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Cisplatin- and Paclitaxel-Induced Apoptosis of Ovarian Carcinoma Cells and the Relationship between Bax and Bak Up-Regulation and the Functional Status of p53

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ABSTRACT

We investigated the roles of p53 and Bcl-2 homologues in the induction of apoptosis by cisplatin and paclitaxel in wild-type p53-expressing human ovarian carcinoma cells and cisplatin-resistant derivatives that have lost p53 function. Cisplatin induced apoptosis in parental A2780 but not in cisplatin-resistant A2780/cp70 cells, whereas paclitaxel induced apoptosis in both cell lines. Immunoprecipitation of p53 using antibodies specific for p53 conformation (pAb 1620 and pAb 240) showed that there were no relative changes in p53 conformation before and after cisplatin treatment in either cell line. A2780/cp70 cells have lost p53 function, yet they have wild-type p53 gene sequence. However, A2780/cp70 cells constitutively express more p53 in a form detected by pAb 240, an antibody that also detects mutant conformations of p53 that are transcriptionally inactive. There were no changes in levels of Bcl-2, Bcl-X_L, or 24-kDa Bax over 72 hr after exposure to cisplatin or paclitaxel, but each agent led to up-regulation of Bak and 21-kDa Bax in A2780 cells. Paclitaxel, but not cisplatin, increased Bak and 21-kDa Bax levels in A2780/cp70 cells. These data suggest that apoptosis in A2780 and A2780/cp70 is associated with an increased level of Bak and 21 kDa Bax after drug-induced damage and that functional p53 may be required for this effect after cisplatin but not after paclitaxel.

Cisplatin is an important drug in the treatment of ovarian cancer, but one of the major obstacles that limits its effectiveness is the acquisition of drug resistance. To introduce new and effective drugs for the treatment of ovarian cancer, an improved understanding is required of how drug-resistant tumors arise. One mechanism of drug resistance in tumors is the suppression of apoptosis after a cytotoxic insult (Dive and Hickman, 1991). The expression and activity of p53 and the Bcl-2 protein family play important roles in controlling apoptotic responses to drug-induced cellular insults, thus modulating the chemosensitivity of tumor cells (Harris, 1996; Reed *et al*., 1996).

p53 is present within cells normally in a latent form (Hupp and Lane, 1995), which can be activated to become DNA binding and transcriptionally active after DNA damage (see Harris, 1996). This modulation of the transcriptional activity of p53 is thought to be mediated through conformational changes of p53 that are regulated by protein/protein and protein/DNA interactions as well as by phosphorylation events (Hupp *et al*., 1992; Bayle *et al*., 1995; Hupp and Lane, 1995). The p53 conformational changes that may be required for the coupling of DNA damage to an apoptotic response are not known but can be investigated using monoclonal antibodies reactive against epitopes within the different domains within the p53 protein (Milner, 1995). Thus, an activated form of p53 that can bind to DNA in a sequence-specific manner and activate gene transcription can be identified by its increased reactivity with the monoclonal antibody pAb1620 (Hupp and Lane, 1995). The capacity of p53 to bind DNA in a sequence specific manner is lost in mutants of p53 that express an epitope recognized by the monoclonal antibody pAb240. In this way, the transcriptionally functional activated form of wild-type p53 is pAb1620 positive but pAb240 negative, whereas Milner (1995) has shown that wild-type p53 that is transcriptionally inactive and no longer suppresses growth is pAb1620 negative and pAb240 positive.

The Bcl-2 protein suppresses apoptosis induced by a diverse array of stimuli (Vaux *et al*., 1988; Yang and Korsmeyer, 1996). Recently, a number of Bcl-2-related proteins have been identified that contain several regions of homology to Bcl-2 (BH domains; Oltvai and Korsmeyer, 1994). Bcl-X_L,

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ABBREVIATIONS: cisplatin, *cis*-diamminedichloroplatinum(II); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

like Bcl-2, suppresses apoptosis, whereas Bax, for example, counteracts the survival function of Bcl-2 and Bcl-X_L to accelerate apoptosis. These proteins exert their activity via protein/protein interactions as a result of their ability to form homodimers and heterodimers with each other (Hanada *et al*., 1995), and these interactions are thought to dictate the threshold for the engagement of apoptosis. Bak seems to function in the same way as Bax and interacts with both Bcl-2 and Bcl-X_L. Wild-type p53 has been shown to induce up-regulation of bax mRNA (Miyashita *et al*., 1994a; Selvakumaran *et al*., 1994; Miyashita and Reed, 1995) with a concomitant down-regulation of *bcl*-2 mRNA (Miyashita *et al*., 1994b). These changes in gene expression are associated with the induction of apoptosis (Selvakumaran *et al*., 1994). In addition, *bcl*-X_L mRNA has recently been shown to be up-regulated in HT29 cells containing a temperature-sensitive p53 when shifted to the constitutive temperature for wild-type p53 activity (Merchant *et al*., 1996). Currently, an association between p53 transcriptional activity and the expression Bak has not been established.

