Jpn. J. Cancer Res. **93**, 1012–1019, September 2002

Differential Suppression of Human Cervical Cancer Cell Growth by Adenovirus Delivery of p53 *in vitro*: Arrest Phase of Cell Cycle Is Dependent on Cell Line

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It has been reported that overexpression of wild-type p53 protein induces suppression of tumor cell growth *in vivo* and *in vitro*. In this study, we further evaluated the differential effects of p53 delivered in an adenovirus vector on the cell growth, apoptosis and cell cycle progression in cervical cancer cell lines. We constructed a recombinant adenovirus expressing p53 and then delivered this into cervical carcinoma cell lines (CaSki, SiHa, and HeLa, HeLaS3) along with adenovirus expressing β-galactosidase as a negative control. Adenovirus-delivered p53 overexpression resulted in a more significant suppression of cell growth in HPV 18-infected cells (HeLa and HeLaS3) and a lesser suppression in HPV 16-infected cells (CaSki and SiHa). However, no suppression was observed in cells infected with a negative control virus. p53 overexpression also induced apoptosis and cell cycle arrest, as determined by annexin V and propidium iodide staining. In particular, the cell cycle was arrested in the G_2/M phase in CaSki cells. In contrast, cell cycles were arrested in the G_1 phase in HeLa cells, suggesting that the arrest phase is dependent upon the cervical cancer cell line. Taken together, these data support the idea that overexpressed p53 protein plays a differential role in suppressing cervical cancer cell growth through apoptosis and cell cycle arrest in either G_1 or G_2/M phase, depending on the cancer cell line.

Key words: AdCMVp53 — Cervical cancer — Apoptosis — Cell cycle arrest — Gene therapy

Cervical carcinoma is caused mostly by infection with a high-risk group of human papillomaviruses (HPV).[1–3] After high-risk HPV infection, two viral oncogenic proteins, E6 and E7, play a critical role in inducing cervical cancers by interacting with p53 and pRB, respectively, and in inactivating these cellular regulatory proteins.[4–6] The two viral oncogenic proteins, E6 and E7 are commonly expressed in these carcinoma cells and are required for maintaining cancer malignancy.[7] It has been reported that, except for cervical cancers, most cancer development results from *p53* gene mutation.[8] p53 mutation is detected in more than 50% of cancer cells, but rarely in cervical cancer cell types.[9] In most cervical cancers, however, the function of p53 is down-regulated by the E6 protein of HPV 16, whereby E6 binds to p53, resulting in degradation of E6-p53 complexes through the ubiquitin pathway.[10–12] For instance, human cervical cancer cell lines, such as CaSki (HPV 16), SiHa (HPV 16), HeLa (HPV 18) and HeLaS3 (HPV 18) express intact p53 protein. However, the viral E6 protein is required for the continuous growth of HPV-immortalized cells, in which E6 reduces the level of p53 protein, resulting in loss of G_1 arrest.[12, 13] Similarly, E7 of HPV 16 is also associated with inactivation of retinoblastoma tumor suppressor gene product.[14]

The tumor suppressor gene *p53* codes for a transcription factor which regulates oncogene expression, gene transcription, and DNA synthesis and repair systems, as well as apoptosis.[9] As a transcription factor, the p53 protein binds to a specific sequence on the promoter region and then activates the transcription of cellular genes.[15] For instance, *mdm-2* is involved in the negative feedback suppression of *p53* genes.[16] $p21^{WAF1/CIP1}$ inhibits cyclin-CDK (cyclin-dependent kinase) complexes for G_1 arrest.[17] *Bax* induces apoptosis.[18] The main function of p53 is on the check point of the G_1/S cycle, and p53 plays an important role in preventing cancer cell growth.[8] In normal cells, DNA damage induces p53 expression, leading to cell cycle arrest in the G_1 phase and apoptosis, as well as inhibition of DNA replication. However, normal function of p53 is missing in immortalized cancer cells. It is known that dysfunction of p53 is associated with uncontrolled cell growth and promotion to the tumor stage. Previous studies demonstrated that introduction of a wild-type *p53* gene results in growth inhibition of human cancer cells.[19, 20]

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Hamada *et al.* reported that expression of adenovirus-delivered p53 is associated with apoptosis and growth suppression of cervical cancer cells *in vitro* and *in vivo*.[10] The mechanism(s) whereby p53 inhibits cancer cell growth might be related to apoptosis[10, 17, 19] and G_1 arrest in the cell cycle progression.[19, 21] Overall, these studies suggest that a wild-type p53 protein is responsible for cancer cell death through apoptosis and cell cycle arrest in the G_1 phase.

