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Restoration of Wild-Type p53 Activity in p53-null HL-60 Cells Confers Multidrug Sensitivity[1]

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ABSTRACT

HL-60 cells that stably express transfected wild-type (wt) p53 were used to determine whether restoration of wt p53 increased the chemosensitivity of cells that normally lack p53 activity. The wt p53 HL-60 transfectants (SN3 cells) were more sensitive than the parental (S) cells to a number of common anticancer drugs representing various mechanisms of action, whereas HL-60 cells transfected with *p53* genes mutated at codons 248 and 143 were not sensitized. The sensitization ratio due to the transfected wt p53 varied from about 2-fold for cisplatin to over 50-fold for thymidine. Cells treated with the thymidylate synthase inhibitor 5-fluoro-2′-deoxyuridine (FdUrd) were used to study changes in various p53-associated gene expressions. A higher percentage of apoptotic cells among the SN3 cells was observed than among the S cells at each concentration of FdUrd. The S cells had undetectable levels of bax and high levels of bcl-2, whereas the SN3 cells had undetectable levels of bcl-2 levels and appreciable basal levels of bax. After FdUrd treatment of SN3 cells, both p53 and bax levels increased, but the induction of bax was faster than that of p53 and paralleled the appearance of apoptotic DNA laddering. FdUrd treatment induced *p21* expression and increased the G_1 fraction of the SN3 cells but did not induce *p21* or change the phase distribution in the S cells. FdUrd treatment also induced the expression and phosphorylation of cyclin D1 in the SN3 cells but not in the S cells. These results show that transfected wt p53 confers multidrug sensitivity to HL-60 cells by re-adjustment of the expressions of apoptosis genes and displays other properties characteristic of endogenously originated wt p53.

INTRODUCTION

The tumor suppressor p53 is now widely recognized to be involved in a remarkable number of cellular processes, including gene transcription, DNA repair, cell cycling, genomic stability, senescence, and programmed cell death (apoptosis; reviewed in Ref. 1). The loss of function of p53, either through mutation, repression, or loss of expression, is one of the most common events in tumorigenesis. Many *in vitro* studies showing that restoration of p53 activity in cells with mutated p53 either suppresses growth of the cells or induces apoptosis (2–6) suggested the idea that gene therapy aimed at restoring p53 activity in tumors might be a fruitful approach to cancer treatment. In fact, a number of studies have now shown that adenovirus- or retrovirus-driven expression of wt[3] p53 can suppress tumor growth *in vivo* (7–12).

The discovery that malignant cells lacking p53 function appeared to be much more resistant to many cytotoxic agents and radiation than are cells with wt p53 generated additional interest in p53 as a possible determinant of response of tumors to anticancer therapy (13, 14). The current model suggests that when DNA damage caused by drugs or radiation is sensed by some as yet unknown mechanism, the intracellular level or the activity of p53 increases and alters the expressions of genes that are involved in cell growth arrest, programmed cell death, or DNA repair (15, 16). Neither growth arrest nor the p53-dependent apoptotic program is initiated by drug treatment of cells having mutated p53, thereby conferring a survival advantage to such cells in the presence of genotoxic agents. Thus, the functional status of p53 in a tumor may be a major prognostic indicator of the success or failure of cancer chemo- or radiotherapy (17).

Because of possible variation in the amount of vector uptake and resulting gene dosages among tumor cells, it is likely that some cells will not be killed by *p53* gene therapy alone. In that case, the above findings suggest that a multimodality approach combining gene therapy with conventional cytotoxic agents might be a more effective treatment than either one alone, provided that re-introduction of wt p53 at a sublethal level of expression can sensitize the tumor cells to anticancer drugs. There have been few studies on this question, probably because of the lack of experimental systems that express exogenously introduced wt p53 without going into apoptosis or severe growth arrest. Cells transduced with a wt p53 adenovirus expression vector and simultaneously treated with cisplatin underwent a great deal of apoptosis, suggesting that wt p53

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[3] The abbreviations used are: wt, wild type; FdUrd, 5-fluoro-2′-deoxyuridine; 5-FUra, 5-fluorouracil; TUNEL, terminal nucleotidyl transferase end-labeling; RT-PCR, reverse transcription PCR; dThd, thymidine; TS, thymidylate synthase; MMC, mitomycin C; VP-16, etoposide.

sensitizes cells to cisplatin, but the growth of these cells was markedly inhibited by the vector, even without the cisplatin treatment (18). A more recent study showed that a cell line containing the *p53* gene in an inducible expression vector was more sensitive to 5-FUra under *p53* induction conditions (19).

We recently developed HL-60 cells that stably expressed transfected wt p53 with only a small effect on growth rate and clonogenicity (20). These cells provide a potential model system with which to clearly discern the effects of re-introduced wt p53 on the sensitivity of cells to cytotoxic agents without being obscured by cell line differences or complicated by vector-driven overexpression of wt p53. In the present study, we determined the chemosensitivity of these cells relative to the parental p53-null HL-60 cells to various commonly used anticancer agents and characterized other molecular changes associated with re-introduction of wt p53.

MATERIALS AND METHODS

Chemicals. 5-FUra, FdUrd, cisplatin, dThd, MMC, and VP-16 were obtained form the Sigma Chemical Co. (St. Louis, MO). ZD1694 (Tomudex, Raltitrexed) was obtained from the Zeneca Pharmaceutical Co.