Paclitaxel and its derivatives have been recently introduced for the treatment of ovarian cancer and have had some impact on this disease (Gregory and DeLisa, 1993). Paclitaxel stabilizes tubulin polymerization, resulting in cell cycle arrest at mitosis. In the current study, we investigated whether changes in the conformation of p53 and in the levels Bcl-2 family members correlate with changes in sensitivity of ovarian carcinoma cells to cisplatin and paclitaxel. The cell model system used is the human adenocarcinoma-derived ovarian cell line A2780 and the cisplatin-resistant subline A2780/cp70 that was derived after multiple exposure of A2780 to cisplatin (Behrens *et al*., 1987). We have shown previously that A2780 cells depend on p53 function for sensitivity to cisplatin, ionizing radiation, 1-β-D-arabinofuranosylcytosine, and doxorubicin as measured by clonogenic assay (Vasey *et al*., 1996). Both the A2780 and A2780/cp70 cisplatin-resistant subline express wild-type p53 gene sequence (Brown *et al*., 1993). However, the A2780/cp70 cell line shows loss of p53 function as indicated by an abrogated radiation-induced G_1 arrest, reduced $p21^{waf1/cip1}$ transcription, and suppressed cisplatin-induced apoptosis (Brown *et al*., 1993; Anthoney *et al*., 1996). Paclitaxel has been shown to induce apoptosis independently of p53 function in mouse embryonic fibroblasts (Wahl *et al*., 1996). Moreover, introduction of a dominant negative p53 construct did not render A2780 cells resistant to paclitaxel (Vasey *et al*., 1996).

We used immunoprecipitation to investigate whether a relationship exists between conformational changes in p53 and drug resistance in human ovarian carcinoma cells that express wt p53 sequence. We also characterized the expression of Bcl-2, Bcl-X_L, Bax, and Bak in A2780 and A2780/cp70 cells before and after exposure to cisplatin or paclitaxel.

Materials and Methods

Cell lines and drug treatments. The cell lines A2780 and A2780/cp70 (Vasey *et al*., 1996) were grown as monolayers in RPMI 1640 supplemented with 10% fetal calf serum (GIBCO, Paisley, Scotland) at 37° in an atmosphere of 5% CO_2. A2780 and A2780/cp70 cells were treated with 20 μM cisplatin (Sigma, Poole, UK) or 40 μM cisplatin, respectively, or 50 nM paclitaxel (Sigma) for 1 hr. It has been shown previously that less initial DNA damage is induced in A2780/cp70 compared with A2780 by equimolar amounts of cisplatin. Therefore, A2780/cp70 cells were exposed to 40 μM cisplatin for 1 hr to induce the same amount of initial DNA damage as 20 μM will in A2780 (Johnson *et al*., 1994).

For clonogenic drug sensitivity assays, cells were seeded at 10^3 per plate and after 24 hr exposed to different drug concentrations or irradiated with γ-rays from a ^{60}Co source. After incubation for 10 days, colonies were stained and counted. All statistical analyses done on clonogenic assays were performed using the Student's *t* test, with statistical significance set at $p < 0.05$.

Immunoblotting and immunoprecipitation. Cell extracts were prepared at the time points indicated by lysing cells in 12.5 mM HEPES, pH 8.0, 200 mM KCl, 5 mM EDTA, 50 mM NaF, 0.5% Nonidet P-40, 5 μg/ml phenylmethylsulfonyl fluoride, 0.2 μg/ml leupeptin, 2 μg/ml trypsinogen, 0.2 μg/ml aprotinin, and 0.2 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone for 30 min at 4°. Insoluble material was pelleted at 13,000 rpm for 10 min at 4°, and protein concentrations were determined using the BioRad protein assay kit (Milton Keynes, Beds, UK). Fifty micrograms of total cellular protein was separated by SDS-PAGE, and immunoblotting was carried out with the antibodies DO 1 (Amersham International, Bucks, UK) for p53, p21 monoclonal (kind gift from Kathryn Ball, University of Dundee), PARP monoclonal (kindly provided by Dr. K. Caldecott, University of Manchester), 124 for Bcl-2 (DAKO, Bucks, UK), Bcl-X monoclonal (Transduction Laboratories, Affiniti, Exeter, Devon, UK), Bax polyclonal (PharMingen, Cambridge Bioscience, Cambridge, UK), and Bak monoclonal (Amersham). Antibody binding was revealed by peroxidase secondary antibodies and visualized using enhanced chemiluminescence (Amersham).

One hundred micrograms of protein was immunoprecipitated using the DO1, pAb1620, pAb421, and pAb240 monoclonal antibodies (Amersham) and IgG isotype-matched control antibodies (DAKO) and using protein G/Sepharose (Pharmacia, St. Albans, Herts, UK). Immunoprecipitated protein was separated using SDS-PAGE, and the presence of p53 was revealed by Western blotting using CM1 polyclonal antiserum (kind gift from Dr Ted Hupp, University of Dundee) followed by peroxidase-conjugated goat antiserum to rabbit immunoglobulins (DAKO) and enhanced chemiluminescence. Analyses of the ratio of p53 in the pAb1620 reactive and pAb240 reactive conformations was determined by densitometric analysis of the autoradiographs. This was performed using a BioRad GS 700 densitometer and Bio-Rad Molecular Analyst software.

