0026-895X/09/030503-09$3.00/0

MOLECULAR PHARMACOLOGY, **57:**503–511 (2009).

Effect of p53 Status on Sensitivity to Platinum Complexes in a Human Ovarian Cancer Cell Line

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Received July 28, 1999; accepted December 3, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

Wild-type p53 is frequently mutated in late-stage ovarian cancer and has been proposed as a determinant of cisplatin chemosensitivity. We have therefore established a human ovarian cancer cell line differing only in p53 status and characterized its response after treatment with different platinum complexes. The wild-type p53-expressing cell line A2780 was stably transfected with *HPV-16 E6* (E6) or an empty vector (VC) as control. Parental A2780 and VC had similar cisplatin sensitivities, whereas E6 was 3- to 4-fold more sensitive as measured by sulforhodamine B and clonogenic assay. E6 was 2- to 3-fold more sensitive to transplatin and the novel cisplatin analog ZD0473 than VC, whereas the *trans*-platinum analog JM335 was approximately equitoxic. Platinum uptake was similar for all of the cell lines after cisplatin. The removal of platinum-DNA adducts, as measured by atomic absorption spectroscopy, was reduced in E6 compared with VC after cisplatin but similar after JM335. After 10 μM cisplatin, the G_1 population (0–96 h) was reduced in E6 cells compared with VC, whereas the S phase (8–48 h) and G_2 phase (48–96 h) were increased. Similar proportions of VC and E6 cells died by apoptosis, as detected by annexin V binding, but more E6 cells died by necrosis relative to VC. Our results suggest that the loss of functional p53 can increase cisplatin cytotoxicity in A2780, with loss of G_1/S checkpoint control and decreased cisplatin-DNA adduct repair, but these effects can be circumvented by the use of JM335, which forms different DNA-platinum adducts.

The *p53* (TP53) gene is the most frequently mutated gene in human cancer (Hollstein et al., 1991). After genotoxic stress, the wild-type p53 protein is induced and stabilized and transactivates several downstream genes (reviewed in Amundson et al., 1998), including *MDM-2*, encoding an inhibitor of p53 transcriptional activation and a promoter of p53 degradation; *BAX*, encoding a promoter of apoptosis; *GADD-45*, shown to encode a protein involved in DNA repair and G_2 arrest; and $p21^{WAF1/CIP1}$, encoding an effector of the G_1/S and G_2 cell cycle arrests and nucleotide excision repair (NER). p53 interacts directly with other proteins, such as transcription factors involved in DNA repair, and has also been implicated in the negative regulation of some proteins, such as BCL-2, an inhibitor of apoptosis. Thus, p53 induction can arrest cell cycle progression and facilitate DNA repair and/or induce apoptosis.

Wild-type p53 function can be lost by mutation, cytoplasmic sequestration, phosphorylation, changes in conformation, and binding and degradation mediated by cellular proteins (reviewed by Kubbutat and Vousden, 1998). Several viral proteins can also disrupt p53 function, including the E6 protein encoded by HPV-18 and HPV-16 that binds p53 and targets it for degradation via an ubiquitin-mediated pathway (Scheffner et al., 1990).

The platinum-based drugs cisplatin and carboplatin play a major role in the chemotherapy of ovarian cancer (Adams et al., 1998). The cytotoxicity of cisplatin (reviewed by Chu, 1994) is thought to be due to the formation of intrastrand and interstrand cross-links in the DNA, which may induce cell cycle arrest and apoptosis. Cisplatin-DNA adducts have been shown to be repaired predominantly by NER (Chu, 1994). Thus, the cytotoxicity of cisplatin may be influenced by the functional p53 status of a cell through its effects on NER, the cell cycle, or apoptosis.

Given the prevalence of loss of p53 function in human cancer, including 68% of stage 3 and 4 ovarian cancers (Shelling et al., 1995), the role of p53 in cisplatin cytotoxicity is the subject of considerable current interest (Wu and El Diery, 1996; O'Connor et al., 1997). Studies of the role of p53 specifically in human ovarian cancer cell lines have shown either no association (De Feudis et al., 1997) or an association (Wu and El Diery, 1996; Pestell et al., 1998) between wild-type p53 expression and platinum sensitivity. Resistance to cisplatin has been demonstrated in human ovarian cancer cell lines transfected with either the HPV-16 E6 protein (Wu and El

This work was supported by the Cancer Research Campaign, UK. K.E.P. is the recipient of an Institute of Cancer Research Ph.D. studentship.

ABBREVIATIONS: VC, vector control transfected cell line; E6, HPV-16 E6-transfected cell line; SRB, sulforhodamine B; NER, nucleotide excision repair; DMEM, Dulbecco's modified Eagle's medium; PI, propidium iodide; FAAS, flameless atomic absorption spectroscopy; DNA-Pt, DNA-platinum; FITC, fluorescein isothiocyanate.

Diery, 1996) or a dominant-negative mutant p53 (Brown et al., 1993; Herod et al., 1996). However, increased sensitivity to cisplatin has been demonstrated on transfection of HPV-16 E6 protein into wild-type p53-expressing fibroblasts and colon and breast cancer cell lines (Fan et al., 1995; Hawkins et al., 1996).

The aim of the present investigation was to determine whether there is a relationship between p53 status and platinum drug sensitivity using isogenic *HPV-16 E6*-transfected derivatives of the A2780 human ovarian cancer cell line that differ only in p53 status. We investigated platinum drug cytotoxicity and the effects on cell cycle control, DNA repair, and apoptosis in the transfected cell lines. We show in this model system that the loss of p53 leads to enhanced cisplatin chemosensitivity due to the loss of G_1/S checkpoint control, DNA repair, and the involvement of cell death pathways additional to apoptosis. However, sensitivity to the *trans*-platinum analog JM335 is not affected by loss of p53 function in this model system, and this may be due to the different DNA-platinum adducts formed by this compound.

