathway for p53 induction following cisplatin-induced DNA damage in 2780CP cells, and this may represent a significant mechanism of cisplatin resistance. Furthermore, induction of p53 in 2780CP cells by X-rays, but not cisplatin, strongly suggests that independent pathways are involved in p53 regulation for the two DNA-damaging agents.

INTRODUCTION

Ovarian cancer is diagnosed in about 20,000 patients every year and is one of the four leading causes of cancer deaths among women in the United States (1). Although platinum-based antitumor agents (i.e., cisplatin and carboplatin) play critical roles in the treatment of this disease, a major impediment concerns relapse in more than 80% of patients, who fail subsequent challenge with the platinum agent due to the onset of drug resistance in their tumor cells (1, 2). Resistance to cisplatin is characterized in general by a number of mechanisms, which include reduced drug accumulation, increased intracellular GSH, and/or increased repair of cisplatin DNA adducts (3–6). These mechanisms, individually or collectively, reduce the level or persistence of the cytotoxic DNA adducts of cisplatin and may prevent activation of apoptosis (programmed cell death) that is normally seen in tumor cells following drug exposure (7, 8). Conversely, down-regulation of the apoptotic process itself could render cells resistant not only to cisplatin but also to other chemotherapeutic agents (9–11).

Apoptosis is normally an orderly process controlled by a number of regulatory genes. The tumor suppressor p53 is one such gene, the translational product of which is stabilized on DNA damage and causes cells to arrest in the G1 phase of the cell cycle to limit the replication of damaged DNA (12, 13). Increased levels of p53 protein can also trigger programmed cell death through transactivation of the Bax gene (10, 11, 14, 15). This is consistent with a recent report that reduced expression of the Bax gene is associated with cisplatin resistance in an in vitro model of ovarian cancer (16). The intricate control of cellular processes under p53, however, can be severely disrupted by inactivation of the p53 protein through rearrangements, deletions, and missense mutations in the p53 gene (17). The consequence of this is that drug resistance can develop as a result of an absence of p53-mediated apoptotic signals (12, 18, 19).

The crucial role of p53 in drug-induced cytotoxicity is supported by reports that several anticancer agents, such as 5-fluorouracil, etoposide, and Adriamycin, are less active against tumor cells containing mutant rather than wild-type p53 both in vitro (10) and in vivo (11). Similarly, cisplatin is 2–3-fold more effective against wild-type p53-containing Burkitt’s lymphoma cells than against those with mutant p53 (20). Clinically, there is ample evidence to indicate that tumors that rarely exhibit p53 mutations at presentation, such as testicular cancer (21) and childhood acute lymphoblastic leukemia (22), are highly responsive to chemotherapeutic agents, whereas p53 mutation in tumors correlates with poor prognosis (23). Mutations in p53 have also been described in ovarian cancer (24), with mutations in Fédération Internationale des Gynécologistes et Obstétristes grade 1–3 cancers being localized in exons 5–8 between residues 135 and 275 of the DNA-binding domain in approximately 50% of the clinical samples examined (25). As a result of these recent developments, we have undertaken an investigation to define the role of p53 in cisplatin resistance in our 2780CP human ovarian tumor cells. We demonstrate that in these cells and in parental sensitive A2780 cells, X-rays increased p53 levels and induced G1 arrest, whereas cisplatin elicited p53 increases only in sensitive cells but not in the cisplatin-resistant model.

MATERIALS AND METHODS

Tumor Models. The A2780 and the corresponding 2780CP ovarian tumor cells were grown under conditions described in our previous report (26). The A2780 line was established from a patient’s biopsy prior to initiation of any chemotherapeutic regimen (27). The cisplatin-resistant A2780/C30 cells (27, 28) were used to derive the subcolon 2780CP line by growing A2780/C30 cells in cisplatin-free medium until the resistance stabilized. The A2780 and A2780/C30 models were both provided kindly by Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA).