Cells. The development and isolation of the HL-60 cell lines stably expressing wt p53 and mutant p53 and their characteristics are described in detail elsewhere (20). Four cell lines were obtained from this study: parental HL-60 cells transfected with the empty vector pWLNeo, for use as controls, and with expression vectors pC53-SN3 (wt), pC53-SCX3 (mutated at codon 143), and pC53–248 (mutated at codon 248) containing the indicated wt and mutant *p53* cDNAs and the *neomycin phosphotransferase* gene under control of the cytomegalovirus promoter. These transfected cell lines were designated as S, SN3 (HL-60 cells transfected with wild-type p53), SCX3 (HL-60 cells transfected with p53 mutated at codon 143), and 248 (HL-60 cells transfected with p53 mutated at codon 248), respectively. Quantitation of immunohistochemical staining by image analysis showed that the SN3 cells express a low but detectable level of p53 protein, whereas the mutant p53 transfectants SCX3 and 248 have 4- and 10-fold, respectively, higher levels of p53 staining, as would be expected based on the higher stability of mutant forms of p53. The SN3 cells have a slightly longer doubling time than either the S cells or the mutant p53 transfectants (24 h *versus* 22 h) and a lower saturation density in suspension culture than either the parents or the mutant p53 cell lines. The SN3 cells are more sensitive to stress conditions, such as serum depletion, than are the S cells (20). The SN3 cells used in this study were one of three clones that were isolated after transfection of the parental HL-60 cells. As described previously (20), all three clones contained wt p53 and behaved identically with respect to growth rate and increased sensitivity to both serum deprivation and the cancer drug VP-16, supporting the idea that their altered properties were due specifically to expression of wt p53.

Cell Growth Conditions. The HL-60 cells were grown in RPMI 1640 containing 10% dialyzed fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37°C in 5% carbon dioxide in complete humidity.

Drug Cytotoxicity. Cells in medium at a density of 5×10^5 cells/ml were treated with the drug at specified times and concentrations. After treatment, cells were centrifuged at 1000 rpm for 10 min, washed twice with fresh medium without serum, and resuspended in fresh medium with serum to a density of 2×10^5/ml. After the cells were grown for 3 days, cell viability was assayed by the conversion of tetrazolium salts 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide or 2,3-bis(2-methoxy-4-nitro-5-sulfenyl)-5-[(phenylamino)carbonyl]-2*H* tetrazolium hydroxide to the blue formazan products (21) using a CellTiter 96 kit (Promega Corp., Madison, WI).

Flow Cytometry. About 10^6 cells were centrifuged at 1000 rpm for 10 min and washed with PBS. The cells were resuspended in 1 ml of a solution of 20 μg/ml RNase (DNase free) and 20 μg/ml propidium iodide in PBS. The mixture was incubated for 2 h at room temperature. The cells were analyzed on a Becton Dickinson FACScan flow cytometer.

Identification of Apoptotic Cells. Cells were fixed in 4% formaldehyde in PBS at a concentration of 2.5×10^5 cells/ml. Approximately 1.2×10^5 cells were spun onto microscope slides using a cytocentrifuge (Shandon, Pittsburgh, PA). Apoptotic cells were identified by the TUNEL assay (22) with the Apoptag *in situ* apoptosis kit (Oncor, Gaithersburg, MD). The slides were counterstained in 0.5% methyl green (Sigma Chemical Co., St. Louis, MO) to identify nonapoptotic cells. The percentage of apoptotic cells was determined by counting both apoptosis-positive and -negative cells in an image analyzer as described previously (20). A minimum of 200 cells was counted at each concentration of FdUrd.

Analysis of DNA for Apoptotic Fragments. Cells (5×10^6) were harvested by centrifugation and resuspended in 0.5 ml lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, 0.5 mg/ml proteinase K, and 0.15 mg/ml RNase). The lysis solution was extracted with 0.5 ml phenol:chloroform (24:1). After centrifugation, the top aqueous layer was removed, placed into a 1.5-ml tube, and 1.25 ml of 100% ethanol was added along with sodium chloride to a final concentration of 15 mM. DNA was allowed to precipitate at −70°C for 30 min and then centrifuged at 14,000 rpm for 30 min. The DNA pellet was resuspended in 100 μl of 10 mM Tris-EDTA solution. The DNA (25 μg) was loaded onto a 1.8% agarose gel, and electrophoresis was carried out in 1× TBE buffer for 7 h.

Western Blot Analysis. Cell lysis and Western blotting were carried out by standard methods essentially as described previously (23). At each time point, cells were harvested and lysed in 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.5%, 0.5 mg/ml proteinase K, and 0.15 mg/ml RNase. Protein concentration was determined by the bicinchoninic assay (Pierce, Rockford, IL), and 20 μg of total protein were loaded onto each lane of the gels. Antibody DO-7 (Dako) was used to detect p53. Anti-human monoclonal antibodies for Western blot analysis of cyclin D1 (#14561C), bcl-2 (#14831A), and bax (#65091A) were obtained from PharMingen (San Diego, CA).