Detection of apoptosis by flow cytometry. Apoptotic cells were detected as described previously (Chapman *et al*., 1995). Exponentially growing cells were treated with 20 or 40 μM cisplatin for 1 hr or untreated and harvested 72 hr later. Monolayer cells were trypsinized, combined with suspension cells, and fixed in 1% formaldehyde for 15 min on ice. The cells were resuspended in PBS and 70% ethanol and stored at 4° before detection of nonrandom DNA strand breaks. Cells were rehydrated in PBS, and aliquots of 1×10^6 cells were incubated for 30 min at 37° with cacodylate buffer (0.2 M potassium cacodylate, 2.5 mM Tris·HCl, pH 6.6, 2.5 mM $CoCl_2$, 0.25 mg/ml bovine serum albumin, 5 units of terminal deoxynucleotidyl transferase/10^6 cells, 0.5 nmol of bromo-dUTP/10^6 cells). After washing in PBS, cells were incubated for 30 min at room temperature in the dark with 4× standard saline citrate and 0.1% Triton X-100 containing 5% fat-free dried milk (Marvel) and 5 mg/ml fluoresceinated avidin. After a further wash in PBS and 0.1% Triton X-100, cells were resuspended in PBS and stained with propidium iodide. Cellular fluorescence was detected using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Results

Cisplatin- and paclitaxel-induced cell death and loss of clonogenicity in human ovarian carcinoma cell lines. We investigated the association between p53 and the induction of apoptosis by cisplatin and paclitaxel in A2780

human ovarian carcinoma cells that express wild-type p53 and the A2780/cp70 cisplatin-resistant derivative that despite having wild-type p53 sequence has lost p53 function. We have shown using clonogenic assays that the A2780 cell line is sensitive to and the A2780/cp70 cell line is resistant to a number of DNA-damaging agents, including cisplatin, ionizing radiation, and adriamycin, but both are sensitive to the effects of paclitaxel (Table 1). We then further characterized these observations using a widely recognized indicator of apoptosis; the cleavage of the 117-kDa PARP protein to an 85-kDa subunit by caspase 3 (CPP-32) that is activated when the apoptotic process is executed. As shown in Fig. 1A, both A2780 and A2780/cp70 cells express the 117-kDa PARP protein. Treatment of the A2780 cell line for 1 hr with either cisplatin (20 μM for A2780 cells) or 50 nM paclitaxel resulted in a time-dependent cleavage of the 117-kDa PARP to the smaller 85-kDa product. Cleavage was first seen at 24 hr after exposure to cisplatin, with further increases in the level of the 85-kDa cleavage product seen over the next 48 hr. It has been shown previously that less initial DNA damage is induced in A2780/cp70 compared with A2780 by equimolar amounts of cisplatin. Therefore, A2780/cp70 cells were exposed to 40 μM cisplatin for 1 hr to induce the same amount of initial DNA damage as 20 μM causes in A2780 (Johnson *et al*., 1994). Treatment of A2780/cp70 cells with 40 μM cisplatin does not induce apoptosis (Anthoney *et al*., 1996). In agreement with this and as shown in Fig. 1A, there was no PARP cleavage in A2780/cp70 cells treated with cisplatin. These cells are 4-fold more resistant to cisplatin than are A2780 cells, as measured by clonogenic assay. However, treatment of A2780/cp70 cells with paclitaxel did result in PARP cleavage. A2780 and A2780/cp70 cells are equally sensitive to paclitaxel treatment (Table 1). These observations are compatible with the levels of cisplatin-induced apoptosis when another indicator was used; as shown in Fig. 1B, A2780 cells undergo DNA fragmentation, as detected by flow cytometry and the terminal deoxynucleotidyl transferase assay, in a time- and cisplatin concentration-dependent manner, whereas very little DNA fragmentation is detectable in cisplatin-treated A2780/cp70 cells.

The conformation of p53 is altered in drug-resistant cells. p53 function or functions depend on the conformation of p53 protein (Hupp and Lane, 1995; Milner, 1995). The conformation of p53 expressed in the drug-resistant A2780/cp70 cells was compared with the parental drug-sensitive A2780 cells by immunoprecipitation using the conformation-specific monoclonal antibodies (pAb1620 and pAb240; Fig. 2). In addition, the DO1 and pAb421 monoclonal antibodies, which recognize epitopes that are independent of protein conformation, were used to measure total cellular p53 content. In exponentially growing A2780 cells, immunoreactivity was observed to the DO1 and pAb421 antibodies. Immunoreactivity with pAb1620 (which recognizes a wild-type conformation of p53, that is transcriptionally active) and to pAb240 (which is reactive against a transcriptionally inactive wild-type conformation and to certain mutant forms of p53 that also are transcriptionally inactive) was not detectable (Table 2). This is consistent with A2780 expressing wild-type p53 and having functional p53 activity (Brown *et al*., 1993; Anthoney *et al*., 1996). Exponentially growing A2780/cp70 cells exhibited immunoreactivity to all four antibodies, suggesting that a greater proportion of p53 was in pAb240 reactive conformation (Table 2). We also examined the conformation of p53 in another ovarian cell line, OV1P,

TABLE 1
Sensitivity of A2780 and A2780/cp70 cells to cytotoxic agents measured by clonogenic assay

Cell line	Cisplatin[a]	Adriamycin[a]	Taxol[a]	IR (SF2)[b]
A2780	5×10^{-6}	1.1×10^{-8}	9×10^{-9}	0.23
A2780/cp70	2×10^{-5}	5.6×10^{-8}	1×10^{-8}	0.66

[a] Values are ID_{80} (M) on clonogenic assays for 1-hr exposure to drug.
[b] SF2 is the surviving fraction at 2-Gy ionizing radiation (γ-ray ^{60}Co source).