In this study, we constructed a recombinant AdCMVp53 and evaluated the differential effect of adenoviral delivery of p53 on growth inhibition in human cervical cancer cell lines *in vitro*. We observed that p53 delivery results in a more significant growth inhibition in HPV 18-infected cells and a lesser growth inhibition in HPV 16-infected cells. This inhibition appears to be mediated by p53-induced apoptosis and cell cycle arrest. Moreover, the arrest phase is likely to be dependent upon the cervical cancer cell line used. Thus, adenoviral delivery of p53 plays a differential role in suppressing cervical cancer cell growth by inducing apoptosis in addition to cell cycle arrest in either the G_1 or G_2/M phase depending on the cell line.

MATERIALS AND METHODS

Cells and culture conditions Four HPV-infected human cervical cancer cell lines (HPV 16-positive cells, CaSki and SiHa cells; and HPV 18-positive cells, HeLa and HeLaS3 cells) and 293 cells were purchased from the American Type Culture Collection (ATCC), Manassas, VA. The cells were maintained in Dulbecco's modified minimal essential medium (Life Technologies/GIBCO-BRL, Paisley, Scotland) supplemented with 10% fetal bovine serum, 0.37% sodium bicarbonate, 30 m*M* HEPES and streptomycin/penicillin (cDMEM).

Construction of recombinant p53 adenoviruses Replication-defective recombinant adenovirus vectors were used. A 1.7 kb wild-type *p53* gene (a kind gift from KIST, Taejon, Korea) with the HCMV promoter sequence was cloned into the *Eco*RV and *Xba*1 sites of pΔE1sp1B. The resulting pΔE1/CMVp53 was cotransfected with pBHGE3 vector into 293 cells. Recombinant adenoviral vector (AdCMVp53) containing a human wild-type *p53* gene under the control of the cytomegalovirus promoter was selected. In detail, recombinant p53 adenoviruses were isolated from a single plaque and expanded in 293 cells and then purified by double CsCl gradient ultracentrifugation (25 000 rpm, 4°C, 2 h) as described by Graham *et al*.[22] The final infectious titers were determined to be 10^8 plaque-forming units (pfu) per ml. AdCMVLacZ was provided by Dr. Jae-Ho Lee of Seoul National University, Seoul, Korea. To determine transfection efficiency, AdCMVLacZ was used as previously described.[10] In particular, 1-day virally infected cells were fixed with 0.5% glutaraldehyde and stained with X-gal. The number of blue-stained cells was counted under a microscope (200 magnification).

Titration of AdCMVp53 virus The virus titer was determined by plaque-forming assays in 293 cells as previously described.[22] Briefly, 293 cells (10^5 cells/well) were inoculated into a 12-well plate and cultured for 24 h. When 90% confluency was achieved, cells were infected with recombinant adenoviruses. For adsorption, the cell plates were shaken 6 times for 1 h and then 1 ml of cDMEM was added. Cell cytopathic effect (CPE) was observed at the time intervals of 12, 24, 26 and 48 h using a converted microscope.

Western blot assay For immunoblot assay, 30 μg of cell extracts was separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to a cellulose membrane. The blotted membrane was blocked with 5% skim milk and reacted with mouse anti-human p53 DO7 (Novocastra, Newcastle, UK). This was followed by reaction with horseradish peroxidase-conjugated goat anti-mouse IgG (Novocastra). For each step, the membrane was washed with TBST (Tris-buffered saline with 0.1% Tween-20) 3 times. Protein bands were visualized using an ECL Kit according to the manufacturer's protocol (Amersham, Arlington Heights, IL).

Viable cell count Cells were cultured for 4 days, treated with trypsin-EDTA, collected, washed with phosphate-buffered saline (PBS) and counted under a microscope. Cell viability was confirmed by trypan blue dye exclusion.

