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A p53 GROWTH ARREST PROTECTS FIBROBLASTS FROM ANTICANCER AGENTS

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1. ABSTRACT

Reversible inhibitors of the cell cycle such as the TGF-betas have been exploited to protect dividing cells from exposure to anticancer drugs and radiation. Here, rat embryo fibroblast (REF) lines expressing different p53 mutations were used to test whether the p53 growth arrest could also chemoprotect cells from high doses of anticancer drugs. Whereas the doubling times of the different REF lines at 37°C were similar, cells bearing temperature-sensitive mutations (mouse 135V or human 143A) were growth arrested at 31°C. Temperature-dependent p53 activity was associated with increased levels of MDM2 and p21/WAF1, and the induction of an integrated p53-responsive luciferase gene. The REF lines exhibited similar sensitivities to common anticancer drugs when grown at 37°C. However, when exposed to the same agents following transient incubation at 31°C, the p53-arrested cells exhibited a marked survival advantage as shown by colony-forming assays. Chemoprotection was not universal, in that colony formation was not enhanced significantly after treatment with cisplatin or 5-fluorouracil, two drugs which can cause cellular damage throughout the cell cycle. Like other negative growth regulators, an activated p53 checkpoint may mediate the survival of cells exposed to drugs that target DNA synthesis or mitosis.

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2. INTRODUCTION

Negative regulators of the cell cycle have attracted interest in strategies designed to protect normal tissues from the adverse effects of radiation and chemotherapy. An arrest of the cell cycle in G1 phase, for example, has been exploited to protect cells from subsequent exposure to drugs that target DNA synthesis or mitosis. Most experimental strategies exhibit several common features that could be considered prerequisites for achieving "chemoprotection." First, the target cell population should be growth arrested before exposure to the drug or other cellular insult. Second, the effects of the growth arrest should be reversible. Chemoprotected cells that are normally proliferating need to re-enter the cell cycle and divide at appropriate rates once chemotherapy or radiation is discontinued. Third, the chemoprotecting agent should be preferential for normal cells relative to malignant cells. In some cases, differential chemoprotection has been achieved simply by targeting the arresting agent to the desired sites (1). Recently, we and others have utilized members of the TGF-beta (TGFB) superfamily of reversible growth inhibitors to increase the viability of cells exposed to chemotherapy drugs *in vitro* and *in vivo* (1-5). Results in our laboratory with TGFB3 and cultures of CCL64 epithelial cells indicated that the TGFB3 arrest could protect cells from drugs active predominantly in S phase (cytosine beta-D-arabino-furanoside hydrochloride [Ara-c]) or M phase (taxol) of the cell cycle. In contrast, TGFB3 was ineffective against drugs such as cisplatin and doxorubicin, which act via multiple mechanisms throughout the cell cycle (5).

The observation that regulatory factors such as IL-11 (6) and the TGFBs can promote the survival of cells exposed to anticancer drugs suggests the possibility that other strategies (chemicals, genes, regulatory factors) that arrest cell growth could also be used for chemoprotection. Alternative approaches could then be compared for efficacy on the different tissues (bone marrow, oral mucosa,

intestines, gonads, hair follicles) most susceptible to the adverse effects of chemotherapy in cancer patients. In the present study, we have focused on the p53 tumor suppressor as one model system to test whether other reversible arrests of the cell cycle can be utilized to chemoprotect cells. A key aspect of the study is the use of temperature-sensitive (ts) p53 mutations (mouse 135V and human 143A) to arrest fibroblasts prior to exposure to anticancer drugs, which include mitotic inhibitors (vinblastine, taxol, etoposide), antimetabolites (Ara-c, 5-fluorouracil [5FU]), and a carboplatinum agent (cisplatin).

3. MATERIALS AND METHODS

3.1 Cell lines

Low passage rat embryo fibroblasts (REFs) were seeded into 10 cm plastic dishes in Dulbecco's Modified Eagle Medium (DMEM) from GibcoBRL (Grand Island, NY) supplemented with L-glutamine and 10% fetal bovine serum from Sigma Chemical Company (St. Louis, MO). Cells were grown to 60-80% confluency, and were then transfected to generate stable transformed cell lines using a CellPhect transfection kit from Pharmacia (Piscataway, NJ). Plasmid constructs for the expression of C-MYC and activated H-RAS were kindly provided by Dr. John Haley of OSI Pharmaceuticals. Plasmids for the expression of human p53 proteins in mammalian cells were as follows: pC53-C1N$_3$ (for wild-type [wt] p53), pC53-CX3$_3$ (for mutation 143A), pC53-Cx22AN$_3$ (for mutation 175H), pC53-248$_3$ (for mutation 248W) and plasmid pC53-4.2N$_3$ (for mutation 273H). p53 constructs have been previously described (7). REF lines were cotransfected with the H-RAS plasmid, plus either the C-MYC plasmid or one of the p53 mutation plasmids, and selected in 800 microgram/ml G418 (Gibco/BRL). After 10-14 days of selection, G418-resistant colonies with a transformed morphology were picked using cloning cylinders from Bellco Glass Company (Vineland, NJ), and were seeded into 12-well plates for further expansion. A1-5 cells, containing murine p53 mutation 135V (8), were kindly provided by Dr. Arnold Levine. Levels of p53 expression in the lines were examined using a commercial p53 mutant-selective ELISA assay from Oncogene Research Products/Calbiochem (Cambridge, San Diego, CA), following the manufacturer's instructions. Results on select lines were corroborated by western blots or immunoprecipitations of [^{35}S]-labeled lysates (below). Cell division times at 37°C and 31°C were determined by seeding cells into 6-well plates followed by allowing cells to attach at 37°C for 3 hours, at which time half of the plates were shifted to 31°C. Every day thereafter, cells counts were made in duplicate, from which division times at both temperatures were calculated.

