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Cisplatin, Camptothecin, and Taxol Sensitivities of Cells with p53-Associated Multidrug Resistance

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SUMMARY

With the use of clonogenic survival assays, we show that wild-type p53-expressing A2780 human ovarian cell lines transfected with a dominant negative mutant p53 gene (codon 143, valine to alanine) acquired cross-resistance to ionizing radiation, cisplatin, doxorubicin, and 1-β-D-arabinofuranosylcytosine. However, these mutant p53-transfected cell lines retained sensitivity to taxol and camptothecin. We also show that immature thymocytes from mice with the p53 gene genetically inactivated showed reduced ability to undergo apoptosis after treatment with ionizing radiation and cisplatin compared with wild-type mice. However, taxol-induced apoptosis in thymocytes does not seem to be dependent on p53 status. Camptothecin also induced apoptosis in a p53-independent manner in thymocytes at low doses but in a p53-dependent manner at high doses. These data suggest that taxoids and camptothecin analogs could have activity in tumors that have aberrant p53 function and provide a rationale for the clinical observations of responsiveness of refractory ovarian cancer to these drugs.

The presence of wild-type *TP53* gene function is important for the sensitivity of certain cell types to chemotherapeutic drugs. In addition to its involvement in a DNA damage-induced G1/S cell cycle checkpoint (1), wild-type p53 seems to play a critical role in the coupling of DNA damage to the apoptotic pathway (2, 3). Functional inactivation of p53 through introduction of a dominant negative mutant of the *TP53* gene into cells can cause loss of p53 function and increased resistance to ionizing radiation as measured with clonogenic assays (4). However, increased resistance to ionizing radiation and other DNA-damaging agents is not always induced by loss of p53 function (5–7), suggesting that p53-mediated cell death is dependent on genetic context and cell type. One possible explanation for these apparently contradictory results is that in cells permissive for p53-mediated apoptosis, the loss of p53 function confers resistance, whereas in cells in which p53-mediated apoptosis is overridden [e.g., by bcl2 or related proteins (8)], the effects on the sensitivity of p53-mediated cell cycle arrest may become dominant. The human ovarian adenocarcinoma cell line A2780 was originally derived from an untreated patient and is relatively sensitive to DNA-damaging agents (9). This cell line has wild-type *TP53* gene sequence and demonstrates a functional G1 arrest after irradiation (6). We have previously shown that p53 function seems to be a major determinant of ionizing radiation sensitivity in these cells (4); therefore, A2780 is a suitable human tumor model system with which to examine patterns of cross-resistance to chemotherapeutic agents that are associated with the function of p53. We examined the patterns of cross-resistance to ionizing radiation, cisplatin, doxorubicin, and ara-C of A2780 cells with differing p53 functional status. Taxoids and topoisomerase I inhibitors have been reported to be effective in ovarian tumors that recur after cisplatin treatment (10, 11). Therefore, we examined whether cytotoxicity of these drugs is dependent on p53 status.

Thymocytes from mice with the *TP53* gene genetically inactivated have been extensively studied as a model system for examination of p53-dependent drug-induced apoptosis (2, 3). Cisplatin-induced apoptosis has not been examined in such *TP53*-null mice. We have previously shown that cisplatin-induced apoptosis occurred in proliferating but not in quiescent rat thymocytes (12). In contrast, apoptosis was induced by etoposide regardless of proliferation status. In the current study, we show that p53 is necessary for cisplatin-induced apoptosis in murine thymocytes that are enriched for proliferating cells. Furthermore, to investigate the requirement for proliferation in the induction of apoptosis by these drugs, we compared the ability of taxol and camptothecin to induce apoptosis in proliferating and quiescent thymocyte populations in the presence and absence of p53 expression.

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ABBREVIATION: ara-C, 1-β-D-arabinofuranosylcytosine.