FACS analysis CaSki or HeLa cells were mock-infected or infected with adenovirus expressing p53 or LacZ at a multiplicity of infection (MOI) of 50. After 2 days of incubation, cells were trypsinized and washed twice with PBS. The cells were stained with annexin V-FITC conjugate or propidium iodide. Samples were then analyzed using FACS (Becton Dickinson, San Jose, CA). For DNA contents, cell debris and fixation artifacts were gated out and the G_0/G_1, S, and G_2/M populations were quantified using the CellQuest program.

RESULTS

Infection of cells with AdCMVp53 and AdCMVLacZ, and protein expression levels To examine whether the AdCMVp53 vector overexpresses p53 protein in CaSki, SiHa, HeLa and HeLaS3 cell lines, cells were infected with AdCMVLacZ or AdCMVp53 for 2 days. The cell extracts were obtained and run on SDS-PAGE for western blot assay. As shown in Fig. 1A, expression of p53 protein was observed in all cervical cancer cell lines infected with AdCMVp53. Furthermore, no significant differences in p53 protein levels were detected in these cells. In contrast, there was no expression of p53 in mock-infected cells or

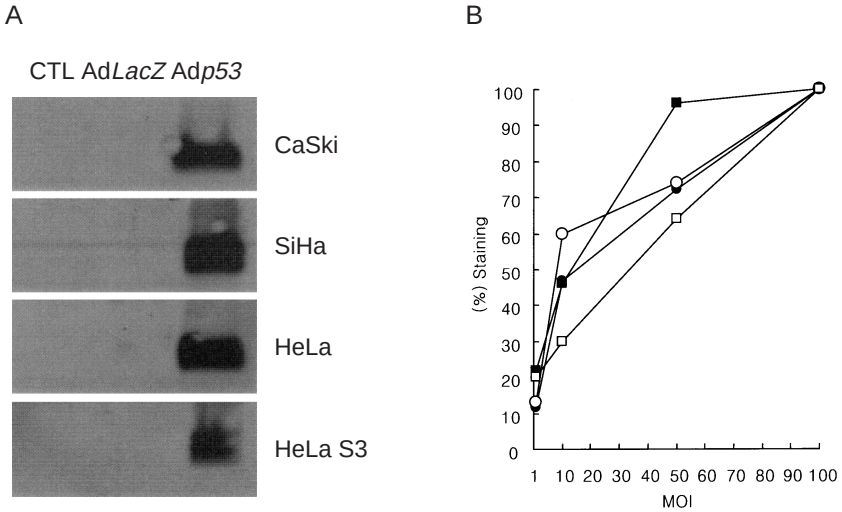

Fig. 1. (A) p53 induction in cervical cancer cell lines following AdCMVp53 infection *in vitro*. Cells were mock-infected or infected with adenoviruses encoding p53 or β-galactosidase at 50 MOI. At 2 days after infection, the cells were collected and cell extracts (30 μg) were run on 10% SDS-PAGE. Immunoblot analysis was performed using 1:1000 sera specific for p53. (B) Transfection efficiency of AdCMVLacZ in cervical cell lines. Cells were infected with AdCMVLacZ at different MOI(s) ranging from 1 to 100. At 1 day after infection, the cells were stained and blue-stained cells were counted. ● CaSki, ○ SiHa, ■ HeLa, □ HeLaS3.

