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Independent Pathways of p53 Induction by Cisplatin and X-Rays in a Cisplatin-resistant Ovarian Tumor Cell Line[1]

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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 $p21^{Waf1/Cip1}$ protein and led to arrest of cells in G_1 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 (1–20 μM) resulted in time- and dose-dependent increases in p53, together with coordinate increases in $p21^{Waf1/Cip1}$. 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,[3] 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 G_1 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- to 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 Gynaecologistes et Obstetristes 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 G_1 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 subcloned 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; IC_{50}, 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.

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 adduct 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. Exponentially 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 [^{32}P]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, GE5F (TGCCGTGTTCCAGTTGCTTT, forward) and GE5R (TCCAAATACTCCACACGCAA, reverse); exon 6, GE6F (CAGATAGCGATGGTGAGCAG, forward) and GE6R (GCCACTGACAACCACCCTTA, reverse); exon 7, GE7F (TGCCACAGGTCTCCCCAAGG, forward) and WG7R (AGGGGTCAGCGGCAAGCAGA, reverse); exon 8, GE8F (ACCTGATTTCCTTACTGCCT, forward) and GE8R (GAGGCAAGGAAAGGTGATAA, reverse); and exon 9, GE9F (GTAAGCAAGCAGGACAAGAA, forward) and GE9R (ACGGCATTTTGAGTGTTAGA, 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 samples were electrophoresed for 18 h at 4.5 W of constant power. Gels were dried and visualized by autoradiography. The SSCP variants were identified, eluted from the gel in water at 55°C for 2 h, amplified by PCR, and sequenced directly or after cloning.

Determination of *p53* Status by a Yeast Functional Assay. The assay was performed essentially as described (32, 33). The mRNA was isolated from exponentially growing cells using the Dynabeads mRNA Direct Kit (Dynal, Lake Success, NY), and cDNA prepared using RT-1 (CGGGAGGTAGAC) as the primer for reverse transcription and a First Strand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). The *p53* cDNA was amplified by PCR using P3 and P4 primers (33), and the products were mixed with the pRDI-22 gapped *p53* expression vector (provided generously by Dr. Richard Iggo, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). The mixture was used to transfect a *Saccharomyces cerevisiae* strain ylG397 that contained a *p53*-responsive promoter 5′ of the adenine gene. After 48–72 h at 35°C, the plates were scored for white, pink, or red colonies. As controls, EBV-transformed lymphoblastoid cell lines from patients with Li-Fraumeni syndrome were used: the A1-000 line contained a temperature-sensitive heterozygous mutation at codon 133 of *p53* (34), A14-000 expressed an inactivating heterozygous mutation at codon 282 (35), and A15-010 was wild type.[4]

Evaluation of Cell Cycle Arrest. Attached cells in an exponential-growth phase in 100-mm tissue culture plates were exposed to X-rays (12.6 Gy) as described above and postincubated at 37°C with or without the mitotic inhibitor nocodazole (0.4 μg/ml) to prevent cells from recycling (24, 36). After 24 h, cells (1×10^6) were washed with ice-cold PBS, resuspended in 1 ml of cold PBS, fixed by dropwise addition of 2.6 ml of absolute ethanol, and left at −20°C overnight. The fixed cells were then centrifuged, rinsed in PBS, and resuspended in 1 ml of 0.04% pepsin (Sigma) in 0.01 N HCl. After 20 min at room temperature, the cells were pelleted by centrifugation and washed in PBS. Cells were finally resuspended in a solution of propidium iodide (10 μg/ml) containing 200 units/ml RNase A (Sigma) and 0.1% NP40, incubated for 2–3 h at 37°C in the dark, and analyzed on a Becton Dickinson flow cytometer.