Materials and Methods

Cell lines. The A2780 cell line was originally isolated from an ovarian adenocarcinoma of an untreated patient and is relatively sensitive to cisplatin and radiation *in vitro* (9). The A2780 line has been previously shown to express only wild-type *TP53* gene sequences (6). Mutant *TP53* cDNA (codon 143, valine to alanine) was transfected into the A2780 cells (A2780/mp53), and clones isolated that express mutant p53 protein and lack a radiation-induced G1 arrest (4). Vector-only transfectants of the A2780 cells were also isolated (A2780/v). Plating efficiencies were 25% for A2780/v and 21% for A2780/mp53, and there was no obvious effect of p53 status on colony size. We observed unstable expression of the transfected mutant *TP53* gene, so throughout the study the loss of a G1 checkpoint after irradiation was routinely confirmed. To minimize this problem, cells were frequently regrown from frozen stocks. All cell lines were maintained as monolayers in RPMI 1640 medium with 10% fetal calf serum and grown at 37° in 95% air/5% CO_2. Cells were checked monthly for *Mycoplasma* contamination.

Clonogenic drug sensitivity assays and cell cycle/proliferation analysis. For clonogenic drug sensitivity assays, cells were seeded at 10^3 cells/plate and after 24 hr exposed to drug for the times shown or irradiated with γ-rays from a ^{60}Co source. For each dose point, 10–20 separate platings were assayed. After incubation of the plates for 10 days, colonies were stained and counted. All statistical analyses done on the clonogenic assays were performed using the Student's *t* test, with statistical significance set at $p < 0.05$.

DNA synthesis was assessed by incorporation of bromodeoxyuridine and flow cytometric analysis as previously described (6).

Drug-induced apoptosis in thymocytes. Four- to 5-week-old male p53 wild-type mice and p53-null mice (13) were used to obtain thymocyte suspensions as described previously (14). Thymocytes (10^7/ml) were incubated for ≤20 hr in the presence or absence of methylprednisolone, cisplatin, camptothecin, or taxol in RPMI 1640 medium containing 10% fetal calf serum at 37° in a humidified atmosphere of 95% air/5% CO_2. Thymocytes were also irradiated (4 Gy) through exposure to γ-rays from a ^{137}Cs source at 3.8 Gy/min before incubation.

Enrichment for proliferating and quiescent cell subpopulations. Discontinuous Percoll gradients were used to separate normal and apoptotic thymocytes as previously described (15). The gradients were calibrated using density marker beads (Sigma Chemical, Poole, Dorset, UK), and the buoyant densities at the 0–60%, 60–70%, 70–80%, and 80–100% interfaces were 1.063, 1.075, 1.099, and 1.119 g/ml, respectively. Cells were removed from these interfaces and mixed with five volumes of RPMI 1640, washed twice, and resuspended in RPMI 1640 containing 10% fetal calf serum. Cells from fractions enriched for proliferating and quiescent cells were used for experiments. The proportion of cells within the various phases of the cell cycle after Percoll separation were estimated by staining cells for the incorporation of 5-bromodeoxyuridine and staining for the proliferation-associated nuclear antigen Ki-67 (Dako, Bucks, UK) (16). Cells were analyzed using a FACS Vantage flow cytometer (Becton Dickinson, San Jose, CA) and Lysis II software (Becton Dickinson).

Quantification of apoptosis. The proportion of apoptotic cells within thymocyte cultures was determined as described previously (14). Briefly, one drop of cell suspension was mixed with 10 μg/ml acridine orange solution (10 μg/ml in phosphate-buffered saline), and cells were viewed using an Olympus BH-2 microscope with a fluorescence attachment. Cells that exhibited bright-green fluorescent condensed nuclei (intact or fragmented) were considered to be apoptotic and were expressed as a percentage of the total cell number.

Thymocytes from the Percoll fractions were incubated with the bisbenzimidazole dye Hoechst 33342 and propidium iodide essentially as described previously (17). Briefly, 1×10^6 cells were incubated with Hoechst 33342 (1.5 mg/ml) in RPMI 1640 containing 10% fetal bovine serum at 37° for 10 min. The cells were then cooled to 4°, centrifuged at 200 × *g* for 5 min, and resuspended in phosphate-buffered saline containing propidium iodide (5 mg/ml). Viable thymocytes have a relatively high forward light scatter (indicative of a large size) and exhibit a low blue fluorescence with Hoechst 33342. In contrast, apoptotic cells have lower forward light scatter (indicative of a smaller size) and exhibit a higher blue fluorescence. Mouse thymocytes were analyzed 20 hr after exposure to the anticancer agents.