Quantitative RT-PCR Analysis of *p21* Expression. Quantitation of *p21* gene expression was carried out by a relative RT-PCR method as described previously (24). In brief, RNA extracted from cells is converted to cDNA using random hexamers. Serial dilutions of the cDNA solution are PCR amplified with appropriate primers specific for the gene of interest (*p21* in this case) and an internal reference gene (β-*actin*). Relative gene expression values are obtained by taking the ratio

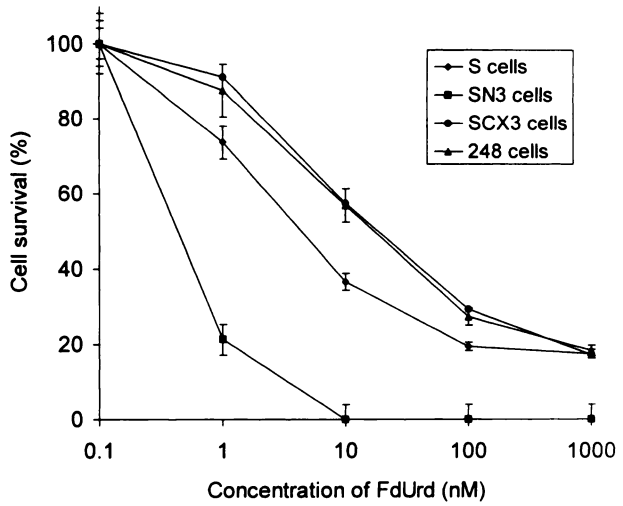

Fig. 1 Representative survival curves of HL-60 cells treated with FdUrd. Cells were incubated with FdUrd at the indicated concentrations for 5 days, and the cell viability was determined. LD_{50} is the point on the survival curves corresponding to 50% survival. Cell viability was determined by a colorimetric assay measuring the extent of transformation of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide to its formazan derivative, as described in "Materials and Methods." The points represent an average of two LD_{50} determinations. Bars, SE.

of the PCR products of the gene of interest and the reference gene within their respective linear ranges of amplification. PCR primers for amplification of *p21* were as follows: 5′-primer (94–114) 5′-GATGTCCGTCAGAACCCATGC-3′; and 3′-primer (496–516) 5′-AATACGACTCACTATAGGGACATGCTGGTCTGCCGCCGTTT-3′. Primers for β-*actin* were described previously (24). The PCR conditions were 1 min at 96°C, 1 min at 65°C, and 1 min at 72°C for 30 cycles.

Image Analysis. Determination and quantitation of band densities from Western blots and analysis of immunocytochemical staining intensity from the TUNEL assay were performed using the image analysis system described previously (20).

RESULTS

Effects of Cytotoxic Agents on Parental HL-60 Cells (S), wt p53 (SN3), and Mutant p53 Transfectants (SCX3 and 248). The cells were treated with a panel of drugs, and cell survival as a function of drug concentration was determined. The cytotoxicities of drugs are expressed as the concentration necessary to reduce cell viability by 50% (LD_{50}), as determined by the tetrazolium-based assay (22). Representative cytotoxicity curves obtained by plotting the percentage of surviving cells as a function of drug concentration are shown in Fig. 1 for FdUrd. The LD_{50}s are obtained from the curves by extrapolating the point of 50% viability to the concentration axis. Fig. 1 shows that the SN3 cells are about 10-fold more sensitive to FdUrd than either the S, SCX3, or 248 cells. The LD_{50}s of FdUrd and other drugs (cytotoxicity curves not shown) against each of the cell lines are presented in Table 1. The cytotoxic potency of most of the drugs was 8–12-fold greater against the SN3 cells than the cells lacking p53. However, there were two exceptions; the activity of cisplatin showed little more than a 2-fold increment against the SN3 cells compared with the S cells, whereas dThd had a remarkably larger differential cytotoxicity against the S and SN3 cells than did any of the other agents. This nucleoside had a strong cytotoxic effect against the SN3 cells (LD_{50}, 2 μM) but had no effect at all on the S cells at concentrations up to 100 μM, corresponding to a sensitivity ratio greater than 50.

Characterization of the Responses of SN3 and S Cells to a Cytotoxic Drug (FdUrd). To establish the role of wt p53 in the sensitization of the SN3 cells to drugs, we analyzed the cells for responses considered on the basis of previous work to be characteristic of wt p53 cells. FdUrd was used as the test drug in these experiments because it has a well-known mechanism of action involving inhibition of TS (25). Inhibition of TS produces a thymineless state, which results in single- and double-stranded breaks in DNA (26) that might be detected by a p53-dependent, DNA damage-sensing mechanism.