Western Analysis. Cells were exposed to cisplatin (1–20 μM for 2 h at 37°C) or X-rays (3.1, 6.3, or 12.6 Gy in air at room temperature) and postincubated at 37°C in drug-free medium for 4–72 h. The cells were then washed with PBS and lysed for 10 min on ice with 1 ml of lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin]. The lysates were collected by microcentrifugation at 4°C, and the protein was determined by the standard Lowry procedure. Five (for p53) or 40 (for p21$^{Waf1/Cip1}$) μg of total cell protein was electrophoresed on a 10% (p53) or 15% (p21$^{Waf1/Cip1}$) SDS-polyacrylamide gel, blotted on a supported nitrocellulose 0.2 μm membrane (Bio-Rad, Hercules, CA), and blocked overnight in TBS-20 buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] containing 3% nonfat milk powder and 0.2% BSA. The membranes were probed for 2 h with either the DO-1 (for mutant and wild-type p53; Oncogene Science, Cambridge, MA) or the sdi1 (for p21$^{Waf1/Cip1}$; PharMingen, San Diego, CA) antibody. The antibody reaction was visualized by the Amersham chemiluminescence procedure using a sheep antimouse horseradish peroxidase as the second antibody (Amersham Corp., Arlington Heights, IL), and quantified by laser densitometry.

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 (IC_{50}, 1.5 Gy) and cisplatin (IC_{50}, 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

Table 1 *Cytotoxicity and biochemical pharmacology of cisplatin against ovarian tumor cells*[a]

	A2780	2780CP
Doubling time (h)	19.0	26.0
Glutathione (nmol/10^6 cells)	7.6	17.0
X-rays IC_{50} (Gy)[b]	1.5	5.0
Radiation resistance factor[c]	1.0	3.3
Cisplatin IC_{50} (μM)[b]		
Continuous exposure	0.22	11.3
2-h exposure	3.9	112
Cisplatin resistance factor[c]		
Continuous exposure	1.0	51.4
2-h exposure	1.0	28.6
2-h drug uptake (100 μM cisplatin) (ng of platinum/mg protein)	83.0	30.6
DNA adducts at 2 h (100 μM cisplatin) (ng of platinum/mg DNA)	48.5	23.9
% DNA repair in 8 h	7.8	15.5
AUC of DNA adducts (ng of platinum × h/mg DNA)[d]	421	200
DNA damage tolerance (ng of platinum × h/mg DNA)[e]	16.4	224

[a] Data are presented as means of two or three independent experiments.
[b] IC_{50} were determined 5 days after exposure to X-rays or cisplatin (5-day continuous or 2-h pulse exposure).
[c] Resistance factor is defined as IC_{50} *versus* 2780CP/IC_{50} *versus* A2780.
[d] AUC was determined by the trapezoidal rule from the beginning of a 2-h 100 μM drug exposure to 8 h postexposure.
[e] DNA damage tolerance is defined as the AUC of DNA adducts extrapolated to the IC_{50} 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 IC_{50} 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 TTT), 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 G_1 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 G_2-M phase and prevents them from contributing to the G_1 population. The flow cytometry data demonstrate 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 G_2-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 G_1 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.

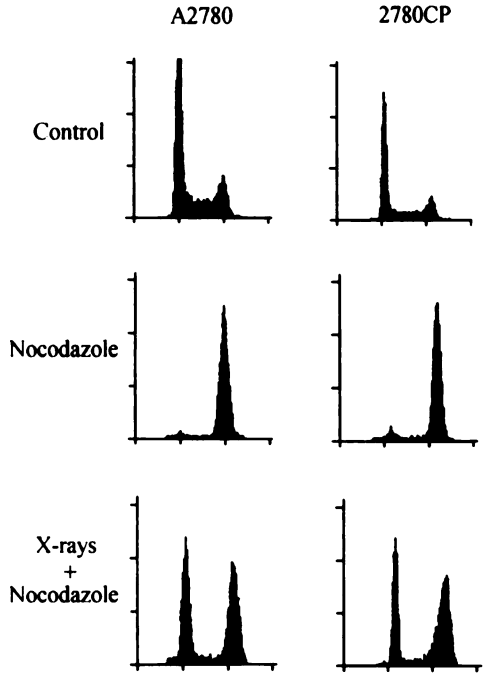

Fig. 1. Arrest of ovarian tumor cells in G_1 by X-rays. Cells in exponential growth phase were exposed to 12.6 Gy and then incubated for 24 h in the presence of the mitotic inhibitor nocodazole (0.4 μg/ml), which prevented cells in G_2-M from reentering G_1. Controls received vehicle or nocodazole only. 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*[a]

Model	Treatment	G_1	S	G_2-M
A2780	Control	60.7	17.4	21.9
	Nocodazole	4.1	2.8	93.2
	X-rays + nocodazole	44.3	4.7	51.1
2780CP	Control	62.4	17.3	20.2
	Nocodazole	6.7	4.0	89.3
	X-rays + nocodazole	33.2	5.5	61.2

[a] 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.