Results

Clonogenic sensitivities of A2780 mutant *TP53* gene transfectants. The human ovarian cell line A2780 expresses wild-type *TP53* gene sequences and has functional p53-mediated G1 arrest after irradiation (6). A2780/mp53 cells are A2780 transfectants that express a dominant negative mutant of *TP53* (codon 143, valine to alanine) (4). These mutant p53 transfectants have lost p53 function, as measured by radiation-induced G1 arrest, compared with vector-only A2780/v transfectants (Table 1). Clonogenic survival curves shows that A2780/mp53 cells are more resistant than vector-only controls to ionizing radiation (Fig. 1A), cisplatin (Fig. 1B), doxorubicin (Fig. 1C), and ara-C (Fig. 1D) but not to taxol or camptothecin (Fig. 1, E and F). The resistance to ionizing radiation confirms our previous observations (4). We previously observed a trend of increased resistance to a 24-hr exposure to cisplatin in A2780/mp53 cells (6). In the current study, we observed a significant increase in resistance to a 1-hr exposure to cisplatin. The fold resistance at the concentration of drug required to reduce colony formation by 80% (ID_{80}) is summarized in Table 1. Compared with vector-only controls, the mutant transfectants were found to be significantly more resistant to doxorubicin (2.8-fold), cisplatin (2.5-

TABLE 1
Cell lines used and relative resistance factors

Cell line	Description	S-phase cells after irradiation[a]	Fold resistance[b]					
			I.R.[c]	Cisplatin	Doxorubicin	ara-C	Taxol	Camptothecin
		%						
A2780/v	Vector-only transfectants of A2780	33 (9)	1	1	1	1	1	1
A2780/mp53	Mutant p53 (codon 143, valine to alanine) transfectants of A2780	97 (7)	2.3[d]	2.5[d]	2.8[d]	2.3[d]	1.1	1.0

[a] Percentage of cells in S phase as determined by pulsed bromodeoxyuridine labeling, 24 hr after 2-Gy ionizing radiation relative to untreated controls. Values shown are the mean of quadruplicate experiments with 20,000 cells counted in each experiment. Numbers in parentheses are standard errors.

[b] Fold resistance shown is calculated at the concentration of drug required to reduce colony formation by 80% except for ionizing radiation, for which the surviving fraction at 2 Gy is used. Statistical significance was determined using a Student's *t* test, with statistical significance set at $p < 0.05$.

[c] Ionizing radiation.

[d] Samples that are significantly different.