Apoptosis in FdUrd-treated SN3 and S Cells. Cells with wt p53 have been shown to have a greater tendency to go into apoptosis upon treatment with DNA-damaging agents than cells that lack p53 activity (13, 14, 27). To compare their apoptotic propensities, the S and SN3 cells were exposed to FdUrd and analyzed with the TUNEL *in situ* immunohistochemistry-based staining method. Both the S and SN3 cells became apoptotic upon FdUrd treatment, but at each concentration of FdUrd, the fraction of SN3 cells in apoptosis was markedly higher than that of the S cells. The extent of apoptosis in the cell lines as a function of FdUrd concentration is quantitatively represented in Fig. 2. The detection of apotosis by the *in situ* method was confirmed by observing DNA laddering effects at the higher doses of FdUrd. Fig. 3 shows FdUrd treatment of both the S and SN3 cells causing DNA laddering, which is distinct in the SN3 cells at 6 h of drug exposure but is detectable in the S cells only at 12 h.

p53 Levels in Cells Exposed to FdUrd. Many previous studies have shown that exposure of cells containing wt p53 to cytotoxic agents that damage DNA causes accumulation of p53 (1). To find out whether the transfected wt p53 showed this behavior in response to cytotoxic agents, p53 levels were assayed by Western blotting in SN3 cells at various times after FdUrd treatment. Fig. 4 shows that the untreated SN3 cells had a basal level expression of p53 that began to increase after exposure to FdUrd, reaching a maximum at about 36 h. The S cells did not have any band corresponding to p53 at any of the time periods.

bax and bcl2 Expression. bax and bcl2 modulate the apoptotic pathway, with bcl-2 being an inhibitor and bax a stimulator of apoptosis (28, 29). To determine whether the greater apoptotic propensity of the SN3 cells was associated with changes in bcl-2 or bax, the intracellular levels of these proteins were measured by Western blotting. Fig. 5*A* shows that the p53-null S cells expressed a detectable basal level of bcl2, whereas bcl2 was undetectable in the wtp53 SN3 cells. Treatment with FdUrd did not cause any change in bcl2 levels in the S cells. The inverse was observed with bax levels. As shown in Fig. 5*B*, bax was expressed in untreated SN3 cells but not in the S cells. bax increased further in SN3 cells upon exposure to FdUrd, with the maximal increase in bax at 6 h corresponding to the time of appearance of the DNA ladder in these cells (Fig. 3), followed by apparent decay of the bax back to the basal levels. A small induction of bax was observed in the S cells after 24 h of FdUrd exposure (Fig. 5*B*), reaching a maximum at 36 h to a level of about 25% of the basal level in the SN3 cells.

Table 1 Drug sensitivity of HL-60 cells as a function of their p53 status

Drug	LD_{50} values[a]				
	SN3 cells	S cells	248 cells	SCX3 cells	Sensitivity ratio (S:SN3)
FdUrd[b]	16 ± 3 nM	160 ± 30 nM			10
FdUrd[c]	0.7 ± 0.4 nM	8.0 ± 2.0 nM	12 ± 4 nM	12 ± 3 nM	11
ZD1694[c,d]	1.0 ± 0.2 nM	7.0 ± 1.6 nM	10.0 ± 1.9 nM	10.0 ± 1.4 nM	7
VP-16[b]	1.0 ± 0.2 μM	10.0 ± 1.3 μM	8.2 ± 1.6 μM	8.1 ± 0.2 μM	10
VP-16[c]	15 ± 3.5 nM	170 ± 4 nM	120 ± 4 nM	80 ± 3 nM	11
Cisplatin[e]	2.5 ± 1.1 μM	5.6 ± 1.4 μM			2.2
dThd[c]	2.0 ± 1.4 μM	>0.1 mM			>50
MMC[c]	4.0 ± 2.1 nM	50.0 ± 1.8 nM			12
5-FUra[c]	8.0 ± 5.0 μM	70 ± 14 μM			9

[a] Cell viability was determined by the tetrazolium-based assay using 2,3-bis(2-methoxy-4-nitro-5-sulfenyl)-5-[(phenylamino)carbonyl]-2*H* tetrazolium hydroxide as the hydrogen acceptor.
[b] Twenty-four-h exposure to the drug.
[c] Five-day exposure to the drug.
[d] ZD1694 (Tomudex, Raltitrexed) is a folate-analogue inhibitor of TS manufactured by the Zeneca Co.
[e] One-h exposure to the drug.

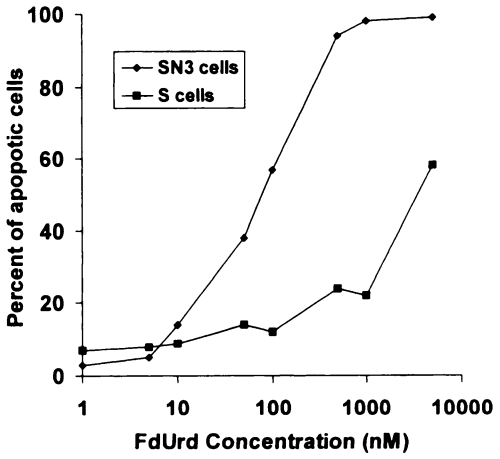

Fig. 2 Dose-response relationship for induction of apoptosis by FdUrd in SN3 and S cells. Cells were exposed to FdUrd for 24 h, cultured in drug-free medium for 96 h, and fixed and stained for apoptosis as described in "Materials and Methods." The percentage of apoptotic cells was determined by digital image analysis as described previously (20) after TUNEL staining of the cells.