Materials and Methods

Cell Culture and Transfection. The A2780 human ovarian carcinoma cell line was obtained from the American Type Culture Collection (Eva et al., 1982). Cells were grown as attached monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4500 mg/l glucose, 10% FCS (Life Technologies, Paisley, UK), 200 mM L-glutamine, and 1× nonessential amino acids (Life Technologies) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Regular checks were carried out for *Mycoplasma* using a polymerase chain reaction-based assay (Stratagene, Cambridge, UK). Cells were transfected with either an empty vector expressing puromycin resistance alone (plasmid F179) or a vector expressing HPV-16 E6 protein and puromycin resistance (plasmid F192), using the liposomal reagent *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP; Boehringer-Mannheim Biochemicals, Indianapolis, IN). Plasmid F179 is a dual cassette eukaryotic expression vector in which transcription of the inserted cDNA is driven from the cytomegalovirus enhancer/promoter in one cassette and expression of the puromycin resistance gene *pac* is driven by the SV40 promoter in the other cassette. A plasmid origin of replication and ampicillin-resistance gene were included for propagation in bacteria. Plasmid F192 contains the coding region for the HPV-16 E6 protein cloned into F179, in which the open reading frame is in the sense orientation with respect to the cytomegalovirus promoter. Cells were grown in medium containing 1 μg/ml puromycin, and clones were picked, amplified, and characterized. Doubling times were determined by seeding 10^5 cells/well onto six-well plates and counting at 24-h intervals for 4 days.

Radiation Treatment. The induction of p53 protein and $p21^{WAF1/CIP1}$ mRNA was determined at 4 h, and G_1/S arrest was measured at 16 h, after 5-Gy irradiation. Radiation was delivered using a ^{60}Co source with a source-to-flask distance of 40 cm and a dose rate of 1.36 Gy/min.

Immunoblotting. Cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 0.2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium orthovanadate) for 30 min on ice and centrifuged, and the supernatant was recovered. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Chester, UK). Samples (50 μg protein) were separated on 12% SDS-polyacrylamide gels, and the proteins were transferred electrophoretically to Hybond-C extranitrocellulose membranes (Amersham, Little Chalfont, UK). Immunodetection of p53 was carried out using DO-1, a mouse monoclonal antibody that binds wild-type and mutant p53 proteins (Santa Cruz Biotechnology, Santa Cruz, CA). Binding of the primary antibody was visualized by incubation with an anti-mouse horseradish peroxidase-linked secondary antibody (Amersham) and followed by enhanced chemiluminescence detection (Pierce) using appropriate film.

Northern Blotting. RNA was prepared as previously described (Chirgwin et al., 1979). Briefly, cells were lysed in GIT buffer (4 M guanidine isothiocyanate, 3 M sodium acetate, pH 6, and 0.12 M β-mercaptoethanol). The extract was centrifuged for 21 h at 170,000*g* through a 5.7 M cesium chloride gradient, and the resulting RNA pellet was dissolved in 0.3 M sodium acetate, ethanol precipitated, washed in 70% ethanol, and redissolved in water. RNA was quantified by A_{260} measurement. RNA (20 μg) was separated on a 1.2% agarose gel in the presence of formaldehyde and transferred by capillary action in 10× SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) to nylon Hybond N membranes (Amersham). RNA was fixed by UV cross-linking at 1200 J/m^2 (Stratagene) and baking at 80°C for 2 h.

The probes used for hybridization were as follows: $p21^{WAF1/CIP1}$ full-length human cDNA (2.12 kb) obtained from an *Eco*RI restriction of pZL-WAF1, *HPV-16 E6* cDNA (1.3 kb), and rat *GAPDH* cDNA fragment (1.3 kb) obtained as a *Pst*I fragment from a pUC-9 based vector into which a *Pst*I fragment had been subcloned from pr-GAPDH-13 (Fort et al., 1985). These cDNA probes were random prime labeled with α-^{32}P-dCTP using a DNA labeling kit (Pharmacia Biotech, St. Albans, UK). Probes were separated from unincorporated ^{32}P before hybridization using Microspin S-300 HR columns (Pharmacia Biotech).

Membranes were hybridized overnight with the radiolabeled

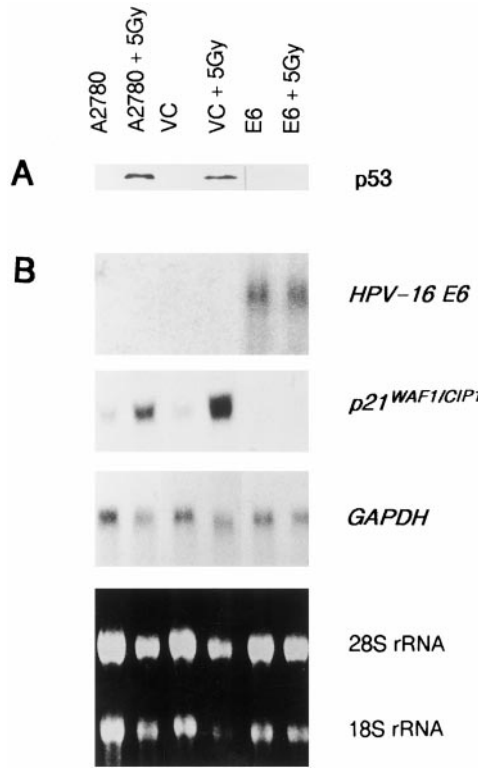

Fig. 1. Characterization of the VC and E6-transfected A2780 cell lines. A, a typical immunoblot showing induction of p53 protein expression after irradiation. Protein was extracted from exponentially growing control cells and from cells 4 h after irradiation with 5 Gy and immunoblotted as described in *Materials and Methods*. B, representative Northern blot showing *HPV-16 E6* and $p21^{WAF1/CIP1}$ mRNA expression after irradiation. RNA was extracted from exponentially growing cells and cells 4 h after irradiation with 5 Gy and analyzed as detailed in *Materials and Methods*. *GAPDH* mRNA, 18S rRNA, and 28S rRNA are shown as loading controls.

probe in Church and Gilbert's buffer (7% SDS, 0.25 M disodium hydrogen phosphate, pH 7.2) at 65°C. After hybridization, the membranes were washed twice in 2× SSC, 0.1% SDS, and 0.1% sodium pyrophosphate for 10 min and once in 0.1× SSC, 0.1% SDS, and 0.1% sodium pyrophosphate for 30 min, all at 65°C. Bands were visualized on film (Kodak X-Omat AR), and loading and transfer efficiency was checked using *GAPDH* mRNA, 18S rRNA, and 28S rRNA.