Cytotoxicity and Biochemical Pharmacology Studies. Cells (A2780, 400 cells/well; 2780CP, 3000 cells/well) were aliquoted in 96-well plates and allowed to attach and grow for 2 days before being exposed to cisplatin (Sigma Chemical Co., St. Louis, MO). The relative sensitivities of the cell lines to the platinum complex were evaluated 5 days later using the MTT assay, as

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3 The abbreviations used are: GSH, glutathione; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC50, concentration or dose of an agent to reduce final absorbance in MTT cytotoxicity assay to 50% of control; AUC, area under the adduct versus time curve; SSCP, single-strand conformation polymorphism.

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reported previously (26). Sensitivity to X-rays was determined in a similar manner. Briefly, cells (1,200 A2780 cells/well or 36,000 2780CP cells/well) were aliquoted in 24-well plates, incubated for 2 days, and then irradiated (1.8 Gy/min) from a Philips RT 250 kV orthovoltage X-ray generator. A 3-mm lead-shielding jig allowed four wells to be irradiated at a time, with adjacent wells receiving negligible amount (<2%) of the dose. Cytotoxicity was assessed 5 days later by the MTT assay. Evaluations in attached cells of cellular cisplatin uptake, DNA adduction formation, DNA adduct repair, and endogenous GSH levels were conducted as detailed in our previous reports (26, 29).

Determination of p53 Status by SSCP and DNA Sequencing: Exponential growing cells were used to isolate DNA by the standard phenol-chloroform extraction procedure, as reported previously (30). Exons 4–9 of p53 in the DNA were amplified by PCR and were examined electrophoretically by SSCP analysis, essentially as reported (31). The 15-µl reactions contained 0.1 µg of genomic DNA; 10 pmol of primer; 0.2 mM dATP, dGTP, dTTP, and d[32P]dCTP; 0.1 Unit of AmpliTaq DNA polymerase (Perkin-Elmer Corp.); and 1.5 µl of PCR buffer (Perkin-Elmer). The primers used were: exon 5, GESF (TGCCGCGTCTCCGTTCT, forward) and GE5R (TCCAAATCTC-CAACGCGA, reverse); exon 6, GESF (CAGATAGCGATGGTGAGCAG, forward) and GE6R (GCCACTGACAACCACCCTTA, reverse); exon 7, GE7F (TGCCAGGTTCTGCTCCAGG, forward) and WG7R (AGGGTG-CAGCGGCAAGCAGA, reverse); exon 8, GE8F (ACCTGATTTCCTTACT-GTGCA, forward) and WG8R (GCCACTGACACCGCAAGA, reverse) and GE9R ( AGGGCTTGTCTGGTCTTGA, reverse). The reaction was heated at 94°C for 5 min, followed by 35 cycles at 94, 62, and 72°C for 1 min each, with a final extension at 72°C for 5 min. An aliquot of the PCR reaction was diluted with an equal volume of formamide, with 20 mM EDTA and 0.05% bromphenol blue; denatured at 95°C for 3 min; rapidly cooled on ice; and loaded on 0.5× mutation detection enhancement gel (FMC Bioproducts, Rockland, ME). Gels were prepared in Tris-borate-EDTA, as reported by Orita et al. (31), and Gels were run for 2–3 h at 37°C in the dark, and analyzed on a Becton Dickinson flow cytometer.

RESULTS

Cytotoxicity and Biochemical Pharmacology of Cisplatin in Ovarian Tumor Models. Differences between parental A2780 and variant 2780CP cells were readily apparent at the level of growth time and GSH content: the variant 2780CP line displayed a 37% longer cell doubling time and a more than 2-fold greater intracellular GSH concentration (Table 1). From an antitumor viewpoint, A2780 cells were highly sensitive to both X-rays (IC50, 1.5 Gy) and cisplatin (IC50, 0.22 or 3.9 µM for continuous or 2-h drug exposure, respectively). In comparison, 2780CP cells were substantially less sensitive to cisplatin, resulting in resistance factors of 28.6 for a 2-h exposure and 51.4 for continuous exposure. In contrast, 2780CP cells were only 3-fold cross-resistant to X-rays. Differences between the two cell lines were also observed at the biochemical pharmacological level. Following a 2-h 100 µM cisplatin exposure, the 2780CP tumor cell line accumulated about 60% less cisplatin and formed approximately 50% less DNA adducts compared to levels found in sensitive cells (Table 1). Repair of DNA adducts, on the other hand, was 2-fold greater in 2780CP cells compared to parental A2780 cells. From these data, it was possible to calculate AUC (adduct versus time curve) as an estimate of exposure of cells to the cytotoxic DNA lesion. The AUC values were determined 5 days after exposure to X-rays or cisplatin (5-day continuous or 2-h pulse exposure).