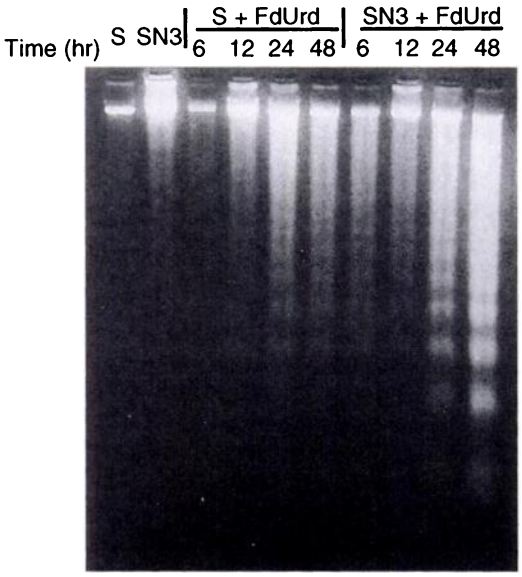

Fig. 3 Time courses of DNA fragmentation in the S and SN3 cells upon exposure to FdUrd. The cells were treated with 10^{-6} M FdUrd, and aliquots were removed at the times indicated for DNA fragment size analysis, as described in "Materials and Methods." The data are representative of two additional experiments.

Cell Cycle Distributions of Cells Before and After FdUrd Treatment. In its cell cycle checkpoint role, wt p53 promotes growth arrest in G_1 after cells have been challenged with drugs or radiation (1, 15, 30). To determine whether when transfected the wt p53 is capable of promoting G_1 arrest, cell cycle distributions were measured by fluorescence-activated cell sorter analysis after exposure of cultures of S and SN3 cells to FdUrd. As shown in Fig. 6*A*, the S-phase population in unsynchronized but logarithmically growing SN3 cells increased by about 10% over 16 h, whereas the G_1 population decreased. However, treatment with FdUrd reversed this trend and caused an increase in G_1 at the expense of the S phase. At the beginning of logarithmic growth, the G_1-S ratio in untreated cultures of SN3 cells was about 1.3 (52% G_1 and 40% S), declining to 0.7 after 16 h of growth. However, in the presence of FdUrd, the G_1-S ratio of the SN3 cells was about 2.5 after 16 h. By contrast, the initial G_1-S ratio of 0.9 in cultures of S cells did not change appreciably with time and was also unaffected by FdUrd treatment (Fig. 6*B*). A small G_2 population found in both S and SN3 cells (5 and 8%, respectively) was eliminated by FdUrd treatment within 8 h (data not shown).

Expression of WAF1/CIP1 (p21). The induction of the cyclin-dependent kinase inhibitor p21 by wt p53 is thought to be responsible for cell cycle arrest in G_1 after exposure to cytotoxic agents (1, 31–33). To test for induction of *p21* gene expression, relative *p21* mRNA levels were measured at various times following FdUrd treatment. The RT-PCR assay (Fig. 7) showed a 14-fold increase of *p21* mRNA in the SN3 cells. However, exposure to FdUrd did not cause any increase in *p21* expression in the S cells.

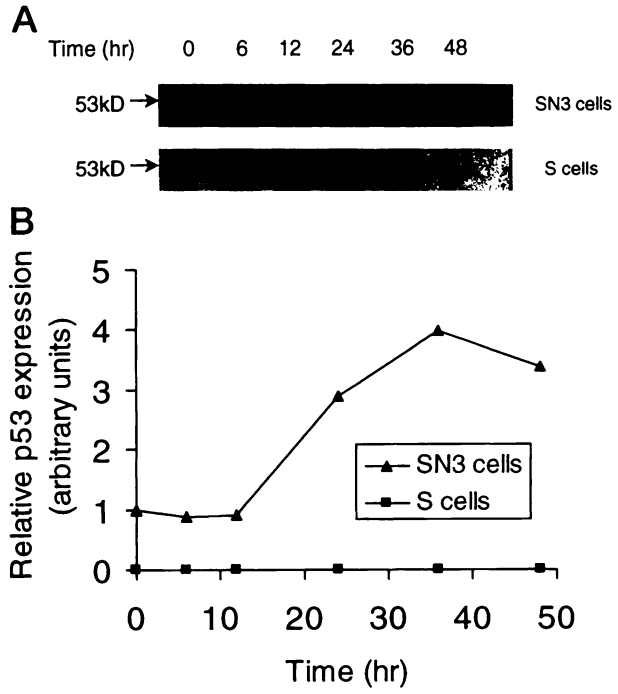

Fig. 4 The effect of FdUrd on the expression of p53 as a function of time. In *A*, S and SN3 cells were exposed to 10 nM FdUrd for 0–48 h. The p53 protein level in each aliquot of cells was analyzed by Western blotting using antibody DO-7 (Dako). The total protein in each cell isolate was determined as described in "Materials and Methods," and the same amount was loaded onto all of the lanes. *B*, band intensities were quantitated by densitometry scanning, and the data were plotted as ratios relative to the $t = 0$ value for SN3 cells.