3.2 Protein analyses

For the analyses of p53 and p21 expression by western blotting, REF cells were seeded into multiple 10 cm dishes (1-3 million cells/dish). Following an overnight incubation at 37°C, half of the plates were shifted to 31°C. Plates were incubated at the indicated temperatures for 24 hours, then washed twice in cold PBS and lysed *in situ* in 1 ml of a buffer composed of 150 mM NaCl, 50 mM Tris-Cl pH 8, 5 mM EDTA, 1% NP-40, 100 microgram/ml phenylmethylsulfonylfluoride (PMSF), 0.2% aprotinin and 1 microgram/ml leupeptin. Plates were kept on ice for 30 minutes, followed by scraping and transfer of the lysate to eppendorf tubes. Cellular debris was pelleted by a 15 minute spin in a microcentrifuge at 4°C, and protein concentrations in the supernatant were determined using a Bradford assay from Biorad (Hercules, CA). Proteins were resolved by electrophoresis in 8-12% polyacrylamide gels, and transferred to nitrocellulose (Trans-Blot transfer medium, Bio-Rad). Filters were washed, blocked and probed as described in the ECL detection manual from Amersham (Arlington Heights, IL). p53 was detected using a combination of PAb 421 and DO-1 antibodies (both from Calbiochem), each at 1 microgram/ml. Rat p21/WAF1 was detected with culture supernatant mp21-22AA, generously provided by Marilee Burrell and Dr. David Hill from Oncogene Research Products/Calbiochem

For analyses of [^{35}S]-radiolabeled proteins by immunoprecipitation, cells were seeded into 10 cm dishes and temperature shifted as before, then washed in PBS, and incubated with 5 mls of DMEM medium lacking methionine and cysteine from ICN (Costa Mesa, CA) containing 10% dialyzed fetal calf serum for two hours. The medium was then replaced with two mls of the same medium, containing 100 microCi Tran^{35}S-label (ICN; 800-1000 Ci/mmol) and incubated for 2-4 hours. Cells were lysed as above, and desired proteins were immunoprecipitated using antibodies preadsorbed to protein G sepharose beads from Pharmacia. Antibody 2A10 (9) was used to detect MDM2. Proteins were resolved by electrophoresis in 8-12% polyacrylamide gels and detected by autoradiography.

For studies of the effect of temperature shifts on the expression of an integrated p53-responsive luciferase gene, plasmid pLH3 was transfected into the REF cells. Plasmid pLH3 encodes resistance to hygromycin-B, and contains a DNA sequence spanning four ribosomal gene cluster (RGC) sites (10) located upstream of the SV40 minimal promoter, which drives expression of the firefly luciferase gene. After 10-14 days of selection in 200 U/ml hygromycin-B from Calbiochem, 30-100 surviving colonies were trypsinized and pooled. The cells were expanded and seeded into quadruplicate wells of white 96-well plates from Becton Dickinson (Franklin Lakes, NJ). After overnight incubation at 37°C, 6 of the plates were shifted to 31°C. At 2-3 hour time-points, one plate at each temperature was processed for luciferase activity using an assay from Promega (Madison, WI). Light production was quantitated by reading the plates in a ML1000 microplate luminometer from Dynatech Laboratories, set to "Medium Gain." Readings from a single plate were averaged, standard deviations were calculated, and 31°C/37°C induction ratios were determined.

Table 1. Cell division times of cloned REF lines at 37°C and 31°C.

Cell Line[a] (Clone)	Division Times (in hours)	
	37°C	31°C
C-MYC (C)	21	22
C-MYC (D)	13	26
135V (A1-5)	22	80
143A (C)	21	120
143A (D)	17	70
175H (B)	20	38
175H (D)	15	39
248W (A)	13	31
248W (B)	14	28

a, All REF lines also express mutant H-RAS (13).