<table>
<thead>
<tr>
<th>Table 1 Cytotoxicity and biochemical pharmacology of cisplatin against ovarian tumor cells</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Doubling time (h)</td>
</tr>
<tr>
<td>Glutathione (nmol/106 cells)</td>
</tr>
<tr>
<td>X-rays IC50 (Gy)</td>
</tr>
<tr>
<td>Radiation resistance factor</td>
</tr>
<tr>
<td>Cisplatin IC50 (µM)</td>
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<tr>
<td>Continuous exposure</td>
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<tr>
<td>2-h exposure</td>
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<tr>
<td>Cisplatin resistance factor</td>
</tr>
<tr>
<td>2-h drug uptake (100 µM cisplatin) DNA adducts at 2 h (100 µM cisplatin) (ng of platinum/mg DNA)</td>
</tr>
<tr>
<td>DNA damage repair in 8 h</td>
</tr>
<tr>
<td>AUC of DNA adducts</td>
</tr>
<tr>
<td>(ng of platinum x h/mg DNA)</td>
</tr>
</tbody>
</table>

* Data are presented as means of two or three independent experiments.

a IC50 were determined 5 days after exposure to X-rays or cisplatin (5-day continuous or 2-h pulse exposure).

b Resistance factor is defined as IC50 (versus 2780CP)/IC50 (versus A2780).

c AUC was determined by the trapezoidal rule from the beginning of a 2-h 100 µM drug exposure to 8 h posttreatment.

d DNA damage tolerance is defined as the AUC of DNA adducts extrapolated to the IC50 of cisplatin obtained using 2-h drug exposures.

4 G. Lozano, unpublished data.
for these lesions in A2780 cells was approximately 2-fold greater than that found in the 2780CP line. At the IC50 cisplatin concentration, the AUC provides an indication of DNA damage tolerance, which is defined as a level of exposure to DNA lesions that will kill 50% of exposed cells (37). The results indicate that 2780CP cells can tolerate almost a 14 times greater level of exposure to DNA lesions than can parental cells (Table 1). Thus, the mechanism of resistance of 2780CP cells to cisplatin is multifactorial, which includes increased GSH levels, reduced drug accumulation, reduced DNA adduct formation, increased DNA repair, and increased DNA damage tolerance.

SSCP and DNA Sequencing Analysis for p53 Status. The correlation between p53 mutation in tumors and poor prognosis is well documented (23). To determine whether resistance of 2780CP cells to cisplatin was associated with a mutant p53 status, exons 5–9 of the p53 gene were examined by SSCP analysis. The DNA from 2780CP cells did indeed show an exon 5 SSCP variant. Sequencing of the PCR product from the exon 5 variant indicated a transverse mutation in codon 172 (GTT to TGT), which results in substitution of valine with phenylalanine in the p53 protein (data not shown). These data demonstrate unequivocally that a p53 mutation is present in 2780CP cells. No SSCP variants were identified in DNA from the A2780 cell line.

Effect of X-Rays on Cell Cycle Kinetics. Mutation in the p53 gene may lead to a loss in protein function and thereby contribute to cisplatin resistance in 2780CP cells. Thus, p53 function was investigated using the ability of X-rays to activate the G1 checkpoint in p53-proficient cells only (12, 13). Exponentially growing cells were exposed to 12.6 Gy and incubated for 24 h with nocodazole, a mitotic inhibitor that arrests cells in the G2-M phase and prevents them from contributing to the G0 population. The flow cytometry data demonstrated that untreated A2780 and 2780CP control cells had similar cell cycle distribution profiles (Fig. 1; Table 2). As expected, exposure to nocodazole alone caused cells to arrest in the G2-M phase. In contrast, exposure of A2780 or 2780CP cells to X-rays, followed by a 24-h postincubation period in the presence of nocodazole, induced G1 arrest, with a concomitant decrease in numbers of cells in the S phase. According to these data, both sensitive and resistant cells have wild-type p53 function.