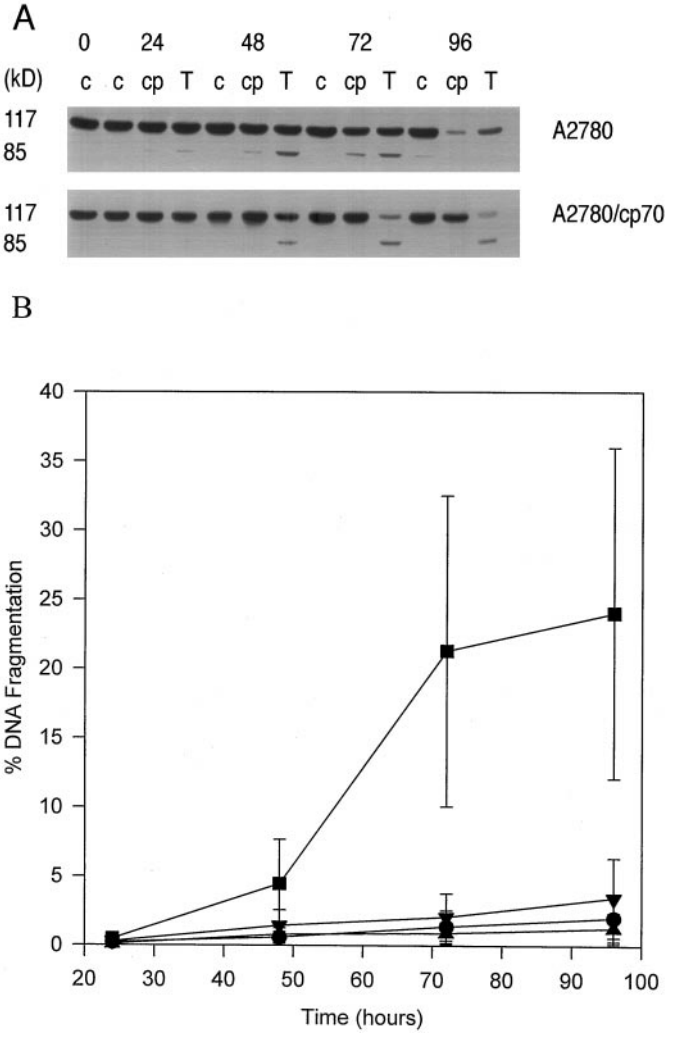

Fig. 1. Cisplatin and paclitaxel induce apoptosis in human ovarian carcinoma cell lines. A, Immunoblots of A2780 cells and A2780/cp70 cells are shown stained for the PARP protein. Cell extracts were prepared after the indicated times after 1-hr exposures to drug as follows: A2780 cells were treated with 20 μM cisplatin (*cp*) and A2780/cp70 with 40 μM cisplatin; both cell lines were treated with 50 nM paclitaxel (*T*). Control cell extracts (*c*) were also prepared at 24-, 48-, 72-, and 96-hr time points. Western blots were immunostained with the PARP monoclonal antibody followed by enhanced chemiluminescence. B, Control A2780 (●) and A2780/cp70 (▲) cells and A2780 (■) and A2780/cp70 (▼) cells treated with cisplatin as above were fixed as described in Materials and Methods. DNA strand breaks were detected by terminal deoxynucleotidyl transferase and biotin-dUTP nick end fluorescent labeling using flow cytometry; increases in strand breaks in cells are indicators of apoptosis. Quantification of the apoptotic cell population was performed using the Cell Quest Program. Values are the mean of four experiments, with 15,000 cells analyzed per experiment. *Error bars*, standard errors of the mean.

which contains a heterozygous mutation of the p53 gene (Brown *et al.*, 1993). The proportion of p53 in a pAb240 immunoprecipitatable form was higher still than that seen for A2780/cp70 cells (Fig. 2). Densitometric analyses of autoradiographs from three experiments showed that the ratio of pAb240 to pAb1620 positive p53 was 7.3 in OV1P cells compared with 2.7 in A2780/cp70 cells (Table 2). Together, these results are consistent with p53 having wild-type gene sequence in the A2780/cp70 cells (Brown *et al.*, 1993) but the loss of p53 function being associated with increased p53 in a transcriptionally inactive conformation (pAb240 positive). The mechanism causing this conformational change that occurred during the derivation of this cell line by long term exposure to cisplatin is unclear, although it may be linked to the loss of hMLH-1 expression observed in these cells (Anthoney *et al.*, 1996).

We also examined the conformation of p53 in A2780 and A2780/cp70 cells 2, 4, or 24 hr after the 1-hr exposure to cisplatin. At the early time points, there was no change in p53 conformation or protein level in either cell line (Fig. 2 and data not shown). This argues against a conformation change of p53 occurring as a result of cisplatin directly interacting with p53 protein. At 24 hr, there was increased immunoreactivity to pAb1620, pAb421, and DO1 in A2780 cells, but pAb240 reactivity remained barely detectable. In A2780/cp70 cells, cisplatin treatment induced an increased p53 immunoreactivity to all four antibodies. Densitometric analyses of autoradiographs from three repeat experiments show that in drug-resistant A2780/cp70 cells, the ratio of pAb240 to pAb1620 positive p53 was 8.1 compared with 1.1 in drug-sensitive A2780 cells 24 hr after exposure to cisplatin (Table 2). This shows that a greater proportion of p53 molecules are in the pAb240 reactive conformation in A2780/cp70 drug-resistant cells. The presence of pAb240 reactive p53 in A2780/cp70 cells is associated with a reduced apoptotic response to cisplatin and may be indicative of the mechanism by which these (wild-type p53 expressing) cells show loss of p53 function.