3.3 Chemoprotection colony assays

To study the effects of a p53 growth arrest on cell survival, it was first necessary to generate IC_{50} cytotoxicity plots for the REF lines exposed to different chemotherapeutic agents. The drugs chosen for study were cytosine beta-D-arabino-furanoside hydrochloride (Ara-c), etoposide, 5-FU, taxol, cisplatin and vinblastine (sulfate salt). The drugs were suspended in 100% DMSO at 3-25 mg/ml, aliquoted, and stored at –70°C until use, except for cisplatin, which was freshly dissolved in DMSO prior to each experiment. All drugs were purchased from Sigma, except for taxol (ICN). First, cells were seeded into 96-well plates (250-1,500 cells/well) in 100 microliter of medium. Twenty-four hours later, two-fold serial dilutions of the drugs in medium were added to each REF line in triplicate wells, to give a final volume of 200 microliter. Cells were exposed to the drugs for 24 hours, followed by one PBS wash, the addition of 200 microliter fresh medium without drugs, and incubation at 37°C for an additional 48 hours. More stringent washing resulted in sloughing and unacceptable levels of cell loss. The agent 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, from Sigma) was used as a measure of cell viability. Fifty microliter of a 0.2% solution of MTT in PBS was added directly to the medium of the wells; the plates were then returned to 37°C for two hours. The media was aspirated, and the metabolized MTT formazan product was solubilized with 100 microliter of ethanol:acetone (50:50). Plates were read at 540 nm in a microplate reader from Bio-Tek Instruments (Winooski, VT), background absorbance from wells without MTT was subtracted, and cytotoxicity curves were plotted.

For colony assays to assess chemoprotection, each cell line was seeded into four 6-well plates at 10-30K cells/well. The majority of experiments assayed five different lines. All plates were left at 37°C for three hours for cell attachment to occur, at which time 2 of the plates were moved to 31°C incubators. Since all drug and control additions were done at both temperatures, and in duplicates, this format allowed for a maximum of 5 drug concentrations plus the no drug (DMSO-only) controls to be tested. After preincubation of plates at the designated temperatures for 20 hours (to allow for growth arrests), the cells were exposed to the agents at the chosen concentrations for 24 hours at the same temperatures. The wells were washed once with PBS, re-fed with fresh medium without drugs, and returned to the 37°C or 31°C incubators for a final 18 hour incubation. The cells were then washed, trypsinized, and a known fraction were seeded into 10 cm dishes. All 10 cm dishes were incubated at 37°C for 10-14 days, to allow colony formation to occur, at which time the dishes were stained with crystal violet or MTT, and the colonies were counted in duplicates. Final colony counts reflect normalization for the fraction of cells seeded. Results are shown in two ways: first, as a percentage of the colonies formed after drug exposure, relative to the same line incubated without drug on the y axis, versus drug concentration on the x axis. Separate plots were generated for cells exposed at 37°C vs 31°C, and the slopes were compared. Secondly, the IC_{50} values were calculated for the different cell lines exposed to the various drugs at 37°C and 31°C and placed into a table.

4. RESULTS

The two most commonly studied temperature-sensitive (ts) p53 mutations, mouse 135V (8) and human 143A (11), were used in these studies. Constitutive "hot-spot" p53 mutations 175H and 248W served as negative controls, as did C-MYC, which (like mutations in p53) can collaborate with H-RAS to immortalize primary REF cells. The 135V and 143A mutations are ts for conformation, with incubation at 37-39.5°C (the nonpermissive temperature) resulting in a mutant (mt) conformation, and incubation at 31-32.5°C (the permissive temperature) resulting in a predominantly wild-type (wt) conformation. In cells bearing such mutations, growth at the permissive temperature correlates with increased localization of p53 to the nucleus, enhanced binding of p53 to DNA oligonucleotides containing p53-consensus sites, and increased transactivation of p53-responsive genes (8). Mutation 143A has been identified in a human gastric tumor line (12).

Immortalized REF lines were derived expressing activated H-RAS, plus either C-MYC, or one of four p53 mutations (135V, 143A, 175H and 248W). For simplicity, cloned lines are designated with either C-MYC, or one of the p53 mutations, with the clone designation in parentheses. All cell lines exhibited a transformed morphology with doubling times at 37°C of 13-22 hours (table 1), although the 143A-expressing cells were flatter and less refractile than the other lines. Cells transfected with wt p53 constructs became growth arrested and permanent cell lines could not be derived. Temperature downshifts to 31°C resulted in increased doubling times for all of the lines, although cells expressing C-MYC, 175H or 248W continued to grow rapidly and acidify the medium at this temperature. By contrast, the lines expressing p53 mutations 135V or 143A either failed to divide noticeably, or grew with division times of 70 hours or greater. Similar results for K562 cells bearing 143A have been reported previously (11). The growth inhibition for lines with ts p53 mutations could be reversed by returning the cultures