Fig. 1. Arrest of ovarian tumor cells in G1 by X-rays. Cells in exponential growth phase were exposed to 12.6 Gy and then incubated for 24 h in the presence of the mitotic inhibitor nocodazole (0.4 μg/ml), which prevented cells in G2-M from reentering G1. Controls received vehicle or nocodazole only. Cells were harvested and analyzed by flow cytometry.

Table 2 Cell-cycle distribution of subpopulations as a percentage of ovarian tumor cells exposed to X-rays with or without nocodazole.

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment</th>
<th>G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>Control</td>
<td>60.7</td>
<td>17.4</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td>Nocodazole</td>
<td>4.1</td>
<td>2.8</td>
<td>93.2</td>
</tr>
<tr>
<td>2780CP</td>
<td>X-rays + nocodazole</td>
<td>44.3</td>
<td>4.7</td>
<td>51.1</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>62.4</td>
<td>17.3</td>
<td>20.2</td>
</tr>
<tr>
<td></td>
<td>Nocodazole</td>
<td>6.7</td>
<td>4.0</td>
<td>89.3</td>
</tr>
<tr>
<td></td>
<td>X-rays + nocodazole</td>
<td>33.2</td>
<td>5.5</td>
<td>61.2</td>
</tr>
</tbody>
</table>

*p Attached cells in an exponential-growth phase were exposed to X-rays (12.6 Gy) and postincubated at 37°C with or without the mitotic inhibitor nocodazole (0.4 μg/ml). After 24 h, the cells were analyzed by flow cytometry.

Fig. 2. Coordinate increases in p53 and p21Waf1/Cip1 by X-rays in the A2780 and 2780CP ovarian tumor models. Cells in exponential growth phase were exposed to either 0 (Lanes 1 and 5), 3.1 (Lanes 2 and 6), 6.3 (Lanes 3 and 7), or 12.6 Gy (Lanes 4 and 8) and then reincubated. After 4 h, cells were harvested, and protein was extracted and examined for p53 and p21Waf1/Cip1 levels by the Western technique.
**Table 3** Analysis of p53 function using the homologous recombination assay in yeast

<table>
<thead>
<tr>
<th>Cell line</th>
<th>White</th>
<th>Red</th>
<th>Pink</th>
<th>% wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>A15-010</td>
<td>295</td>
<td>12</td>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>A14-000</td>
<td>260</td>
<td>259</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>A1-000</td>
<td>70</td>
<td>6</td>
<td>117</td>
<td>36</td>
</tr>
<tr>
<td>A2780</td>
<td>119</td>
<td>5</td>
<td>0</td>
<td>96</td>
</tr>
<tr>
<td>2780CP</td>
<td>228</td>
<td>0</td>
<td>346</td>
<td>40</td>
</tr>
</tbody>
</table>

*p53 mRNA from tumor cells was reverse transcribed, amplified by PCR, and cotransformed into yeast with a gapped p53 expression vector. The yeast was then grown at 35°C for 2-3 days and colonies counted. Data are presented as a mean of two independent experiments.*

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Because increases in p53 levels can result in p53-mediated transcription of p21\(^{Waf1/Cip1}\) gene, induction of p21\(^{Waf1/Cip1}\) was also determined in a separate study. Cells were exposed for 2 h to 20 \(\mu M\) cisplatin, and protein levels were examined 24 h later. As in previous experiments, cisplatin caused greater increases in p53 protein in A2780 cells than in the 2780CP model (Fig. 5). Corresponding levels of p21\(^{Waf1/Cip1}\) were negligible in control cells but increased substantially by cisplatin in A2780 cells only (Fig. 5). It is noteworthy that p21\(^{Waf1/Cip1}\) levels were elevated only in parallel with the induction of p53, which is consistent with a p53-mediated effect.