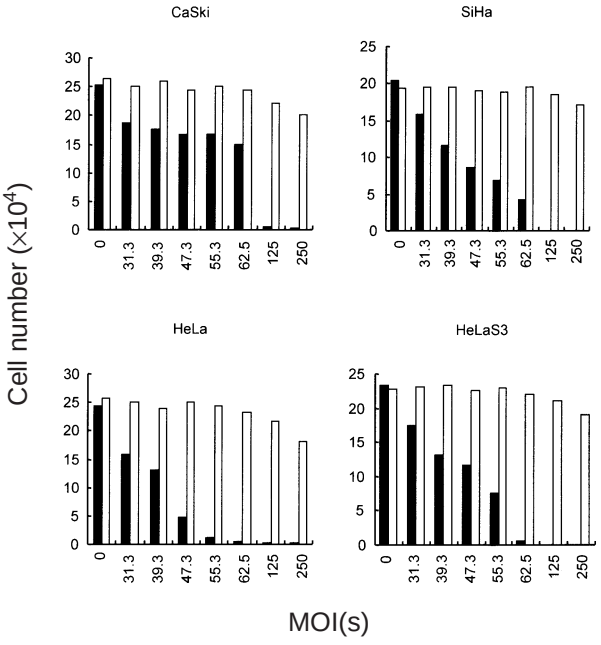

Fig. 2. Growth-inhibitory effects of adenovirus p53 delivery in various cervical carcinoma cell lines at different MOI(s). Cells (10^5 cells/well) were cultured in 12-well plates in triplicate overnight and infected with adenoviruses expressing p53 and LacZ at increasing MOI(s). After infection, cells were cultured for 2 days and trypsinized for counting under a microscope. The mean values of cell counts in triplicate were plotted. Open bar, AdCMVLacZ; closed bar, AdCMVp53.

cells infected with AdCMVLacZ. This confirms that at an MOI of 50 AdCMVp53 induces a similar amount of p53 overexpression in these human cervical cancer cell lines. To further evaluate transfection efficiency in these cell lines, cells were infected with different amounts of AdCMVLacZ ranging from 1 to 100 MOI for 1 day. As shown in Fig. 1B, infection of cells at 100 MOI resulted in 100% X-gal-staining cells in four different cell lines. Furthermore, infection of CaSki, SiHa, and HeLaS3 cells at an MOI of 50 showed 60–75% transfection efficiency. In particular, HeLa cells showed almost 100% transfection efficiency at an MOI of 50. Taken together, these data suggest that recombinant AdCMV vectors can infect these different cell lines in a similar fashion.

Growth-inhibitory effects of adenovirus *p53* gene delivery in various cervical carcinoma cell lines To evaluate the differential effect of exogenous wild-type p53 expression on the growth of the uterine cervix carcinoma cell lines, we transfected four different cervical cancer cell types with AdCMVp53 for 2 days at an increasing MOI. As shown in Fig. 2, in all cervical carcinoma cell types tested, infection with an increasing number of recombinant adenoviruses (31.3, 39.3, 47.3, 55.3, 62.5, 125 and 250 MOI) resulted in inhibition of cell growth in an MOI-dependent manner. In particular, complete inhibition of cell growth was observed at 125 MOI in both CaSki and SiHa cell lines (HPV 16-infected). However, complete inhibition of cell growth was detected at 62.5 MOI in HeLa and HeLaS3 cell lines (HPV 18-infected), a one-fold

lower MOI (from 125 to 62.5 MOI) than that of HPV 16-infected cervical cancer cell lines, CaSki and SiHa cells. When the cell growth state at 50 MOI was compared between HeLa and CaSki cells under a microscope, a greater extent of cell death was observed in HeLa cells, as compared to CaSki cells (Fig. 3). These data support the idea that there is a significant difference in sensitivity to p53 between HeLa and CaSki cells. In contrast, at these MOI(s), no suppression of cell growth was observed when cells were infected with recombinant adenovirus expressing β-gal as a negative control, suggesting that inhibition of cell growth is mediated solely by exogenous p53 expression. Moreover, when cervical cancer cells were infected with AdCMVp53 for increasing incubation times, complete inhibition of cell growth was observed over time (Fig. 4). In contrast, infection with AdCMVLacZ showed increased cell growth in a manner similar to the negative control group. This further supports the view that p53 expression is responsible for cell growth inhibition in cervical cancer cells.

Exogenous p53-induced apoptosis in HeLa and CaSki cells We next focused on HeLa and CaSki cells, as these two types of cells displayed a different sensitivity to p53. To investigate whether overexpression of p53 protein induces apoptosis in these two cell lines, we infected cells with AdCMVp53 at an MOI of 50 and then performed annexin V staining and FACS analyses. As shown in Fig. 5, infection of CaSki and HeLa cells with a recombinant p53 adenovirus resulted in a large increase in the proportion of cells staining with annexin V, suggesting that exogenous p53 overexpression induces apoptosis in CaSki and HeLa cells. In contrast, little staining with annexin V was displayed in control or AdCMVLacZ-infected cells (Fig. 5). These data support the view that apoptosis is induced by adenovirus *p53* gene delivery in cancer cells.