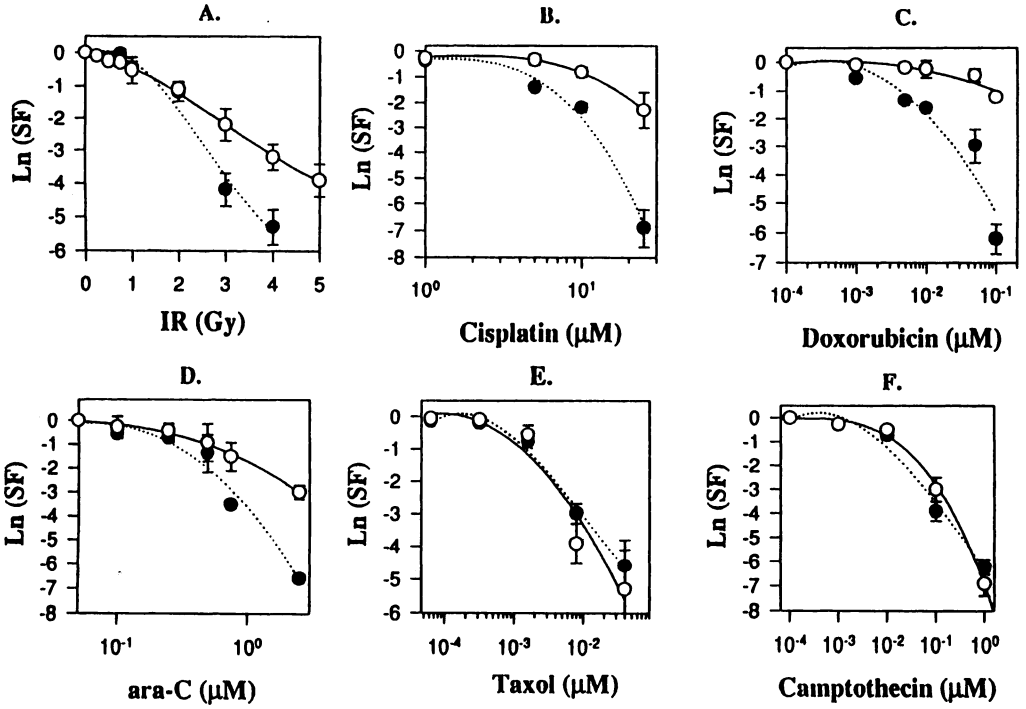

Fig. 1. Clonogenic assays of A2780/v and A2780/mp53. The natural log of the surviving fraction, *Ln (SF)*, is shown for varying doses of (A) ionizing radiation, (B) cisplatin (1 hr), (C) doxorubicin (24 hr), (D) ara-C (1 hr), (E) taxol (24 hr), and (F) camptothecin (1 hr). ●, A2780/v. ○, A2780/mp53. Individual values represent the average of at least 10 clonogenic assays using independent mutant p53 transfectants and vector-only transfectants. *Curves*, second-order linear regressions; *error bars*, standard error.

fold), and ara-C (2.3-fold). However, the mutant p53 transfectants are not more resistant to camptothecin (1.0-fold) or taxol (1.1-fold) compared with the vector-only controls. The data shown are for a 24-hr exposure to taxol; however, a 1- or 3-hr exposure to taxol also showed no significant difference in sensitivity compared with vector-only controls. Throughout these experiments, it was confirmed that the A2780/mp53 transfectants had abrogated radiation-induced G1 arrest, whereas the A2780/v cells retained the G1 arrest.

Induction of apoptosis in mouse thymocytes by cisplatin, taxol, and camptothecin. We examined cisplatin-induced apoptosis in thymocytes from mice that were either homozygous wild-type at the *p53* locus or homozygous null for *p53* (13). As previously observed, we confirmed that >90% of thymocytes undergo apoptosis in both wild-type and *p53*-null mice 24 hr after treatment with 10 μM methylprednisolone, whereas at 24 hr after ionizing radiation with 4 Gy, apoptosis is observed in the wild-type *p53*-expressing mice and not in the *p53*-null mice (Table 2). Cisplatin induces a dose-dependent increase in apoptosis in thymocytes from wild-type mice that is markedly reduced in the *p53*-null mice. It has previously been shown that cisplatin induces apoptosis in proliferating rat thymocytes and not in nonproliferating thymocytes (14). The thymocytes studied in the current study have been enriched for proliferating thymocytes, but the cell populations still contain a high proportion of nonproliferating cells. In the nonseparated cells, 13% were in S phase, as measured through bromodeoxyuridine incorporation, whereas after enrichment for proliferating cells, 30% were in S phase. Therefore, a 2–3-fold enrichment for proliferating cells is achieved through Percoll fractionation. In addition, the Percoll gradients allow removal from the cell suspension of cells that are already dead (see Table 2, *Control*).