Drug Sensitivity Assays. Cisplatin (*cis*-[$PtCl_2(NH_3)_2$]); ZD0473, formerly known as AMD473 and JM473 (*cis*-[$PtCl_2(NH_3)$-(C_5H_4N-2′CH_3)]; Holford et al., 1998); and transplatin (*trans*-[$PtCl_2(NH_3)_2$]) and JM335 (*trans*-[$PtCl_2(OH)_2(NH_3)$-(c-$C_6H_{11}NH_2$)]; Mellish et al., 1995) were all from Johnson Matthey Technology Center (Reading, Berkshire, UK) and were prepared in 0.9% saline at 1 mM (cisplatin and ZD0473) or 0.5 mM (transplatin and JM335). Cytotoxicity was assessed using the SRB growth delay assay as previously described (Skehan et al., 1990). Briefly, between 3.5×10^4 and 5×10^4 cells were seeded onto each well of 96-well plates (Falcon, Becton-Dickinson, Cowley, UK) and allowed to attach overnight. Serial dilutions of platinum drugs were added to quadruplicate wells for 96 h, and cells were stained with 0.4% SRB in 1% acetic acid. IC_{50} values were determined as the drug concentration that reduced the cell density to 50% of that in untreated control wells. Cytotoxicity was also assessed using clonogenic survival by incubating cells with five doses of cisplatin or JM335 for 2 h and then plating at 10^2, 10^3, and 10^4 cells/well onto 6-well tissue culture plates (Falcon). The plates were incubated at 37°C for 10 to 14 days until visible colonies (50+ cells) had formed. Colonies were fixed with methanol and stained with 0.5% methylene blue before counting.

Measurement of Platinum Accumulation. Exponentially growing cells were exposed to 100 μM cisplatin for 2 h. Cells were washed twice with ice-cold PBS, scraped and harvested in 500 μl of ice-cold water, and sonicated with 2× 5-s pulses on a Soniprep 150 (Fisons) set on half-maximum power. Protein was measured by the bicinchoninic acid protein assay (Pierce). Platinum content was determined by flameless atomic absorption spectroscopy (FAAS) using models HGA700 and 1100B (Perkin-Elmer Cetus, Norwalk, CT).

Determination of G_1/S Checkpoint Integrity. Exponentially growing cells were irradiated and 16 h later pulse labeled for 4 h with 10 μM bromodeoxyuridine, recovered, and fixed in ice-cold 70% ethanol. Cellular DNA was partially denatured in 2.5 M HCl containing 0.2 mg/ml pepsin for 20 min. Cells were then washed and resuspended in blocking buffer (1% BSA, 0.5% Tween 20 in PBS) containing a primary mouse monoclonal anti-bromodeoxyuridine antibody (DAKO, Glostrup, Denmark) for 1 h and then washed and resuspended in blocking buffer containing a fluorescein isothiocyanate-conjugated anti-mouse antibody (Sigma, Poole, UK) for 30 min. Finally, cells were stained overnight in PBS containing 10 μg/ml propidium iodide (PI) before flow cytometric analysis on a Coulter EPICS elite ESP flow cytometer (Beckman Coulter, High Wycombe, UK). The percentage of cells arresting in G_1/S after irradiation was determined by calculating the proportion of cells not entering S phase after irradiation relative to unirradiated normal cycling cells.

Flow Cytometry. Cells were incubated for 2 h with 10 or 25 μM cisplatin, washed with PBS, and then grown under standard conditions. Cells were fixed in ice-cold 70% ethanol at 0, 8, 16, 24, 48, 72, and 96 h after initial cisplatin treatment. The cells were stained

TABLE 1
Platinum drug sensitivity of transfected A2780 human ovarian cancer cells determined by SRB growth delay assay

Cell Line	IC_{50}			
	Cisplatin[a]	Transplatin[a]	ZD0473[a]	JM335[a]
	μM			
Parental A2780	1.58 ± 0.06*	N.D.	N.D.	N.D.
VC	1.90 ± 0.08*,**	129 ± 20.8***	7.73 ± 0.37**	1.87 ± 0.13*
E6	0.63 ± 0.07**	50.9 ± 11.1***	2.42 ± 0.14	1.25 ± 0.20*

[a] Values are mean ± S.E. for data from three independent experiments with four values for each.
* No significant difference between values.
** $P < .01$.
*** $P < .05$.
N.D., not determined.

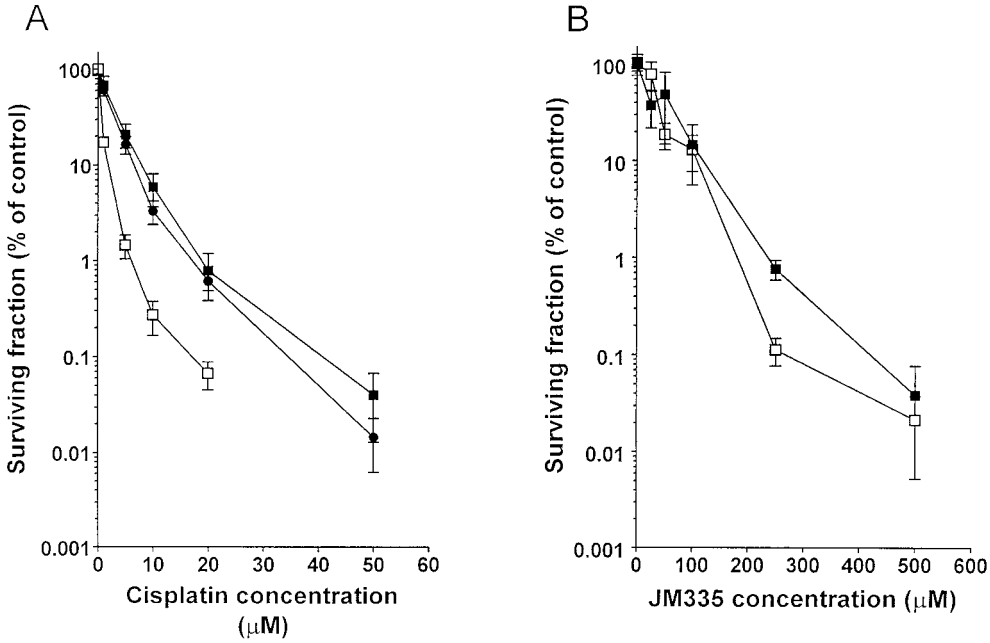

Fig. 2. Cisplatin (A) and JM335 (B) cytotoxicity in transfected A2780 cell lines. ●, A2780. ■, VC. □, E6. Values are the mean ± S.E. (n = 6–9) of data from two or three independent experiments. Determined by clonogenic survival after 2-h drug exposure as described in *Materials and Methods*.

overnight in PBS containing 10 μg/ml PI and 50 μg/ml RNase before flow cytometric analysis as described earlier.