Exogenous p53-induced cell cycle arrest in either G_1, S or G_2/M phase depending on cell line We also tested for cell cycle arrest in CaSki and HeLa cells. To determine if overexpression of p53 has any effect on cell cycle perturbation, cell cycle analysis was performed by propidium iodide staining. When cells were infected with recombinant p53 adenovirus, there was a significant alteration in cell cycle progression in both CaSki and HeLa cells (Fig. 6). In particular, the cell cycle was arrested at the G_1 phase by *p53* gene delivery in HeLa cells, but at the G_2/M phase in CaSki cells. In contrast, infection with negative control adenovirus resulted in a normal cell cycle progression, similar to that in mock-infected cells. Table I shows cell cycle arrest patterns at 1, 2 and 3 days post infection with

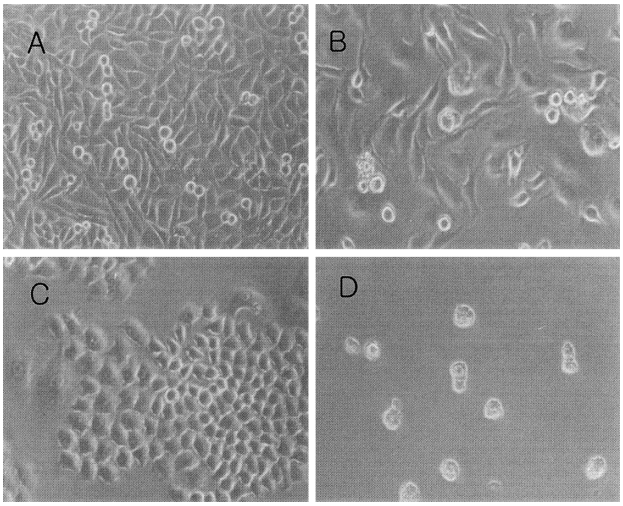

Fig. 3. Microscopic state of cells at 4 days after AdCMVp53 and AdCMVLacZ infection in CaSki and HeLa cells. CaSki and HeLa cells (10^5 cells/well) were cultured in 12-well plates in triplicate overnight, and mock-infected or infected with adenoviruses expressing p53 or LacZ at 50 MOI for 4 days. Cells were photographed under a microscope. A, CaSki (AdCMVLacZ-infected); B, CaSki (AdCMVp53-infected); C, HeLa (AdCMV-LacZ-infected); D, HeLa (AdCMVp53-infected).

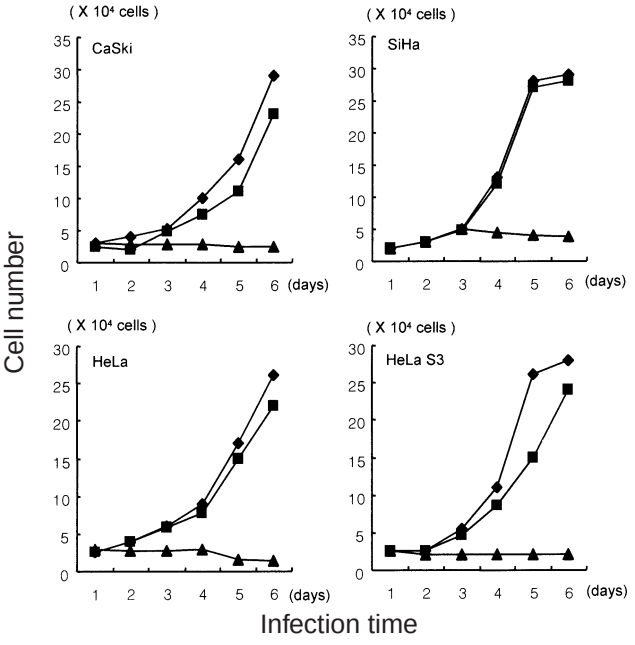

Fig. 4. Growth-inhibitory effects of adenovirus p53 delivery in various cervical carcinoma cell lines. Cells (10^5 cells/well) were cultured in 12-well plates in triplicate overnight and infected with adenoviruses expressing p53 and LacZ at 50 MOI. After infection, cells were cultured for various periods and then trypsinized for counting under a microscope. The mean cell counts in triplicate were plotted. ◆ mock-infected, ■ AdCMVLacZ-infected, ▲ AdCMVp53-infected.

