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p53 expression overcomes *p21$^{WAF1/CIP1}$*-mediated G_1 arrest and induces apoptosis in human cancer cells

Shunsuke Kagawa[1], Toshiyoshi Fujiwara[1], Akio Hizuta[1], Tatsuji Yasuda[2], Wei-Wei Zhang[3], Jack A Roth[3] and Noriaki Tanaka[1]

[1]*First Department of Surgery,* [2]*Department of Cell Chemistry, Okayama University Medical School, Okayama 700, Japan;* [3]*Department of Thoracic and Cardiovascular Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA*

The *p21$^{WAF1/CIP1}$* gene, which encodes a cyclin-dependent kinase inhibitor, may be critical for tumor suppressor gene *p53*-induced cell cycle arrest. The *p53* gene is known to regulate G_1 checkpoint, which can either induce G_1 arrest or initiate apoptosis. To directly examine the role of *p21$^{WAF1/CIP1}$* in the control of *p53* function, we have introduced human *p21$^{WAF1/CIP1}$* gene into a *p53*-deficient human non-small cell lung cancer cell line H1299 using a *p21$^{WAF1/CIP1}$*-expressing adenoviral vector (AdCMVp21). Infection with AdCMVp21 resulted in high levels of *p21$^{WAF1/CIP1}$* expression and significantly suppressed the growth of H1299 cells through the G_1 arrest of the cell cycle. In contrast, transient expression of the wild-type *p53* gene by a recombinant adenoviral vector (AdCMVp53) in H1299 cells induced apoptotic cell death and resulted in a rapid loss of cell viability. We then examined the effects of combined infection with AdCMVp21 and AdCMVp53 on H1299 cells to explore the dominant function of these molecules. Interestingly, introduction of exogenous *p53* overcame *p21$^{WAF1/CIP1}$*-mediated cell cycle arrest at G_1 and induced apoptosis, although viral-transduced *p21$^{WAF1/CIP1}$* expression level was unaffected. These observations suggest that *p53* expression converts a *p21$^{WAF1/CIP1}$*-mediated growth arrest into apoptosis. The result was repeated with two additional human colon adenocarcinoma cell lines with the different *p53* status, mutant *p53*-expressing DLD-1 and wild-type *p53*-expressing LoVo, suggesting that this phemonenon is a general event among human cancer cells. Thus, *p53*-mediated apoptotic pathway is dominant over the growth arrest pathway, indicating that *p53* may be an essential upstream mediator of *p21$^{WAF1/CIP1}$* in the regulation of a cell process leading either to growth arrest or to apoptotic suicide.

Keywords: *p53*; *p21$^{WAF1/CIP1}$*; apoptosis; G_1 arrest; adenoviral vector

Introduction

Apoptosis is defined by morphologic changes, such as condensation and margination of chromatin concomitant with cell shrinkage, that are clearly distinct from necrosis; these changes are followed by a characteristic intranucleosomal DNA fragmentation resulting from cleavage of nuclear DNA. Although apoptosis is a part of normal development involving nonpathologic cell death, it also is involved in the cytotoxic mechanism of anticancer treatments. Recent studies have demonstrated that at least two pathways regulate apoptosis: one dependent on the tumor suppressor gene *p53* and the other *p53*-independent (Clarke *et al*., 1993). Inactivation of *p53* by point mutation or deletion is among the commonest genetic abnormalities in human cancer. Overexpression of wild-type *p53* induces apoptotic cell death of tumor cells devoid of wild-type *p53* or expressing mutant *p53*, although the response depends on cell type and experimental conditions (Fujiwara *et al*., 1993). To develop specific strategies for anticancer therapy, it may be important to explore the molecular basis of *p53*-mediated apoptosis.

Cloning and characterization of the *p21$^{WAF1/CIP1}$* gene, which is induced by p53, have revealed that its protein product p21 potently regulates the cell cycle by inhibiting cyclin-dependent kinases (Cdk) required for progression from the G_1 to the S phase (Harper *et al*., 1993; El-Deiry *et al*., 1993), and also inhibits the ability of proliferating-cell nuclear antigen to activate DNA polymerase δ, resulting in inhibition of DNA replication (Waga *et al*., 1994). In addition, it has been shown that *p21$^{WAF1/CIP1}$* is induced following upregulation of *p53* expression in the process of apoptosis triggered by DNA damage (El-Deiry *et al*., 1994). Recent studies using *p21*-deficient fibroblasts obtained from mice lacking *p21$^{WAF1/CIP1}$* as well as the *p21*-deficient human colon cancer cells created by a homologous deletion of *p21$^{WAF1/CIP1}$* have demonstrated that *p21$^{WAF1/CIP1}$* is a critical mediator of *p53*-dependent G_1 arrest associated with DNA damage; the finding that *p21*-null mouse thymocytes are sensitive to γ-irradiation-induced apoptosis, however, suggests that *p53*-dependent apoptosis may not require *p21$^{WAF1/CIP1}$* (Deng *et al*., 1995; Waldman *et al*., 1995). Indeed, forced expression of the Cdk inhibitors has been reported to protect against apoptosis in differentiating murine myocytes (Wang and Walsh, 1996) and in human colon cancer cells treated with prostaglandin A_2 (Gorospe *et al*., 1996).