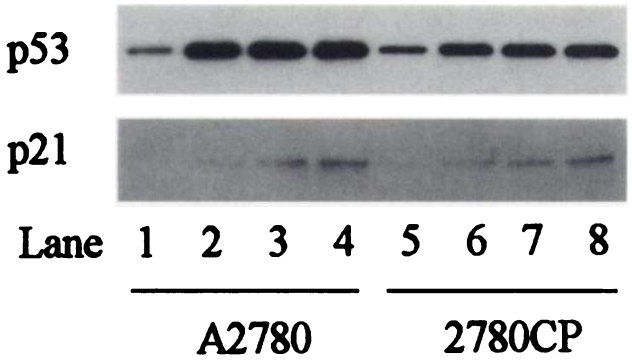

Fig. 2. Coordinate increases in p53 and $p21^{Waf1/Cip1}$ by X-rays in the A2780 and 2780CP ovarian tumor models. Cells in exponential growth phase were exposed to either 0 (*Lanes 1* and *5*), 3.1 (*Lanes 2* and *6*), 6.3 (*Lanes 3* and *7*), or 12.6 Gy (*Lanes 4* and *8*) and then reincubated. After 4 h, cells were harvested, and protein was extracted and examined for p53 and $p21^{Waf1/Cip1}$ levels by the Western technique.

To determine whether the activation of the G_1 checkpoint was mediated through p53 and the p53-regulated $p21^{Waf1/Cip1}$ gene, A2780 and 2780CP cells were exposed to X-rays (3.1, 6.3, or 12.6 Gy), and levels of p53 and $p21^{Waf1/Cip1}$ were examined 4 h later. The immunoblots, shown in Fig. 2, indicate that X-rays induced p53 in both tumor models. Levels of $p21^{Waf1/Cip1}$ were also increased in both cell lines by X-rays in a dose-dependent manner. These results demonstrate that X-rays induce p53, and also $p21^{Waf1/Cip1}$, in both parental and cisplatin-resistant 2780CP cells to similar extents. Thus, the regulation of p53 and p53-dependent transactivation function following DNA damage by X-rays appears to be normal in A2780 and 2780CP cells.

Yeast Functional Assay for *p53*. The ability to transactivate $p21^{Waf1/Cip1}$ in A2780 and 2780CP cells and induce G_1 arrest suggests that p53 is functionally competent in the two models. To confirm this, we examined the functional integrity of p53 in yeast (32, 33). Homologous recombination in yeast between the reverse transcription-PCR product spanning exons 4–10 of *p53* from tumor cells and the gapped *p53* plasmid restores *p53* sequence and activates gene expression. Repair of the gap by wild-type *p53* results in white yeast colonies. Repair by inactivating mutant *p53*, on the other hand, produces red colonies. Similarly, temperature-sensitive *p53* mutants generate pink colonies. The Li-Fraumeni cell lines gave colonies of the appropriate color and distribution that were consistent with the presence of wild-type *p53* (A15–010 cells), heterozygous inactive mutant *p53* (A14–000), or heterozygous temperature-sensitive mutant *p53* (A1-000; Table 3). With A2780 cells, the yeast functional assay revealed about 96% white colonies, indicating that *p53* was wild-type in these cells. In contrast, the reverse transcription-PCR

Table 3 *Analysis of p53 function using the homologous recombination assay in yeast*[a]

Cell line	Number of colonies			% wild type
	White	Red	Pink	
A15-010	295	12	0	96
A14-000	260	259	0	50
A1-000	70	6	117	36
A2780	119	5	0	96
2780CP	228	0	346	40

[a] 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.