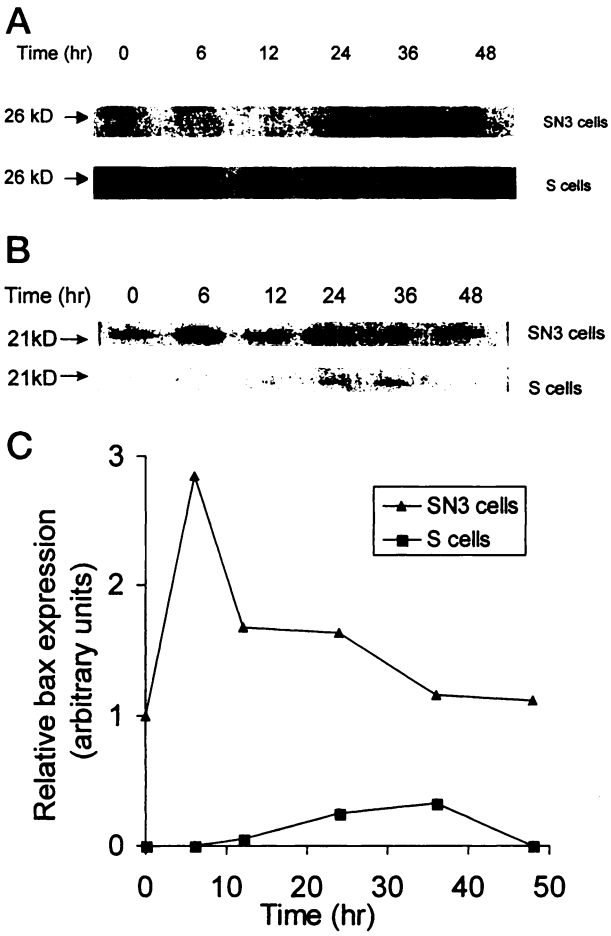

Fig. 5 bcl-2 and bax expression in S and SN3 cells and the effects of FdUrd treatment on bcl-2 and bax levels. In *A*, S and SN3 cells were exposed to 10 nM FdUrd for 0–48 h, and the bcl-2 protein level was assayed using purified mouse anti-human monoclonal antibody #14831A from PharMingen. In *B*, S and SN3 cells were treated with 10 nM FdUrd for 0–48 h, and the bax protein level was assayed using purified mouse anti-human bax monoclonal antibody clone G321-73 (#65091A) from PharMingen. Each lane of the gel contains the same amount of total protein. *C*, band intensities were quantitated by densitometry scanning, and the data were plotted as ratios relative to the $t = 0$ value for SN3 cells.

Cyclin D1 Expression. Endogenously originated wt p53 was found to increase the intracellular levels of cyclin D1 through prior induction of p21 (34). To determine whether the transfected p53 would affect the expression of cyclin D1, Western blots were performed using a cyclin D1-specific antibody. The SN3 cells had somewhat lower basal levels of cyclin D1 (Fig. 8). Treatment with FdUrd caused cyclin D1 content of SN3 cells to increase, whereas the levels in S cells were unchanged or underwent only a very small increase. Multiple cyclin D1 antibody-reactive bands appeared at about 12 h in the SN3 cells. The induction of cyclin D1 was maximum at 24 h of FdUrd exposure.

DISCUSSION

In a previous report, we described transfection of HL-60 cells with a plasmid bearing wt p53 and isolation of cells that stably expressed wt p53 (SN3 cells) at a level that caused only a small inhibition of cell growth and colony forming ability (20). The amount of wt p53 expression in the SN3 cells was apparently low enough not to cause appreciable amounts of spontaneous apoptosis but was sufficient to induce gene expressions associated with monocytic differentiation and to confer increased sensitivity to serum deprivation stress and to the anticancer drug VP-16 (20). In the present study, we used these cells as a model system for predicting how the chemosensitivity of tumors might be affected by re-introduction of wt p53. The parental HL-60 cells lack any p53 expression due to major deletions in the gene (35), thereby precluding any possible dominant-negative effects of mutated p53 proteins on the activity of the wt p53.

To test the previous suggestion that wt p53 cells might respond differently to agents that cause different types of DNA damage (27), the drugs to be compared against the p53-positive and -negative cell lines were selected to represent various mechanisms of action. These included TS inhibition (FdUrd, D1694, and 5-FUra), TS inhibition and incorporation into RNA (5-FUra), inhibition of pyrimidine biosynthesis (dThd), topoisomerase II inhibition (VP-16), DNA alkylation (MMC), and DNA platination (cisplatin). The salient observation of this study is that the SN3 cells were more sensitive to all of the agents in Table 1 than either p53-null S cells or mutant p53 transfectants, with sensitivity enhancement ratios ranging from

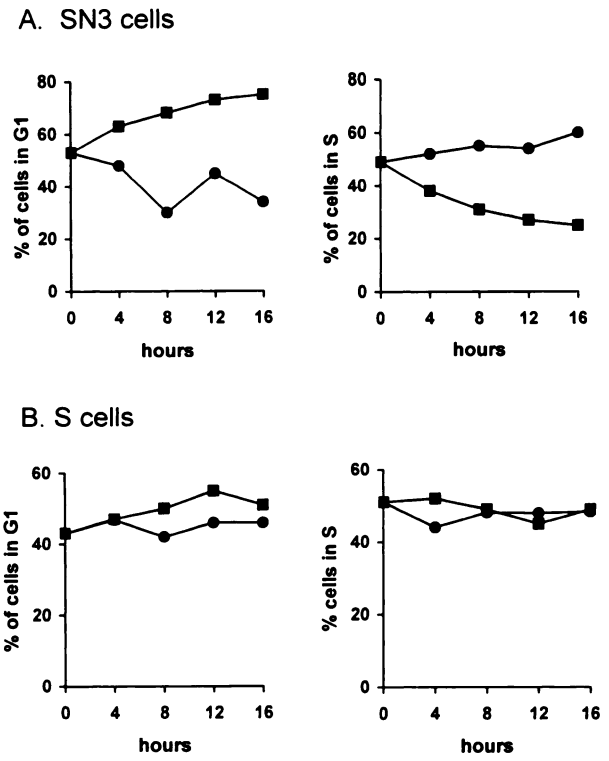

Fig. 6 Cell cycle distribution of SN3 and S cells as a function of time treated with FdUrd (■) and untreated (●). Logarithmically growing cells (10^5) were placed into fresh medium in the presence or absence of 10 nM FdUrd and analyzed by fluorescence-activated cell sorter at 0–16 h time points. The data are representative of two additional experiments.