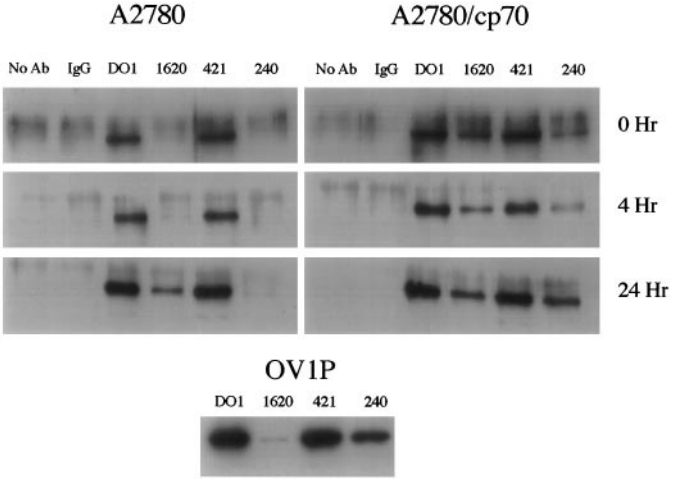

Fig. 2. p53 conformation analysis in A2780 and A2780/cp70 cells. A2780 and A2780/cp70 cells and cells treated with cisplatin for 1 hr as described in legend to Fig. 1. Cell extracts were prepared from control cells and from cells 4 and 24 hr after treatment. One hundred micrograms of protein was immunoprecipitated using the DO1, pAb1620, pAb421, and pAb240 monoclonal antibodies and IgG isotype-matched control antibodies (*Ab*). Immunoprecipitated protein was separated using SDS-PAGE, and the presence of p53 revealed by Western blotting using CM1 polyclonal antiserum and enhanced chemiluminescence.

TABLE 2
Ratio of pAb240 to pAb1620 p53 immunoprecipitated from A2780 and A2780/cp70 cells

The ratios have been normalized for total levels of immunoprecipitable p53 protein.

Time after exposure to cisplatin (hr)	A2780	A2780/cp70
Control	N.D.[a]	2.7
4	N.D.[a]	2.6
24	1.1	8.1

[a] pAb240 immunoprecipitable p53 was not detectable, and therefore a ratio could not be calculated.

The site-specific DNA binding activity of p53 can be activated using the monoclonal antibody pAb421 (Hupp *et al.*, 1992, 1995; Hupp and Lane, 1994) or by phosphorylation of p53 by protein kinase C at the carboxyl-terminal site or sites (Ser371, Ser376, and Ser378; see Hupp and Lane, 1995). Phosphorylation of p53 *in vitro* with protein kinase C inhibits the binding of the pAb421 antibody to this epitope. Therefore, pAb421 immunoreactivity can be used to assess the phosphorylation status of p53 at this site (Hupp TR, personal communication), which may then suggest a mechanism by which p53 is transcriptionally inactive in A2780/cp70 cells. Lysates were prepared from untreated A2780 and A2780/cp70 cells or from these cells 24 hr after a 1-hr exposure to cisplatin. These cell extracts were first immunodepleted of pAb421 reactive p53 by four sequential immunoprecipitations using pAb421. The p53 protein remaining in the cell lysate was then immunoprecipitated with the DO1 p53 antibody. Thus, we can assess the amount of p53 remaining in the cell lysate after immunodepletion and therefore pAb421 negative. As shown in Fig. 3, which is representative of six independent experiments, there was no difference between the amount of p53 remaining in A2780 and A2780/cp70 cell lysates after pAb421 immunodepletion, indicating that the phosphorylation status and therefore the ability of the p53 in both cell lines to bind to DNA are the same. Thus, changes in the pAb421 epitope that have been reported to control the ability of p53 to bind to DNA seem to not play a role in regulating p53 DNA binding capacity and therefore p53 activity in this cell system.

We next investigated the up-regulation of the $p21^{Waf\text{-}1/Cip\text{-}1}$ cyclin-dependent kinase inhibitor protein in the A2780 and A2780/cp70 cell lines before and after treatment with cisplatin or paclitaxel. As shown in Fig. 4, A2780 cells express high constitutive levels of $p21^{Waf\text{-}1/Cip\text{-}1}$ protein, which is indica-

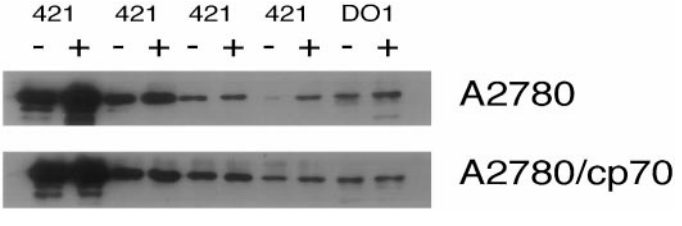

Fig. 3. Immunodepletion of p53 from A2780 and A2780/cp70 cells. Cells were treated with cisplatin as described in the legend to Fig. 1. Untreated (−) and cisplatin-treated (+) cell lysates were prepared 24 hr after exposure to cisplatin as described in Materials and Methods. Two hundred micrograms of protein was immunoprecipitated four times sequentially using pAb421, followed by a final immunoprecipitation using DO1. Immunoprecipitated protein was separated using SDS-PAGE, and the presence of p53 was revealed by Western blotting using CM1 polyclonal antiserum and enhanced chemiluminescence.

tive of a functional p53. Elevated levels of $p21^{Waf\text{-}1/Cip\text{-}1}$ were detectable 24 hr after 1-hr exposures to either 20 μM cisplatin or 50 nM paclitaxel. The elevation of $p21^{Waf\text{-}1/Cip\text{-}1}$ in A2780 cells was sustained over a 72-hr time period (Fig. 4). The decreased levels of $p21^{Waf\text{-}1/Cip\text{-}1}$ seen at the 96-hr time point may be due to the presence of apoptotic cells at this time (see above). In contrast, A2780/cp70 cells expressed very low levels of $p21^{Waf\text{-}1/Cip\text{-}1}$ protein, and there was no elevation in $p21^{Waf\text{-}1/Cip\text{-}1}$ levels after treatment with 40 μM cisplatin. Equivalent levels of protein loading was confirmed by Ponceau S staining of the filters (data not shown) as well as by reprobing the blots for actin.

