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p53 Inhibits Adriamycin-Induced Down-Regulation of Cyclin D1 Expression in Human Cancer Cells

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The tumor suppressor p53 gene product is an essential component of the cytotoxic pathway triggered by DNA-damaging stimuli such as chemotherapeutic agents and ionizing radiation. We previously demonstrated that adenovirus-mediated wild-type p53 gene transfer could enhance the cytotoxic actions of chemotherapeutic drugs both *in vitro* and *in vivo;* however, the molecular mechanism of this chemosensitization is still unclear. Cyclin D1 is a major regulator of the progression of cells into the proliferative stage of the cell cycle. Here we show that infection with an adenovirus vector expressing the wild-type p53 gene (Ad-p53) caused an increase in cyclin D1 protein levels in human colorectal cancer cell lines DLD-1 and SW620; treatment with the anti-cancer drug adriamycin, however, down-regulated their cyclin D1 protein expression in a dose-dependent manner. The suppression of cyclin D1 expression following adriamycin treatment could be blocked by simultaneous Ad-p53 infection. Furthermore, DLD-1 and SW620 cells transfected with the cyclin D1 expression construct displayed increased sensitivity to adriamycin compared to that of the vector-transfected control. Our results suggest that ectopic wild-type p53 gene transfer results in increased cyclin D1 expression and, consequently, sensitizes human colorectal cancer cells to chemotherapeutic agents.

Key Words: **p53; cyclin D1; adenovirus vector; adriamycin; colorectal cancer.**

Most solid tumors, including colorectal adenocarcinomas, are poorly sensitive to DNA-damaging chemotherapeutic agents. This drug resistance likely explains the significant proportion of treatment failure in patients with advanced colorectal neoplasms. Recent advances in our understanding of the cell death pathway induced by chemotherapeutic agents have revealed the importance of p53-mediated apoptosis in the therapeutic response of human tumors (1, 2). The tumor suppressor p53 is a nuclear transcriptional factor that is activated by DNA damage, and mutation of p53, which is the most common genetic change in human cancers, leads to disruption of its pro-apoptotic function (3). Therefore, it is possible that p53 could influence the cellular outcome of DNA-damaging chemotherapy. Indeed, we previously demonstrated that restoration of normal p53 function by ectopically transducing the wild-type p53 (wt-p53) gene could induce chemosensitivity in human non-small cell lung cancer and colorectal cancer cell lines *in vitro* and *in vivo* (4, 5); the precise molecular mechanism, however, remains to be elucidated.

Cyclin D1 is a G1 cyclin that controls the transition of the cell cycle from G1 to S phase. Cyclin D1 in association with its catalytic subunit cyclin-dependent kinase (cdk) 4 or cdk6, controls the phosphorylation and inactivation of the retinoblastoma gene product (pRb), which leads to cell cycle progression and DNA synthesis (6). Cyclin D1 is transcriptionally induced by various oncogenic signals such as Ras family members and the β-catenin/APC pathway (7–10). Overexpression of cyclin D1 is often associated with poor survival of patients with cancers of the breast (11), esophagus (12), colon (13) and pancreas (14). In addition, expression of antisense cyclin D1 cDNA inhibits the growth of human cancer cell lines *in vitro* and *in vivo* (15, 16), suggesting a critical role for cyclin D1 in tumorigenesis. However, conversely, cyclin D1 overexpression has been reported to induce apoptosis of certain types of cells such as neural and mammary epithelial cells (17, 18). Cyclin D1-overexpressing human breast cancer cells were also demonstrated to be more sensitive to ionizing radiation than parental cells (19). These observations suggest that the effects of increased cyclin D1 expression in particular cell types are highly context dependent.

In the present study, we demonstrated that adenovirus-mediated wt-p53 gene transfer into mutant p53-

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expressing human colon cancer cells resulted in cyclin D1 overexpression, whereas treatment with a DNA-damaging chemotherapeutic drug, adriamycin, decreased cyclin D1 protein levels in a dose-dependent manner. Furthermore, infection with a p53-expressing adenovirus could prevent adriamycin-induced down-regulation of cyclin D1 expression. Our data provide a novel and significant insight into the mechanism of p53-mediated chemosensitization in human colorectal cancer cells.