AdCMVp53. The arrest profiles of p53 were consistent over the time points. In agreement with this, adenovirus *p53* gene delivery resulted in a dramatic decrease in the G_2/M cell population in HeLa cells (Table I). However, in the case of CaSki cells, a significant decrease in G_1 cell population was observed. Overall, these data suggest that exogenous p53 expression induces cell cycle arrest in either G_1 or G_2/M phase depending on the cervical cancer cell line.

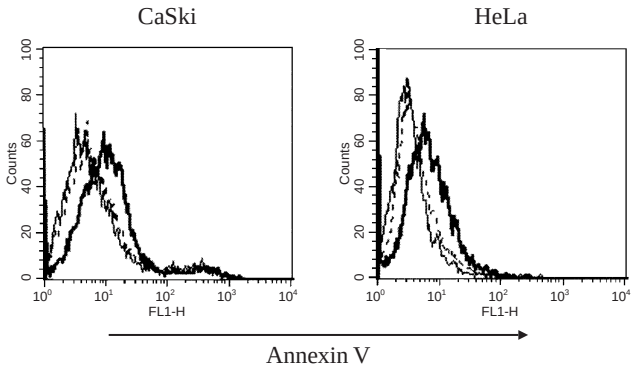

Fig. 5. Effects of AdCMVp53 on apoptosis. CaSki and HeLa cells (10^5 cells/well) were cultured in 12-well plates in triplicate overnight, and mock-infected or infected with adenoviruses expressing p53 or LacZ at an MOI of 50 for 2 days. For apoptosis analysis, cells were stained with annexin V. Stained cells were analyzed via FACS analysis. Thin line, negative control; dotted line, AdCMVLacZ; thick line, AdCMVp53.

DISCUSSION

Human papilloma virus is commonly observed in the cervical cancer tissues and is a main cause of cervical cancers in humans. Growth of cervical carcinoma cells depends on continuous expression of the E6 and E7 oncogenic proteins of high-risk HPV types, including type 16.[23] In HPV type 16, mutations on the open reading frame of E6 or E7, or on their upstream sequences, alter the oncogenicity of the virus, suggesting an important role of these viral proteins in oncogenesis. The cells infected with HPV type 16 have a very low p53 expression level.[6, 24] In most cervical cancers, the function of p53 is down-regulated by the E6 protein of HPV 16; E6 binds to p53 and leads to degradation of E6-p53 complexes.[10–12] Exogenous p53 overexpression results in degradation of viral E6 proteins and decreases the survival of cancer cells.[25] A previous study also showed that p53 binds E6 oncoprotein to decrease the tumorigenic properties of E6.[24] Introduction of a wild-type *p53* gene into cells is associated with growth inhibition of human cancer cells.[18, 19, 26] Moreover, the efficacy of virus vector-delivered p53 therapy has been proved clinically.[27] We also observed a significant growth suppression of cervical cancer cells when AdCMVp53 was delivered into the cells. In particular, HPV 18-infected cell types were more susceptible to p53-mediated cell growth inhibition than HPV 16-infected cell types. In one study, HPV 16-infected cells (SiHa, 1–2 copies of HPV per cell) were more susceptible to growth inhibition exerted by adeno-associated virus, as