Expression of Bcl-2 and homologs during cisplatin- and paclitaxel-induced apoptosis. The Bcl-2 family of proteins control the disposition of a cell to undergo apoptosis (Yang and Korsmeyer, 1996). We investigated the levels of expression of four Bcl-2 family proteins, Bcl-2 and Bcl-X_L (suppressors of apoptosis) and Bak and Bax (promoters of apoptosis), before and after exposure of A2780 and A2780/cp70 cells to cisplatin and paclitaxel. A2780 cells and A2780/cp70 cells both express Bcl-2, but higher levels were detected in A2780/cp70 cells (Fig. 5A); a similar level of Bcl-X_L was noted in the two cell lines (Fig. 5A). There was no change in the expression of either of these two proteins after drug treatment (Fig. 5A).

The proapoptotic protein Bak was expressed at a higher constitutive level in the drug-sensitive A2780 cells compared with A2780/cp70 cells (data not shown). Treatment of A2780 cells with cisplatin (20 μM for 1 hr) and paclitaxel (50 nM for 1 hr) resulted in increased levels of Bak after 24 hr. These elevations in the level of Bak expression were at a time when apoptotic cells were first detectable (24 hr). Bak was also elevated by treatment of A2780/cp70 cells with paclitaxel but not cisplatin (Fig. 5B), although the elevated level of expression was not as substantial as that seen in A2780 cells after similar treatment. Equivalent protein loading was confirmed by reprobing the blots for actin (Fig. 5B).

A 24-kDa form of the Bax protein was also expressed in both cell lines and, like Bak, at a higher protein level in A2780 cells compared with A2780/cp70 cells (data not shown). The level of expression of this 24-kDa form of Bax did not change after treatment of either cell line with cisplatin or paclitaxel (Fig. 5B). A 21-kDa form of Bax has previously been shown to induce apoptosis (Lowe *et al*., 1993); constitutive expression of this form of Bax was not detectable in A2780 or A2780/cp70 cells. Treatment of A2780 cells with cisplatin or paclitaxel resulted in the appearance of the 21-kDa form of Bax at the 48-hr time point when PARP cleavage and DNA fragmentation (see Fig. 1, A and B) were easily detectable. The 21-kDa form of Bax was undetectable at all time points in A2780/cp70 cells treated with cisplatin; however, a moderate elevation in the 21-kDa Bax was seen when A2780/cp70 cells were treated with Paclitaxel (Fig. 5B). This elevation can be seen clearly when the blot is overexposed (not shown).

Discussion

Cisplatin is an important drug in the treatment of ovarian cancer, but the acquisition of drug resistance by tumor cells is a major problem preventing the successful outcome of cancer chemotherapy. Paclitaxel has recently been introduced for the treatment of cisplatin-resistant ovarian tumors and has had some impact in the treatment of this disease. There are multiple mechanisms that could contribute to resistance to cisplatin; these include reduced platinum accumulation, increased detoxification (via increased levels of glutathione and metallothioneins), and increased DNA-platinum adduct removal. These mechanisms will affect the level of DNA damage induced in the cell. However, it also has become apparent that cells can differ in their cytotoxic response to the same levels of DNA damage. The p53 protein is a sequence-specific transcription factor that plays an important role in coupling DNA damage to growth arrest and/or the apoptotic response of a cell after DNA damage. In addition, molecules such as Bcl-2 and Bcl-X_L act downstream of DNA damage to modulate the threshold at which a drug-treated cell will undergo apoptotic cell death (Dole *et al*., 1995; Miyashita and Reed, 1995). Notably, Bax, a proapoptotic member of the Bcl-2 family, has been shown to be a transcriptional target of p53 in some cell types (Oltvai *et al*., 1993).

p53 plays a role in coupling DNA damage, including that imposed by cisplatin to growth arrest or apoptosis depending on cellular context (Clarke *et al*., 1993; Lowe *et al*., 1993; Vasey *et al*., 1996). The human A2780/cp70 cell line has acquired resistance to cisplatin after multiple exposures of the parental A2780 cells to cisplatin *in vitro*. We have previously shown for A2780/cp70 cells that despite expressing wild-type p53 gene sequence (Brown *et al*., 1993), the protein seems to be transcriptionally inactive (Fig. 4; Brown *et al*., 1993), but these cells retain sensitivity to paclitaxel (Table 1 and Fig. 1A). Perego *et al*. (1996) have similarly shown that cisplatin-resistant IGROV-1 ovarian carcinoma cells express a transcriptionally inactive p53 protein; in this study, however, in contrast to A2780/cp70 cells, p53 in IGROV-1 cells contained two mutations in the DNA binding domain.

Wahl *et al*. (1996) demonstrated that human fibroblasts, which lack p53 or whose p53 is inactivated by the HPV16 E6 protein, are more sensitive to paclitaxel-induced apoptosis.