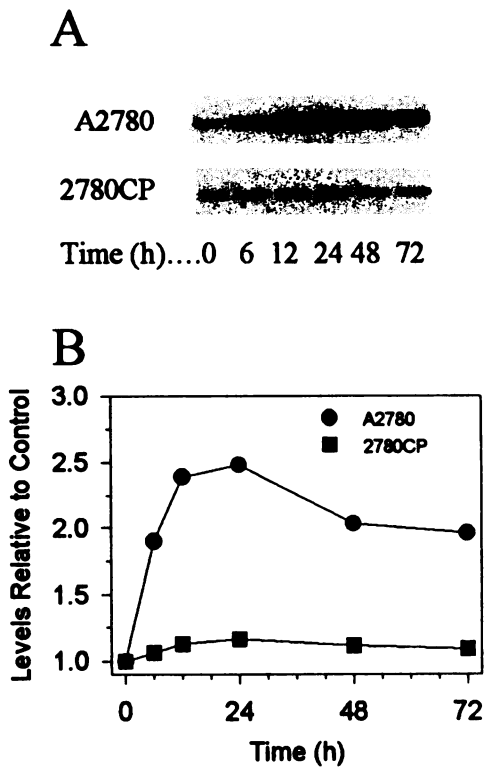

Fig. 3. Temporal increases in p53 by cisplatin in ovarian tumor cell lines. Cells in exponential growth phase were exposed to 20 μM cisplatin for 2 h and then incubated for up to 72 h without the drug. Cells were harvested at selected time points, and protein was extracted and examined for p53 levels by the Western technique. *A*, immunoblot analysis of p53. *B*, levels of p53 estimated from immunoblots by laser densitometry.

product of mRNA from 2780CP cells yielded colonies that were about 40% white and 60% pink. These results indicate that resistant cells have a heterozygous temperature-sensitive mutation in their *p53*.

Effect of Cisplatin on p53 and p21$^{Waf1/Cip1}$ Levels. To examine whether the differential cytotoxic response of A2780 and 2780CP cells to cisplatin correlated with differences in p53 induction, cells were exposed to a cisplatin concentration (20 μM; 2 h) that is clinically relevant (38), and cellular extracts subjected to Western analysis. In parental A2780 cells, induction of p53 was readily apparent (Fig. 3): levels increased rapidly, peaked at 24 h posttreatment, and then decayed approximately 35–40% by 72 h. Induction of p53 in A2780 cells was dependent on cisplatin concentration, with increased levels being observed after 24 h at drug concentrations as low as 1 μM (Fig. 4). In contrast, treatment of 2780CP cells with cisplatin produced minimal increases in p53 levels (Figs. 3 and 4). These data suggest that the pathway involved in p53 induction by cisplatin is defective in 2780CP cells.

Because increases in p53 levels can result in p53-mediated transactivation 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 μM 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.