Both taxol and camptothecin induce an increase in the number of apoptotic thymocytes from wild-type mice compared with untreated controls (Table 3). Unseparated and proliferation-enriched thymocytes show a concentration-dependent increase in camptothecin-induced apoptosis. No significant difference in apoptosis induced by taxol was observed between thymocytes from wild-type mice and those from *p53*-null mice in either unseparated or proliferation-enriched cells. Interestingly, camptothecin-induced apoptosis at 0.1 μM also seems to be independent of *p53* status; however, at a concentration of 10 μM, camptothecin-induced apoptosis is reduced in the thymocytes from the *p53*-null mice. The *p53*-independent apoptosis induced by 0.1 μM camptothecin is observed after treatment of thymocytes for 24 and 48 hr (Table 4). The differences in apoptosis that we observed were not due to camptothecin-induced effects on thymocyte total cell number.

TABLE 2

Cisplatin-induced apoptosis in proliferating thymocytes of *p53* wild-type and *p53*-null mice

Treatment[a]	p53 genotype[b]	Apoptotic cells[c]
		%
Control	+/+	29, 30, 29 (29)
	−/−	25, 27, 22 (25)
Methylprednisolone (10 μM)	+/+	94, 87 (90)
	−/−	88, 87 (88)
γ-Rays (4 Gy)	+/+	88, 83 (86)
	−/−	31, 31 (31)
Cisplatin (10 μM)	+/+	47 (47)
	−/−	30 (30)
Cisplatin (25 μM)	+/+	50, 55 (52)
	−/−	34, 37 (36)
Cisplatin (50 μM)	+/+	61, 62, 62 (62)
	−/−	34, 55, 35 (41)

[a] Thymocytes were treated with methylprednisolone for 24 hr and cisplatin for 2 hr.
[b] +/+, Wild-type mice; −/− homozygous p53-null mice.
[c] Percentage of apoptotic thymocytes observed 24 hr after treatment as measured by flow cytometry (see Materials and Methods). Values in parentheses are mean.

TABLE 3

Taxol- and camptothecin-induced apoptosis in thymocytes of *p53* wild-type and *p53*-null mice

Treatment	p53 genotype[a]	Apoptosis: unseparated[b]	Apoptosis: proliferating[c]
		%	
Untreated	+/+	30	9
	−/−	33	13.5
Taxol (0.1 μM)	+/+	46 (2.3)	33.3
	−/−	44 (2)	27
Taxol (1 μM)	+/+	46 (1.4)	34.3
	−/−	49 (2.4)	30.6
Taxol (10 μM)	+/+	50 (2.8)	39.3
	−/−	44 (2.3)	33
Camptothecin (0.1 μM)	+/+	41 (1.8)	56.8
	−/−	43 (2.3)	50
Camptothecin (1 μM)	+/+	60 (1.6)	54.5
	−/−	48 (3.3)	56.4
Camptothecin (10 μM)	+/+	79 (2.3)	67
	−/−	57 (1.3)	46.6

[a] +/+, wild-type mice; −/− homozygous p53-null mice.
[b] Percentage of apoptosis in unseparated thymocytes 24 hr after treatment (13% of cells were in S phase). Numbers in parentheses are standard errors.
[c] Percentage of apoptosis as measured by morphology (see Materials and Methods) in thymocytes enriched for proliferating cells (30% of cells were in S phase).

TABLE 4

Camptothecin-induced apoptosis in thymocytes

Treatment[a]	p53 genotype[b]	Cell number[c]	Apoptosis: proliferating[d]
		$\times 10^6$	%
24 hr			
Untreated	+/+	8.3	28
	−/−	9.6	16.5
Camptothecin (0.1 μM)	+/+	6.7	41
	−/−	7.5	35
Camptothecin (1 μM)	+/+	5.5	62
	−/−	7.0	44.5
Camptothecin (10 μM)	+/+	6.0	82
	−/−	6.5	50
48 hr			
Untreated	+/+	8.9	44
	−/−	9.6	48
Camptothecin (0.1 μM)	+/+	5.6	56
	−/−	5.6	55
Camptothecin (1 μM)	+/+	5.0	94
	−/−	4.9	84
Camptothecin (10 μM)	+/+	4.3	100
	−/−	5.1	95

[a] Thymocytes were treated with camptothecin for the times shown.
[b] +/+, wild-type mice; −/− homozygous p53-null mice.
[c] The cell number observed after an initial seeding of 10^7 cells.
[d] Percentage of apoptosis as measured by morphology (see Materials and Methods) in thymocytes enriched for proliferating cells (30% of cells were in S phase).