MATERIALS AND METHODS

Cells and culture conditions. The human colon cancer cell lines DLD-1, which is homozygous for a mutation in p53 at codon 241 (ser to phe), and SW620, which carries a homozygously-mutated p53 gene at codons 273 and 309 (arg to his and pro to ser, respectively), were routinely propagated in monolayer cultures in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 25 mM HEPES, 100 units/ml penicillin and 100 mg/ml streptomycin. The transformed embryonic kidney cell line 293 was cultured in DMEM (GIBCO, Grand Island, NY) with high glucose (4.5 g/l), supplemented with 10% FCS and penicillin/streptomycin.

Reagents. Adriamycin (Sigma Chemical Co., St. Louis, MO) was prepared as a 0.05 mg/ml stock solution in phosphate-buffered saline (PBS) and stored at −20°C. PBS alone was added to control cultures.

Adenoviruses. The recombinant, replication-deficient adenovirus vector expressing human p53 cDNA was constructed and characterized previously (4, 5, 20). The resultant virus was termed Ad-p53. An E1A-deleted adenovirus vector lacking a cDNA insert (dl312) was used as a control vector. These viruses were obtained by lysing infected 293 cells. Titers of the viral stocks were determined with a plaque-forming assay using 293 cells.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from mock-, dl312- and Ad-p53-infected DLD-1 SW620 cells using RNAzol (Cinna/BioTecx, Friendswood, TX) in a single-step phenol-extraction method, and used as templates. Reverse transcription was performed at 22°C for 10 min and then at 42°C for 20 min using 2.0 μg/ml of RNA per reaction to ensure that the amount of amplified DNA was proportional to the specific mRNA in the original sample. PCR was performed with specific primers in volumes of 50 μl according to the protocol provided by the supplier (PCR kit; Perkin-Elmer Cetus, Norwalk, CT). The primers used for cyclin D1 and GAPDH were as follows: cyclin D1 sense, 5′-GAAGCT-GTGCATCTACACCG-3′; cyclin D1 antisense, 5′-GGCCTTGGG-GTCCATGTTCT-3′; GAPDH sense, 5′-CCATCTTCCAGGAGCG-AGA-3′, and GAPDH antisense, 5′-AGTGATGGCATGGACTGTGG-3′. The amplification reaction involved denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and elongation at 72°C for 2 min using a thermal cycler (Perkin-Elmer, Foster City, CA) for 30 cycles. The density of PCR products visualized by ethidium bromide staining was quantified using an image analyzing program, NIH Image, with GAPDH as the internal control. Quantitative assessment was performed by comparing the amplified signal intensity in serially diluted RT products.

Western blot analysis. Whole cell extracts were prepared and protein concentrations were determined using the Bio-Rad protein determination method (Bio-Rad, Hercules, CA). Equal amounts of whole cell extracts (60 mg) were boiled for 5 min and electrophoresed under reducing conditions on a 12.5% (w/v) polyacrylamide gel. Proteins were electrophoretically transferred to hybond-polyvinylidene difluoride (PVDF) transfer membranes and the expression levels were measured using the following antibodies: primary rabbit antibody against cyclin D1 (Santa Cruz Biotech, Santa Cruz, CA), mouse monoclonal antibodies against human p53 (Oncogene Science, Manhasset, NY) and human actin (Amersham, Arlington Heights, IL). An Amersham ECL chemiluminescent Western system (Amersham Japan) was used to detect secondary probes. Quantitative data were obtained by an image analyzer and expressed as percentages of the density of untreated cells.

Plasmid construction and gene transfer. To construct an expression plasmid for cyclin D1, the human cyclin D1 cDNA was amplified by PCR (21) and inserted into the pcDNA3.1(+) plasmid (Invitrogen Corporation, Carlsbad, CA). The resultant plasmid was named pcDNA3.1(+)/cyclin D1. DLD-1 and SW620 cells were plated at a density of 2×10^5 cells/well in a 6-well plate and incubated until the cells were 70% confluent. A mixture of 5 μg of pcDNA3.1(+)/cyclin D1 or control pcDNA3.1(+) without cyclin D1, and 25 μl of LipofectACE Reagent (Life Technologies, Inc., Grand Island, NY) in 200 μl of serum-free medium was then added to the culture according to the protocol provided by the manufacturer. The transfected cells were assessed for expression of sufficient levels of cyclin D1 by Western blot analysis using anti-cyclin D1 antibody.