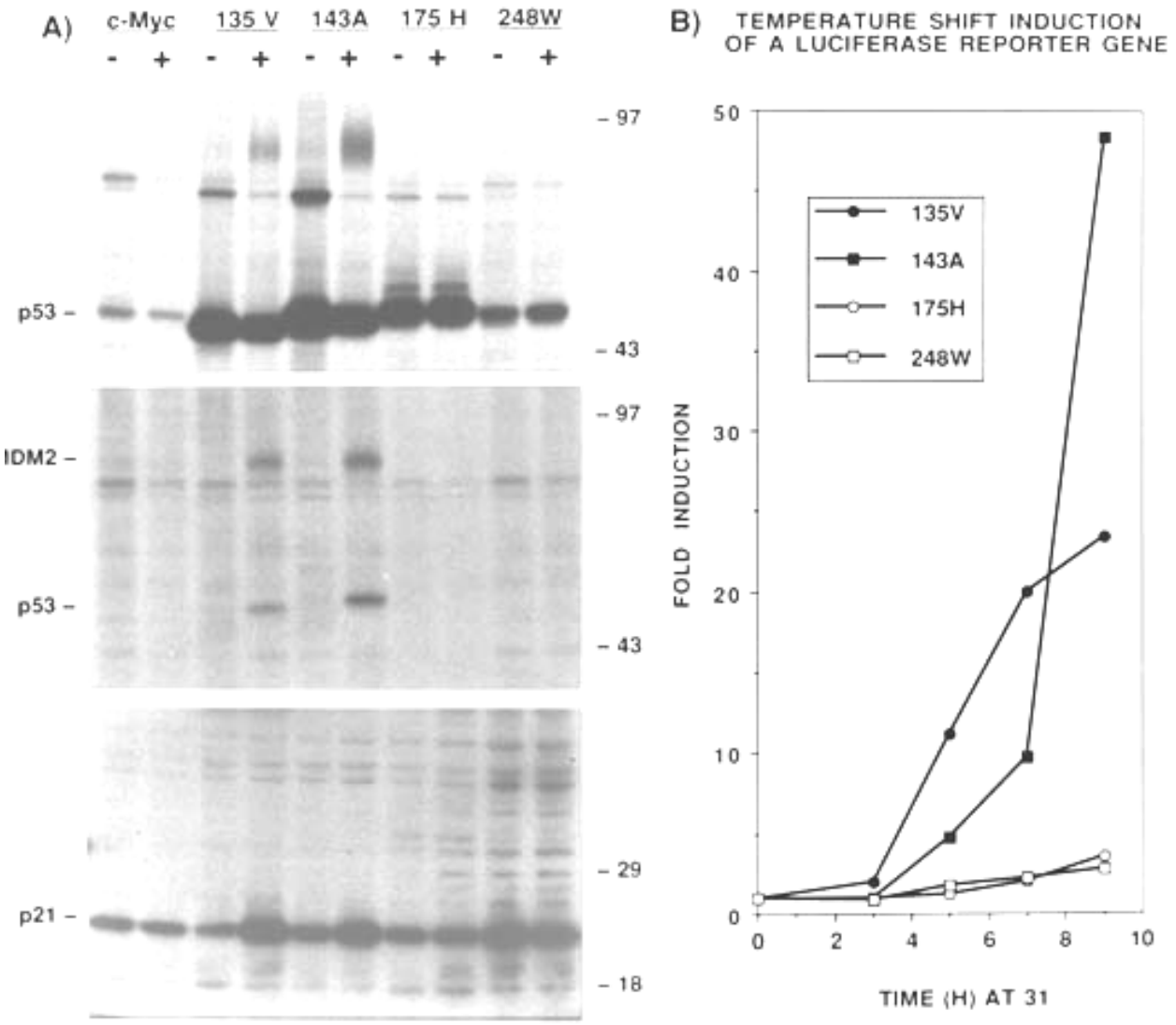

Figure 1. Activation of wild-type p53 responsive genes in REF cells bearing the ts p53 mutations 135V and 143A. A (Top), Five REF lines were labeled at either 37°C (- lanes) or 31°C (+ lanes) with [^{35}S]-methionine, lysed, and an aliquot was immunoprecipitated with antibody 421 to detect p53. The numbers on the right represent molecular weight markers in kDa. (Center) A second aliquot of lysates was precipitated with antibody 2A10 to detect endogenous MDM2. (Bottom), A western blot of unlabeled lysates was probed to detect endogenous p21^{Waf1}. B, p53 mutations 135V and 143A are ts for the expression of integrated wt p53-responsive luciferase genes. REF cells expressing the p53 mutations shown were transfected with plasmid pLH3, containing a firefly luciferase gene expressed from a minimal SV40 promoter containing four p53 consensus sites (10). After selection in hygromycin, cells were seeded into white 96-well microtiter plates and incubated at 37°C. Sixteen hours later (time 0), half of the plates were placed at 31°C. At 2 to 3 hour timepoints thereafter, one plate at each temperature was processed for luciferase activity. The results are plotted as fold induction, defined as light signal (31°C/37°C). Each point is the mean of quadruplicate wells, with all coefficients of variation < 20.

to 37°C (see below). Incubation at 31°C did not result in detectable levels of apoptosis in any of the lines, based on studies of cell viability, and a comparison of DNA samples from cells maintained at either temperature (data not shown).