DNA-Platinum (DNA-Pt) Binding and Removal. Exponentially growing cells were exposed to 100 μM cisplatin or JM335 for 2 h, washed once with PBS, and further incubated in normal medium for 0, 3, or 22 h. DNA was extracted by a high salt extraction method (Miller et al., 1988). Cells were detached by trypsinization and resuspended in TE-9 buffer (0.5 M Tris, pH 9.0, 20 mM EDTA, pH 7.0, 10 mM NaCl) containing 1% SDS and 0.5 mg/ml proteinase K and incubated overnight at 37°C. Saturated NaCl (0.25 volume) was added, the solution was shaken vigorously, and the protein was spun down at 500*g* for 15 min. The supernatant was recovered, and the DNA was ethanol precipitated. The DNA was redissolved in 3 ml of TE-9 buffer containing 0.1 mg/ml RNase and incubated at 37°C for 1 h. The DNA was ethanol precipitated, washed, and redissolved in 350 μl of DNase-free water and quantified using the A_{260}. Platinum content was determined by FAAS using models HGA700 and 1100B (Perkin-Elmer Cetus).

Trypan Blue Exclusion Assay. Exponentially growing cells were incubated for 2 h with cisplatin, washed once with PBS, and exposed to fresh medium. Detached cells were removed from duplicate flasks 24, 48, 72, and 96 h after the addition of cisplatin. Trypan blue (final concentration, 0.8%) was added to the detached cells, and after 5 min, the cells were mounted in a hemocytometer and the number of trypan blue excluding cells was counted.

Annexin V Binding. Exponentially growing cells were exposed to cisplatin for 2 h and washed once with PBS before the addition of fresh medium. Attached and detached cells were recovered 5, 24, and 48 h after initial drug exposure. The cells were resuspended in annexin V binding buffer (Clontech, Palo Alto, CA). PI (2.5 μg/ml) and annexin V-fluorescein isothiocyanate (1 μg/ml; Clontech) were added, and the cells were left at room temperature for 15 min before flow cytometric analysis.

Light Microscopy. Attached and detached cells were recovered 24 and 48 h after 2-h cisplatin exposure and fixed in 2% glutaraldehyde in 0.05% sucrose (pH 7.3). Pellets were postfixed for 1 h in 1% osmium tetroxide, dehydrated through a graded series of ethanols, infiltrated, and embedded in Epon. For light microscopy, sections of 1 μm thickness were cut and stained with toluidene blue.

Significance Tests. Levels of significance were determined using the two-tailed paired Student's *t* test (Prism; GraphPAD Software, San Diego, CA). Differences in cytotoxicity were compared using IC_{50} values.

Results

Characterization of VC and E6 Transfectants of A2780. The wild-type p53-expressing cell line, A2780 (Brown et al., 1993; Pestell et al., 1998), was transfected with either a vector expressing puromycin resistance alone or a vector

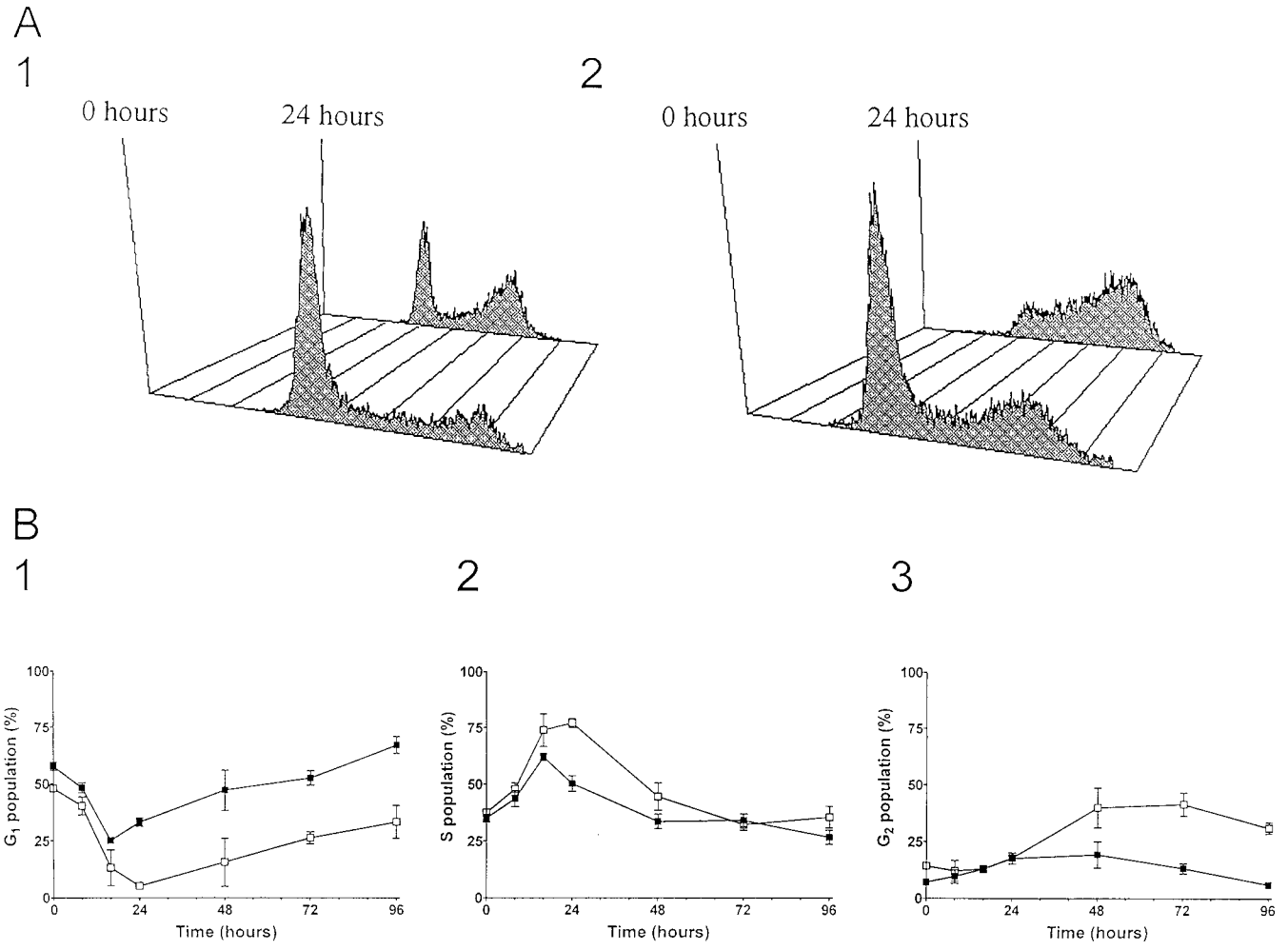

Fig. 3. Cell cycle distribution after 10 μM cisplatin exposure in VC and E6. A, representative PI fluorescence histograms for VC (1) and E6 (2) for control and 24 h after treatment. B, percentage of cells in G_1 (1), S (2), and G_2 (3) phases of the cell cycle. ■, VC. □, E6. Values are the mean ± S.E. (n = 4) of data from two independent experiments. Cells were treated as detailed in *Materials and Methods*.

expressing puromycin resistance and *HPV-16 E6*. A2780 vector control and *HPV-16 E6* transfectants were initially screened for either p53 protein induction (controls) or lack of expression (E6) after irradiation (5 Gy), and a number of suitable clones were selected for further characterization. One control clone (VC), functionally wild-type for p53, and one *HPV-16 E6*-transfected clone (E6), functionally null for p53, were chosen for detailed investigations.