RESULTS AND DISCUSSION

A replication-deficient adenovirus vector carrying a wt-p53 gene under the control of the cytomegalovirus (CMV) promoter (Ad-p53) was generated to achieve efficient gene transfer into human cancer cells (20). Monolayer cultures of mutant p53-expressing DLD-1 and SW620 human colorectal cancer cells were infected with either control dl312 adenovirus or Ad-p53 at various multiplicities of infection (MOI). Western blot analysis performed 24 h after infection showed increased expression of exogenous p53 and endogenous cyclin D1, in a dose-dependent manner (Fig. 1A). Densitometric analysis indicated that cyclin D1 protein levels were increased 4.5- and 2.3-fold in DLD-1 and SW620 cells, respectively, when infected with Ad-p53 at an MOI of 30. Infection with control dl312 vector had no effect on cyclin D1 expression in either cell line. Moreover, time-course experiments demonstrated that cyclin D1 protein levels were up-regulated within 8 h after Ad-p53 infection and remained elevated for up to 48 h (data not shown). We previously examined the effects of Ad-p53 infection on the cell cycle by flow cytometry analysis (22). DLD-1 and SW620 cells were infected with Ad-p53, harvested at 24 and 36 h post-infection, and then assayed for DNA content by propidium iodine staining. The Ad-p53-infected cells showed a decreased fraction of S-phase cells resulting from an accumulation of most cells in the G_0/G_1 phase 24 h after infection. In addition, cells exhibited a DNA content less than the diploid G_0/G_1 peak, indicating apoptosis-specific nuclear fragmentation 36 h after infection.

To examine whether induction of cyclin D1 protein levels by wt-p53 gene transfer was due to a concomitant increase in cyclin D1 gene expression, we next analyzed cyclin D1 mRNA levels by semi-quantitative RT-PCR analysis. As shown in Fig. 1B, both cell lines expressed detectable levels of cyclin D1 mRNA; how-