In the present study, to gain a further insight of *p21$^{WAF1/CIP1}$* functions in the regulation of the cell cycle and apoptosis by *p53*, we used a recombinant adenoviral vector expressing the human *p53* or *p21$^{WAF1/CIP1}$* gene. We found that *p21$^{WAF1/CIP1}$* overexpression induces a stable growth arrest in three human cancer cell lines differing their *p53* status, and

Correspondence: T Fujiwara
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also explored the effects of ectopic wild-type *p53* expression on this *p21*$^{WAF1/CIP1}$-mediated G_1 arrest.

Results

Effect of p53 or p21$^{WAF1/CIP1}$ *expression on human cancer cell lines*

Replication-deficient adenoviral vectors were chosen to achieve efficient gene transfer into cells at all stages of the cell cycle. Monolayer *p53*-deficient H1299 human non-small cell lung cancer cells were infected at a multiplicity of infection (MOI) of 50 with either AdRSVLuc, AdCMVp53, or AdCMVp21 for 24 h. Western blot analysis was performed to compare the levels of each protein (Figure 1). AdCMVp53-infected H1299 cells showed high levels of both p53 and p21 proteins 48 h after infection, suggesting that the virally transduced p53 induced endogenous *p21*$^{WAF1/CIP1}$. In contrast, only p21 protein was detected in AdCMVp21-infected cells. Neither parental nor AdRSVLuc-infected H1299 cells expressed detectable p53 or p21 protein. Although detectable levels of p53 or p21 protein were observed a day after infection, the kinetics of p53 or p21 expression showed a distinctly different pattern. The level of p53, as previously reported (Zhang *et al*., 1994), reached a maximum 3 days after AdCMVp53 infection followed by a rapid decrease; AdCMVp21, however, caused a gradual increase of p21 protein expression, which was maintained for at least 15 days (data not shown).

To assess the effects of AdCMVp53 or AdCMVp21 infection, cells which remained as an attached monolayer were assessed for viability by measuring trypan blue uptake for 6 days following infection with AdCMVp53 or AdCMVp21 for 24 h. A rapid loss of cell viability due to massive cell death, as evidenced by floating, highly light-refractile cells, was observed in AdCMVp53-infected H1299 cells, while AdCMVp21 significantly reduced cell growth rates and induced morphologic changes such as an increased cytoplasmic-to-nuclear ratio (Figure 2). AdRSVLuc-infected H1299 cells retained both a morphology and a growth rate similar to uninfected cells.

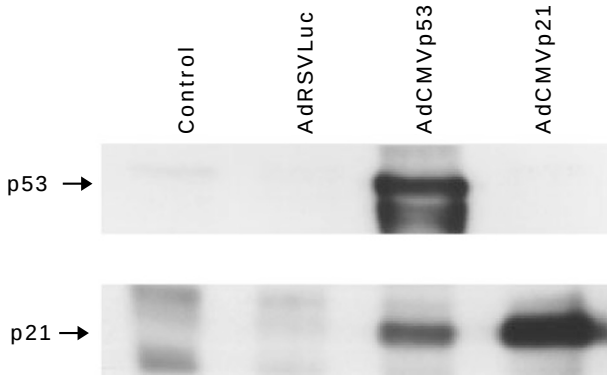
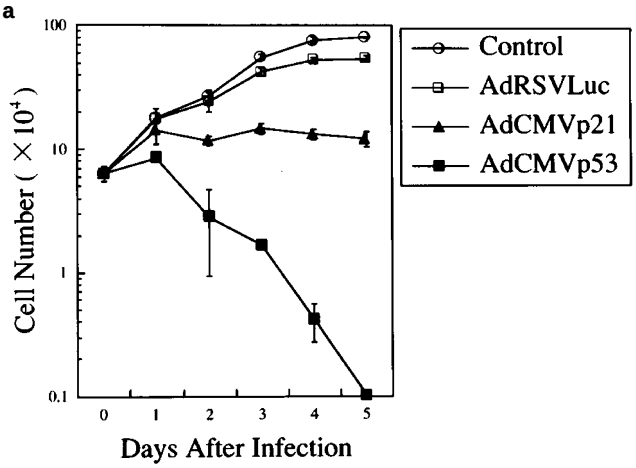