Activation of wt p53 results in increased transcription of a set of p53-responsive genes (9, 14), leading to increased levels of the corresponding proteins. When metabolically-labeled lysates of REF lines were immunoprecipitated with an antibody (PAb 421) to p53, significantly higher levels of p53 were observed for the cells transformed with p53 mutations, relative to the cell lines transformed with C-MYC and expressing only endogenous rat p53 (figure 1A, top). A coprecipitating band in the 70 kDa range, presumably hsc70 (7), was seen

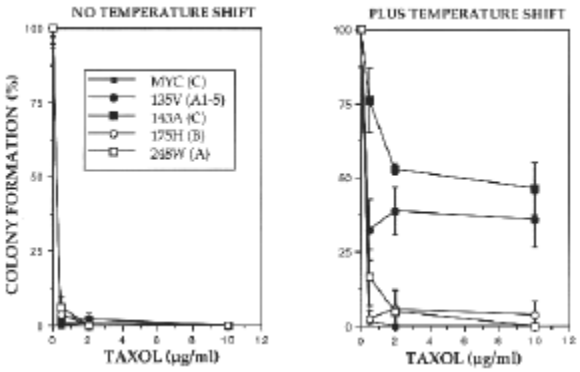

Figure 2. Transient induction of wt p53 activity by temperature shift correlates with chemoprotection from high doses of taxol. Left, the REF clones shown were exposed to different doses of taxol for 24 hours at 37°C (no temperature shift), washed and processed for colony assays as described in Methods. Right, the same lines were processed similarly, except that drug addition occurred after temperature shift to 31°C. After trypsinization and seeding, colonies were stained and counted ten to fourteen days later. The points shown represent the mean (with standard deviation [SD]) from triplicate plates from one of two independent experiments with similar results.

for cells expressing 135V, 143A or 175H at 37°C; when incubated at 31°C, this band was reduced in intensity only for cells expressing 135V or 143A. By contrast, a protein in the 90 kDa range was observed to coprecipitate preferentially with cells expressing either 135V or 143A at 31°C. The size of the protein and its expression pattern was highly suggestive of MDM2, which increases in quantity in response to p53 activation (9). MDM2 has been shown to form a physical complex with p53, and to inhibit p53 transactivation functions in a manner suggestive of a negative feedback mechanism. Accordingly, precipitation of the lysates with antibody 2A10, which recognizes rat MDM2 (9), revealed increased MDM2 levels in the 135V and 143A lines at 31°C (figure 1A, middle). As expected, the MDM2-coprecipitating proteins in the 50 kDa range were identified as p53, based upon western blots of anti-MDM2 precipitates probed with horseradish peroxidase-conjugated antibodies to p53 (data not shown).

The cell doubling times and MDM2 expression patterns were consistent with the temperature-dependent expression of wt p53 functions in the lines bearing p53 mutations 135V or 143A. Western blots of whole cell lysates, probed with an antibody to p21/WAF1, revealed increased p21 expression in the cells presumed to express wt p53 (figure 1A, bottom). Unlike the results with MDM2, detectable baseline levels of p21 were observed for all the REF lines, consistent with previous observations that wt p53 signaling is not required for the expression of p21 (15).

p53-responsive reporter genes have proven useful for studies of chemicals, proteins and mutations which impinge on p53 functions, and could conceivably be used to identify novel p53-activating mechanisms. Here, a wt p53-responsive luciferase gene (see Materials and Methods) was used to confirm the ts nature of mutations 135V and 143A, and to follow the acquisition of wt p53 transactivation properties as a function of time at 31°C. Such reporter genes generally provide variable p53-independent baseline signals, which can be increased from 10 to >1000-fold by the presence of wt p53 ([11], and data not shown). Incubation at 31°C for five hours was sufficient to trigger increased levels of luciferase expression in the 135V and 143A populations, which peaked at nine hours (figure 1B). At later timepoints, the 31°C/37°C values decreased, due to the continued growth of the cells at 37°C and consequent increases in the luciferase baselines. In the chemoprotection assays below, cells were preincubated at 31°C for 20 hours prior to the addition of the chemotherapy agents.

4.1 Protection from taxol

Many chemotherapy drugs inhibit tumor growth by interfering, either directly or indirectly, with DNA replication or mitosis. Irrespective of whether the killing of these normal populations is via necrosis or apoptosis, the induction of a temporary growth arrest (outside of S or M phase) during the period of drug exposure might result in enhanced survival. Briefly, a 20 hours pre-incubation at 31°C was used to arrest the cells with ts p53 mutations, prior to co-incubation with chemotherapy drugs for 24 hours, also at 31°C. After the drugs were washed out of the cultures, the designated plates were returned to 31°C for an additional 18 hours. The reasoning behind extending the protection "window" beyond the point of drug removal was to allow for the intracellular drug concentrations to decrease before allowing the cells to resume cell division at 37°C. The cells were trypsinized, diluted and replated, such that individual cells gave rise to colonies. The assay thus requires that a protected cell will retain not only viability, but be able undergo a sufficient number of rounds of replication ($\geq$ 6) to be counted.