Wild-type p53 protein is normally present in low concentrations in cells due to rapid turnover (Oren et al., 1981) but can be induced and stabilized after DNA damage (Fritsche et al., 1993). As shown in Fig. 1A, constitutive levels of p53 protein were barely detectable in parental A2780, VC, or E6 cell lines. However, 4 h after 5-Gy irradiation, levels of p53 protein were increased to a similar extent in parental A2780 and VC. By contrast, there was no detectable p53 protein observed in E6 after this treatment.

Many of the biological effects of wild-type p53 are achieved through transactivation of downstream genes, including $p21^{WAF1/CIP1}$. We measured induction of this gene by Northern blotting 4 h after irradiation as an assay of p53 function. Constitutive expression of $p21^{WAF1/CIP1}$ mRNA was readily detectable in parental A2780 and VC but not in E6 cells (Fig. 1B). After irradiation, expression was induced to similar levels in parental A2780 and VC, but no induction was seen in E6. To confirm that loss of p53 function in E6 cells was a consequence of *HPV-16 E6* transfection, expression of *HPV-16 E6* mRNA was determined. *HPV-16 E6* mRNA was not detectable in parental A2780 and VC but was present in E6 cells (Fig. 1B). *HPV-16 E6* mRNA levels did not change after irradiation in any of the cell lines.

One of the downstream events mediated by wild-type p53 after DNA damage is G_1/S cell cycle arrest (Kuerbitz et al., 1992). The extent of this arrest can be measured by the proportion of bromodeoxyuridine incorporated into S-phase cells after DNA damage relative to that incorporated by undamaged cells. Parental A2780 and VC cells were arrested in G_1 16 to 20 h after 5 Gy, with 20 to 30% of cells incorporating bromodeoxyuridine and thus actively synthesizing DNA (data not shown). In comparison, approximately 70% E6 cells could be seen to be incorporating bromodeoxyuridine, and thus actively cycling, after 5 Gy, indicative of compromised G_1/S arrest capacity (data not shown). VC and E6 exhibited similar doubling times (21.8 ± 2.3 and 20.5 ± 2.2 h, respectively, mean ± S.E., $n = 4$), although slightly slower than the parental cell line (18.0 ± 0.0 h, mean ± S.E., $n = 4$).

Sensitivity to Platinum Compounds. The sensitivities of parental A2780, VC, and E6 to cisplatin, transplatin, ZD0473, and JM335 were determined by SRB growth delay assay. The cisplatin IC_{50} value after 96-h continuous exposure (Table 1) was similar for both parental A2780 and VC ($P = .31$). However, E6 showed an approximately 3- to 4-fold increase in sensitivity ($P < .01$ compared with VC). This increased sensitivity was confirmed by clonogenic survival assay (Fig. 2A; $P < .05$, comparing VC and E6). Further confirmation was provided by a clone showing a partial loss of wild-type p53 function where the IC_{50} value after a 96-h continuous exposure to cisplatin was 1.18 ± 0.05 μM (mean ± S.E., $n = 3$), i.e., intermediate between that of E6 and VC. These changes in chemosensitivity were not due to alterations in accumulation of cisplatin, which was similar for parental A2780, VC, and E6 (310 ± 38.3, 318 ± 16.5, and 311 ± 20.6 ng Pt/mg protein, respectively; mean platinum uptake after 2-h exposure to 100 μM cisplatin ± S.E., $n = 3$). There also was a 2- to 3-fold increase in transplatin and ZD0473 cytotoxicity in E6 compared with VC, as determined by 96-h SRB growth delay assay (Table 1; $P < .05$ and < 0.01, respectively). By contrast, cytotoxicities were similar for JM335 in VC and E6 (Table 1; $P = .102$), and this was confirmed by clonogenic survival assay (Fig. 2B; $P = .780$).

Cell Cycle Distribution after Cisplatin Treatment. Cell cycle distribution was determined after equimolar (10 μM) cisplatin treatment (Fig. 3) in VC and E6. Between 16 and 48 h after cisplatin treatment, there were considerably fewer cells in the G_1 phase in E6 compared with VC cells, consistent with its compromised G_1/S checkpoint (Fig. 3B, 1). Both VC and E6 showed an increase in cells in S phase after cisplatin treatment, reaching maximum levels at 16 h after treatment, but this increase was greater in the E6 cells (Fig. 3B, 2). There was a greater increase in cells in G_2/M phase 48 to 72 h after cisplatin treatment in E6 versus VC (Fig. 3B, 3). Exposure to 10 μM cisplatin in E6 and 25 μM cisplatin in VC results in an approximately equitoxic dose, and similar results were obtained (data not shown).

Platinum Removal from DNA after Cisplatin and JM335 Treatment. To examine the ability of VC and E6 to repair DNA-Pt adducts, we determined total levels of platinum bound to DNA by FAAS after 100 μM platinum treatment. Similar levels of DNA-Pt binding were seen in VC and E6 immediately after cisplatin treatment (Fig. 4A). However, at 5 and 24 h after the start of treatment, a greater amount