Figure 1 Western blot analysis of p53 and p21 levels in H1299 human lung cancer cells infected with AdCMVp53, AdCMVp21 or AdRSVLuc. Cells were infected with each viral vector at 50 MOI. Whole-cell extracts were prepared, and equal amounts of protein were loaded into each lane. Blots were then probed using the monoclonal antibodies against p53 or p21 and visualized using enhanced chemiluminescence

Growth suppression mediated by p21$^{WAF1/CIP1}$ *and p53-mediated cell death are caused by G_1 arrest and apoptosis*

To further study the mechanism of *p21*$^{WAF1/CIP1}$-induced growth arrest and *p53*-mediated cell death, H1299 cells were infected with either AdCMVp21 or AdCMVp53, harvested at 48 h post-infection, and then assayed for DNA content by flow cytometry and for intranucleosomal DNA cleavage. Representative cell cycle profiles of cells stained with propidium iodide are shown in Figure 3a. Neither untreated proliferating cells nor AdRSVLuc-infected cells showed a specific pattern in the cell cycle distribution; the cells infected with AdCMVp53, however, exhibited a DNA content less than the diploid, G_0/G_1 peak, indicating nuclear fragmentation. In contrast, after AdCMVp21 infection, cells showed a decreased fraction of S-phase cells resulting from an accumulation of most cells at G_0/G_1, indicative of G_1 arrest. This *p21*-mediated block of the cell cycle apparently continued as long as p21 was



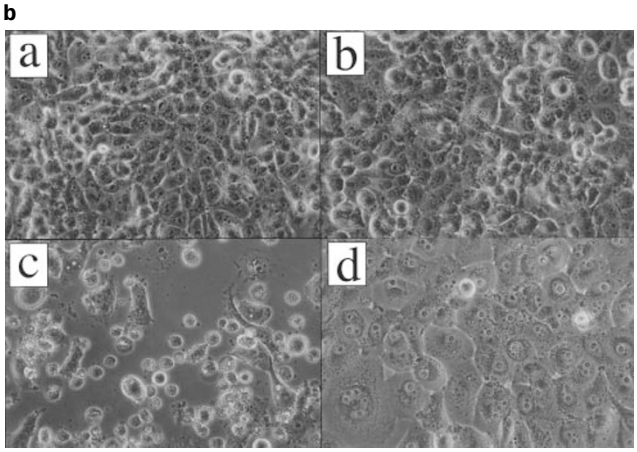
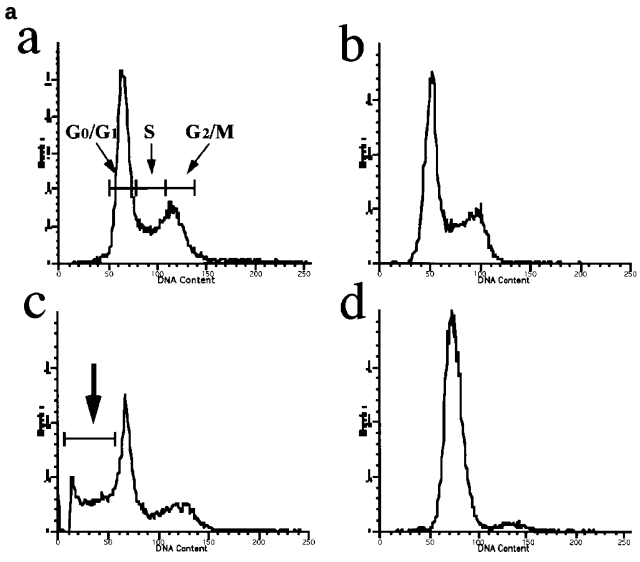


Figure 2 (**a**) Effect of exogenous *p53* and *p21*$^{WAF1/CIP1}$ expression on the growth of H1299 cells. H1299 cells cultured as a monolayer (5×10^4) were infected with AdRSVLuc, AdCMVp53 or AdCMVp21 at a MOI of 50 for 24 h. The growth of H1299 cells were determined by counting cell numbers each day after infection. The mean ± standard deviation of three different wells is shown. (**b**) Phase-contrast photomicrographs (×200) of uninfected cells (panel a), AdRSVLuc-infected cells (panel b), AdCMVp53-infected cells (panel c), and AdCMVp21-infected cells (panel d) 48 h after infection. The majority of AdCMVp53-infected cells are floating, undergoing cell death, while most of the AdCMVp21-infected cells became greatly enlarged in average diameter compared with uninfected or AdRSVLuc-infected cells