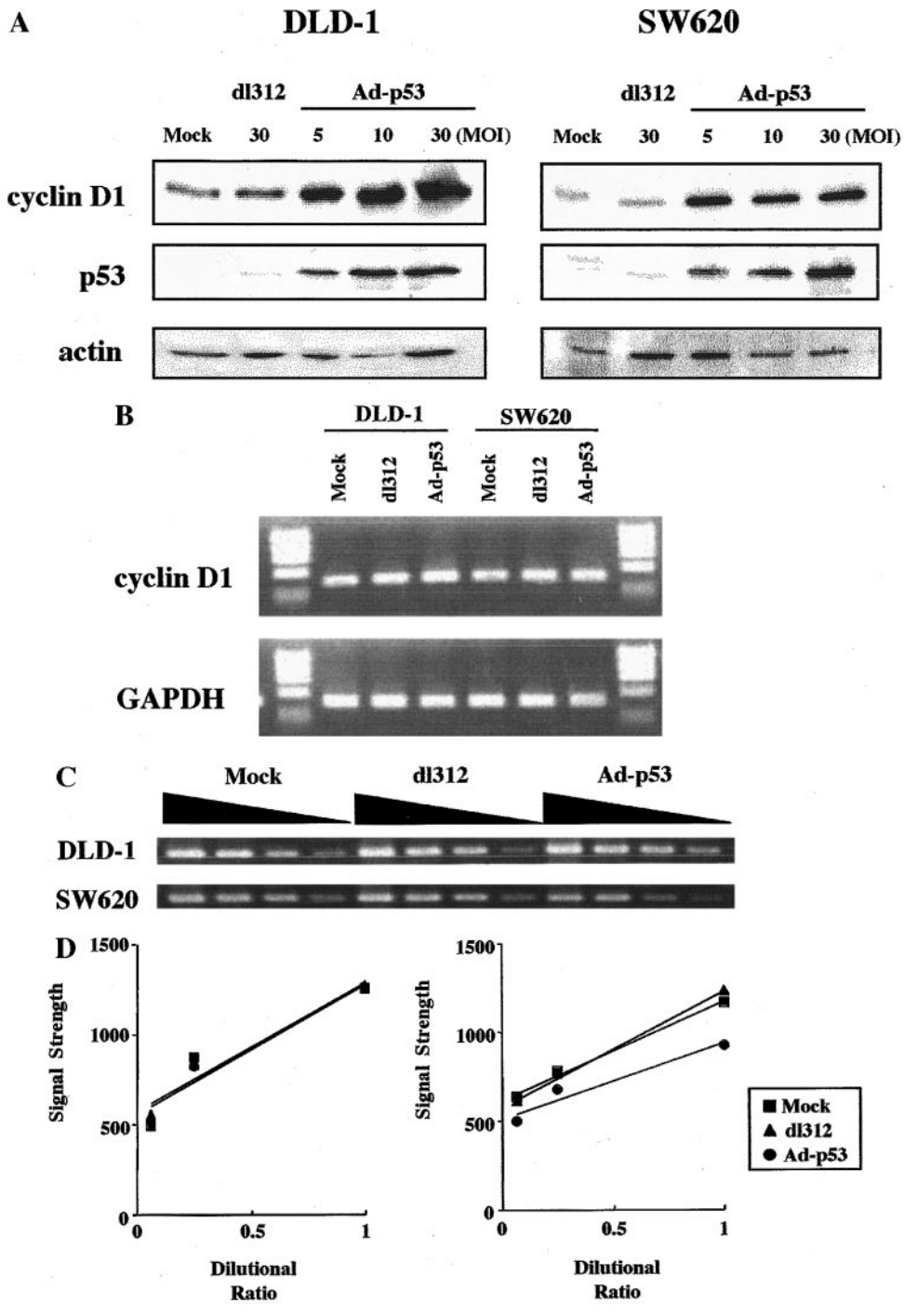

FIG. 1. (A) Cyclin D1 and p53 protein levels were determined by Western blot analysis of proteins from Ad-p53-infected DLD-1 and SW620 human colorectal cancer cells. Cells were infected with dl312 (30 MOI) or Ad-p53 (5, 10, or 30 MOI). Equivalent amounts of extracts obtained from whole cell lysates 24 h after infection were loaded, probed with anti-cyclin D1 or anti-p53 antibody, and then visualized with an ECL detection system. Equal loading of samples was confirmed by stripping each blot and reprobing with anti-actin antiserum. (B) Semi-quantitative RT-PCR analysis of cyclin D1 mRNA expression in DLD-1 and SW620 human cancer cells after Ad-p53 infection. Cells were exposed to either dl312 (30 MOI) or Ad-p53 (30 MOI) for 24 h and harvested. RT-PCR was performed with primers specific for the human cyclin D1 sequence, or primers that recognized GAPDH sequences as an internal control. PCR amplification was continued for 30 cycles. (C) Quantitative assessment of cyclin D1 mRNA expression. Serially diluted RT products (1, 4, 16, 64-fold dilution) were analyzed by PCR. (D) Relationship between the signal strength of the amplified products and the dilutional ratio was illustrated.