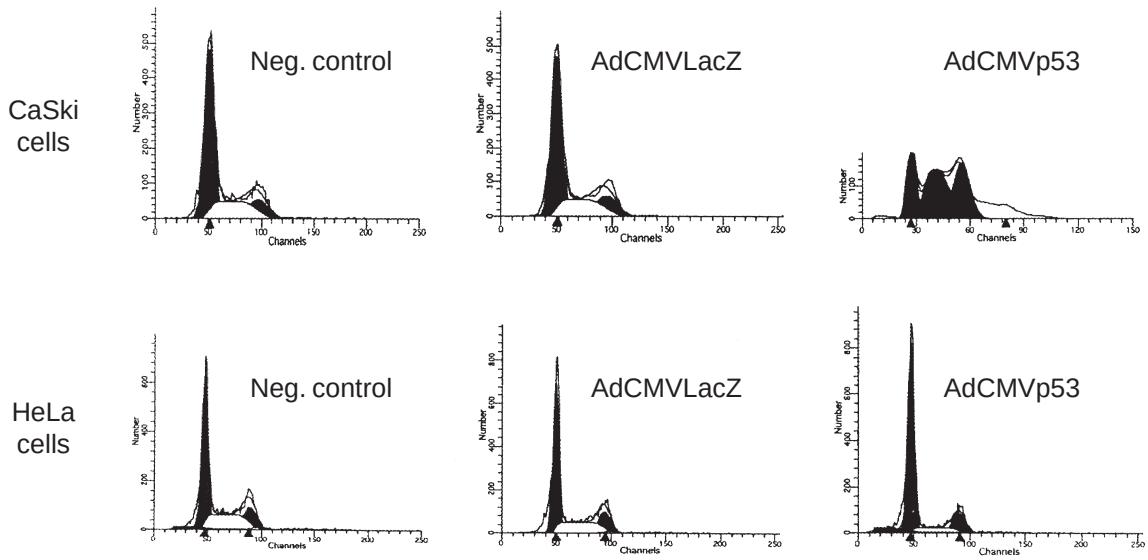

Fig. 6. Cell cycle arrest in either G_1 or G_2/M phase by AdCMVp53 in CaSki and HeLa cells. Cells (10^5 cells/well) were cultured in 12-well plates overnight in triplicate, and mock-infected or infected with adenoviruses expressing p53 or LacZ at 50 MOI for 2 days. Cell cycle distribution was determined by propidium iodide staining.

Table I. Percent of Cells Distributed in Cell Cycle Phases

		CaSki cells			HeLa cells		
		G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
Day 1	Mock-infected	59.03	27.13	13.84	50.12	31.85	18.03
	AdCMVLacZ	60.27	22.14	17.59	59.19	25.81	14.99
	AdCMVp53	37.80	35.62	29.57	69.50	22.14	8.35
Day 2	Mock-infected	60.95	25.17	13.87	52.12	31.51	16.37
	AdCMVLacZ	60.21	24.32	15.47	55.72	27.65	16.64
	AdCMVp53	17.33	45.22	37.46	72.06	12.40	15.54
Day 3	Mock-infected	62.54	24.24	13.22	55.47	22.15	22.38
	AdCMVLacZ	60.73	30.35	8.92	49.31	30.75	19.93
	AdCMVp53	9.72	69.54	20.74	75.68	11.00	13.32

Cells were cultured in 12-well plates (10^5 cells/well) and mock-infected or infected with adenoviruses expressing p53 or LacZ at 50 MOI. Percent of cells distributed at various stages of the cell cycle was measured by flow cytometry. This was performed in triplicate and data are presented as mean values.

compared to HPV 18-infected cells (HeLa, 50 copies of HPV per cells).[28] The authors speculated that cells with a smaller HPV copy number might be more susceptible to growth inhibition by adeno-associated virus. In this case, however, this is unlikely, as both CaSki (600 copies of HPV per cell) and SiHa cells (1–2 copies of HPV per cell) showed similar susceptibility to p53-mediated growth inhibition. Furthermore, no suppression of cancer cell growth was observed after infection of the cells with AdCMV-LacZ, supporting the view that the effects were not mediated by virus itself. Our data are compatible with previous findings in cervical cancer cells.[10]