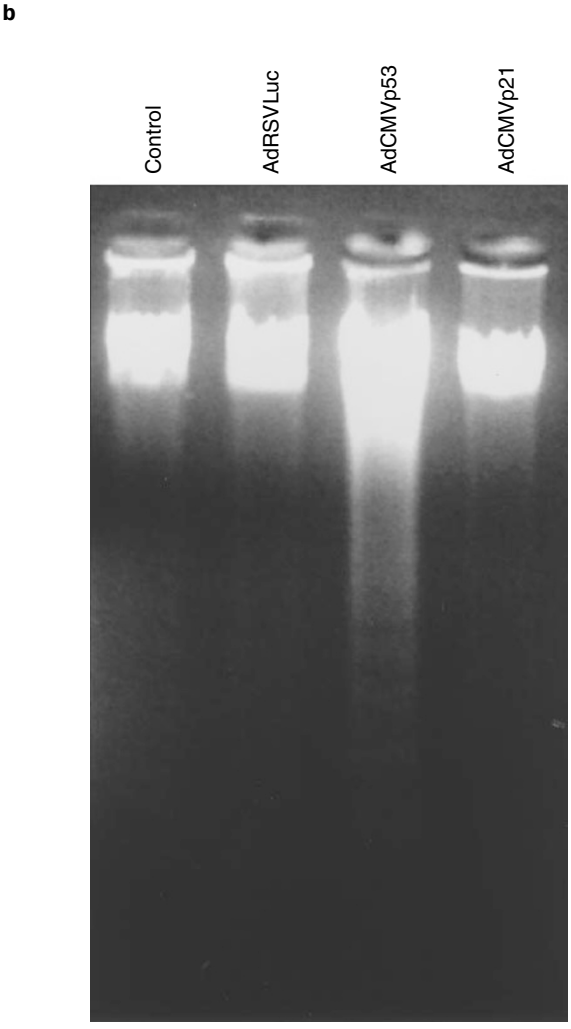


Figure 3 (**a**) Cell-cycle analysis of H1299 cell lines infected with AdCMVp53 and AdCMVp21. H1299 cells (5×10^5) were infected with AdCMVp53, AdCMVp21 or AdRSVLuc at 50 MOI for 48 h and subjected to flow cytometry. Uninfected cells (panel a), AdRSVLuc-infected cells (panel b), AdCMVp53-infected cells (panel c) and AdCMVp21-infected cells (panel d) are shown. The histograms indicate cells in G_0/G_1, S, and G_2/M phases of the cell cycle as described in panel a. The arrow in panel c indicates the population of cells in the sub-G1 group (apoptotic cells). (**b**) Cells were infected with AdCMVp53, AdCMVp21 or AdRSVLuc at 50 MOI. After 48 h of infection, DNA was isolated and subjected to agarose gel electrophoresis. A low-molecular-size DNA ladder, which is characteristic of cells undergoing apoptosis, was observed only in AdCMVp53-infected H1299 cells. No internucleosomal DNA fragmentation was observed in AdCMVp21-infected cells

induced (data not shown). Although the morphologic changes of *p53*-transduced H1299 cells resembled those identified in the apoptotic death pathway, isolated DNA was analysed by electrophoresis through an agarose gel to obtain an additional indication of apoptosis (Figure 3b). The characteristic DNA ladder, a hallmark of apoptotic cell death, appeared after AdCMVp53 infection, but not in cells similarly infected with AdCMVp21.

We also looked for the effect of AdCMVp53 and AdCMVp21 on human colon carcinoma cell lines differing in their *p53* status: mutant *p53*-expressing DLD-1 and LoVo which contained wild-type *p53*. Flowcytometric analysis has demonstrated that infection with AdCMV*p53* induced a complete apoptosis in DLD-1 and LoVo cells by 48 h, whereas cell cycle arrest at the G_1 phase was observed in both cell lines after AdCMVp21 infection (data not shown).

Apoptosis mediated by p53 *can overcome* $p21^{WAF1/CIP1}$*-induced G_1 arrest*

To investigate the effect of $p21^{WAF1/CIP1}$ overexpression on *p53*-mediated apoptotic cell death in three human cancer cell lines, AdCMVp21 and AdCMVp53 infections were performed in sequence. Five $\times$ 10^4 H1299 cells cultured as a monolayer were infected with AdCMVp21 at a MOI of 50 for 24 h to be arrested in G_1, further exposed to either AdCMVp53 or AdRSVLuc in the same condition (50 MOI) 2 days after the first infection, and then assessed for cell viability over 5 days. Double infection with both AdCMVp21 and AdCMVp53 was carried out at a MOI of 100 for each viruses on DLD-1 and LoVo colon cancer cells. Previous work has showed that the range of adenovirus infectivity varies among cell types (Zhang *et al*., 1994). A preliminary titration study using serially diluted viral stocks demonstrated that infectious condition at a MOI of 100 achieved an efficient effect on these two cell lines. The growth rate of H1299, DLD-1, and LoVo cells was significantly reduced after the first AdCMVp21 infection and most cells stopped growing by 48 h; however, following additional AdCMVp53 transduction, viability of cells rapidly declined over 72 h (Figure 4), suggesting that infection with AdCMV*p21* prior to AdCMVp53 showed little effect on AdCMVp53-mediated cell killing. There was no apparent toxicity in AdCMVp21/AdRSVLuc-infected cells, and cells showed no morphologic changes compared to uninfected cells (data not shown).