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

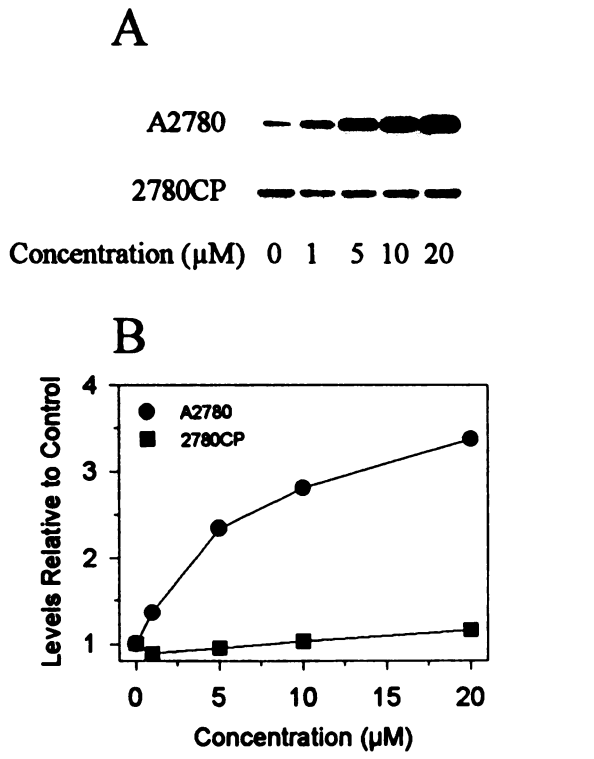

Fig. 4. Concentration-dependent increases in p53 by cisplatin in ovarian tumor cell lines. Cells in exponential growth phase were exposed to 0–20 μM cisplatin for 2 h, washed, and then incubated in drug-free medium. Cells were harvested 24 h later, and protein was extracted and examined for p53 levels by the Western technique. *A*, immunoblot analysis of p53. B, levels of p53 estimated from immunoblots by laser densitometry.

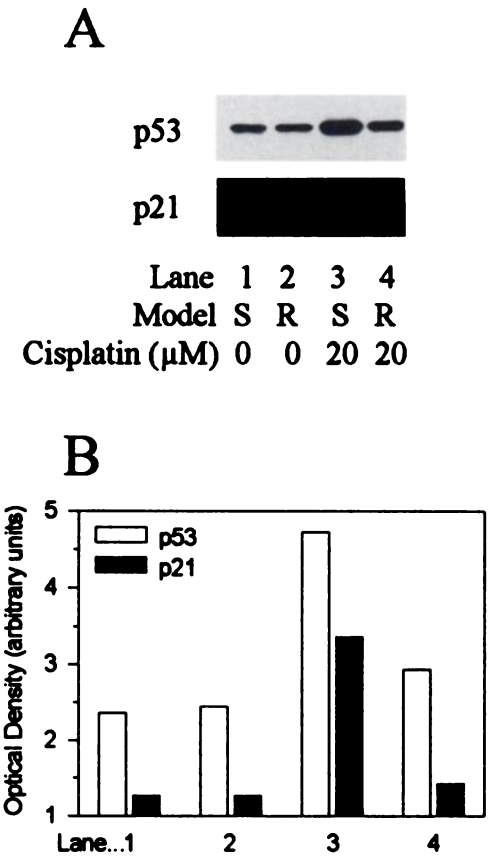

Fig. 5. Coordinate induction of p53 and p21$^{Waf1/Cip1}$ by cisplatin in the sensitive (*S;* A2780) and resistant (*R;* 2780CP) ovarian tumor models. Cells in exponential growth phase were exposed to a clinically relevant concentration of cisplatin (20 μM) for 2 h, washed, and then incubated in drug-free medium. Cells were harvested 24 h later, and protein was extracted and examined for p53 and p21$^{Waf1/Cip1}$ levels by the Western technique. *A,* immunoblot analysis of p53 and p21$^{Waf1/Cip1}$. *B,* levels of p53 estimated from immunoblots by laser densitometry.

2-h exposure protocol. The results indicate that DNA damage tolerance is a major mechanism of resistance in 2780CP cells. This conclusion is consistent with that reported recently with a panel of human ovarian tumor models (37).

In an attempt to determine the underlying basis for the substantial increase in DNA damage tolerance in 2780CP cells, we have focused our attention on *p53*. The rationale for this was based on reports that mutation in *p53* can deregulate cell cycle control and apoptosis (13, 19) and contribute to cisplatin resistance (20). Consistent with these reports is our finding that sensitive A2780 cells have wild-type *p53,* whereas resistant 2780CP cells do indeed harbor a heterozygous mutation at codon 172 of *p53* gene. However, contrary to expectation, gene mutation in resistant cells did not disrupt p53 function. This was clearly evident from data demonstrating that X-ray exposure of both A2780 and 2780CP models led to induction of p53, coordinate increases in p21$^{Waf1/Cip1}$, and activation of the G_1-checkpoint, which are all characteristics associated with p53-proficient cells exposed to DNA-damaging agents (12, 36, 41–45). However, the temperature-sensitive nature of mutant p53 in 2780CP cells suggests that the protein has partial activity, and this could explain its lack of a dominant-negative effect on wild-type p53. It is interesting to note that, although our present investigations and those of Fan *et al.* (20) suggest that loss of p53 function can lead to cisplatin resistance, other reports provide evidence to the contrary. Thus, inactivation of wild-type p53 by human papilloma virus 16 E6 transfections in MCF-7 breast tumor cells (46) and normal human foreskin fibroblasts (47) increased cisplatin sensitivity. These data suggest that other cellular factors or events may work in concert with p53 dysfunction to dictate the final cytotoxic response to cisplatin.