Wild-type p53 induces apoptosis to eliminate abnormal cells.[29] No inhibitory properties of p53 against DNA replication or synthesis have been observed in either cervical cancer cell lines infected with HPV 16 or HPV 18, or cervical cancer cells with p53 mutation that blocks normal p53 function.[12] Exogenous p53 expression is directly related to inhibition of NF-κB activity in human colon cancer cells.[30] For example, combination therapy using aspirin plus p53 further decreased NF-κB activity, which is associated with prevention of apoptosis. More recent studies have demonstrated that combination of p53 therapy with radiation or chemotherapy is more effective for suppressing cancer cell growth, as compared to single therapy.[31, 32] In p53-mediated apoptosis, apoptotic protease activating factor-1 (APAF-1) plays an important role as a p53 downstream effector, as confirmed by cDNA microarray.[33] We also observed that p53 overexpression induces apoptosis in CaSki and HeLa cells, as determined by annexin V staining. This accords well with our observation that both CaSki and HeLa cells displayed cell death upon adenovirus p53 delivery (Fig. 3). These findings confirm the notion that wild-type p53 is responsible for apoptosis and cell death in cancer cells.

Cell cycle checkpoints are among the multiple mechanisms that eukaryotic cells employ to maintain their genomic integrity and to reduce tumor formation. The p53 protein induces cell cycle arrest in the G_1 phase and apoptosis through induction of the expression of WAF1/p21, which has growth-inhibitory ability.[34] In particular, when delivered in a virus vector form, p53 induces G_1 arrest in osteoblastoma cells.[21] The mechanism(s) whereby p53 inhibits cancer cell growth might be related to G_1 arrest in the cell cycle.[20, 21] Here, we observed that p53 delivery induced G_1 arrest in HeLa cells. In contrast, G_2/M phase arrest was caused by p53 overexpression in CaSki cells. In particular, S phase arrest also occurred in CaSki cells, suggesting that p53 might arrest cells in the S phase. If this is the case, there should be a decrease of cell number in the G_2/M phase by AdCMVp53, as compared to negative control groups. However, we observed an increased cell number in the G_2/M phase. It is possible that cells in the S phase might be a retarded cell population due to cell arrest in the G_2/M phase. This should be further examined. Taken together, these data suggest that the arrest phase is dependent upon the cervical cancer cell line examined. However, how adenovirus-delivered p53 behaves differently in HeLa and CaSki cells is presently unclear. In one study, treatment with adeno-associated virus induced different levels of cell growth inhibition among cervical cancer cell types,[28] suggesting a unique nature of each cervical cancer cell line. However, the mechanism(s) whereby p53 induces cell cycle arrest in different phases in cervical cells remains to be determined.

The p53 protein plays an important role in maintaining genome integrity through induction of cell growth arrest for repair of DNA damage, or of apoptosis for eliminating cells with irreparable damage.[29, 35] For example, DNA strand breaks trigger the accumulation of p53 protein in

cells.[35] In our studies, overexpression of p53 was accomplished by infecting cells with adenovirus expressing p53. Overexpressed p53 might transactivate its downstream genes, such as *p21*, based upon the previous report that p21, a downstream regulator of p53, plays an inhibitory role in cell growth.[20, 36] In our study, however, no induction of p21 was observed in the presence of AdCMVp53 infection (data not shown), suggesting that p53 might act in some other way in these HPV-infected cervical cancer cell lines. In these cells, endogenous p53 is exhausted as the viral E6 protein binds to p53, resulting in degradation of p53-E6 complexes via the ubiquitin pathway. It is likely that when a high concentration of p53 protein is administered, the p53 protein level is sufficiently supplemented. This is supported by our observation that p53 was detected strongly in cells infected with AdCMVp53, as compared to negative controls. The amount of p53 is likely to be enough to bind E6 and then to reduce the oncogenic activity of E6. This suggests a potentially powerful strategy for gene therapy against HPV-infected cervical cancers.

In sum, we observed that adenovirus delivery of a wild-type *p53* gene exerts a differential effect in suppressing cervical cancer cell growth through apoptosis and cell cycle arrest. In particular, G_1 arrest was observed in HeLa cells, whereas G_2/M arrest was seen in CaSki cells, suggesting that the arrest phase is dependent on the cervical cancer cell line. Thus, these data support the view that overexpressed p53 plays an important role in suppressing cancer cell growth by inducing apoptosis as well as cell cycle arrest in either the G_1 or G_2/M phase, depending on the cancer cell line.

(Received April 8, 2002/Revised June 19, 2002/Accepted July 1, 2002)

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