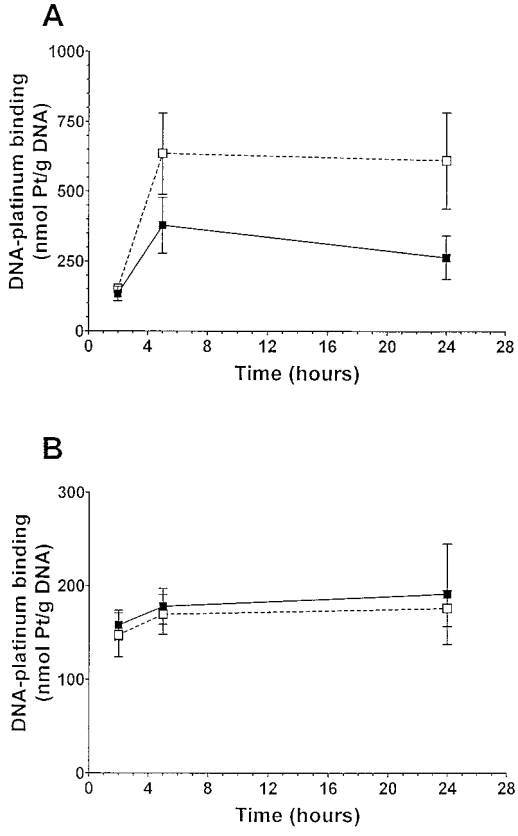

Fig. 4. DNA-Pt binding after 100 μM cisplatin (A) or JM335 (B) exposure in VC and E6. ■, VC. □, E6. Values are mean ± S.E. ($n = 3$ or 4) of data from two independent experiments. Cells were treated as detailed in *Materials and Methods*.

of DNA-Pt binding was seen in E6 compared with VC, suggesting compromised DNA repair kinetics in E6 (Fig. 4A; $P = .08$ at 24 h). The *trans*-platinum analog JM335 has been demonstrated to form different DNA-Pt adducts from cisplatin (Mellish et al., 1995), and therefore we looked at total levels of platinum bound to DNA after JM335 treatment. In contrast to cisplatin, there was no difference in the amount of adducts at any time point after JM335 treatment (Fig. 4B; $P = .93$ at 24 h).

Apoptosis after Cisplatin Treatment. After equimolar (10 μM) doses of cisplatin, there was slightly greater cell detachment in E6 compared with VC, as measured by trypan blue exclusion of detached cells (data not shown). However, this was not apparent at an equitoxic dose of cisplatin (25 μM for VC versus 10 μM for E6, data not shown). Both detached and attached cells were assayed by a flow cytometric annexin V binding assay (reviewed by Van Engeland et al., 1998). Representative results are shown in Fig. 5B. Annexin V binds phosphatidyl serine residues that are asymmetrically distributed to the inner plasma membrane but move to the outer plasma membrane early in apoptosis. An increase in the annexin V-positive population in the attached cells was seen over time after 10 μM cisplatin treatment for both VC and E6 (data not shown). Similar results were seen at equimolar 25 μM and equitoxic doses of cisplatin (10 μM for E6 versus 25 μM for VC). The proportion of annexin V-positive/PI-negative versus annexin V-positive/PI-positive detached cells was higher for VC cells than for E6 cells (compare Fig. 5C with Fig. 5F). This suggests that some of the E6 cells detaching may not be dying via a classic apoptotic pathway. Cell morphology was investigated 24 h after cisplatin treatment (Fig. 6). The majority of detached VC cells show classic apoptotic morphology with cell shrinkage and chromatin condensation (Fig. 6C). In comparison, a smaller proportion of detached E6 cells showed classic apoptotic morphology at 24 h, and there were a number of swollen cells with fragmented nuclei and many vesicles (Fig. 6F). Cell swelling was also apparent in the attached transfected E6 cells 24 h after treatment (Fig. 6E). At 48 h after cisplatin treatment, all the detached E6 cells showed this morphology or were fragmented (data not shown). This swollen/enlarged cell morphology was rarely seen in either attached or detached VC cell lines.

Discussion

The role of p53 in ovarian cancer chemosensitivity remains unclear. We therefore established isogenic A2780 human