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 *p21$^{Waf1/Cip1}$* (13). Indeed, coordinate increases in p21$^{Waf1/Cip1}$ 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

1. Schilder, R. J., and Ozols, R. F. New therapies for ovarian cancer. Cancer Invest., *10:* 307–315, 1992.
2. Ozols, R. F. Chemotherapy for advanced epithelial ovarian cancer. Hematol. Oncol. Clin. North Am., *6:* 879–894, 1992.
3. Behrens, B. C., Hamilton, T. C., Masuda, H., Grotzinger, K. R., Whang-Peng, J., Louie, K. G., Knutsen, T., McCoy, W. M., Young, R. C., and Ozols, R. F. Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell and its use in evaluation of platinum analogues. Cancer Res., *47:* 414–418, 1987.
4. Eastman, A., Schulte, N., Sheibani, N., and Sorenson, C. M. Mechanisms of resistance to platinum drugs. *In:* M. Nicolini (ed.), Platinum and Other Metal Coordination Compounds in Cancer Chemotherapy, pp. 178–196. Boston: Martinus Nijhoff, 1988.

5. Kelley, S. L., and Rozencweig, M. Resistance to platinum compounds: mechanisms and beyond. Eur. J. Cancer Clin. Oncol., *25:* 1135–1140, 1989.
6. Andrews, P. A., and Howell, S. B. Cellular Pharmacology of cisplatin: perspectives on mechanisms of acquired resistance. Cancer Cells, *2:* 35–43, 1990.
7. Eastman, A. Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells, *2:* 275–280, 1990.
8. Demarcq, C., Bunch, R. T., Creswell, D., and Eastman, A. The role of cell cycle progression in cisplatin-induced apoptosis in Chinese hamster ovary cells. Cell Growth Differ., *5:* 983–993, 1994.
9. Hickman, J. A., Potten, C. S., Merritt, A. J., and Fisher, T. C. Apoptosis and cancer chemotherapy. Philos. Trans. R. Soc. Lond. B Biol. Sci., *345:* 319–325, 1994.
10. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. *p53*-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell, *74:* 957–967, 1993.
11. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. *p53* status and the efficacy of cancer therapy *in vivo*. Science (Washington DC), *266:* 807–810, 1994.
12. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. Cancer Res., *51:* 6304–6311, 1991.
13. Hainaut, P. The tumor suppressor protein *p53:* a receptor to genotoxic stress that controls cell growth and survival. Curr. Opin. Oncol., *7:* 76–82, 1995.
14. Miyashita, T., and Reed, J. C. Tumor suppressor *p53* is a direct transcriptional activator of the human *bax* gene. Cell, *80:* 293–299, 1995.
15. Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Van Dyke, T. *p53*-dependent apoptosis suppresses tumor growth and progression *in vivo*. Cell, *78:* 703–711, 1994.
16. Perego, P., Giarola, M., Righetti, S. C., Supino, R., Caserini, C., Delia, D., Pierotti, M. A., Miyashita, T., Reed, J. C., and Zunino, F. Association between cisplatin resistance and mutation of *p53* gene and reduced *bax* expression in ovarian carcinoma cell systems. Cancer Res., *56:* 556–562, 1996.
17. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. *p53* mutations in human cancers. Science (Washington DC), *253:* 49–53, 1991.
18. Oltval, Z. N., and Korsemeyer, S. J. Checkpoints of dueling dimers foil death wishes. Cell, *79:* 189–192, 1994.
19. Hartwell, L. H., and Kastan, M. B. Cell cycle control and cancer. Science (Washington DC), *266:* 1821–1828, 1994.