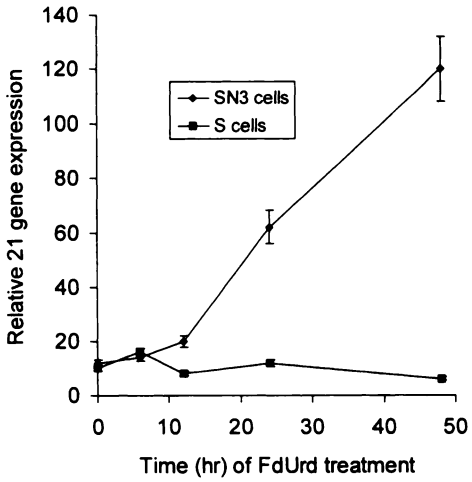

Fig. 7 The effect of FdUrd on *p21* expression as a function of time. S and SN3 cells were exposed to 10 nM FdUrd for 0–48 h. Levels of *p21* mRNA were quantitated by RT-PCR as described in "Materials and Methods." PCR reactions using primers specific for *p21* and β-*actin* were performed with serial dilutions of cDNA solutions prepared from RNA extracted from the cells at each time point. Slopes were calculated from plots of *p21* and β-*actin* PCR products *versus* the respective volumes of the cDNA solution used for each gene. Slope values were used only if the linearity coefficient of the data from at least three consecutive PCR reactions was greater than 0.9. The relative gene expression value is the ratio of the slopes of the *p21* and β-*actin* curves. Bars, SE.

2-fold to more than 50-fold. It is now generally accepted that wt p53 sensitizes cells to DNA-damaging agents by increasing their propensity for apoptosis (1, 36), and consistent with this hypothesis, the SN3 cells had a greater percentage of apoptotic cells than the S cells at all concentrations of FdUrd.

To begin characterizing the mechanism by which p53 lowered the apoptotic threshold of the HL-60 cells, we first examined the levels of bcl-2 and bax the in the S and SN3 cells. The ratio between the antiapoptotic molecule bcl-2 and the proapoptotic molecule bax has been proposed to be a critical determinant of apoptotic propensity in cells (29). Previous studies had indicated that the expressions of the *bcl-2* family genes are p53 regulated (37, 38), and indeed, we found that the expression of exogenously introduced wt p53 in the HL-60 cells, even at levels that were insufficient appreciably to inhibit growth rate, had caused a dramatic re-adjustment of the bcl-2/bax balance in the direction favoring apoptosis. The further increase in bax in the SN3 cells after 6 h of exposure to FdUrd, in synchrony with the appearance of apoptotic DNA fragmentation (Fig. 3), is consistent with previous reports showing p53-dependent apoptosis in response to genotoxic stress correlating with induction of bax (39). The question has been raised whether the increased induction of gene expressions by p53 in response to DNA damage is due to increases in p53 protein levels or whether the p53 protein becomes altered in some way that enhances its transcriptional activity (40). The observation that p53 levels did not begin to increase noticeably until considerably after the beginning of DNA laddering indicates that the rapid induction of bax expression and apoptosis in the SN3 cells after drug treatment does not require prior accumulation of p53 but only the presence of wt p53. Surprisingly, an induction of bax was also detectable in the S cells after about 12 h of FdUrd exposure. Whether this induction of bax is responsible for initiating apoptosis in the S cells is not clear, because the maximum amount of bax was lower than even the basal level of bax in the SN3 cells, whereas the considerable levels of bcl-2 in S cells might be expected to block the activity of any small amount of bax that is generated (41). Nevertheless, this observation shows that other mechanisms besides p53-stimulated transcriptional up-regulation exist for the induction of bax expression following DNA damage.

The activation of apoptosis by wt p53 in the SN3 cells appears to lower LD_{50}s of DNA-damaging agents by about one order of magnitude, based on the observation that the restoration of wt p53 sensitized HL-60 cells to most of the drugs (with the exception of cisplatin and dThd) to a similar extent (~10-fold), despite differences among these agents in either metabolic pathways, intracellular targets, or types of DNA damage. The somewhat lower sensitization of the cells toward cisplatin by wt p53 (~2–3-fold) may be due to the induction of additional genes that decrease the apparent DNA damage caused by this agent. Cisplatin has been shown to be an efficient inducer of the p53-regulated gene *GADD45* (42), which is thought to be associated with DNA repair (43). Thus, DNA damage may be more rapidly repaired in cisplatin-treated cells, resulting in lower cytotoxicity. Conversely, the remarkably large sensitivity enhancement of the SN3 cells to dThd suggests that additional mechanisms of p53-mediated cytotoxicity are triggered by this agent. dThd