H1299, DLD-1, and LoVo cells were sequentially infected with two types of adenoviral vectors in the same experimental condition described above, and studied by a flow cytometry after the second infection to follow the cell-cycle events during *p53*-mediated apoptosis. We examined whether cells arrested in G_1 phase promote cell cycle again in the process of apoptosis. As shown in Figure 5, cells expressing $p21^{WAF1/CIP1}$ constitutively stayed in G_1 phase at least for

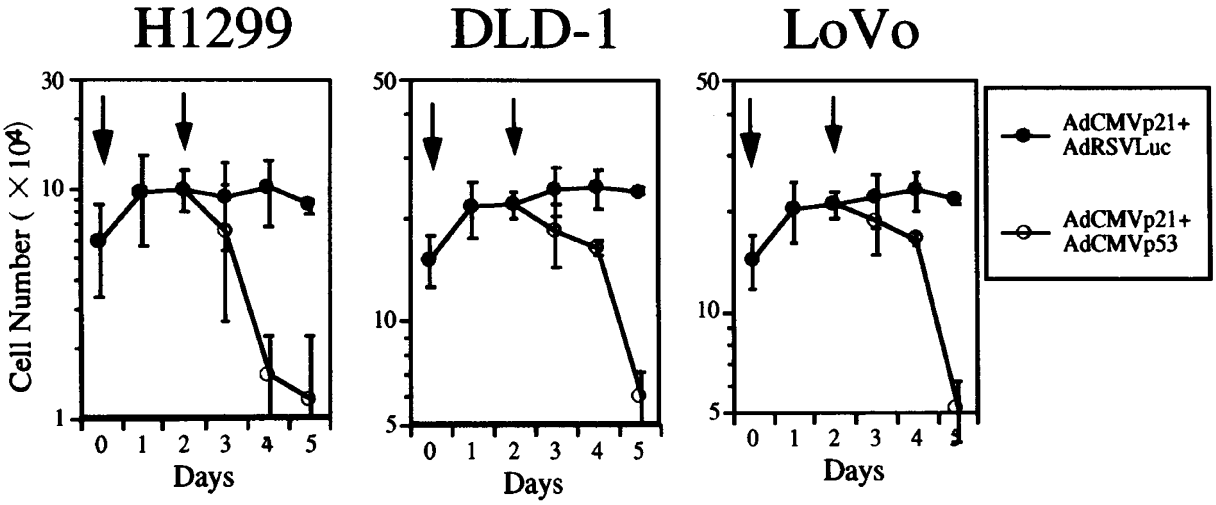


Figure 4 Cells (5×10^4) cultured as a monolayer were infected with AdCMVp21 at a MOI of 50 (H1299) or 100 (DLD-1 and LoVo) for 24 h, further exposed to either AdCMVp53 or AdRSVLuc at a MOI of 50 (H1299) or 100 (DLD-1 and LoVo) 2 days after the first infection, and assessed for cell viability over 5 days

48 h after a second AdRSVLuc infection, while, following addition of AdCMVp53, most cells moved to the G_0/G_1 subfraction, indicating apoptotic cell death. Apoptotic cell death was not preceded by premature entry into S phase, suggesting that AdCMVp53 infection resulted in a direct shift of DNA profile to a sub-G_0/G_1 position without cell cycle progression.

p53 gene transfer did not affect prior transduced p21 protein expression level

To examine whether expression of an adenoviral-transduced p21 protein is affected by subsequent AdCMVp53 infection, extracts of cells sequentially infected with the two adenoviral vectors were subjected to Western analysis. H1299 cells co-infected with AdCMVp21 and AdCMVp53 showed high levels of p21 protein, similarly to those with AdCMVp21 infection alone, suggesting that AdCMVp53 infection did not affect AdCMVp21-infected exogenous *p21*$^{WAF1/CIP1}$ expression (Figure 6).

Discussion

Apoptosis, also known as programmed cell death, plays a major role in development, tissue regeneration, and the cytotoxicity of anticancer therapy. To understand apoptotic cell death, it seems important to define the links between cell proliferation and cell death, and to clarify the mechanism by which a cell chooses either life or death. The balance between these two processes decides the fate of each cell and apparently plays a critical role to determine the therapeutic efficacy of anticancer therapy. Levels of p53 protein increase in response to DNA-damaging anticancer stimuli, and the augmented p53 levels resulted in inhibition of cell division or apoptosis. Induction of p21 protein has been reported to occur in cells undergoing either *p53*-mediated G_1 arrest or apoptosis (El-Deiry *et al*., 1994), suggesting that p21 might mediate some or all of the known functions of p53. The study presented here employed an adenoviral vector expressing *p53* or *p21*$^{WAF1/CIP1}$ to assess the specific impact of these genes on cell-cycle control as well as apoptosis.