Results in figure 2 are shown for taxol, an agent that acts at the level of mitosis, through an enhancement of tubulin polymerization and consequent stabilization of microtubules (16). At 31°C, ts p53 mutations mediated a growth arrest that clearly protected the 135V and 143A-expressing lines from this drug. All REF lines exposed to taxol at 37°C exhibited an inhibition of colony formation that was >90%, even at the lowest concentration tested (0.5 microgram/ml; figure 2, left). When the MYC, 175H and 248W-expressing clones were exposed at 31°C, plating efficiency in response to 0.5 microgram/ml improved slightly (figure 2, right), but virtually no colonies formed from cells exposed to 2 or 10 microgram/ml. Similar results were observed with two additional clones, 175H (D), and 248W (B) (not shown). By contrast, the 135V and 143A-expressing cells retained $\geq$37% colony forming

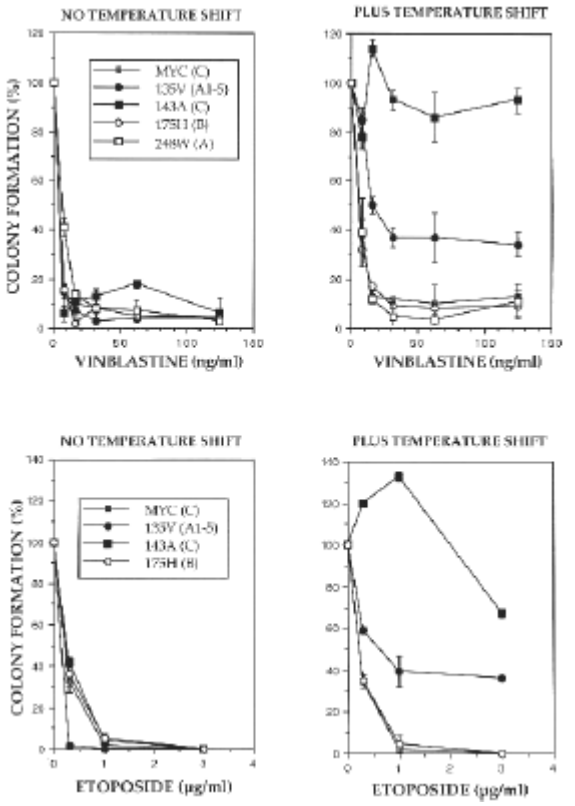

Figure 3. Induction of wt p53 activity correlates with chemoprotection from vinblastine and etoposide. Top, the REF clones shown were exposed to vinblastine at 37°C or 31°C, and processed for colony assays. Data points represent the mean (with SD) from triplicate plates from one of three independent experiments with similar outcomes. Bottom, Lines were exposed to the indicated doses of etoposide at both temperatures and processed similarly. The data points represent the mean (with SD) from triplicate plates from one of two independent experiments with similar outcomes.

efficiency when exposed to drug at 31°C. An additional clone, 143A (D) was similar to the 135V (A1-5) line in its survival profile (not shown). Visual inspection of the stained dishes revealed that colony sizes derived from the treated versus untreated cells were not different (not shown).

4.2 Protection from vinblastine, etoposide and Ara-C

Vinblastine acts to prevent the polymerization of tubulin dimers, disrupting the formation of microtubules. While the primary cytotoxicities of vinblastine and other vinca alkaloids occur in metaphase, the agents may also act in other phases of the cell cycle (16). Vinblastine was a potent inhibitor of colony formation, generating >80% inhibition at 15 ng/ml, for all REF lines exposed at 37°C (figure 3, top). REF lines expressing MYC, 175H or 248W were similarly inhibited when exposed at 31°C. By contrast, the 143A line retained >85% of its clonogenicity at 31°C, after drug exposures of up to 125 ng/ml, whereas protection of the 135V (A1-5) line plateaued at about 40%. Thus, enhanced colony formation after drug exposure correlated with wt p53-mediated growth arrests, and was observed over a range of vinblastine concentrations (12.5 to 125 ng/ml). Similar results using vinblastine were seen in 2 subsequent experiments, in which the concentrations of drug tested were 0, 2, 10 and 150 ng/ml (summarized in table 2).

The p53-mediated growth arrest also correlated with chemoprotection from etoposide, an epipodophyllotoxin that is thought to exert its major cytotoxic effects by inducing DNA strand breaks consequent to interactions with topoisomerase II (16). The highest concentration of etoposide tested (3 microgram/ml) was sufficient to inhibit 100% colony formation in all of the lines exposed at 37°C, as well as for the control lines exposed at 31°C (figure 3, bottom). By contrast, the 143A and 135V-expressing lines retained significant clonogenicity (70% and 38%, respectively) when exposed to 3 microgram/ml drug at 31°C. Relative to the DMSO-only control plates, a slight increase in clonogenicity following drug exposures of 0.5 and 1 microgram/ml was observed for the 143A-expressing line. The mechanism underlying this repeatable observation is unknown, and has been observed previously using TGFB3 as the arresting agent on CCL64 epithelial cells (5).