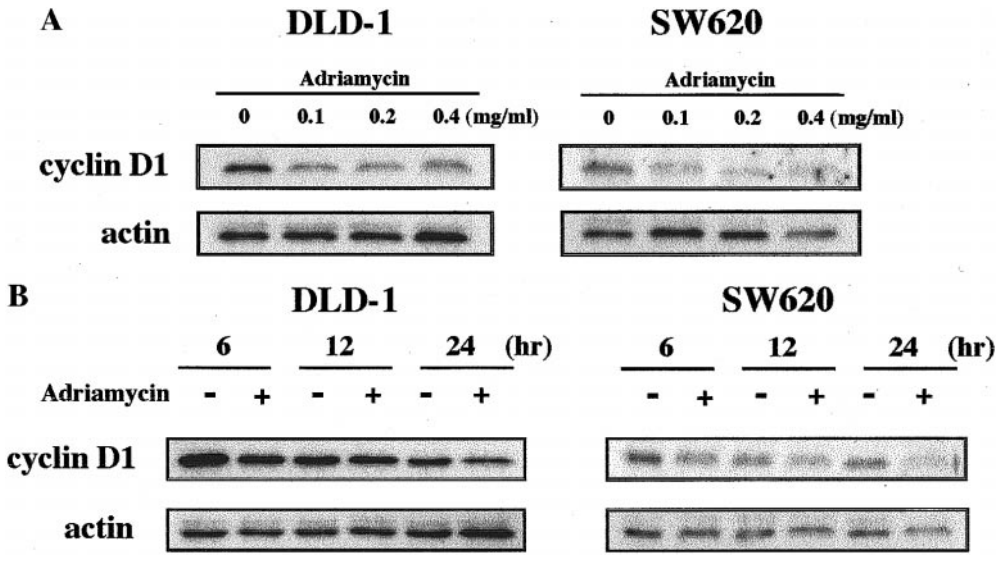

FIG. 2. (A) Effect of adriamycin on cyclin D1 protein expression levels. DLD-1 and SW620 cells were treated with adriamycin at the indicated concentrations. After 24 h of treatment, cell lysates were analyzed for cyclin D1 protein levels by Western blot analysis. Equal loading of samples was confirmed by stripping each blot and reprobing with anti-actin antiserum. (B) Time-course experiments of cyclin D1 protein expression after adriamycin treatment. DLD-1 and SW620 cells were treated with 0.4 mg/ml of adriamycin and harvested at the indicated time points. Cyclin D1 and actin protein levels were determined by Western blot analysis.

ever, there was no significant change in the levels of cyclin D1 mRNA in Ad-p53-infected DLD-1 and SW620 cells, suggesting that p53-mediated regulation of cyclin D1 expression is unlikely to occur at the transcriptional level. To generate linear dilution curves for quantitation of cyclin D1 mRNA expression, RT products were sirially diluted and then analyzed by PCR (Fig. 1C). As shown in Fig. 1D, the relation between the dilution and the signal strength of the amplified product was reproducibly almost linear for both cell lines.

Adriamycin is a chemotherapeutic agent that is commonly used for various types of cancer. It has been previously shown that adriamycin treatment induces apoptotic cell death of tumor cells in a p53-dependent manner (23). We next determined whether treatment with adriamycin could alter the expression of cyclin D1 in DLD-1 and SW620 cells. Western blotting demonstrated that adriamycin markedly decreased cyclin D1 protein levels in a dose-dependent fashion 24 h after drug treatment (Fig. 2A). In addition, as shown in Fig. 2B, cyclin D1 levels were down-regulated as early as 6 h after adriamycin treatment and remained decreased for at least 24 h, whereas the levels of cyclin D1 protein remained constant in untreated cells.

Several studies have demonstrated that overexpression of cyclin D1 can enhance apoptotic cell death in particular types of cells after exposure to DNA-damaging stimuli (17–19). These observations suggest that the decrease in cyclin D1 protein following adriamycin treatment is likely to be the mechanism of cellular resistance to adriamycin in human colorectal cancer cells. If this is indeed the case, cyclin D1 overexpression would be expected to increase adriamycin sensitivity in these cell lines. To test this possibility, we generated stable transfectants of DLD-1 and SW620 cells overexpressing human cyclin D1. As shown in Fig. 3A, Western blot analysis clearly demonstrated increased cyclin D1 protein expression in cells transfected with the pcDNA3.1(+)/D1 plasmid (DLD-1/D1 and SW620/D1) compared to that in cells harboring the control plasmid pcDNA3.1(+) (DLD-1/Neo and SW620/Neo).

To assess the impact of gain of cyclin D1 on the chemosensitivity of DLD-1 and SW620 cell lines, we incubated cultures of transfectants with increasing concentrations of adriamycin, and cell viability was assessed by measuring trypan blue uptake 24 h after treatment. The addition of adriamycin to cells resulted in a significantly higher chemosensitivity of DLD-1/D1 and SW620/D1 cells compared to that of DLD-1/Neo and SW620/Neo cells ($P < 0.05$) (Fig. 3B). These results suggest that overexpression of cyclin D1 can, at least partially, sensitize DLD-1 and SW620 cells to adriamycin treatment.

Finally, we determined whether overexpression of the wt-p53 gene could overcome adriamycin-induced down-regulation of cyclin D1 expression. For this purpose, DLD-1 and SW620 cells were infected with Ad-p53 for 1 h at MOIs of 10 and 50, respectively, and then exposed to adriamycin at 0.4 mg/ml for 24 h. Western blot analysis showed that Ad-p53 infection apparently blocked adriamycin-induced down-regulation of cyclin D1 expression, resulting in significantly increased levels of cyclin D1 protein (Fig. 4).