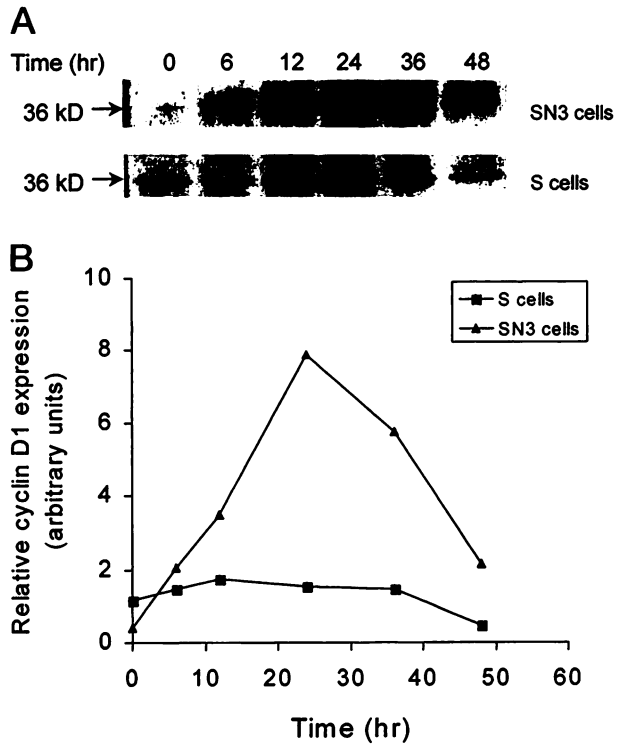

Fig. 8 Basal levels of cyclin D1 in S and SN3 cells and effects of FdUrd treatment. In *A*, the cells were exposed to 10 nM FdUrd for 0–48 h, and the cyclin D1 protein level was assayed using purified mouse anti-human monoclonal cyclin D1 antibody #14561C from PharMingen. The same amount of protein was loaded onto all of the lanes. *B*, band intensities were quantitated by densitometry scanning, and the data were plotted as ratios relative to the $t = 0$ value for S cells.

probably produces DNA damage similar to that of other DNA-directed antimetabolites because it inhibits DNA synthesis via the inhibition of several enzymes of pyrimidine biosynthesis (44), resulting in cell growth arrest and lethality to cells at high doses (45). However, nucleotide pool imbalances can also trigger p53-dependent apoptosis (46), and thus it could be speculated that the increased levels of dThd nucleotides and the deficiency of deoxycytidine nucleotides caused by excess dThd (47) result in an extra layer of p53-dependent apoptotic response in addition to that of the DNA damage response. Regardless of the actual mechanism, this observation suggests that dThd might be an effective agent specifically against tumors that harbor wt p53.

In addition to enhancing apoptosis, the other well-known function of p53 is to act as a cell cycle checkpoint gene and, upon sensing DNA damage, to induce cell cycle arrest by increasing the expression of the cyclin-dependent kinase inhibitor p21 (32, 33). Consistent with this function, the G_1-S ratio was substantially increased in cultures of SN3 cells after FdUrd treatment but was essentially unchanged in cultures of S cells. The increase of *p21* mRNA in the SN3 cells was roughly in synchrony with the increase of wt p53, suggesting that the induction of *p21* expression, in contrast to that of bax, is regulated by the amount of p53 protein in the cell. Interestingly, in spite of the presence of wt p53, basal level *p21* expression in the SN3 cells was not noticeably higher than that of the S cells, thus probably accounting for the fact that the growth rate of the SN3 cells is not appreciably inhibited. This finding suggests that the intracellular level of p53 needs to reach a certain threshold before *p21* expression is induced.

The discovery that wt p53 markedly increased the expression of cyclin D1 (34) was initially surprising because cyclin D1 was thought to promote phosphorylation of pRb, whereas growth inhibition in the presence of wt p53 was shown to be associated with hypophosphorylation of pRb (48). The explanation for this effect is not clear, but we also found in this study that transfected wt p53 induced a substantial increase in cyclin D1 levels in the SN3 cells after FdUrd treatment, approximately in concert with the induction of p21 expression. The appearance of slower-migrating cyclin D1 bands in the SN3 cells during the course of FdUrd treatment indicates the formation of phosphorylated products and thus suggests the induction of other protein kinase activities by wt p53. The function of cyclin D1 phosphorylation is not clear, but it has been proposed to regulate the association of the cyclin with other proteins (49).

In summary, the re-introduction of wt p53 into p53-deficient cells caused the cells to acquire "multidrug sensitivity" through a p53-induced re-adjustment of the levels of apoptosis enzymes. These results support the idea that gene therapy strategies aimed at introducing wt p53 into tumors in combination with conventional therapy with cytotoxic agents may be a more effective approach to cancer treatment than either one alone. Because the transfected wt p53 was shown to be capable of simulating functions ascribed to endogenously generated wt p53, the isogeneic cell system of S and SN3 cell system may be a unique model system: (*a*) for determining the effect of p53 status on the activity of anticancer agents; and (*b*) for studying p53-dependent and p53-independent pathways of apoptosis.

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