The main toxicity of Ara-C, an antimetabolite, arises from the ability of its active form (Ara-CTP) to be incorporated into DNA, and to act as a competitive inhibitor of DNA polymerase. These effects are consistent with the drug's selective toxicity for cells in S phase (16). Ara-C was tested at five concentrations ranging from 25-500 ng/ml, against six cell lines (in triplicate) from Table I: MYC (C), 135V (A1-5), 143A (C), 143A (D), 175H (D) and 248W (B). For all of the lines exposed at 37°C, >75% inhibition of colony formation was seen for the two highest concentrations (250 and 500 ng/ml) of Ara-C tested (table 2). When added to cells at 31°C, the same two concentrations inhibited colony formation by >95%, but only for the control cell lines. As was observed with the previous three agents, the 135V and 143A mutations enhanced viability when cells were exposed at the permissive temperature.

4.3 p53 enhances survival weakly for cells treated with 5-FU, and does not protect from cisplatin

The compounds shown in figures 2 and 3 are known to exert their maximum cytotoxicities in the S or M phases of the cell cycle (16). Since p53 has been shown to arrest cells in G_1 (8), the results are consistent with the idea that a transient arrest of the cell cycle, outside of S or M phase, can be exploited to protect cells from a subsequent exposure to chemotherapy. However, not all anticancer drugs are restricted in their actions to particular phases of the cell cycle. We reasoned that a p53-mediated growth arrest might fail to protect from such agents, and might even exacerbate toxicity. Two agents that were not

Table 2. Estimated IC_{50} measurements calculated from multiple colony assays, including representative data shown in figures 2-4, for REF cell lines treated with drugs shown.

Cell Line	Taxol[a]		Vinblastine		Etoposide		Ara-c		5-FU		Cisplatin	
	37°C	31°C	37°C	31°C	37°C	31°C	37°C	31°C	37°C	31°C	37°C	31°C
MYC (C)	0.2	0.2	5.0	10	0.5	0.6	0.02	0.04	0.2	0.4	0.01	0.01
135V(A15)	0.2	2	5.0	20	0.1	0.5	0.05	> 0.5	1.2	4.0	0.01	0.01
143A (C)	0.2	> 10	5.0	> 125	0.2	> 3.0	0.05	> 0.5	0.6	2.5	0.01	0.01
175H (D)	0.2	0.2	5.0	10	0.2	0.4	0.04	0.05	0.3	0.5	0.01	0.01
248W (A)	0.2	0.2	7.5	10	0.2	0.4	0.04	0.05	0.2	0.6	<0.01	0.05

[a] All values correspond to (microgram/ml), except for vinblastine (ng/ml).
[b] IC_{50} concentrations higher than maximum drug concentrations tested.

significantly affected in their cytotoxicity profiles by wt p53 function were cisplatin and 5-FU.

The antitumor activity of cisplatin involves the crosslinking of intracellular molecules, primarily DNA. Cells in all stages of the cell cycle are susceptible, although cells in G_1 may be the most affected (16). Virtually no protection was conferred by wt p53 for this agent. Five concentrations of cisplatin (0, 0.1, 1, 5, 10 and 20 microgram/ml) were tested figure 4, as well as lower concentrations in subsequent experiments, and the cytotoxicity plots at both temperatures were virtually superimposable.

Active metabolites of 5-fluorouracil (5-FU), a pyrimidine analog, are incorporated into both RNA and DNA (16). Incorporation into RNA causes defects in nuclear processing and polyadenylation, whereas incorporation into DNA causes strand breakage and fragmentation. Based on the initial cytotoxicity curves, three concentrations of 5-FU (0.5, 5 and 50 microgram/ml) were chosen for testing in colony assays against the five REF lines in figure 4. Concentrations of 5-FU $\geq$5 microgram/ml were sufficient to inhibit colony formation by >90% for all the lines exposed at 37°C; the same concentrations at 31°C inhibited colony formation 100% for the cells lacking ts mutations (summarized in table 2). At 31°C, colony-forming efficiency was reduced to 35% and 20% for the 143A and 135V-expressing lines, respectively, but only at 5 microgram/ml. Chemoprotection was not observed at 50 microgram/ml.