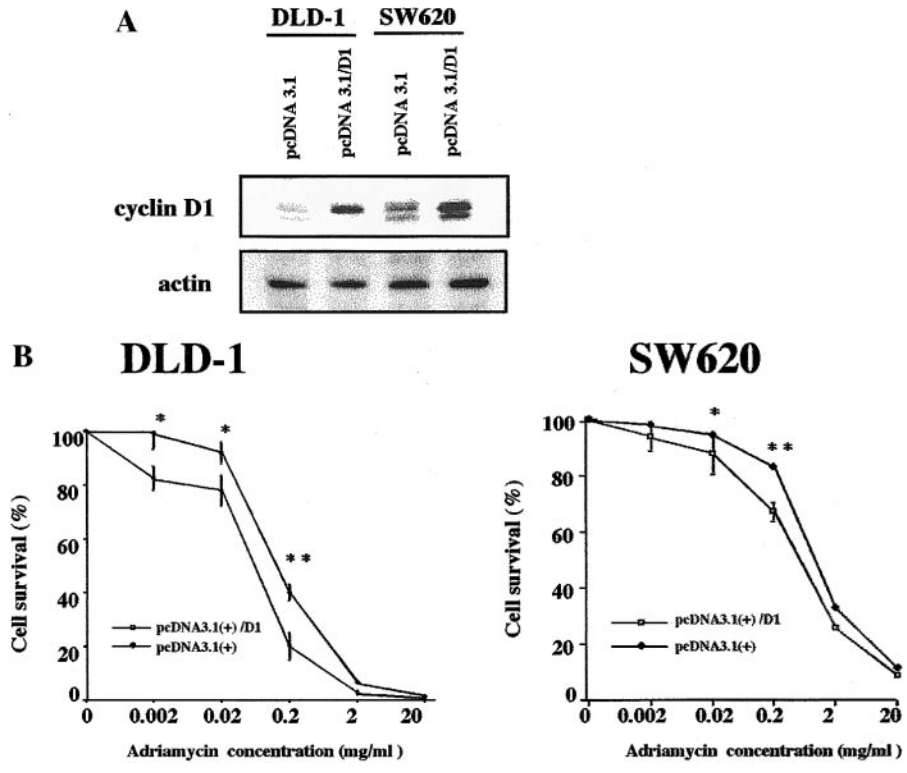

FIG. 3. (A) Western blot analysis of ectopically-transduced cyclin D1 in DLD-1 and SW620 cells stably transfected with the pcDNA3.1(+)/D1 plasmid. Cells were transfected with either the control pcDNA3.1(+) plasmid or the pcDNA3.1(+)/D1 plasmid, and selected in 500 mg/ml of geneticin for at least 30 days. Extracts were prepared and analyzed by Western blotting with anti-human cyclin D1 antibody as probes. Control vector-transfected cells exhibited baseline cyclin D1 expression, while intense cyclin D1 protein expression was detected in cells expressing exogenous cyclin D1. (B) Effect of ectopically-transduced cyclin D1 on adriamycin-induced cytotoxicity. Cells transfected either with pcDNA3.1(+) or pcDNA3.1(+)/D1 were exposed to adriamycin at the indicated concentrations. Cell viability was assessed by measuring trypan blue uptake 24 h after treatment. The results were expressed as a percentage of the mock-treated control. Statistical analysis was performed with Student's *t*-test. Data represent the mean ± SD of triplicate determinations. $*P < 0.05$, $**P < 0.01$.

In the present study, we demonstrated that treatment with adriamycin reduced endogenous cyclin D1 protein expression levels in mutant p53-expressing human colorectal cancer cells, and that this could be inhibited by adenovirus-mediated wt-p53 gene transfer in a transcription-independent manner. Progression of