Increased levels of *p21*$^{WAF1/CIP1}$ expression are known to be associated with cell-cycle arrest in normal cells under physiologic conditions (Harper *et al*., 1993). We have demonstrated that overexpression of *p21*$^{WAF1/CIP1}$ itself induces a cell-cycle arrest of three human cancer cell lines differing in their *p53* status (H1299, DLD-1, and LoVo) at G_1 phase, and that *p21*$^{WAF1/CIP1}$ expression alone is not sufficient to trigger the apoptotic process. The failure to induce apoptosis by overexpression of *p21*$^{WAF1/CIP1}$ previously has been reported (Yang *et al*., 1995; Chen *et al*., 1995); our system using these cells, however, is unique because cells expressing *p53* underwent complete apoptotic cell death in spite of *p53*-induced endogenous *p21*$^{WAF1/CIP1}$ expression.

What determines whether a particular cell chooses cycle arrest or suicide is not fully understood. Signals that positively regulate the cell growth, such as activation of the proto-oncogene c-*myc* and the overexpression of E2F, abrogate *p53*-induced G_1 arrest and trigger apoptosis (Hermeking *et al*., 1994; Wu and Levine, 1994). In contrast, excess retinoblastoma (Rb) protein, which negatively regulates the cell cycle, has been shown to suppress *p53*-mediated apoptosis (Haupt *et al*., 1995). These findings support the hypothesis that signal conflicts may trigger apoptosis and that cells successfully arrested in G_1 may be resistant to apoptosis. A link between decreased levels of p21 and an increased tendency toward apoptosis has been recently reported in a growth factor-dependent murine hematopoietic cell line (Canman *et al*., 1995). In addition, increased levels of Cdk inhibitors such as *p21*$^{WAF1/CIP1}$ and *p16*^{INK4A} fail to block Rb phosphorylation, rendering Rb constitutively active, and inhibiting apoptosis during myocyte differentiation (Wang and Walsh, 1996). These observations suggest that alteration in *p21*$^{WAF1/CIP1}$ expression may account for occurrence of growth arrest rather than apoptosis, and led us to examine the effect of augmented *p21*$^{WAF1/CIP1}$ expression on *p53*-mediated apoptosis.

Our experiments demonstrated that *p53* induction following $p21^{WAF1/CIP1}$ overexpression resulted in rapid and massive death of cells arrested in the G_1 phase. In other words, *p53*-mediated apoptosis was not inhibited by $p21^{WAF1/CIP1}$-induced G_1 arrest. Recent studies have showed that the specific cell-cycle changes were