20. Fan, S., El-Deiry, W. S., Bae, I., Freeman, J., Jondle, D., Bhatia, K., Fornace, A. J., Jr., Magrath, I., Kohn, K. W., and O'Connor, P. M. *p53* gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. Cancer Res., *54:* 5824–5830, 1994.
21. Heimdal, K., Lothe, R. A., Lystad, S., Holm, R., Fossa, S. D., and Borresen, A. L. No germline TP53 mutations detected in familial and bilateral testicular cancer. Genes Chromosomes Cancer (Phila.), *6:* 92–97, 1993.
22. Jonveaux, P., and Berger, R. Infrequent mutations in the *p53* gene in primary human T-cell acute lymphoblastic leukemia. Leukemia (Baltimore), *5:* 839–840, 1991.
23. Thorlacius, S., Borresen, A. L., and Eyfjord, J. E. Somatic *p53* mutations in human breast carcinomas in an Icelandic population: a prognostic factor. Cancer Res., *53:* 1637–1641, 1993.
24. Yaginuma, Y., and Westphal, H. Abnormal structure and expression of the *p53* gene in human ovarian carcinoma cell lines. Cancer Res., *52:* 4196–4199, 1992.
25. Zheng, J., Benedict, W. F., Xu, H-J., Hu, S-X., Kim, T. M., Velicescu, M., Wan, M., Cofer, K. F., and Dubeau, L. Genetic disparity between morphologically benign cysts contiguous to ovarian carcinomas and solitary cystadenomas. J. Natl. Cancer Inst., *87:* 1146–1153, 1995.
26. Yoshida, M., Khokhar, A. R., and Siddik, Z. H. Biochemical pharmacology of homologous alicyclic mixed amine platinum(II) complexes in sensitive and resistant tumor cell lines. Cancer Res., *54:* 3468–3473, 1994.
27. Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C., and Anderson, M. E. High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of glutathione synthesis. Proc. Natl. Acad. Sci. USA, *89:* 3070–3074, 1992.
28. Hamaguchi, K., Godwin, A. K., Yakushiji, M., O'Dwyer, P. J., Ozols, R. F., and Hamilton, T. C. Cross-resistance to diverse drugs is associated with primary cisplatin resistance in ovarian cancer cell lines. Cancer Res., *53:* 5225–5232, 1993.
29. Kido, Y., Khokhar, A. R., and Siddik, Z. H. Glutathione-mediated modulation of tetraplatin activity against sensitive and resistant tumor cells. Biochem. Pharmacol., *47:* 1635–1642, 1994.
30. Kido, Y., Khokhar, A. R., Al-Baker, S., and Siddik, Z. H. Modulation of cytotoxicity and cellular pharmacology of 1,2-diaminocyclohexane platinum(IV) complexes mediated by axial and equatorial ligands. Cancer Res., *53:* 4567–4572, 1993.
31. Orita, M., Suzuki, Y., Sekiya, T., and Hayashi, K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics, *5:* 874–879, 1989.
32. Ishioka, C., Frebourg, T., Yan, Y-X., Vidal, M., Friend, S. H., Schmidt, S., and Iggo, R. Screening patients for heterozygous *p53* mutations using a functional assay in yeast. Nat. Genet., *5:* 124–129, 1993.
33. Flaman, J-M., Frebourg, T., Moreau, V., Charbonnier, F., Martin, C., Chappuis, P., Sappino, A-P., Limacher, J-M., Bron, L., Benhattar, J., Tada, M., Van Meir, E. G., Estreicher, A., and Iggo, R. D. A simple *p53* functional assay for screening cell lines, blood, and tumors. Proc. Natl. Acad. Sci. USA, *92:* 3963–3967, 1995.
34. Law, J. C., Strong, L. C., Chidambaram, A., and Ferrell, R. E. A germ line mutation in exon 5 of the *p53* gene in an extended cancer family. Cancer Res., *51:* 6385–6387, 1991.
35. Brugieres, L., Gardes, M., Moutou, C., Chompret, A., Meresse, V., Martin, A., Poisson, N., Flamant, F., Bonaiti-Pellie, C., Lemerle, J., and Feunteun, J. Screening for germ line *p53* mutations in children with malignant tumors and a family history of cancer. Cancer Res., *53:* 452–455, 1993.