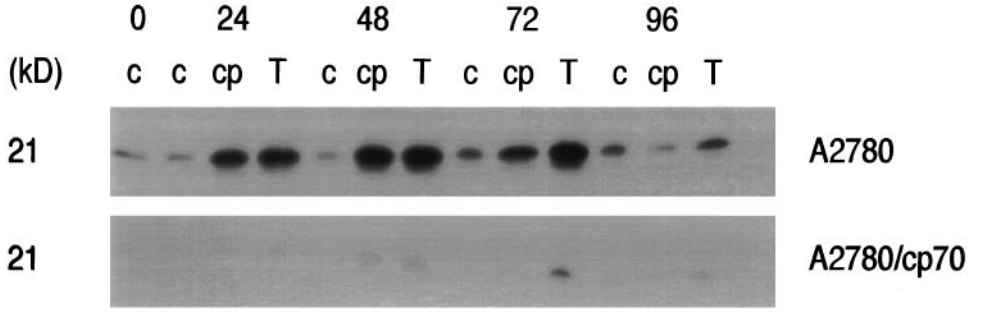

Fig. 4. Wild-type p53 function is abrogated in A2780/cp70 cells. Cells were treated with cisplatin and paclitaxel as described in the legend to Fig. 1. Immunoblots of A2780 cells and A2780/cp70 cells were stained for the presence of p21 by Western blotting and enhanced chemiluminescence.

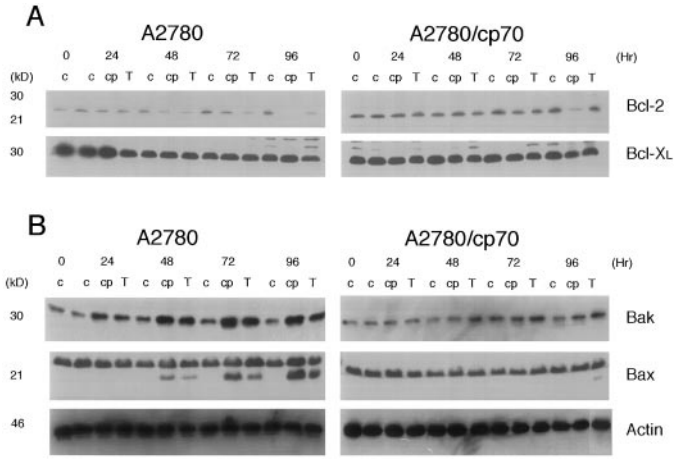

Fig. 5. Levels of expression of Bcl-2, Bcl-X_L, Bak, and Bax proteins in human ovarian carcinoma cell lines. A2780 and A2780/cp70 cell extracts were prepared at the indicated times after 1-hr exposures to drug as described in the legend to Fig. 1. A2780 (*left*) and A2780/cp70 (*right*) Western blots were immunostained for Bcl-2, Bcl-X_L and Bak, and Bax and actin using enhanced chemiluminescence.

These cells undergo a G_2/M block followed by apoptosis in response to paclitaxel treatment. In contrast, p53-expressing cells undergo G_1 arrest after the paclitaxel treatment but only after completion of mitosis; thus, paclitaxel induces cell cycle arrest and apoptosis depending on p53 status. The mechanism or mechanisms of paclitaxel-induced G_1 arrest in these cells containing functional p53 is unclear because no increase in p53 or p21 was detected 4 hr after paclitaxel treatment but may result from DNA damage induced by an aberrant mitosis rather than direct DNA damage by paclitaxel (Hupp and Lane, 1994). In cells expressing wild-type p53 paclitaxel may therefore initiate the apoptotic pathway via a p53-independent mechanism, and this seems to be the case in A2780/cp70 cells. The function of p53 as a transcriptional activator is regulated by protein conformation that in turn is thought to be controlled by protein/protein interactions and post-translational modifications. A higher proportion of the total cellular pool of p53 molecules immunoprecipitated from A2780/cp70 cells exists in a pAb240 conformation (Table 2). The pAb240 antibody recognizes denatured and mutant p53 protein and wild-type p53 in a transcriptionally inactive conformation. A2780 cells treated with cisplatin expressed a pAb1620 positive population of p53 molecules consistent with these cells expressing a functional p53 protein. The absence of relative changes in immunoreactivity with the conformational specific antibodies after treatment with cisplatin argues against a DNA-induced conformational "flip" or a direct interaction of cisplatin with p53 protein. Taken together, these data suggest that an inherently altered conformation of p53 may be associated with the observed abrogated p53 function and an altered sensitivity to cisplatin for A2780/cp70 cells. There is evidence that pAb240 reactive p53 may act as a dominant negative mutant (Gannon *et al*., 1990), and this could be a possible mechanism by which the p53 is transcriptionally inactivated in A2780/cp70 cells because these cells express both pAb240 and pAb1620 immunoprecipitatable p53. Interestingly, transfection of the V143A mutant p53 (which is a dominant negative mutant) into A2780 increases resistance of these cells to DNA-damaging agents but not paclitaxel (Vasey *et al*., 1996), and pAb240 reactive p53 becomes detectable (Jones NA, unpublished observations).