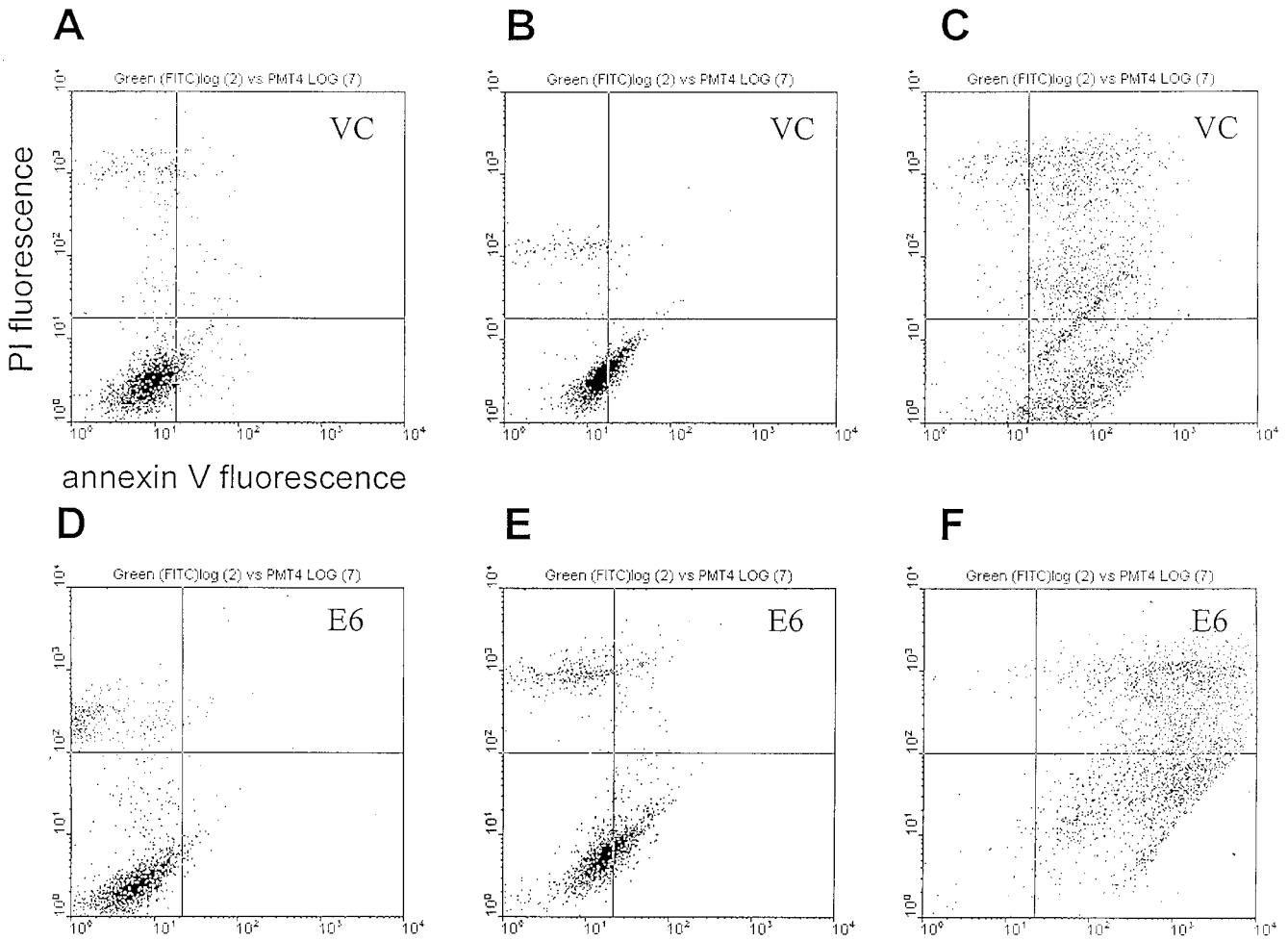

Fig. 5. Quantification of apoptosis after 10 μM cisplatin exposure in VC and E6. Representative annexin V/PI fluorescence scattergrams showing VC control (A), attached (B), and detached (C) cells at 24 h after treatment and E6 control (D), attached (E), and detached cells (F) at 24 h after treatment. Similar results were obtained in repeat experiments.

ovarian cell lines that differ only in p53 function and investigated the effects of platinum-based chemotherapeutic compounds in this model.

A p53-deficient cell line was created by the transfection of *HPV-16 E6*, a protein that binds p53 and targets it for degradation via an ubiquitin-mediated pathway (Scheffner et al., 1990). The E6-transfected cell line was shown to be approximately 3- to 4-fold more sensitive to cisplatin by two different assays of cytotoxicity. It was also more sensitive to transplatin and the novel platinum agent ZD0473, developed to overcome certain types of acquired cisplatin resistance (Holford et al., 1998) and currently in phase I clinical trial. Increased sensitivity to cisplatin has been observed on transfection of *HPV-16 E6* into human wild-type p53-expressing fibroblasts (Hawkins et al., 1996) and colon and breast cancer cell lines (Fan et al., 1995). However, increased cisplatin resistance has been reported on transfection of a dominant negative mutant p53 into A2780 cells (Brown et al., 1993; Herod et al., 1996), and no change in sensitivity was observed after the transfection of either a dominant negative mutant of p53 or wild-type p53 into the p53-null SKOV-3 human ovarian cancer cell line (Graniela Siré et al., 1995). Transfection of HPV-16 E6 into the PA1 human ovarian cancer cell line resulted in cisplatin resistance (Wu and El Diery, 1996).

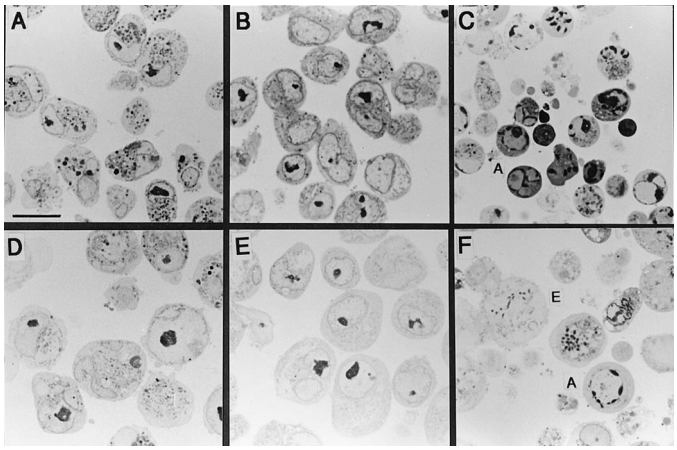

Fig. 6. Cell morphology after 10 μM cisplatin exposure in VC and E6. Representative results are shown for VC control (A), attached (B), and detached (C) cells at 24 h after treatment and E6 control (D), attached (E), and detached (F) cells at 24 h after treatment. Typical morphology of cells undergoing apoptotic (A) and necrotic (E) cell death is indicated. A 10-μm scale bar is shown in A. Cells were treated as detailed in *Materials and Methods*.

A summary of the effects of p53 status on cisplatin chemosensitivity recorded in the literature are reported in Table 2. Previous attempts to correlate p53 status and cisplatin sensitivity in human-derived ovarian cancer cell lines have shown an association between expression of wild-type protein and increased sensitivity (O'Connor et al., 1997; Pestell et al., 1998) or no association (De Feudis et al., 1997).

Of particular interest, because of the use of the same parental cell line as the present study, was the increased resistance to cisplatin seen on transfection of dominant negative mutant *p53* into the A2780 human ovarian cancer cell line (Brown et al., 1993; Herod et al., 1996). This disparity in outcome may reflect the differences between dominant negative p53 effects on chemosensitivity versus the null phenotype. Creation of a p53-deficient A2780 derivative, as described here, eliminates any complicating gain of function mutants (Chin et al., 1992), and there is no residual functional wild-type p53 protein present in the transfected cells as may occur with transfection of mutant p53 into wild-type backgrounds. However, use of HPV16 E6 may have limitations due to the interaction E6 may have with a number of other proteins (e.g., Keen et al., 1994).

The VC and E6 cell lines showed similar sensitivities to the novel *trans*-platinum compound JM335 as measured by two different assays of cytotoxicity, suggesting differences in the mechanism of action between JM335 and the other platinum compounds investigated. This is supported by the observations that JM335 shows comparable cytotoxicity to cisplatin, is more than 50-fold more potent than transplatin, and can overcome acquired cisplatin resistance arising from reduced accumulation or enhanced removal/tolerance of DNA-Pt adducts (Kelland et al., 1994). The clinical development of this compound has, however, been stopped due to lack of potency in vivo.