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**DISCUSSION**

The mechanism of cisplatin resistance is characterized as multifactorial, with reduced cisplatin uptake, reduced DNA adduct formation, increased DNA adduct repair, and increased intracellular GSH being cited the most often (3–6, 27, 39, 40). In the present investigation, the cisplatin-resistant 2780CP model was no different and displayed these mechanisms also. We found that these mechanisms collectively lead to a 2-fold reduction in exposure of resistant cells to the cytotoxic lesion, as evidenced by the difference in AUC of DNA adducts between sensitive and resistant cells. This difference in adduct exposure, however, is not consistent with the 29- to 51-fold resistance of 2780CP cells to cisplatin, thereby suggesting that other mechanisms of cisplatin resistance may be more important in this model. In accord with this view, we have noted a major biochemical pharmacological difference between the sensitive and resistant cell lines: 2780CP cells can tolerate about 14-fold greater DNA damage than parental A2780 cells. The 2-fold reduction in AUC of DNA adducts and the 14-fold increase in DNA damage tolerance combine to give a theoretical resistance factor of 28. Interestingly, this value, which is derived from results using a 2-h drug exposure, is almost identical to the resistance factor of 29 obtained experimentally from IC\(_{50}\) values using a similar...
Another major difference noted between sensitive and resistant cells was their ability to respond to cisplatin-induced DNA damage through p53. Cisplatin could readily induce p53 in A2780 cells but not in resistant cells. This indicates that the signal transduction pathway involved in p53 induction was defective in 2780CP cells. Because X-rays were capable of inducing p53 in both sensitive and resistant tumor models, it is reasonable to conclude that induction of p53 by cisplatin and X-rays must occur through distinctly different signal transduction pathways. Analogous conclusions have been reached recently by Artuso et al. (48) and Zhang et al. (49) from their studies with ataxia telangiectasia cells, in which a converse effect was reported. These authors found that p53 induction was poor following exposure to ionizing radiation but was comparable to that in normal cells when exposed to methylmethane sulfonate, cisplatin, or UV light.

Once p53 is induced, it can transactivate a number of genes, including p21Waf1/Cip1 (13). Indeed, coordinate increases in p21Waf1/Cip1 were observed in both A2780 and 2780CP cells by X-rays, but only in A2780 cells by cisplatin. This indicates that p53 induction is of paramount importance for p53-mediated transactivation functions. Moreover, induction of p53 appears to be mandatory also for p53-mediated apoptotic functions (41). Thus, similar inductions of p53 by X-rays in both A2780 and 2780CP cells may account for the low cross-resistance of 2780CP cells to this ionizing radiation, whereas a lack of p53 induction by cisplatin in the 2780CP line is strongly implicated in the resistance of this model to the platinum agent. It is highly likely that in the absence of p53-mediated apoptotic signals, platinum-induced DNA lesions accumulate and account for the observed increase in 2780CP cells of DNA damage tolerance to cisplatin; this would be consistent with currently held concepts on the role of p53 in drug-induced cell death (10, 12, 19). Induction of p53 can involve up-regulation of transcriptional, translational, or post-translational activity (12, 41, 42, 50). What is not known at this stage is whether the lack of p53 induction in resistant cells by cisplatin is due to a pathway-specific signaling defect in any of these or other p53 regulatory mechanisms.

In summary, DNA damage tolerance is the major mechanism of cisplatin resistance in ovarian 2780CP tumor cells. These cells possess a normal and a mutated p53 allele, but the mutation does not affect p53 functions and, therefore, is not directly responsible for resistance. Our data, however, are consistent with the premise that down-regulation in the signaling pathway for p53 induction is a significant factor in the resistance of the 2780CP model to cisplatin. Moreover, the differential effect of cisplatin and X-rays on p53 levels in this model strongly points to the existence of independent pathways of p53 induction by these two agents.

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REFERENCES


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