Sequence-specific DNA binding of p53 can be activated by phosphorylation at a carboxyl-terminal site (Hupp *et al*., 1994; Hupp and Lane 1994). pAb421 binds to this region of p53 when it is in a nonphosphorylated state and hence can be used to determine the phosphorylation status of these carboxyl-terminal site or sites. A sequential immunoprecipitation assay exploiting the above reactivity of pAb 421 suggested that there was no difference in the phosphorylation status of p53 between A2780 and A2780/cp70 cells either before or after treatment with cisplatin (see Fig. 3). Phosphorylation changes of p53 at this important regulatory site do not, therefore, seem to explain the difference in the transcriptional activity of p53 in A2780 compared with A2780/cp70 cells as inferred by the lack of p21 protein up-regulation after cisplatin treatment of A2780/cp70 cells. In addition, we have shown that p53-mediated transcriptional activity, measured using luciferase reporter constructs, is significantly depressed in A2780/cp70 cells compared with A2780 cells (Kim YT and Brown R, unpublished observations). *O*-Glycosylation also has been reported to activate p53 transcriptional activity in EB-1 colon carcinoma cells, and this is associated with a loss of pAb421 reactivity (Han *et al*., 1996). However, high levels of pAb421 reactive p53 are detectable in both A2780 and A2780/cp70 cells (see above and Figs. 2 and 3), so it is probable that glycosylation at this site is not important in the control of p53 activity in this cell model system.

The absolute requirement for p53-mediated transcription in the coupling of DNA damage to apoptosis remains controversial and seems to be heavily dependent on the cellular context. On the one hand, the up-regulation of p21 by p53 in certain scenarios promotes growth arrest, with time for DNA damage repair rather than apoptosis. On the other hand, at least three transcriptional targets of p53 have been identified that may affect the ability of the cell to commit suicide. p53 can transcriptionally up-regulate IGF BP-3 (Schwarze and Hawley, 1995), potentially reducing a survival signal provided by IGF-1. In addition, p53 has been shown to transcriptionally repress the expression of Bcl-2 and transcriptionally activate Bax (Selvakumaran *et al*., 1994; Lieberman *et al*., 1995), a suppressor and promoter of apoptosis, respectively. Whether the Bcl-2 homolog Bcl-X_L and the Bax homolog Bak are transcriptionally regulated by p53 is not known. High levels of expression of Bcl-2 in ovarian carcinoma cells has been shown to confer resistance to cisplatin (Eliopoulos *et al*., 1995). In the current study, we confirm that A2780/cp70 drug-resistant cells express Bcl-2 to a higher level than the sensitive A2780 cells, indicating a role for Bcl-2 in chemoresistance of ovarian carcinoma cells to cisplatin. The level of expression of Bcl-X_L was similar in both A2780 and A2780/cp70 cells. Up-regulation or ectopic expression of Bcl-X_L protein has been associated with resistance to apoptosis in a number of tumor cell lines (Schwarze and Hawley, 1995; Gauthier *et al*., 1996; Han *et al*., 1996). In addition, increased expression of Bcl-X_L has been correlated with drug resistance in a number of solid tumors (Reed *et al*., 1996). There was no evidence of reduced expression of either Bcl-2 or Bcl-X_L after treatment with cisplatin or paclitaxel in the A2780 cells compared with the A2780/cp70 cells, which may argue against a role for Bcl-X_L in the drug-resistant phenotype of

the A2780/cp70 cells. However, as for all of the Bcl-2 family members, further work is under way to determine their cellular location, their binding partnerships, and whether these are disrupted before apoptosis.

Consistent with the role that Bax and Bak play in increasing the sensitivity of cells to chemotherapeutic agents, A2780 cells were shown to constitutively express more Bak and 24 kDa Bax than the resistant A2780/cp70 cells. In agreement with this observation, Perego *et al*. (1996) demonstrated that the level of expression of *Bax* mRNA was higher in IGROV-1 ovarian carcinoma cells that expressed a transcriptionally active p53. Drug-induced increases in the levels of Bak and a 21-kDa Bax protein were observed whenever apoptosis was induced by paclitaxel or cisplatin in A2780 and A2780/cp70 cells, and these increases were particularly striking when A2780 cells were treated with cisplatin. Previously, Thomas *et al*. (1996) observed multiple bands (24, 21, and 18 kDa) on a Western blot of B cells using a human-specific polyclonal anti-bax antibody. They suggested that the lowest molecular weight band that appeared only after drug treatment could be due to proteolysis of Bax rather than transcriptional upregulation of the *Bax* gene. It is also possible that the 21-kDa form of Bax that we observed only after paclitaxel or cisplatin treatment could be a cleavage product of 24-kDa Bax.

Preliminary data suggest that increased levels of Bak may be a common event preceding drug-induced apoptosis in these cell lines because exposure of A2780 and A2780/cp70 cells to a lethal concentration of the nongenotoxic compound *N*-methylformamide (Dibner *et al*., 1985) elevated Bak in both cell lines. Because paclitaxel induces apoptosis in these cell lines in a p53-independent manner (Vasey *et al*., 1996) but still causes elevations in 21-kDa Bax in A2780 cells but only a moderate increase when A2780/cp70 cells are treated with paclitaxel, it seems most likely that there are p53-dependent and -independent controls of the expression of Bax. The data also suggest that p53 function is not required for Bak-mediated apoptosis in these cell lines. The requirement of Bak and Bax for cisplatin-induced apoptosis of A2780 cells in being tested. The precise mechanism by which Bak and Bax induce apoptosis must be resolved, although it seems likely that the rheostat is shifted such that homodimeric forms of Bak and Bax predominate over heterodimers with Bcl-2 and Bcl-X_L according to the model suggested by Korsmeyer (Oltvai *et al*., 1993).

The work presented suggests that in the ovarian cell lines studied, the two proapoptotic proteins Bax and Bak have an important controlling influence on drug-induced apoptosis. Paclitaxel is able to regulate the expression of these two proteins via p53-dependent and -independent routes, and these observations infer a mechanism by which cisplatin-resistant tumors retain sensitivity to paclitaxel.

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