Differences in platinum uptake after drug treatment may influence cisplatin sensitivity (Andrews et al., 1988). However, platinum accumulation was similar for the cell lines, demonstrating this was not involved in the differences in platinum sensitivity in the present study.

p53 plays a key role in the G_1/S cell cycle arrest after DNA damage (Kuerbitz et al., 1992) through induction of $p21^{WAF1/CIP1}$ protein. In contrast to the VC cells, the E6 cells described here were seen to have no $p21^{WAF1/CIP1}$ mRNA induction after irradiation and minimal G_1/S arrest. Differences in cell cycle distribution after cisplatin treatment were also seen between VC and E6. The presence of fewer E6 cells in G_1 phase

TABLE 2
Summary of the effects of altering p53 status on cisplatin sensitivity

Cell Line	p53 Status of Cell Line[a]	Transfection[b]	Change in Cisplatin Sensitivity	Reference
Human foreskin fibroblasts	WT	*HPV-16 E6*	Sensitive	Hawkins et al., 1996
MCF-7 (breast)	WT	*HPV-16 E6*	Sensitive	Fan et al., 1995
		Dom neg p53	Sensitive	Fan et al., 1995
RKO (colon)	WT	*HPV-16 E6*	Sensitive	Fan et al., 1995
PA-1 (ovarian)	WT	*HPV-16 E6*	Resistant	Wu and El Diery, 1996
HCT116 (colon)	WT	*HPV-16 E6*	Sensitive	Fan et al., 1997
Lymphoblastoid	WT	Dom neg p53	Resistant	Piovesan et al., 1998
A2780 (ovarian)	WT	Dom neg p53	Resistant	Brown et al., 1993; Herod et al., 1996
AsPC-1 (pancreatic)	MT	WT p53	No change	Kimura et al., 1997
HL60	MT	WT p53	Sensitive	Ju et al., 1998
SKOV-3 (ovarian)	Null	WT p53	No change	Graniela Siré et al., 1995
		Dom neg p53	No change	

[a] p53 status of cell line before transfection: WT, wild type; MT, mutant.
[b] Gene transfected: Dom neg p53: dominant negative *p53* mutant transcript; WT, wild-type p53 transcript.

and more E6 cells in S phase was indicative of a loss of G_1/S checkpoint integrity in this p53-deficient cell line.

p53 is also involved in facilitating a G_2 arrest (Amundson et al., 1998) through induction of $p21^{WAF1/CIP1}$, 14-3-3σ proteins, and GADD-45. There was, however, no evidence of a reduced capacity to arrest in G_2 phase of the cell cycle in the E6 compared with VC cells. In fact, there was evidence of a greater number of E6 cells in the G_2 phase at later time points after cisplatin treatment, possibly as a consequence of increased cell transit through the G_1/S checkpoint and a greater number of cells entering the G_2 phase.

In addition to cell cycle checkpoint control, p53 has been implicated in control of DNA repair via several routes (Amundson et al., 1998). Increased levels of p53 protein result in transactivation of *GADD-45*, encoding a protein hypothesized to have a role in NER, and $p21^{WAF1/CIP1}$, encoding a postulated effector of NER. p53 protein also interacts directly with transcription factors involved in NER. To compare the ability of VC and E6 to remove DNA-Pt adducts after cisplatin or JM335 treatment, we looked at levels of platinum bound to DNA by FAAS. Similar levels of DNA-Pt binding were seen in VC and E6 immediately after cisplatin treatment, as would be anticipated from the comparable rates of uptake. However, greater DNA-Pt binding was seen in E6 cells at later time points, suggesting compromised DNA repair kinetics in E6 cells. This method does not distinguish between decreased DNA-Pt binding as a consequence of DNA repair versus DNA synthesis. However, at the high dose used (100 μM), it is unlikely DNA synthesis was occurring. In addition, this method measures all the DNA-Pt adducts formed and does not distinguish between the different types of repair taking place; however, it is known that the majority of cisplatin-DNA adducts are repaired by NER (Chu, 1994). Reduced repair of a cisplatin-treated reporter plasmid has been seen in E6-transfected MCF-7 (Fan et al., 1995) and RKO cells (Smith et al., 1995) relative to wild-type controls.

In contrast to cisplatin, there was no difference in DNA-Pt adduct formation and removal for VC and E6 after JM335 treatment, which may explain the similar cytotoxicities seen in the two cell lines. JM335 does not form the 1,2-intrastrand guanine-guanine adduct, which is the major adduct formed by cisplatin, and was unique among seven platinum compounds studied, including transplatin, in forming both interstrand cross-links and single-strand DNA breaks in some human ovarian cancer cell lines (Mellish et al., 1995). The formation of different adducts from those seen after cisplatin treatment is a possible explanation for the differences in repair of cisplatin and JM335 adducts seen in VC and E6.

A third major function of p53 is its involvement in the apoptotic pathway (Amundson et al., 1998). Three methods were used to assess apoptosis after cisplatin treatment. Trypan blue exclusion by cells demonstrates membrane integrity and in detached cells is indicative of recent detachment. At equimolar doses of cisplatin, greater detachment of cells was seen in E6, as could be expected due to the greater toxicity of cisplatin in these cells. However, this method does not distinguish between apoptotic and necrotic cell death. Flow cytometric analysis of annexin V binding does distinguish between apoptotic and necrotic cell death (Van Engeland et al., 1998). Similar proportions of VC and E6 cells were shown to be dying by apoptosis; however, an additional population of E6 cells appeared to be dying by a necrotic route.

Examination of cell morphology confirmed that the majority of VC cells died via apoptosis and also supported the view that although some E6 cells died by apoptosis, a proportion appeared swollen with deformed nuclei and decondensed chromatin. Similar morphology has been seen in cells lacking functional p53 and $p21^{WAF1/CIP1}$ after ionizing radiation; these cells proceed into mitosis more readily that wild-type cells and subsequently die through failure of cytokinesis (Waldman et al., 1996; Bunz et al., 1998). Thus, the differences in cell death observed in VC versus E6 cells may be due to differences in cell cycle control between the cell lines. The VC cells were able to arrest at the G_1/S phase of the cell cycle, enabling the cells to either repair the DNA damage or undergo apoptosis. By contrast, the E6 cells showed reduced G_1/S arrest capacity, permitting increased transit through S phase of the cell cycle with an accumulation of damaged DNA, and potentially unsuccessful mitosis, leading to mitotic catastrophe and necrotic cell death.

In conclusion, we demonstrated that transfection of E6 into the A2780 human ovarian cancer cell line results in increased sensitivity to cisplatin, transplatin, and ZD0473. This increased sensitivity can be attributed to a number of factors, including reduced capacity to arrest at a G_1/S phase checkpoint, reduced repair of DNA-Pt adducts, and increased cell death via a necrotic route. However, transfection of E6 into A2780 did not affect sensitivity to the transplatin analog JM335. The differences seen between cisplatin and JM335 may be due to the different DNA-Pt adducts formed, as there was no difference in DNA-Pt repair kinetics between the vector control and E6-transfected cell lines.

Acknowledgments

We thank Dr. B. Vogelstein for providing the $p21^{WAF1/CIP1}$ cDNA, Dr. T. Crook for providing the *HPV-16 E6* cDNA, and Dr. M. Crompton for providing the *GAPDH* cDNA. We thank David Robertson for the preparation of the cell sections and light microscopy. We also thank Prof. P. Workman and Dr. M. Ormerod for helpful discussions during the course of this study.

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