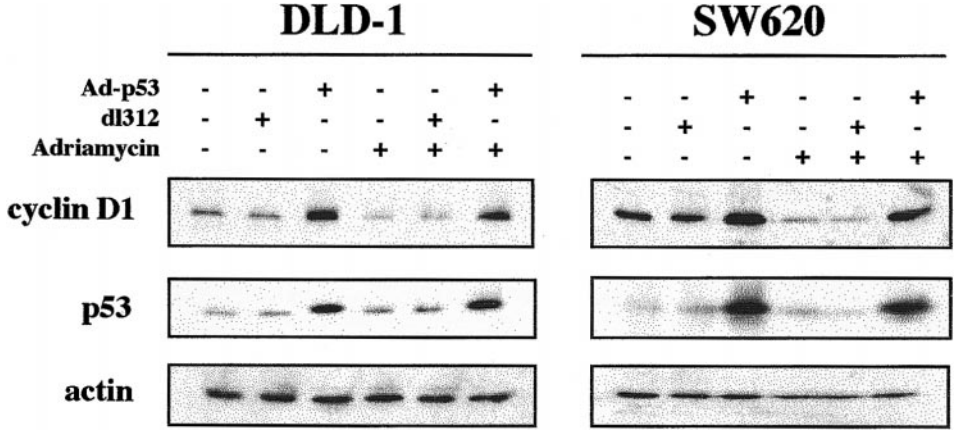

FIG. 4. Effect of Ad-p53 infection on adriamycin-induced down-regulation of cyclin D1 protein expression. DLD-1 and SW620 cells were infected with either dl312 or Ad-p53, at 10 or 50 MOI, respectively, for 1 h and then subjected to treatment with 0.4 mg/ml of adriamycin for an additional 24 h. Equivalent amounts of extracts obtained from whole cell lysates were loaded, probed with anti-cyclin D1 or anti-p53 antibody, and then visualized with an ECL detection system. Equal loading of samples was confirmed by stripping each blot and reprobing with anti-actin antiserum.

the cell cycle from G_1 to S phase is facilitated by cyclin D1 in association with either cdk4 or cdk6, whereas the tumor suppressor p53 plays a central role in the G_1-S and G_2-M checkpoints regulating cell cycle transition. Therefore, it can be speculated that overexpression of wt-p53 inhibits G_1 to S transition in p53-deficient cancer cells, which in turn creates a positive feedback loop that accelerates cell cycle progression by inducing cyclin D1 expression. However, up-regulation of cyclin D1 expression can be achieved by infection with as little as 5 MOI of Ad-p53 (Fig. 1A), an amount that could not arrest the cell cycle as shown by flow cytometric analysis (data not shown). In addition, increased cyclin D1 expression was rapidly induced in parallel with p53 up-regulation, although it is known that several hours are needed for transcription-dependent $p21^{WAF1/Cip1}$ induction. Thus, a positive feedback mechanism is unlikely to contribute to the wt-p53-mediated cyclin D1 accumulation. It has been previously reported that pRb stimulates cyclin D1 promoter activity through other transcription factors, such as Sp1 (24); our data, however, indicated that p53 stabilizes cyclin D1 protein in the absence of any transcriptional changes (Fig. 1B).

The abundance of cyclin D, cyclin E, $p21^{WAF1/Cip1}$ and $p27^{KIP1}$ proteins is regulated by the ubiquitin-proteasome pathway. In fact, genotoxic stresses such as ionizing radiation have been demonstrated to trigger rapid proteolysis of cyclin D1, leading to p53-independent G_1 arrest (25). This is in accordance with our finding that treatment of p53-deficient cells with adriamycin caused a significant decrease in cyclin D1 protein stability (Fig. 2). Han *et al.* (18) have reported that when cyclin D1 was overexpressed in normal human mammary epithelial cells, it resulted in growth inhibition as well as enhanced apoptosis associated with increased expression of $p27^{KIP1}$. Moreover, Coco Martin *et al.* (19) demonstrated that the response to a DNA-damaging agent was dependent upon initial levels of cyclin D1. Our transfection experiments indicated that cyclin-D1-expressing tumor cells were more sensitive than control cells to adriamycin (Fig. 3). These findings suggest that increased cyclin D1 expression might be beneficial for chemotherapy.

Interestingly, we noted that introduction of the wt-p53 gene into mutant p53-expressing human cancer cells prevented disruption of cyclin D1 protein by adriamycin and, furthermore, resulted in significantly increased cyclin D1 expression (Fig. 4). Levels of cyclin D1 induced by Ad-p53 infection might not be physiological, as adenovirus-mediated gene transfer is highly efficient; this finding, however, may be of potential importance, and it also suggests that cyclin D1 overexpression may be one mechanism for p53-mediated sensitization of human cancer cells to chemotherapeutic drugs. Gene therapy with p53 induces apoptosis in a wide variety of tumor models as well as human cancers in clinical trials (20, 26, 27). There remains, however, a group of tumor cells that are extremely resistant to p53-mediated apoptosis, highlighting the need for significant therapeutic enhancement. Thus, p53 gene therapy in combination with conventional chemotherapy and/or radiotherapy may be a potentially useful approach for therapies directed against refractory human cancers.

In summary, we have shown for the first time that p53-mediated specific inhibition of chemotherapeutic drug-induced cyclin D1 down-regulation could render chemotherapy more effective in human cancer cells expressing mutated p53. Studies are in progress to identify the precise mechanisms that contribute to p53-induced cyclin D1 up-regulation.

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