At lower concentrations of camptothecin, more apoptosis is observed in the proliferation-enriched thymocytes than in the unseparated population (Table 3). DNA replication has been suggested to be important for the toxicity of this topoisomerase I inhibitor (18), although camptothecin toxicity may not be exclusively dependent on proliferation (19). At the higher concentrations of camptothecin, the unseparated and the proliferation-enriched cell populations become equivalent in the amount of apoptosis observed. This suggests that camptothecin induces apoptosis in a *p53*-independent manner at low doses but in a *p53*-dependent manner at high doses. Furthermore, the data imply that the *p53*-independent camptothecin-induced apoptosis is dependent on cell proliferation, whereas the *p53*-dependent apoptosis is not.

Discussion

We have shown that transfection of a dominant negative mutant *TP53* gene into a human ovarian cell line expressing wild-type *p53* confers increased resistance to ionizing radiation, doxorubicin, cisplatin, and ara-C as measured by clonogenic assay. Interestingly, the mutant *TP53* transfectants are not cross-resistant to taxol or camptothecin. A link between the activity of *p53* and the induction of apoptosis after DNA damage has been previously established in comparative studies of immature rodent thymocytes containing wild-type *p53* or thymocytes from animals with germ-line deletion of the *TP53* gene (2, 3). In these studies, apoptosis was induced using either etoposide or ionizing radiation in wild-type mice but was markedly reduced in *p53*-null mice. To explore further the relationship between different types of DNA damage and *p53*-dependent cellular responses, we examined the effects of cisplatin, camptothecin, and taxol in this well-established thymocyte model. We show reduced cisplatin-induced apoptosis in thymocytes from *p53*-null mice. We observed no difference in taxol-induced apoptosis of thymocytes from wild-type or *p53*-null mice in either unseparated or proliferation-enriched thymocytes. A recent study has shown that fibroblasts with the *p53* gene functionally inactivated become hypersensitive to taxol (20).

An important observation in this study was that camptothecin seems to induce apoptosis of thymocytes in a *p53*-independent manner at lower concentrations but in a *p53*-dependent manner at higher concentrations. Furthermore, at lower concentrations of camptothecin, more apoptosis is observed in the proliferation-enriched thymocytes than in the unseparated population. However, at the higher concentrations of camptothecin, in both cell populations the amount of apoptosis observed by 24 hr become equivalent. In the A2780 cells, a rapidly proliferating ovarian tumor cell line, cytotoxicity to camptothecin seems to be independent of *p53* function. However, this occurred in response to a 1-hr drug exposure, and a *p53* dependence may emerge after longer periods of drug exposure. One mechanism of camptothecin-induced cell death is dependent on DNA replication and has been suggested to be due to collision of the DNA polymerase with the camptothecin/DNA complex and subsequent generation of a double-strand break (18). Based on our data, we suggest that this type of cell death in thymocytes and in the A2780 ovarian tumor cell line is *p53* independent. Both *p53*-dependent and -independent mechanisms of DNA damage-induced apoptosis are known to exist (2, 21). At higher concentrations of camptothecin, it could be speculated that other means of generating an apoptotic signal become important. For example, it is known that topoisomerase I activity is important for RNA transcription, and interactions of the camptothecin/DNA complex with the RNA polymerases could produce DNA strand breaks at a higher camptothecin concentration (19).

There is evidence that dysfunctional *p53* plays an important role in the resistance of tumors to platinum-based chemotherapy (22). If the loss of *p53*-mediated apoptosis is important in treatment failure of ovarian cancer, then drugs that mediate toxicity in *p53*-independent mechanisms have the potential to be very useful. Indeed, taxol and camptoth-

ecin analogs are of increasing interest in the treatment of ovarian tumors that are resistant to cisplatin chemotherapy (10, 11).

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