5. DISCUSSION

The G1 phase of the cell cycle is a period in which cells can reversibly arrest in response to various environmental cues and the activation of cell cycle checkpoints. The arresting pathways do not necessarily utilize identical signaling mechanisms, but appear to share the ability to inhibit cyclin-dependent kinases (such as CDK4) that operate in G1 (reviewed in 17). We had previously reported that a TGFB3 growth arrest could protect epithelial and hematopoietic cells from anticancer drugs *in vitro* and *in vivo*, and to reduce the severity of oral mucositis induced by chemotherapy in an animal model (1, 2). In tissue culture, preincubation of CCL64 epithelial cells with TGFB3 improved survival following exposure to agents (vinblastine, etoposide, taxol, Ara-c) which act predominantly in S or M phase (5). However, the effects of TGFB3 were reduced for the drugs cisplatin and doxorubicin, which are toxic to cells throughout the cell cycle. In the present study, we examined a different growth arrest mechanism (p53) for the chemoprotection of cells exposed to anticancer drugs. We anticipated from previous studies (18, 19) that p53 (or other arrest mechanisms) might differ from the TGFB family regarding the profile of drugs where protection was observed. Here, we report that ts p53 mutations behaved similarly to TGFB: improving survival from drugs which target DNA synthesis or mitosis selectively, but showing little or no effect against drugs active throughout the cell cycle.

The p53 protein functions as part of a cell cycle checkpoint that can be activated in response to DNA damage. Whereas untransformed fibroblasts and many adherent cell lines undergo growth arrest following p53 activation, other cells are triggered into undergoing apoptosis (20-22). Examples include subsets of murine hematopoietic and gastrointestinal cells exposed to ionizing radiation, or cells lacking normal Rb function due to the ectopic expression of E2F or presence of viral oncoproteins. In such cell types, wild-type p53 status has a negative effect on survival, and apoptosis is further increased after drug or radiation treatment. Thus, since one function of p53 is to delete damaged cells from an organism, the choice of cell type is critical for examining any positive effects of p53 on survival. Several groups have previously analyzed the differences between the $p53^{(+/+)}$ and $p53^{(-/-)}$ genotype in a cell background where p53 activation leads to growth arrest. Unlike the present study, where we pre-arrested cells for chemoprotection, these earlier reports examined the effect of anticancer drugs on dividing cultures. Hawkins and coworkers (18) reported that human foreskin fibroblasts (HFF) were more resistant to killing by cisplatin, carboplatin and paclitaxel than derivative lines in which p53 function was abrogated by the human papillomavirus (HPV) E6 protein. Fan and coworkers (19) reported that both MCF-7 and RKO cells were more resistant to cisplatin than derivative lines overexpressing either HPV E6 or p53 mutations. Moreover, abrogation of a checkpoint operative in G2 with the caffeine derivative pentoxfylline led to a further differential killing of the p53 defective cells. Taken

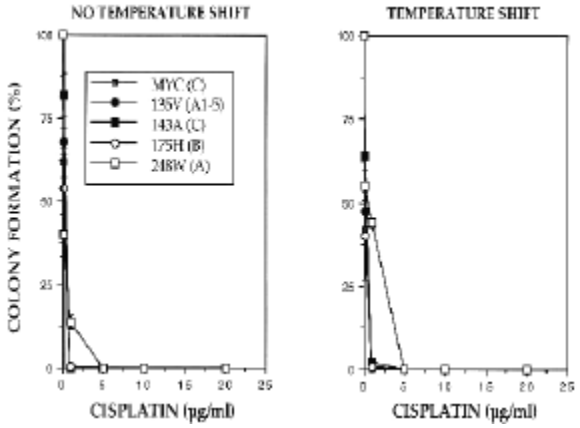

Figure 4. Induction of wt p53 activity fails to chemoprotect from an agent (cisplatin) that can damage cells in G_1 phase. The REF clones indicated were exposed to cisplatin at the doses shown at 37°C or 31°C, and processed in colony forming assays. Data points represent the mean (with SD) from triplicate plates from one of two experiments with similar outcomes.

together with the present study, we would argue that novel mechanisms for p53 activation could be expoited to promote the survival of some lineages exposed to many drugs or radiation. Our inability to protect cells from cisplatin with either p53 or TGFB3 (5) probably relates to the ability of this drug to kill effectively cells in G1 (16), where our pre-arrested cells were synchronized. In addition, our G1-arrested cells do not benefit from the contribution made by p53 to checkpoints in S or G2 phases (23).

A complication of engaging the p53 pathway to chemoprotect the appropriate cells is that p53 is physiologically activated by cellular insults (DNA damage, virus infection, hypoxia). Safer mechanisms of activation would be needed to exploit the p53 growth arrest. The specific DNA binding function of wt p53 is negatively regulated by sequences in its carboxy terminus, which can be truncated to constitutively activate DNA binding (24). Peptides based on c-terminal sequences have been shown to activate p53 for specific DNA binding *in vitro* and *in vivo* (25). Selivanova and coworkers (26) reported that a peptide corresponding to residues 361-382 could activate DNA binding and transcription activation of wt (and some mt) forms of p53. When the peptide was engineered to translocate cell membranes, p53 transactivation increased and the growth of tumor lines was inhibited in a p53-dependent fashion. Thus, an emerging question is whether small molecular weight drugs will be identified which restore normal functions to some p53 mutations, and whether the same agents (or the p53 peptides themselves) would protect normal dividing cells from DNA damaging agents.

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