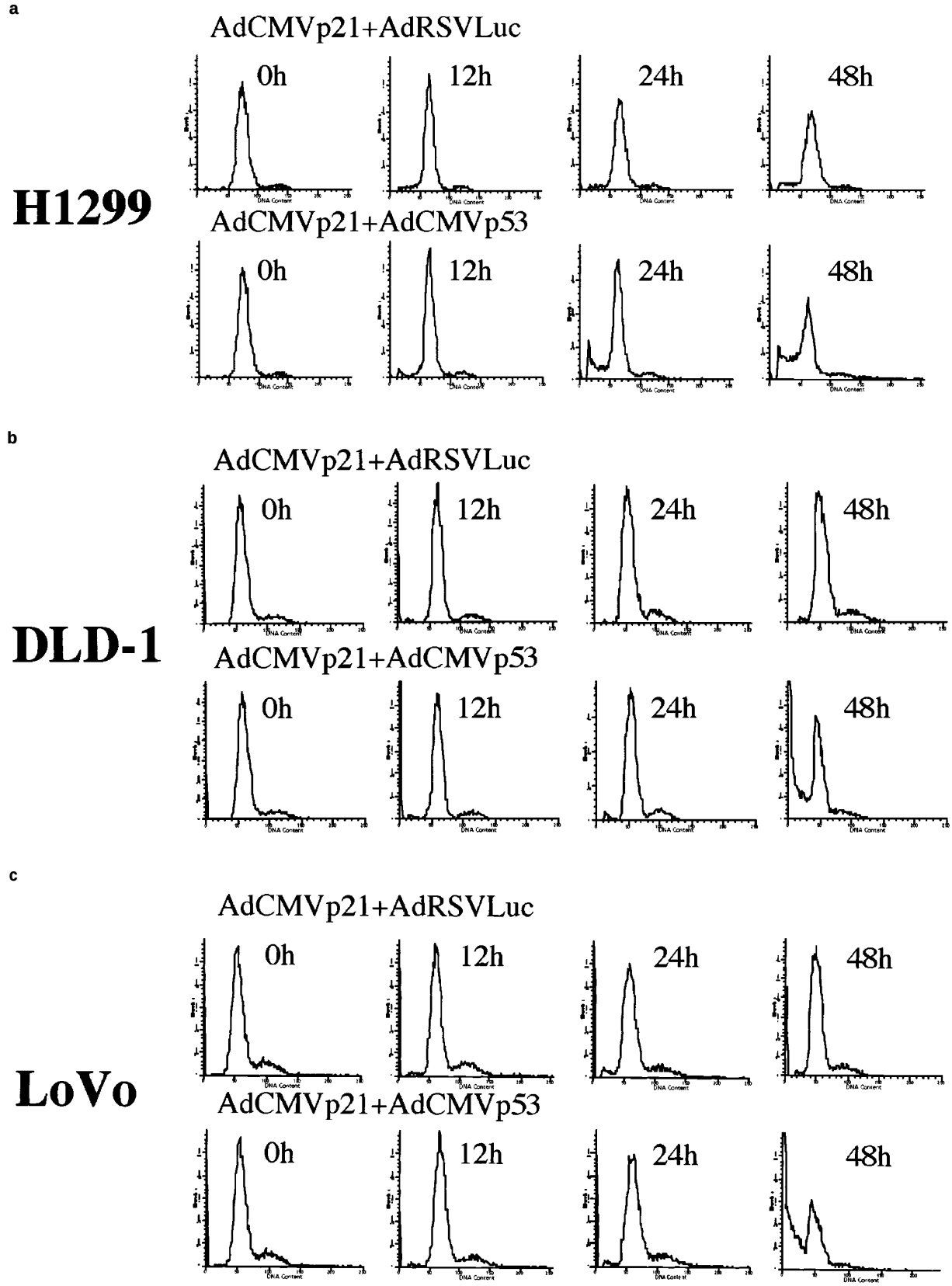


Figure 5 Cells (5×10^5) cultured as a monolayer were first infected with AdCMVp21 at a MOI of 50 (H1299) or 100 (DLD-1 and LoVo) for 24 h, and, 2 days after the first infection, were infected with AdCMVp53 or AdRSVLuc at 50 MOI (H1299) or 100 MOI (DLD-1 and LoVo). Cells were harvested 0, 12, 24, and 48 h after the second infection, and studied by flow cytometry. (**a**) H1299. (**b**) DLD-1. (**c**) LoVo

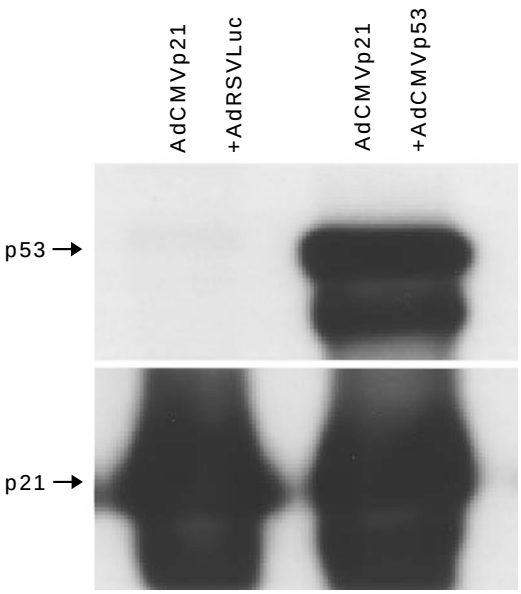


Figure 6 p21 protein expression on H1299 cells serially infected with AdCMVp21 and AdCMVp53. Cell lysates were prepared 48 h after the second AdCMVp53 infection, and then subjected to Western analysis

associated with apoptosis, such as inappropriate S-phase entry without mitosis (Waldman *et al*., 1996); our flow cytometric analysis, however, demonstrated a rapid increase in the sub-G_0/G_1 population following AdCMVp53 infection without any changes of the S-phase cell population. Thus, *p53* might directly induce apoptosis in cells stably remaining in the G_1 phase. These findings clearly suggest that in this system the apoptotic program is not triggered by signal conflict and that the functional activity of *p53* appears to be directly coupled with cell-death induction. To the best of our knowledge, this is the first report that described the mechanism of *p53*-mediated apoptosis completely independent of cell cycling. Moreover, the result was consistent on three human cancer cell lines differing in their genetic status: H1299 cells derived from a human large cell lung carcinoma expressing mutant N-*ras* and deficient of *p53*; DLD-1 cells derived from a human colon carcinoma expressing mutant K-*ras*, mutant *p53* and slightly increased level of c-*myc*; and LoVo cells derived from a human colon carcinoma expressing mutant K-*ras*, wild-type *p53*, and increased level of c-*myc*. Therefore, the dominance of apoptosis over cell cycle arrest is a general phenomenon among human cancer cells, in which ectopic *p53* expression resulted in the apoptotic response.