36. O'Connor, P. M., Jackman, J., Jondle, D., Bhatia, K., Magrath, I., and Kohn, K. W. Role of the *p53* tumor suppressor gene in cell cycle arrest and radiosensitivity of Burkitt's lymphoma cell lines. Cancer Res., *53:* 4776–4780, 1993.
37. Johnson, S. W., Laub, P. B., Beesley, J. S., Ozols, R. F., and Hamilton, T. C. Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines. Cancer Res., *57:* 850–856, 1997.
38. Vermorken, J. B., Van Der Vijgh, W. J. F., Klein, I., Gall, H. E., and Pinedo, H. M. Pharmacokinetics of free platinum species following rapid, 3-hr and 24-hr infusions of *cis*-diamminedichloroplatinum(II) and its therapeutic implications. Eur. J. Cancer Clin. Oncol., *18:* 1069–1074, 1982.
39. Johnson, S. W., Swiggard, P. A., Handel, L. M., Brennan, J. M., Godwin, A. K., Ozols, R. F., and Hamilton, T. C. Relationship between platinum-DNA adduct formation and removal and cisplatin cytotoxicity in cisplatin-sensitive and -resistant human ovarian cancer cells. Cancer Res., *54:* 5911–5916, 1994.
40. Johnson, S. W., Perez, R. P., Godwin, A. K., Yeung, A. T., Handel, L. M., Ozols, R. F., and Hamilton, T. C. Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. Biochem. Pharmacol., *47:* 689–697, 1994.
41. Fritsche, M., Haessler, C., and Bradner, G. Induction of nuclear accumulation of the tumor-suppressor protein *p53* by DNA-damaging agents. Oncogene, *8:* 307–318, 1993.
42. Tishler, R. B., Calderwood, S. K., Coleman, C. N., and Price, B. D. Increases in sequence specific DNA binding by *p53* following treatment with chemotherapeutic agents. Cancer Res., *53:* 2212–2216, 1993.
43. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. *WAF1,* a potential mediator of *p53* tumor suppression. Cell, *75:* 817–825, 1993.
44. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. Cell, *75:* 805–816, 1993.
45. Bae, I., Fan, S., Bhatia, K., Kohn, K. W., Fornace, A. J., Jr., and O'Connor, P. M. Relationships between G_1 arrest and stability of the *p53* and *p21Cip1/Waf1* proteins following γ-irradiation of human lymphoma cells. Cancer Res., *55:* 2387–2393, 1995.
46. Fan, S., Smith, M. L., Rivet, D. J., Duba, D., Zhan, Q., Kohn, K. W., Fornace, A. J., Jr., and O'Connor, P. M. Disruption of *p53* sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. Cancer Res., *55:* 1649–1654, 1995.
47. Hawkins, D. S., Demers, G. W., and Galloway, D. A. Inactivation of *p53* enhances sensitivity to multiple chemotherapeutic agents. Cancer Res., *56:* 892–898, 1996.
48. Artuso, M., Esteve, A., Bresil, H., Vuillaume, M., and Hall, J. The role of the Ataxia telangiectasia gene in the *p53, WAF1/CIP1(p21)*- and *GADD45*-mediated response to DNA damage produced by ionising radiation. Oncogene, *11:* 1427–1435, 1995.
49. Zhang, N., Song, Q., Lu, H., and Lavin, M. F. Induction of *p53* and increased sensitivity to cisplatin in ataxia-telangiectasia cells. Oncogene, *13:* 655–659, 1996.
50. Gotlieb, W. H., Watson, J. M., Rezai, A., Johnson, M., Martinez-Maza, O., and Berek, J. S. Cytokine-induced modulation of tumor suppressor gene expression in ovarian cancer cells: up-regulation of *p53* gene expression and induction of apoptosis by tumor necrosis factor-α. Am. J. Obstet. Gynecol., *170:* 1121–1130, 1994.