Other interesting findings in our experiments are morphologic changes of AdCMVp21-infected H1299 cells such as an increased cytoplasmic-to-nuclear ratio. Cells arrested in G_1 phase by AdCMVp21 also showed increased adherence (data not shown). These are consistent with characteristics of cells with a terminally differentiated or senescent phenotype. Previous studies demonstrated that retinoblastoma and osteosarcoma cells infected with the wild-type *Rb*-expressing retrovirus as well as glioblastoma cells transfected with *p53* exhibited similar morphological changes (Huang *et al*., 1988; Gomez-Manzano *et al*., 1996). These observations suggest that cellular differentiation and senescence can be proposed as one of the tumor suppressor mechanism. Thus, functional exploration of $p21^{WAF1/CIP1}$ itself might have implications for the development of a novel anticancer modality.

In summary, exogenous overexpression of wild-type *p53* in human cancer cells could overcome a stable cell cycle arrest and resulted in a complete apoptotic cell death. Thus, *p53* gene transfer may have a significant clinical implication as a potential therapeutic agent. Recently, a phase I trial of the replication-defective retrovirus-mediated wild-type *p53* gene therapy for non-small cell lung cancer has been reported (Roth *et al*., 1996). The results reported here suggest the possible mechanism of this novel anticancer strategy and may provide a key to the successful cancer therapy.

Materials and methods

Cells and culture conditions

The following cell lines were used: a human non-small cell lung cancer cell line H1299, which has homozygously deleted *p53*; a human colon carcinoma cell line DLD-1, which exhibits a homozygous *p53* gene mutation; and a human colon carcinoma cell line LoVo, which contains wild-type *p53*. These cells were routinely propagated in monolayer culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 25 mM HEPES, 100 units/ml penicillin, and 100 mg/ml streptomycin. The transformed embryonic kidney cell line 293 was grown in Dulbecco's-modified Eagle's Medium (D-MEM) (Gibco, Grand Island, New York) with high glucose (4.5 g/l), supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Recombinant adenoviruses

The recombinant adenoviral vector expressing human *p53* or $p21^{WAF1/CIP1}$ was previously constructed and characterized (Zhang *et al*., 1994; Fujiwara *et al*., 1994; Eastman *et al*., 1995). Briefly, the plasmid containing the cytomegalovirus promoter, the wild-type human *p53* or $p21^{WAF1/CIP1}$ cDNA, and the SV40 polyadenylation signal was cotransfected with pJM17 into 293 cells by liposome/DNA coprecipitation to generate an adenoviral *p53* or $p21^{WAF1/CIP1}$ expression vector. The resultant viruses were named AdCMVp53 or AdCMVp21. An adenoviral vector containing luciferase cDNA (AdRSVLuc) was used as a control vector. Culture supernatants of the viral stocks were quantified by a plaque-forming assay using 293 cells. The viruses were aliquotted and stored at $-80^{\circ}C$.

Western blot analysis

Attached cells collected by trypsinization and cells floating in the culture medium were combined in a 10 ml conical tube and washed twice in cold phosphate-buffered saline (PBS). Cells were then lysed in SDS solubilization buffer (62.5 mM Tris-HCl, pH 6.8, containing 10% glycerol, 5% β-mercaptoethanol, and 2% SDS Sigma, St. Louis, MO). Equal amounts of proteins were boiled for 5 min and electrophoresed under reducing conditions on a 12.5% (w/v) polyacrylamide gel. Proteins were electrophoretically transferred to a Hybond-polyvinylidence difluoride (PVDF) transfer membrane (Amersham, Arlington Heights, Illinois), and incubated with primary antibody against *p53* (Ab-2) or p21WAF1 (Ab-1) (Oncogene Science, Manhasset, New York) followed by peroxidase-linked secondary antibody. An Amersham ECL chemiluminescent western system (Amersham) was used to detect secondary probes.

Flow cytometric analysis

Trypsinized adherent and floating cells were collected, washed twice with cold PBS, and resuspended in PBS containing 0.1% Triton X-100 and 1 g/l RNase for 5 min at RT. Samples were then stained with propidium iodide at 50 mg/ml and analysed in a cell sorter (FACScan, Becton Dickinson, Mountain View, California) for DNA content. Cell debris and fixation artifacts were gated out, and G_1, S, and G_2/M populations were quantified using the ModFit LT program for Mac Ver. 1.01 (Verity Software House, Inc.).

Electrophoretic analysis of DNA fragmentation

Both adherent and floating cells were harvested, washed twice with ice-cold phosphate saline buffer, and incubated at 55°C for 6 h in lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS and 100 mg/ml proteinase K). DNA was extracted twice with equal volumes of phenol-chloroform-isoamylalchol (25:24:1). DNA was then precipitated in ethanol. Samples were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining.

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